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Prolonged Glucose Deprivation Sensitizes Snf1 to Negative Regulation By PKA to Delay Entry into Quiescence

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by
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ABSTRACT

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AMPK, the fuel gauge of the cell, and its upstream kinase (LKB1) have been implicated in cancer prevention and stress response associated with energy exhaustion. In the yeast *Saccharomyces cerevisiae*, Snf1 is the ortholog of mammalian AMPK. In *S. cerevisiae*, Snf1 is activated by phosphorylation of its T-loop at Thr210, primarily by its upstream kinase Sak1, in absence of the preferred carbon source, glucose, or during some other stress responses. Cyclic AMP (cAMP)-dependent protein kinase A (PKA) is involved in nutrient signaling largely antagonistically to Snf1. Using yeast strains of the ∑1278b genetic background, which have a high basal level of cAMP signaling, PKA was previously suggested to downregulate Snf1 by a mechanism that involves phosphorylation of Sak1 on two consensus PKA recognition sites in the non-catalytic C-terminal domain. Sequence analysis suggests that Snf1 and/or its immediate regulators are also targets for negative regulation by PKA in other genetic lineages of *S. cerevisiae* and even in other yeast species. Here, we have investigated the possible existence of an antagonistic relationship between PKA and Snf1 in another *S. cerevisiae* strain lineage,

W303, which has a relatively low basal level of cAMP signaling. In addition to short-term glucose limitation, we monitored Snf1 activation under conditions of long-term carbon stress that normally leads to exit from the mitotic cycle and entry into a quiescent state. We observed that W303 *ira1*Δ mutant cells with increased PKA signaling have significantly reduced levels of Snf1 activation after long-term carbon stress. The quiescence-associated trait of stress resistance, specifically heat-shock survival, was also evaluated. W303 lacking Snf1 or with significantly higher levels of PKA activity due to the *ira1*Δ mutation, did not acquire normal heat-shock resistance, suggesting failure to enter quiescence. This suggests that downregulation of Snf1 represents one mechanism by which PKA inhibits entry into quiescence. Since the ability to enter quiescence offers significant evolutionary advantages, similar relationships between PKA and Snf1 are likely to exist in various fungal species. Moreover, since PKA and Snf1/AMPK are conserved in eukaryotes from yeast to humans, the PKA-AMPK pathway could also regulate quiescence as it pertains to development and tumorigenesis.

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Introduction:

Saccharomyces cerevisiae, also known as baker's yeast, has been utilized in the food industry for centuries, and has been used for scientific research for many years as well. The stability in both haploid and diploid states makes it easy to perform genetic research with, and yeast serves as a powerful model eukaryote, making experimental findings easily applicable and beneficial to the biomedical fields, as well as biotechnology. One important trait is the ability of yeast to readily ferment sugars, even in the presence of oxygen, which makes it ideal for ethanol production (Nelson & Cox, 2008). The phenomenon of aerobic fermentation, termed Crabtree effect, is also interesting because of its relationship to the Warburg effect in mammalian cancer cells, which represents an important process for targeting with drugs (Diaz-Ruiz, Rigoulet, & Devin, 2011; Hardie, 2007a). In this single-celled eukaryote, studying sugar metabolism can also give deeper insights into the molecular etiology of diabetes (Diaz-Ruiz et al., 2011; Hardie, 2007a; Hardie, 2007a; Hedbacker & Carlson, 2008; Nelson & Cox, 2008).

A process that is not well understood is quiescence, a specific state of programmed cell dormancy, which occurs in unicellular and multicellular organisms. The yeast model could provide medically relevant insights into the programmed exit from mitosis and entry into this dormant state. The aim of the present work was to investigate the

interplay between two evolutionarily conserved protein kinases - PKA and Snf1 - during long-term glucose starvation and entry into quiescence (see Fig. 11 and text further below).

Cancerous cells that are in a dormant state in the human body can express traits comparable to those in quiescent yeast cells, such as drug resistance. In addition, the cancerous nature of dormant tumor cells is hard to detect because the current approaches are based on detecting proliferation markers, which are not expressed in dormant states. Unfortunately, since little is known about dormant cancer cells, the markers for this state are not well-defined. Thus, a dormant cancer can appear to be in remission and remain undetected and untreated for years. Recently, studies on dormant cancer have begun to target the entry to and exit from quiescence (Aguirre-Ghiso, 2007; Allan, Vantyghem, Tuck, & Chambers, 2006). Knowledge obtained in the yeast model could be highly beneficial for this area of research. The interactions between PKA and Snf1 could be critical for these transitions in *S. cerevisiae*, and understanding these interactions at the molecular level could provide additional insight into the mammalian system, since PKA and Snf1 (known as AMP-activated protein kinase in mammals) are highly conserved in evolution.

S. cerevisiae Snf1 (sucrose non-fermenting 1) was first identified genetically in a screen for mutants unable to utilize sucrose (Carlson, Osmond, & Botstein, 1981). Further studies indicated that Snf1 is conserved in evolution. The mammalian homolog of Snf1

is known as AMP-activated protein kinase (AMPK). Members of the Snf1/AMPK family play central roles in responses to carbon/energy limitation, which is detected by sensing the levels of "spent" nucleotides such as AMP and/or ADP (Fig. 1). For example, AMP binding to AMPK favors its activated state, in which AMPK is phosphorylated on the T-loop at Thr172 by upstream kinases, such as LKB1, TAK1, and CaMKK (Chandrashekarappa, McCartney, & Schmidt, 2011; Hardie et al., 1998; Hardie, Carling, & Carlson, 1998; Hardie, 2003; Hardie, 2007b; Hardie, 2007d; Hedbacker & Carlson, 2008). AMPK is activated by conditions ranging from glucose limitation to hypoxia. The primary activating kinase for AMPK is LKB1, and defects in LKB1-AMPK signaling have been implicated in tumorigenesis. One function of AMPK is its role in inhibition of cell growth and proliferation in response to decreases in energy availability. When this function is insufficient (e.g. due to mutations in LKB1), the cells show a tendency for inappropriate growth and tumor development. For example, mutations affecting the AMPK pathway have been associated with Peutz-Jeghers syndrome and tuberous sclerosis (Chandrashekarappa et al., 2011; Collins, Reoma, Gamm, & Uhler, 2000; Corradetti, Inoki, Bardeesy, DePinho, & Guan, 2004; Hardie, Carling, & Halford, 1994; Hardie et al., 1998; Hardie et al., 1998; Hardie, 2003; Hardie, 2007b; Hardie, 2007d; Hedbacker & Carlson, 2008) . Acting as the fuel gauge of the cell is not the only role of AMPK though. In addition to energy-limiting conditions, AMPK is activated by other stresses, including oxidative stress and heat shock (Corton, Gillespie, & Hardie, 1994; Hardie, 2004; Kahn, Alquier, Carling, & Hardie, 2005).

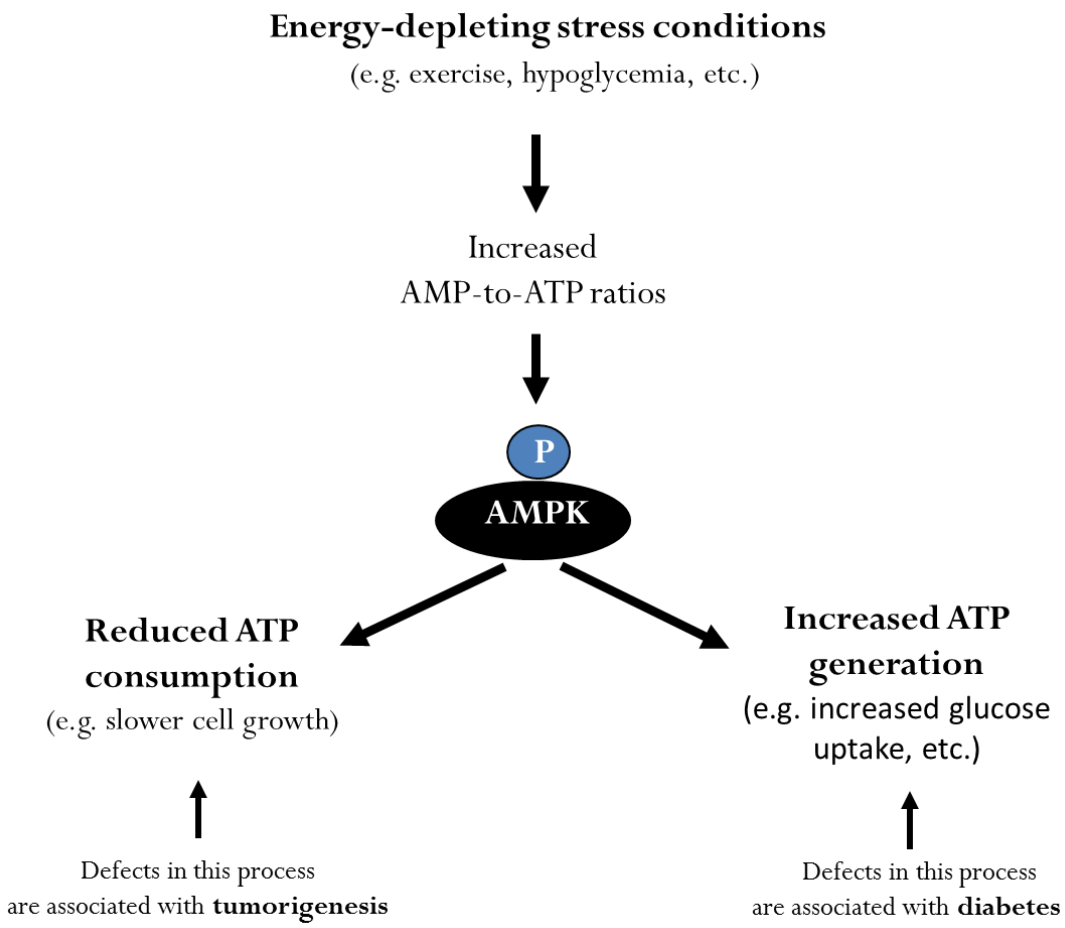

Figure 1: AMP-activated protein kinase (AMPK): the cell's fuel gauge. As available carbon is depleted, the levels of ATP are decreased, while levels of AMP are increased. AMPK becomes phosphorylated at its T-loop threonine (Thr172), in order to activate a metabolic shift, and to replete the ATP generation. This leads to transcription of genes associated with energy storage, while repressing genes associated with promoting prolific growth. Diagram modified from Barrett, L. (2011) PhD Thesis: "Regulation of Stress-Response Protein Kinase Snf1 in *Saccharomyces cerevisiae*".

As mentioned above, the *S. cerevisiae* Snf1 is a structural and functional ortholog of the mammalian AMPK (Mitchelhill et al., 1994). Snf1 is similarly activated by phosphorylation on its T-loop at Thr210 by upstream kinases. There are three Snf1-activating kinases, Sak1, Tos3, and Elm1 in yeast (Fig. 2 and Fig. 6). Snf1 is activated in response to environmental stresses, primarily the depletion of the preferred carbon source - glucose. As glucose is depleted, the ratio of AMP:ATP is increased, and Snf1 is

phosphorylated, although AMP does not activate Snf1 *in vitro* (Adams et al., 2004; Mitchelhill et al., 1994; Wilson, Hawley, & Hardie, 1996; Woods et al., 1994). Besides nutrient depletion, Snf1 is also activated by oxidative, alkaline, ionic, even mechanical stresses, and heat shock (Carlson, Osmond, Neigeborn, & Botstein, 1984; Carlson, 1998; Carlson, 1999; Celenza & Carlson, 1989; Hardie et al., 1994; Hardie, 1999; Hedbacker & Carlson, 2008; Hong & Carlson, 2007; Orlova, Kanter, Krakovich, & Kuchin, 2006; Wilson et al., 1996).

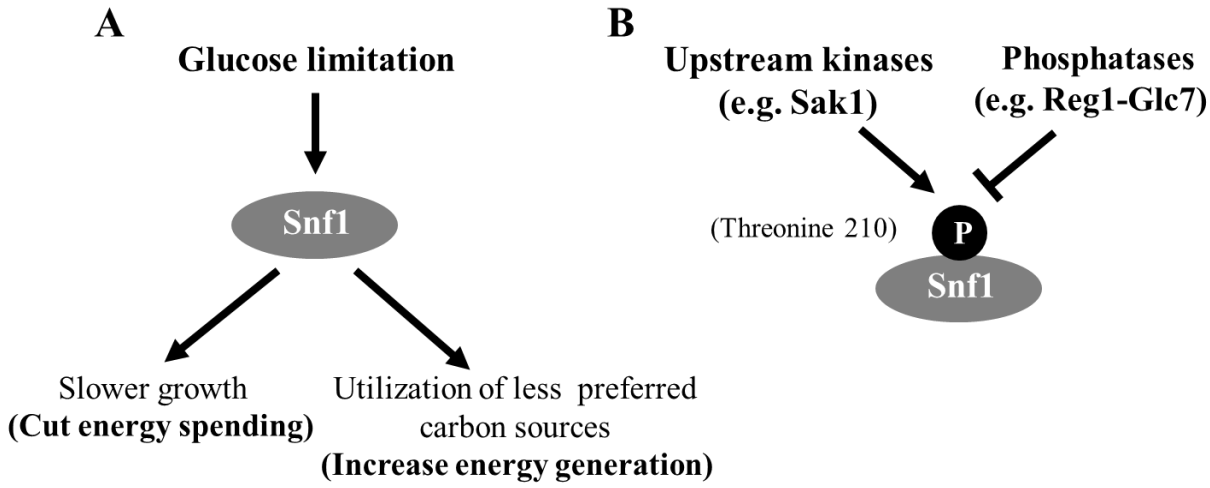

Figure 2: Snf1 is the ortholog of mammalian AMPK (the cell's fuel gauge). **(A)** Snf1 becomes activated under conditions of glucose deprivation. It balances the energy budget by cutting general energy spending while stimulating specific back-up energy-yielding programs. **(B)** Snf1 is activated by phosphorylation of its T-loop threonine (Thr210). The principal Snf1-activating kinase is Sak1. In high glucose, Snf1 is turned off by dephosphorylation. The principal phosphatase is Reg1-Glc7. The mechanisms that modulate the rates of Snf1 activation and inactivation are not completely understood.

Yeast prefers to ferment carbon sources instead of oxidizing them. Glucose and fructose are preferred over other mono-, di- or tri- saccharides like galactose, sucrose or raffinose. Trehalose, which can be made endogenously, is still not preferred over glucose and fructose. Fructose even takes second to glucose. Ultimately, glucose is the preferred carbon source for yeast (Hardie, 2003; Hedbacker & Carlson, 2008;

Santangelo, 2006; Zaman, Lippman, Zhao, & Broach, 2008). *snf1* mutants cannot ferment sucrose (Hedbacker & Carlson, 2008), or utilize other alternative carbon sources like ethanol and glycerol (Carlson et al., 1981; Hardie et al., 1998). Snf1 is repressed in the presence of glucose (Fig. 3 and Fig. 6), but in order to utilize these less preferred carbon sources, yeast must phosphorylate Snf1. In the absence of all carbon or in presence of an alternative carbon source, there is an activation of the stress response (Carlson et al., 1981; Carlson, 1999; De Virgilio, 2012; Hardie, 2007b; Hedbacker & Carlson, 2008; Santangelo, 2006; Zaman et al., 2008).

A low glucose level activates Snf1 to allow the cell to use the alternative carbon that might be available in the extracellular environment. This is critical when the preferred carbon source (glucose) has been exhausted, and to prepare for further insults in the environment, or to buffer itself from the depletion of total carbon, especially in the event that nutrients are not restored (Tamai, Liu, Silar, Sosinowski, & Thiele, 1994). In this case, the cell would then eventually enter into quiescence (De Virgilio, 2012; Gray et al., 2004; Santangelo, 2006; Werner-Washburne, Braun, Johnston, & Singer, 1993; Werner-Washburne, Braun, Crawford, & Peck, 1996). PKA (cAMP-dependent protein kinase A) and Snf1 play opposing roles in response to the presence and absence of glucose. Here, we show that when PKA activity is high, the cell is prevented from entering into quiescence due to the negative regulation of Snf1 by PKA.

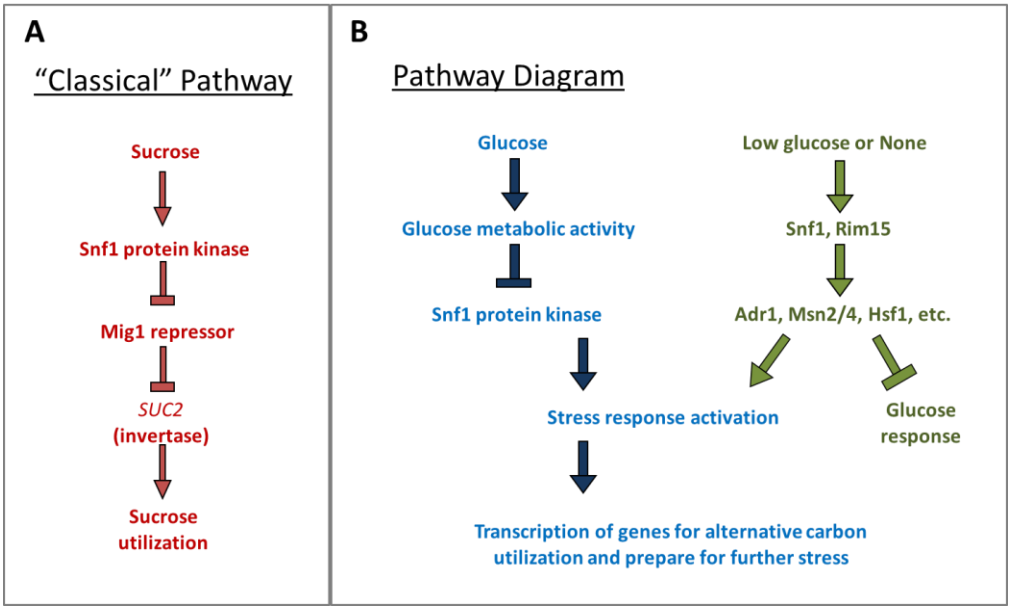

Figure 3: Snf1 is activated in response to glucose limitation. Sucrose non-fermenting protein kinase 1 (Snf1) was discovered in a sucrose utilization screen in 1981 (Carlson et al, 1981). Glucose is the preferred carbon source, but yeast can use others. **(A)** The "classical" pathway in utilization of sucrose. Snf1 is required for yeast to use sucrose. Later it was also determined that Snf1 was activated in other alternative carbon sources, and in stress response. Snf1 protein kinase phosphorylates and inactivates the Mig1 repressor. This allows polymerase to proceed with transcription at the *SUC2* gene which encodes the protein invertase. Invertase is released from the cell, and extracellularly cleaves sucrose into glucose and fructose, which are then brought into the cell by hexose transporters. **(B)** In glucose-repressing conditions, Snf1 is repressed. When Snf1 is inactive, glucose-repressed genes like *SUC2* are not transcribed. The stress response pathways are also repressed by the presence of glucose. When cells are starved for glucose, given alternative carbon sources such as sucrose, or completely deprived of carbon in the habitat, Snf1 will be phosphorylated to regulate a stress response mechanism, and subsequent transcriptional changes. This leads to the up-regulation of genes associated with stress and a down-regulation of genes associated with usage of glucose.

In previous work using Σ1278b, we have shown that a non-catalytic section of the C-terminal domain (CTD) in Snf1-activating protein kinase 1 (Sak1) contains conserved PKA sites (Barrett, Orlova, Maziarz, & Kuchin, 2012; Kennelly & Krebs, 1991; Shabb, 2001). One such conserved site is Ser1074, which is highly phosphorylated in Σ1278b *bcy1Δ* mutants (Barrett et al., 2012; Griffioen & Thevelein, 2002; Kennelly & Krebs, 1991; Shabb, 2001). In that study (Barrett et al., 2012), *bcy1Δ sak1*Δ double mutants were

transformed with plasmids containing mutated forms of Sak1 to determine the PKA effect on Sak1. When combined, two specific point mutations in the C-terminal domain of Sak1, from serine-to-alanine (Sak1-S1074A-S1139A), at these conserved PKA sites, produced partially constitutive phosphorylation at Thr210 on Snf1, which was also elevated compared to the WT (wild type). This suggests that the high PKA level affects Sak1 activity (Barrett et al., 2012).

PKA is a serine/threonine protein kinase. The substrates of PKA have a highly conserved consensus motif, which PKA recognizes (Griffioen & Thevelein, 2002; Kennelly & Krebs, 1991; Nelson & Cox, 2008; Santangelo, 2006; Shabb, 2001). Approximately 80% of the substrates containing the most frequently occurring site are phosphorylated by PKA. Other sites are recognized and phosphorylated with much less frequency and efficiency. These PKA recognition sites are found in numerous proteins in yeast. The PKA pathway interacts with several cross-talking pathways under varied conditions, including in glucose response, stress response, and entry into quiescence (Carlson, 1999; De Virgilio, 2012; Gray et al., 2004; Griffioen & Thevelein, 2002; Hedbacker & Carlson, 2008; Hedbacker & Carlson, 2008; Kennelly & Krebs, 1991; Nelson & Cox, 2008; Rubenstein et al., 2008; Shabb, 2001).

Here, we analyze Snf1 activation under different conditions of temporal carbon stress. To determine the extent of carbon stress and entry into dormancy, we subsequently evaluate a quiescence-associated phenotype (heat-shock survival). This is monitored in

both WT and mutants of W303, a more widely used strain background with relatively lower basal PKA activity than Σ1278b. This made the subtle changes in PKA activity much easier to observe, especially the role of PKA in negatively regulating Snf1 to enter the quiescent state. For high PKA activity, we constructed and isolated haploid W303 *ira1Δ::KAN* mutants in our lab from diploid WT.

Results and Discussion:

The *ira1Δ* mutant in W303 is not different from the wild type in short-term glucose deprivation

We first wished to test whether mutations affecting cAMP signaling in strains with an endogenously lower cAMP-PKA signaling levels would produce an effect on Snf1 activation. Previous results were observed in strains of ∑1278b background, which have a higher endogenous level of cAMP-PKA signaling. We decided to use the W303 background to observe the carbon stress effect on activation of Snf1 in the presence of the *ira1*Δ mutation, which leads to increased cAMP-PKA signaling, as explained below.

Glucose signaling begins at the cell surface and involves the ability to detect carbon in the environment. Glucose response requires activation of the adenylate cyclase and cAMP. PKA (cAMP-dependent protein kinase A) then catalyzes several glucose response reactions. Key nutrients in the environment, e.g. glucose, initiate the signal via the G-protein coupled receptor (GPCR), or one of 18 different hexose sensor-transporters in the cell membrane.

This subsequently activates the Ras G-proteins (or Gpa2 G-protein) (Fig. 4). The GTP-bound form of the G-protein then binds and activates the adenylate cyclase, Cyr1 in *Saccharomyces cerevisiae*. Cyr1 increases levels of cAMP, by cleaving and modifying the bond between the alpha and beta phosphates on ATP (Nelson & Cox, 2008; Santangelo, 2006). The Ras G-protein is turned on by exchanging GDP for GTP, assisted by GEFs (guanine nucleotide exchange factors). The Ras-GEFs in this pathway (Cdc25 and Sdc25) promote Ras signaling and increase levels of cAMP via the adenylate cyclase (Cyr1). The GTP-bound Ras is deactivated by cleaving the gamma phosphate of GTP to make GDP again, assisted by GAPs (GTPase-activating protein). The Ras-GAPs assist in decreasing cAMP levels in this manner. Ras-GAPs Ira1 and Ira2 effectively inhibit Ras signaling. By removing or disrupting the *IRA* genes, the Ras signal can stay constitutively active, and cAMP levels stay elevated.

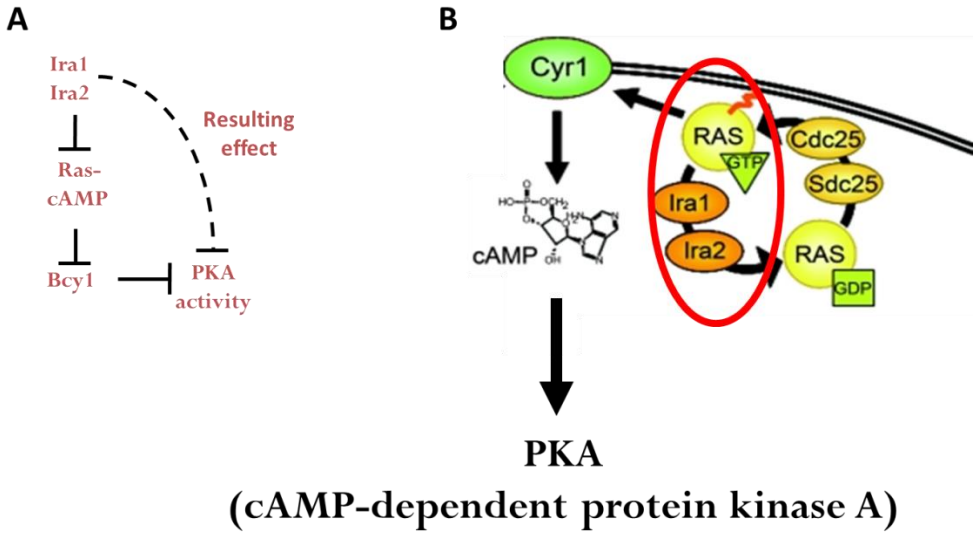

Figure 4: Loss of *IRA1* leads to increased cAMP-PKA activity. **(A)** Schematic of mechanisms controlling PKA (cAMP-dependent protein kinase A), further addressed in B. **(B)** Yeast molecular control of cAMP increase and subsequent PKA activation. Diagram modified from Santangelo (2006). Cdc25 and Sdc25 are Ras-GEFs

which positively regulate the "Ras switch" by assisting the Ras-GTP association. Activated Ras activates the adenylate cyclase Cyr1. Cyr1 cleaves ATP, and modifies the phosphate bond to make cyclic AMP (cAMP). The Ira1/2 Ras-GAP proteins regulate the "Ras switch", by promoting GTP hydrolysis and leading to Ras association with GDP instead of GTP. This inhibits Ras activity and subsequent cAMP production. Elimination of either Ira protein creates a constitutively active Ras. This effect on Ras creates a high cAMP signaling level. cAMP binds the regulatory subunit (Bcy1) of PKA, causing Bcy1 to release the catalytic subunits (Tpk1-3) of PKA (not shown here) for downstream activation of glucose regulated genes.

cAMP can now directly act on the regulatory subunits of cAMP-dependent protein kinase A (PKA). PKA is a tetramer of two Bcy1 regulatory subunits, and any two (of three) catalytic subunits, Tpk1-3. cAMP interacts with the Bcy1 regulatory subunits, causing them to release the Tpk catalytic subunits for downstream phosphorylation (Nelson & Cox, 2008; Santangelo, 2006).

In our W303 mutant, the loss of the *IRA1* gene creates higher Ras-cAMP signaling. This increased the PKA activity by allowing the release of the Tpk catalytic subunits, and a downstream response, stimulating transcription of genes involved in proliferation in the presence of glucose (one of the reasons that Ras is recognized as an oncogene). This acts in opposition to Snf1 protein kinase, which is active in the absence of glucose. It is widely known that the activation of PKA negatively correlates with phosphorylation levels of Snf1 protein at Thr210 in the WT (Aguirre-Ghiso, 2007; Barrett et al., 2012; De Virgilio, 2012; Gray et al., 2004; Nelson & Cox, 2008; Santangelo, 2006; Zaman et al., 2008).

While the relationship between PKA and Snf1 is not well understood, it has been suggested in literature that PKA may phosphorylate Sak1 (Barrett et al., 2012;

Santangelo, 2006). Of the three upstream kinases, Sak1 is the principal kinase for Snf1 activation involved in the carbon deprivation response (De Virgilio, 2012; Gray et al., 2004; Hardie, 1999; Hardie, 2003; Hardie, 2004; Hardie, 2007b; Hardie, 2007c; Hardie, 2007d; Rubenstein et al., 2008; Santangelo, 2006; Werner-Washburne et al., 1996; Wilson et al., 1996). We have also previously showed a direct connection between PKA and Sak1 in Σ1278b strains, via phosphorylation at an ideal conserved consensus motif for PKA that is found within the non-catalytic portion of the CTD in Sak1.

We previously showed that using Σ1278b the *bcy1*Δ single mutant (constitutively active PKA) had lower Thr210 phosphorylation on Snf1, and was seemingly no different from the *ira1*Δ mutant. A single point mutation in Sak1 (HA-Sak1-S1074A) did not result in increased phosphorylation of Snf1 at Thr210 (Barrett et al., 2012). However, in *bcy1Δ sak1*Δ mutant transformed with WT Sak1 protein (HA-Sak1), this protein showed an increase in phosphorylation at Ser1074, detectable with anti-phospho-Ser1074 antibodies. Creating a double mutation at the Sak1 C-terminal non-catalytic domain (Sak1-S1074A, S1139A) resulted in partially constitutive phosphorylation of Snf1 Thr210 (Barrett et al., 2012).

The Σ1278b yeast genetic background is believed to have higher levels of endogenous cAMP-PKA activity (Stanhill, Schick, & Engelberg, 1999), which could lead to increased effects on PKA targets as compared to other strains. The W303 genetic background has a comparatively lower cAMP-PKA basal level, and the regulatory effects of PKA could be

more subtle. To investigate this, we grew W303 cells to mid-log phase in YPD, and then shifted them to equivalent media without glucose (YEP), thus inducing carbon-stress and activation of Snf1. In the Σ1278b strain, the *ira1* mutants had lower levels of Snf1 Thr210 phosphorylation than the WT (Barrett et al., 2012). We observed that deletion of the Ras-GAP gene *IRA1* in the W303 strain background, with lower basal cAMP-PKA, did not affect Thr210 phosphorylation of Snf1 protein kinase after short-term glucose starvation (Fig.5B).

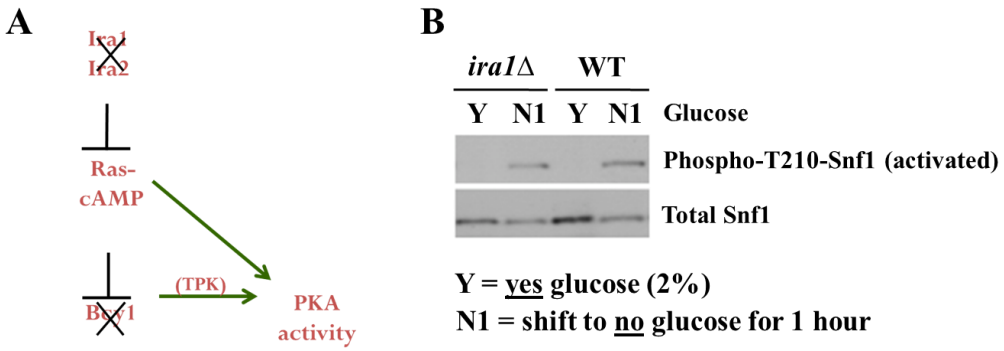

Figure 5: The *ira1*Δ mutation has no effect on Snf1 Thr210 phosphorylation after short-term glucose deprivation. **(A)** A simplified cartoon showing the relationship between some components of the PKA pathway, and why deletion of the *IRA1* gene results in elevated PKA activity. **(B)** Western blot analysis of activated phospho-Thr210-Snf1 (upper panel) and total Snf1 (lower panel). Cells growing exponentially in YEP+2% glucose (Glucose, Y) were shifted to YEP + no glucose for 1 hour (Glucose, N1). There is no obvious difference in Snf1 activation between WT and *ira1*Δ cells.

This was consistent with (though slightly less effective than) the results from *IRA1* deletion in Σ1278b. We previously showed that in Σ1278b strains, PKA negatively regulates Snf1 via conserved sites in Sak1 (Fig. 6) (Barrett et al., 2012). Using W303 instead of Σ1278b reduces or abolishes the effect of elevated PKA on Snf1 activation in response to short-term (1 h) glucose depletion (Fig. 5). This result could suggest two possibilities. First, Ras-cAMP-PKA signaling in W303 is still quite weak even in the *ira1*Δ

mutant. Second, PKA has absolutely no role in Snf1 regulation in W303. As discussed below, this second suggestion is likely to be incorrect.

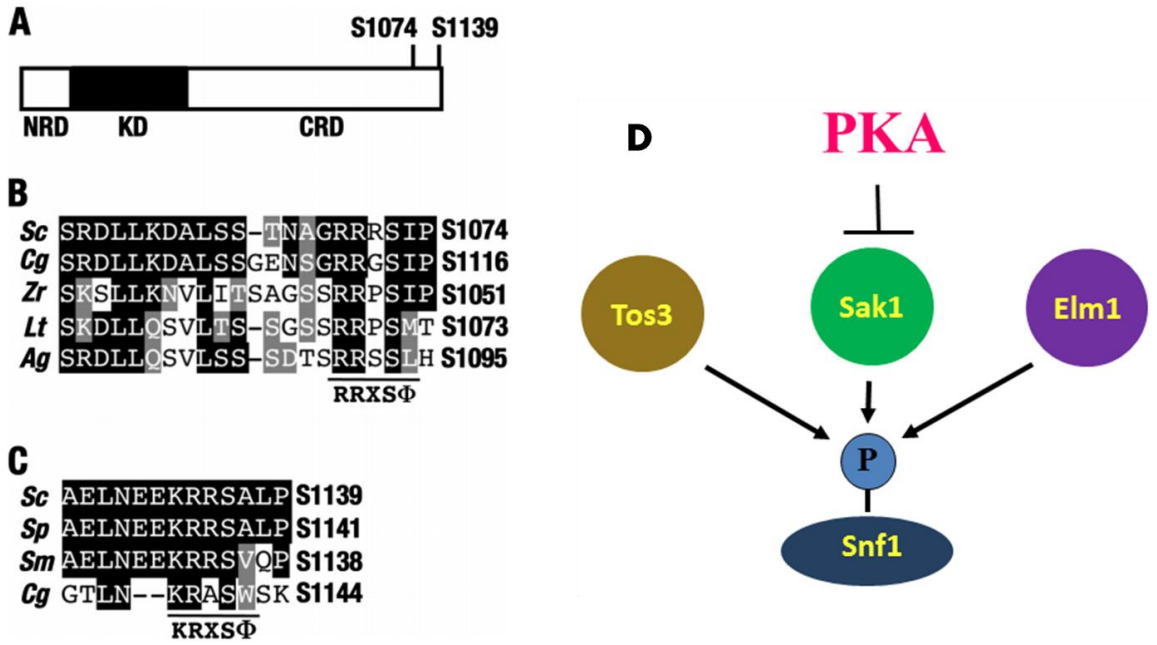

Figure 6: PKA negatively regulates Snf1 via conserved motifs in Sak1. **(A-C)** We have previously investigated the relationship between PKA and the conserved consensus sites in Snf1 (short-term) derepression response in Σ1278b. **(A)** The Sak1 protein kinase has two conserved PKA recognition sites in the non-catalytic CTD, Ser1074 and Ser1139. **(B)** Conservation of the Ser1074 site. These sites are aligned across various species. *Sc*, *Saccharomyces cerevisiae*; *Cg*, *Candida glabrata*; *Zr*, *zygosaccharomyces rouxii*; *Lt*, *Lachancea thermotolerans*; *Ag*, *Ashbya gossipii*. **(C)** Conservation of the Ser1139 site. These sites are aligned across species. *Sc*, *Saccharomyces cerevisiae*; *Sp*, *Saccharomyces paradoxus*; *Sm*, *Saccharomyces mikitae*; *Cg*. Diagram from Barrett et al. (2012) **(D)** Model. PKA negatively regulates Snf1, at least in part, via phosphorylation at Sak1. Other proteins in the Snf1 stress response pathway also carry conserved PKA sites, but Sak1 is the principal activator of Snf1.

<u>The *ira1*Δ mutation has a significant effect on Snf1 after long-term glucose deprivation</u>

The possibility that PKA has absolutely no role in Snf1 regulation in W303 cells did not seem consistent with the presence of the same PKA consensus recognition sites in the same components of the Snf1 pathway. For example, the Ser1074 and Ser1139 sites in the principal Snf1-activating kinase are present in Sak1 in both ∑1278b and W303.

Moreover, these sites appear to be conserved in Sak1 homologs of fungi representing several species and even genera (Fig. 6B,C). This suggested that these sites confer an important evolutionary advantage. It is known that in the wild, microbes spend significant amounts of time in the so-called quiescent state, which allows them to survive through long periods (years) of nutrient deprivation, lack of water, and other unfavorable conditions. Quiescence can be achieved in the laboratory, by depriving cells of an essential nutrient such as carbon for a period of time of about 24 h or longer (Brauer et al., 2008). We therefore decided to test the possibility that in W303 cells, elevated PKA signaling (caused by *ira1*Δ) could affect the activation state of Snf1 after prolonged (rather than short-term) glucose deprivation. To test this, the cells were stressed on a time course varying from 4 hours to 96 hours.

A saturated yeast culture (started in a nutrient-rich medium, such as YPD) grown for approximately a week in the laboratory, was once believed to yield quiescent cells. The cells have shown to be post-proliferative and post-diauxic shift, once in stationary phase (Brauer et al., 2008). This stationary phase was then presumably a G0 state of cellular inactivity. It was also assumed that a substantial enough portion of the cells in the culture were in a state of quiescence, that the culture, as a whole, could be considered uniformly the same.

Yeast can enter a fairly stable non-proliferative state (and remain viable) when starved for specific nutrients. Under a specific combination of events, spores form when

(diploid) yeast is starved of glucose for extended time durations, but this must also be in the presence of an alternative carbon (e.g. acetate), and the loss of at least one other essential nutrient (e.g. nitrogen) (Zaman et al., 2008). It is now understood that haploid and diploid entry into quiescence, or quiescent-like states, is as much a programmed response as apoptosis (Fig. 11), and not as simple as saturated culture density (De Virgilio, 2012; Gray et al., 2004; Hedbacker & Carlson, 2008; Santangelo, 2006; Werner-Washburne et al., 1993; Werner-Washburne et al., 1996; Zaman et al., 2008).

Most of the cells in a post-diauxic growth phase are known to be mostly quiescent (or pre-quiescent), but some subpopulations within the culture may still have some traits associated with proliferation, although the growth is typically negligibly slow. Induction of nutrient stress, by shifting the cells to YEP without glucose, can activate Snf1 protein kinase. Yet Snf1 is potentially activated by the progressive depletion of nutrients, and increase in ethanol, in the saturated culture. Allowing Snf1 to stay active for 24-48 hours of carbon starvation can induce dormancy (Gray et al., 2004; Wilson et al., 1996). This leads to transcription of several genes in response to this current stress, any potential future stress, and also genes in the programmed preparation for quiescence. Carbon loss progressively leads to slowed growth, buffer from further insult (heat, mechanical stress, etc.), and eventually arrested mitotic division (Gray et al., 2004; Hedbacker & Carlson, 2008; Werner-Washburne et al., 1993; Werner-Washburne et al., 1996; Zaman et al., 2008). Eventually yeast express quiescence-associated traits, and non-

proliferative viability, that together allow the cell to respond immediately to the re-introduction of nutrients such as carbon (glucose), post starvation.

In the absence of glucose, Snf1 protein is activated within 5 minutes (Gray et al., 2004; Wilson et al., 1996). Without Snf1, cells cannot utilize the alternative carbon sources in the environment, such as ethanol that results from the catabolism of glucose (Carlson et al., 1981; Hardie et al., 1998). Without Snf1, yeast also do not prepare well for stress, do not enter programmed quiescence (and are also less viable post-insult) (De Virgilio, 2012; Gray et al., 2004; Hardie, 2003; Hedbacker & Carlson, 2008; Santangelo, 2006; Werner-Washburne et al., 1996; Zaman et al., 2008). To test post-stationary state, in our W303 strains, we grew the cells in nutrient media (YPD), and shifted them to YEP (without glucose) for 1, 2, or 4 days, to allow for entry into quiescence.

Deletion of *IRA1* leads to higher PKA activity (Fig. 4). In the above experiments, we have shown the higher PKA activity associated with the *ira1*Δ mutation in W303 does not result in a detectable reduction in phospho-Thr210-Snf1 under short-term carbon stress conditions [1 hour (Fig. 5) and 4 hours (Fig. 7)]. When we stressed the cells much longer, for 24-48 hours without carbon (glucose), the phospho-Thr210-Snf1 level was significantly lower in W303 *ira1*Δ cells than in W303 WT cells, as shown in Figure 7A. To verify that Snf1 activation persists even longer, the 96 hour (4 day) time point was also tested. By four days without carbon, Snf1 was still activated in the WT, and there still was a significant reduction of Snf1 Thr210 phosphorylation in the *ira1*Δ mutant (Fig. 7B).

This suggests that higher cAMP-PKA activity negatively affects Snf1 at time points that are consistent with entry into quiescence in the WT. Thus, PKA could delay the onset of quiescence by inhibiting activation of Snf1 protein kinase (Fig. 8).

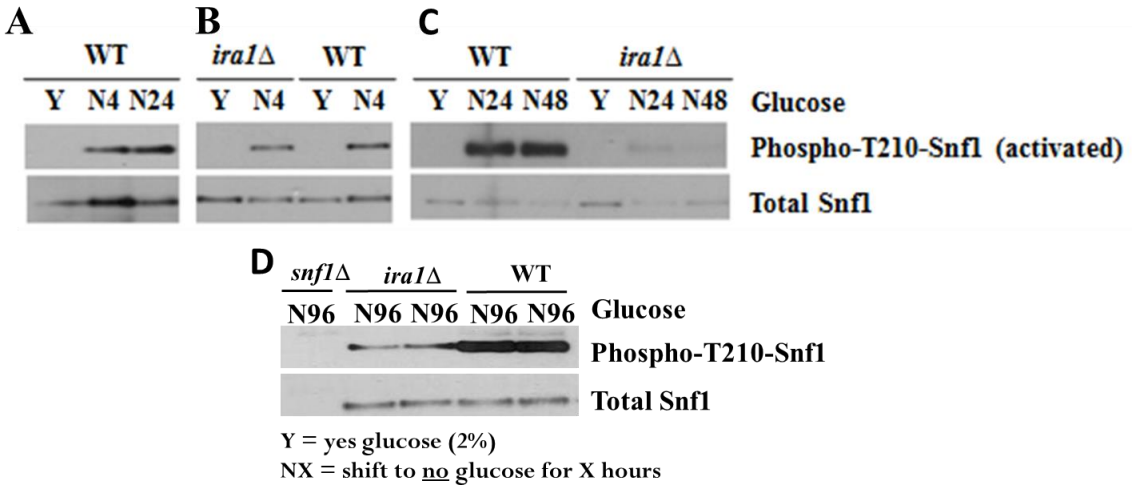

Figure 7: Effects of *ira1*Δ on Snf1 activation in W303 after prolonged glucose deprivation. Western blot analysis of activated phospho-Thr210-Snf1. **(A)** In WT cells, Snf1 activation state persists in no glucose for quite some time. **(B)** After 4 hours in no glucose, there is still no obvious difference in Snf1 activation between WT and *ira1*Δ cells. **(C)** After 24 and 48 hours in no glucose, however, there is a significant difference in the activation state of Snf1 between WT and *ira1*Δ cells. For WT cells, these time scales are normally associated with entry into quiescence. **(D)** This long-term effect is persistent through 96 hours; for WT and *ira1*Δ, each lane represents one (of two) independent biological replicate. Panels A-D represent independent experiments. In all panels, cells growing exponentially in YEP+2% glucose (Glucose, Y) were shifted to YEP + no glucose for the indicated periods of time (Glucose, NX).

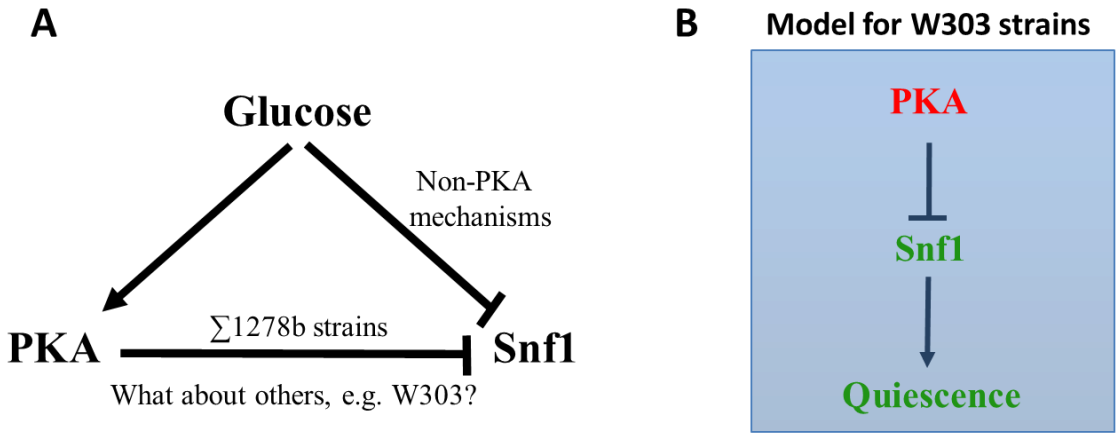

Figure 8: Contribution of PKA to Snf1 regulation. **(A)** PKA mediates a vast array of responses to glucose presence. It is recognized that mutations which lead to hyperactivation of the PKA pathway share common phenotypes with mutations that inactivate the Snf1 pathway, suggesting a role of PKA in Snf1 regulation. We recently presented evidence that PKA contributes to the negative control of Snf1 in strains of the ∑1278b background, whose use is typically limited to studies of filamentous growth. Here, we decided to explore this relationship in a more widely used background, W303. **(B)** The proposed model, in which PKA negatively regulates Snf1 and Snf1-dependent entry into quiescence, a programmed state of dormancy, which occurs in response to prolonged glucose deprivation.

The *ira1*Δ and *snf1*Δ mutants fail to acquire WT levels of heat-shock resistance after prolonged glucose deprivation

PKA negatively regulates Snf1, and Snf1 is required for programmed transition into quiescence. Long-term carbon deprivation leads to growth cessation and activation of the stress response. This long-term deprivation also progressively leads to the quiescent state (Fig. 11). Quiescent yeast (and mammalian) cells have specific traits. To evaluate the induction of stress response sufficient for protection from stress, and for subsequent viability, cells were heat-shocked and then CFU (colony forming units) were evaluated. To observe the roles of Snf1 and PKA in this transition, the tests were done in WT, *ira1*Δ, and *snf1*Δ strains (see Fig. 10 and text further below).

Among the genes activated in the programmed cell progression toward quiescence are those that reinforce the cell wall, and provide stress protection such as heat-shock genes whose products tend to increase over time in the post-stationary intervals (Hsf1, Hsp12, Hsp26, Hsp82, Hsp104), and genes that encode proteins for cell wall synthesis (e.g. Gsc2, beta-glucan synthase) (De Virgilio, 2012; Galdieri, Mehrotra, Yu, & Vancura, 2010). These changes make the cell wall less susceptible to zymolyase digestion and increase certain drug tolerances. As a result, yeast also becomes more thermotolerant and osmotolerant than proliferative state cells. Yeast with quiescence-associated phenotypes have readied themselves for future stress and are buffered from further environmental insult (De Virgilio, 2012; Gray et al., 2004; Klis, Mol, Hellingwerf, & Brul, 2002; Reinders, Burckert, Boller, Wiemken, & De Virgilio, 1998; Smith, Ward, & Garrett, 1998; Tamai et al., 1994; Werner-Washburne et al., 1993; Werner-Washburne et al., 1996; Zaman et al., 2008).

In the long-term starvation experiments (Fig. 7), there was little difference between 24 h and 96 h of nutrient (carbon) stress for the WT. The WT contained high levels of phospho-Thr210-Snf1 from 24 h through 96 h of carbon stress conditions. This high level of phosphorylation was not seen in the *ira1*Δ cells at 24-96 h. This suggests possible impairments of entry into quiescence for cells with higher cAMP-PKA signaling. The next step is to determine if the cells are actually expressing quiescence-associated phenotypes, and not just post-stationary saturation stress (Fig. 9).

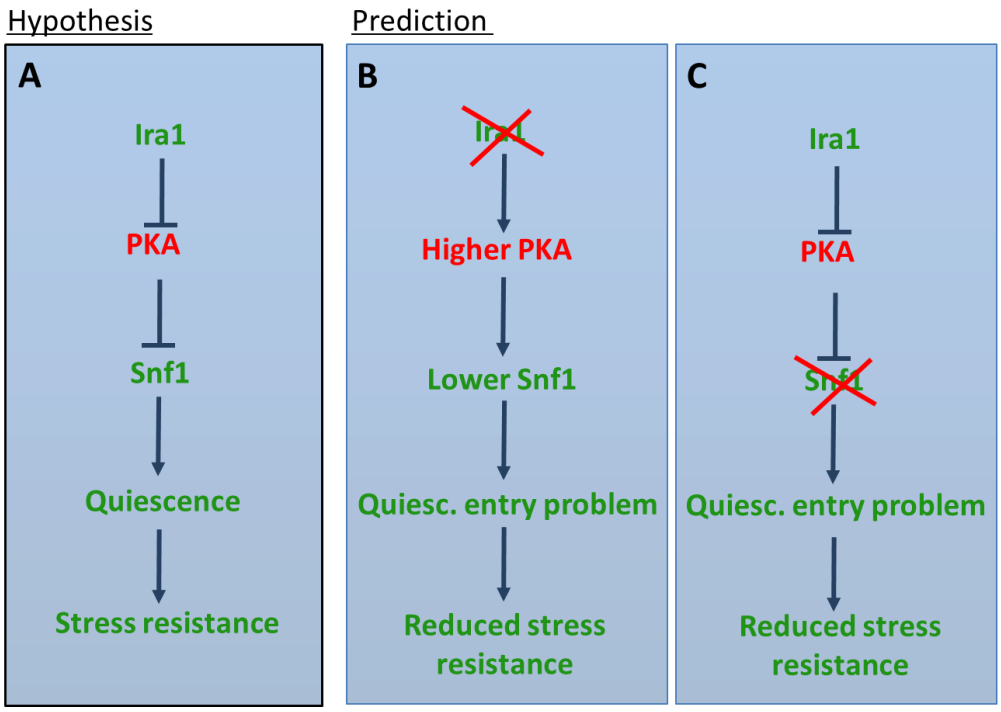

Figure 9: Justification for evaluation of quiescent state by heat shock: cells deficient for the quiescent programming fail thermotolerance testing. **(A)** If the program is allowed to progress as temporally required, the entry into quiescence will provide condensed chromosomes, minimal mass or energy usage, thickened cell wall, and the increased expression of heat shock proteins. (Heat shock protein expression is not necessarily initiated in response to heat.) Thus there would be more thermotolerance in quiescent cultures than in exponentially growing cells. **(B)** Yeast lacking the Ira proteins will have elevated PKA activity, which will downregulate Snf1 and inhibit the subsequent stress response in the cell. (Quiesc. = quiescence) **(C)** Likewise, loss of Snf1 will inhibit capacity to complete programming for quiescence. Potentially these mutants could also be deficient in exit from long-term starvation as well.

To test this, we shifted W303 cultures for 24 or 48 h to carbon stress conditions, and subsequently heat-shocked them. The WT cultures in a proliferative state (not exposed to carbon stress) showed significant loss of viability after a heat-shock exposure of 5 min or longer. The WT cultures that were shifted to no glucose media, and allowed enough time to enter quiescence (24-48 h), had significantly more viable CFU post-heat shock exposure (Fig. 10.). This indicates that the yeast were more thermotolerant and prepared for such an environmental insult as extreme temperature change, and had likely expressed heat shock proteins during the preparation for dormancy. The *ira1*Δ

mutants were not prepared for insult regardless of carbon starvation, as demonstrated by poor viability after heat shock. These cells expressing high cAMP-PKA activity, and poor activation of Snf1 protein kinase, were less prepared to enter into quiescence.

To confirm the requirement of Snf1, we looked at the effect of the *snf1*Δ mutation on thermotolerance as an indicator of quiescence-associated traits (Fig. 10.). The deletion of *SNF1* should inhibit the cell's capacity to activate certain genes required for further protection from insult, including thermotolerance, and entry into quiescence. Indeed, despite exposure to prolonged carbon stress, the *snf1*Δ mutants still showed significant loss of viability post insult.

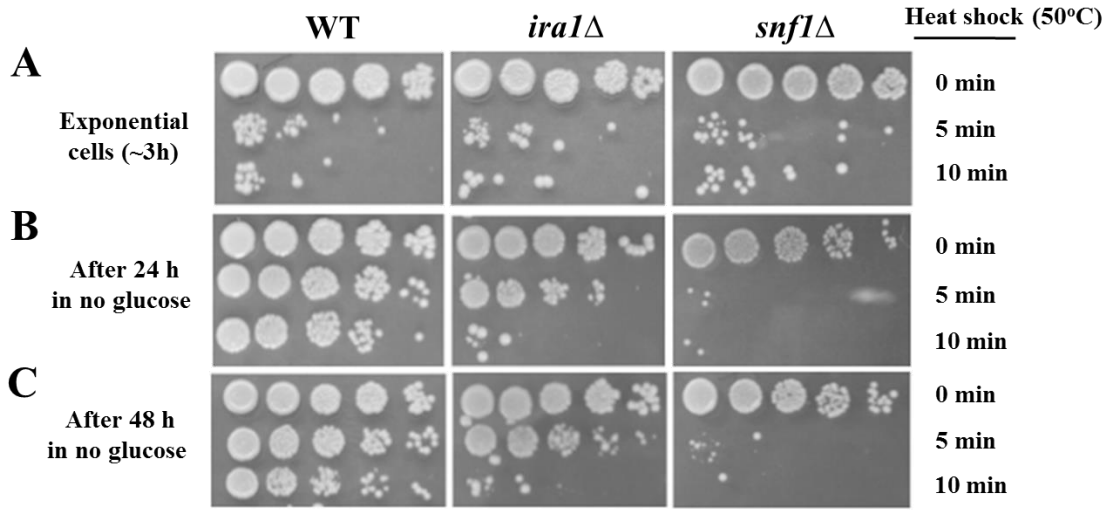

Figure 10: Spotting assay with serial dilutions: the *ira1Δ* and *snf1Δ* mutants exhibit a defect in heat shock resistance. W303 strains were grown to mid-log phase in YPD, then shifted to YEP (without glucose) for **(A)** 0 hours (exponential cells), **(B)** 24 hours (24 h), or **(C)** 48 hours (48 h). Post-starvation, the cultures were heat shocked (50℃) for 0, 5, or 10 min. All cultures were serially diluted and spotted on YPD with 1.7% agar. Cell viability was estimated by observation of CFU (colony forming units).

PKA, a serine/threonine protein kinase, most efficiently recognizes a specific site Arg-Arg-X-Ser. This is the most abundant site and represents more than half of the present PKA sites in yeast. The sites which are recognized and phosphorylated with much less efficiency and frequency include Arg/Lys-X-Ser/Thr and Arg/Lys-X-X-Ser/Thr. The physiological substrate recognition site is found in numerous proteins in the cell. This site has been identified within other kinases, receptors, phosphatases, glucose metabolism components, ion channels, heat shock proteins, and many more. PKA interacts with several overlapping pathways under such conditions as glucose response, stress response, entry into quiescence, and exit from quiescence (Hedbacker & Carlson, 2008; Kennelly & Krebs, 1991; Nelson & Cox, 2008; Shabb, 2001).

The preferred site Arg-Arg-X-Ser is found within Tos3/TAK1, Sak1/LKB1, and Bcy1 (regulatory subunit of PKA). Less efficient sites are also found in Snf1, Reg1, and even the Tpk catalytic subunits of PKA itself. Sak1 is the principal kinase for Snf1 in carbon stress. Tos3 protein kinase also acts on Snf1, and is generally sufficient for growth on other alternative carbon sources. However, without Tos3, yeast is defective for growth on plated media containing glycerol or ethanol (Hedbacker & Carlson, 2008; Kennelly & Krebs, 1991; Shabb, 2001).

In previous work using Σ1278b, a higher basal cAMP-PKA cell lineage, the loss of *IRA1* decreased the Thr210 phosphorylation to a level below that of WT after a standard 1h shift to low glucose (Barrett et al., 2012). Yet in W303, the *ira1*Δ mutant was fairly

comparable to the WT (Fig. 5). This supports that there is a notable difference between *S. cerevisiae* strain backgrounds, as previously acknowledged in Barret et al. 2012 (and in other literature (Stanhill et al., 1999)).

In Σ1278b, we showed that the C-terminal domain (CTD) of the Snf1-activating protein kinase 1 (Sak1) contains conserved PKA sites (Barrett et al., 2012; Griffioen & Thevelein, 2002; Kennelly & Krebs, 1991; Shabb, 2001). PKA is known to phosphorylate Sak1 at two sites within the CTD. Mutations at this region, making it nonphosphorylatable by PKA, can increase *SUC2* transcription near 40 fold in the presence of glucose. The lack of phosphorylation at these sites by PKA led to partially constitutive phosphorylation at Thr210 of Snf1 (Barrett et al., 2012). Thus PKA negatively regulates Snf1, at least in part, by phosphorylating its primary upstream kinase Sak1.

While PKA has many roles in yeast metabolic activity, one that has been studied very little is its role in negatively regulating Snf1 under various conditions. PKA has roles in glycolysis as well as glycogen metabolism, in mitotic control and DNA condensation, transcriptional regulation, and other duties of balanced on-off switching (Barrett et al., 2012; De Virgilio, 2012; Galdieri et al., 2010; Gray et al., 2004; Hardie, 2003; Hedbacker & Carlson, 2008; Nelson & Cox, 2008; Werner-Washburne et al., 1996; Zaman et al., 2008). We show here that another physiologically relevant role for PKA in *Saccharomyces,* once approaching a state of carbon exhaustion, is to negatively regulate Snf1 for programmed transition from post-diauxic shift into quiescent state.

PKA appears to act as an inhibitor of entry into quiescence. Cells expressing constitutively active PKA, via deletion of *BCY1*, lose viability after the diauxic shift (Gray et al., 2004; Werner-Washburne et al., 1996; Zaman et al., 2008), indicating the need to lower the cellular PKA activity in order to allow for the appropriate progression to the post-diauxic phase. The negative control of Snf1 must be removed in order to allow Snf1 to promote preparation for stress, also to allow the entry into quiescence.

Environmental insults on the cell can include temperature stress, oxidative stress, osmotic changes, starvation, etc. Each of these (and others not listed) act on the cell as a general stress. General stresses can lead to reinforcement of the cell wall, and other structures, to minimize their vulnerability to other less preferred conditions. As the yeast is continuously exposed to starvation, the internal activity shifts. For example, production is increased to assist in inhibition of growth and positive setup of cells for quiescence entry (via Rim15 and other major proteins) (De Virgilio, 2012; Gray et al., 2004; Reinders et al., 1998). This overall shift in metabolism includes transcription of genes that assist cell stabilization for long-term preservation and stress tolerance (e.g. via trehalose accumulation) (Crowe, 2007; De Virgilio, 2012; Jain & Roy, 2009; Singer & Lindquist, 1998; Werner-Washburne et al., 1996), increased expression of Hsf1 (and other heat shock proteins mentioned above) in response to nutrient stress (Galdieri et al., 2010; Hahn & Thiele, 2004; Tamai et al., 1994; Werner-Washburne et al., 1993; Werner-Washburne et al., 1996) (but not necessarily in response to heat) (Tamai et al.,

1994). PKA is recognized as having a role in spore formation and carbohydrate storage also stress-induced adjustments. Without the Tpk catalytic subunits of PKA, cells do not sporulate, cannot utilize alternative carbon sources, and lack the capacity to recover from starvation (Cameron, Levin, Zoller, & Wigler, 1988). Due to this and the numerous roles mentioned above in on-off switching, as well as other physiological roles described below, it is more difficult, albeit not impossible, to work with PKA mutants directly.

In the presence of glucose, the PKA regulatory (negative) subunit, Bcy1, localizes almost absolutely in the nucleus. Upon glucose depletion, approximately half of the cellular Bcy1 is translocated to the cytoplasm. The return of this regulatory subunit to the cytoplasm may enable it to interact with cytoplasmic PKA, and to decrease its own activity. PKA mediates a response to glucose and suppresses stress response-induced expression of Rim15, Msn2, and Msn4. Genes associated with growth are transcribed in a PKA-mediated manner. Genes associated with both the stress response, and the promotion of stationary phase, are blocked by Tpk activity, the catalytic subunits of PKA. Thus, in the absence or decreased activity of Tpk the cell would be better prepared for stress-associated preparation for G1 arrest, and entry into quiescence (Adams et al., 2004; Carlson, 1999; Gray et al., 2004; Hedbacker & Carlson, 2008; Werner-Washburne et al., 1993; Werner-Washburne et al., 1996; Zaman et al., 2008).

Snf1, Rim15, and Msn2,4 are directly associated with the transcription of stress induced genes and quiescence entry. Without Rim15 or Snf1, yeast tends to have stationary

phase defects associated with failure to stop at the G1 checkpoint. Rim15 is required for Msn2,4 transcriptional activity. Msn2 is known to bind to the STRE (stress response) element, a conserved consensus motif (5'-CCCT-3') in promoters of stress response genes, and Msn2 is required for Msn4 activation (Galdieri et al., 2010). Snf1 is required for the transcription of Adr1 (used in catabolism of non-fermentable carbons) and the diauxic shift in yeast. Without it, cells cannot use the accumulated ethanol and are not viable post-exponential phase; however, Rim15, Snf1, Adr1, and Msn2,4 are negatively regulated by PKA (De Virgilio, 2012; Galdieri et al., 2010; Gray et al., 2004; Gray et al., 2004; Hardie, 1999; Hardie, 2003; Hedbacker & Carlson, 2008; Santangelo, 2006; Werner-Washburne, Brown, & Braun, 1991; Werner-Washburne et al., 1993; Werner-Washburne et al., 1996; Zaman et al., 2008).

PKA phosphorylates Msn2 on its nuclear localization signal inhibiting its translocation to the nucleus and subsequent transcriptional functions in environmental stress response, and Msn2/4 expression is dependent on Msn2 (Gorner et al., 1998; Gorner et al., 2002; Zaman et al., 2008). Thus, PKA levels must be decreased in order for stress response to be increased, since the *msn2*Δ *msn4*Δ mutant is not able to withstand environmental stresses from heat shock and alkalinity to carbon starvation and oxidative stress (Estruch, 2000). However, yeast lacking Msn2 and Msn4, stress response transcription factors, can compensate for the loss of PKA in preparation for quiescence, and protection from environmental insults (Brauer et al., 2008; Smith et al., 1998; Zaman et al., 2008).

Glucose depletion leads to the translocation of Snf1 from cytoplasm to vacuole and nucleus. This stimulates transcriptional changes and metabolic processes in response to the loss of carbon stress. Among its many roles, Snf1 has also been linked to recruitment of the SAGA complex to promoters of particular genes (e.g. glucose transporters), and subsequent chromatin remodeling activity, at the glucose depletion shift (Galdieri et al., 2010). Snf1 protein kinase is also responsible for activation of Hsf1, which binds the promoters of other heat shock proteins, during carbon stress. Long-term carbon stress activates Msn2 phosphorylation in a manner dependent upon Snf1 activation (Zaman et al., 2008).

Typically with longer stress periods, there is increased expression of proteins that reinforce the cell wall including Gsc2 (beta-1,3-glucan synthase) (De Virgilio, 2012). Gsc2 synthesizes the abundant polysaccharide 1,3-D-beta glycan and is induced by starvation and pH stress (De Virgilio, 2012; Mazur et al., 1995; Serrano, Martin, Casamayor, & Arino, 2006). Gsc2 is important for the plasma membrane general composition, as well as spore wall formation and stability (De Virgilio, 2012; Ishihara et al., 2007; Klis et al., 2002). This proteomic shift and induction of spore formation are also physiological markers of post-diauxic yeast, and preparation for quiescence. Each of these and many others have become consistent with cumulative physiological traits associated with quiescent yeast (De Virgilio, 2012; Gray et al., 2004; Werner-Washburne et al., 1996).

Cells tend to respond differently depending on the type of starvation imposed. If a natural nutrient (glucose, sulfate, phosphate, or ammonia) is withheld from yeast, the mitotic response is not the same as starvation imposed by an unnatural nutrient (auxotrophic requirements such as leucine or uracil). When starved for natural nutrients, as we have used, yeast tends to arrest the cell cycle at G0/G1, recognized by unbudded cells, and based on the nutrient that was withheld, not based on the genotype of the strain (Brauer et al., 2008; De Virgilio, 2012; Gray et al., 2004). (For this reason, glucose-starved yeast, expressing the mutant genotypes we investigated, were able to be consistently compared to each other, and we are able to confidently state that the differences noted were a result of the genotypes in the tested conditions, not the conditions themselves).

Genes whose expression is strongly correlated to yeast growth rate, and those independent of growth rate, have been studied by many researchers over the years. When the transcriptional activity is positively-correlated with growth rate, these are typically genes encoding mitochondrial proteins or proteins involved in ribosome biogenesis, translation, and rRNA metabolism or processing. Those genes negatively-correlated with growth rate are typically involved in other metabolic processes, such as those associated with peroxisomal activity, as well as alcohol and stored carbohydrate metabolic processes (Brauer et al., 2008; De Virgilio, 2012; Gray et al., 2004). Previous literature has shown that the protein synthesis rate is maximized during the exponential phase, allowing for the optimum utilization of resources, with the most opportunistic

and proliferative benefit (Brauer et al., 2008; De Virgilio, 2012; Galdieri et al., 2010; Gray et al., 2004; Hardie et al., 1998; Hardie, 2003; Hardie, 2007d; Hedbacker & Carlson, 2008; Nelson & Cox, 2008; Reinders et al., 1998; Smith et al., 1998; Werner-Washburne et al., 1993; Werner-Washburne et al., 1993; Werner-Washburne et al., 1996; Werner-Washburne et al., 1996; Zaman et al., 2008).

In the progression (Fig. 11) of the cells, in media exhausted of glucose, the number and function of proteins synthesized is significantly decreased and modified. Those proteins associated with growth and proliferation are decreased, and proteins correlated with post-exponential activity (pre-diauxic shift) begin to accumulate. Once the diauxic shift has been reached, the protein activity is again modified to prepare the cell for post-diauxic activity, and the total protein mass is generally restored to levels of exponential growth. However, the ratio of proteins associated with exponential growth, per the proteins associated with post-exponential growth, will be very different. This total protein mass may taper slightly to a level less than the total protein mass of exponential phase cells, as the cell drifts into quiescence. Stationary yeast continue to grow slowly, negligibly, unlike quiescent cells.

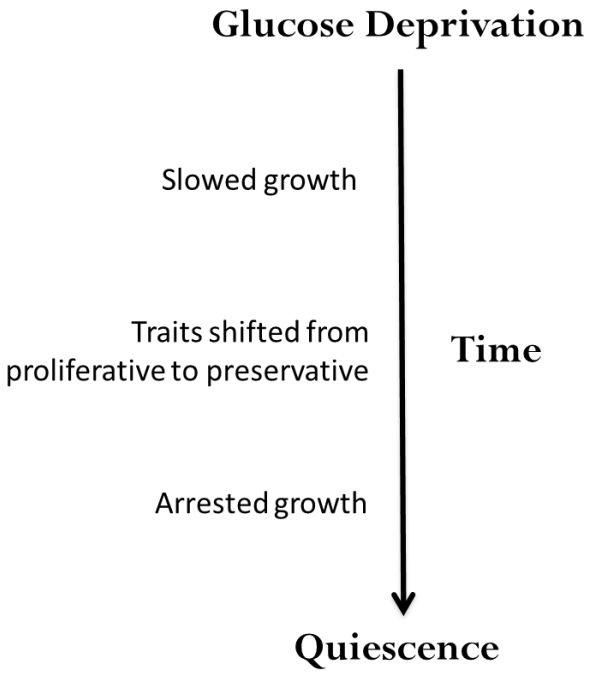

Figure 11: Quiescence is a programmed state of dormancy. Much as apoptosis, exponential phase, or stationary phases of growth, this stage has specific protein markers. These quiescent traits are specific to a particular set of processes. This includes the down-regulation of metabolic activities associated with glucose utilization, proliferative growth, or energy consumption. At the same time, there is a surge in processes associated with stress response. Additionally, traits characteristic of post-quiescent recovery are established. Eventually, growth is fully arrested, but the cell remains viable. These cells have condensed chromosomes (G0, non-replicating), decreased transcription and translation, failure to further accumulate mass or volume, thickened cell wall (resistance to zymolyase), increased thermotolerance and osmotolerance, and increased expression of heat shock proteins. While transcriptional and translational processes are halted at this stage, the degradation of RNA is inhibited, and the ribosomal activity seems to only be repressed, and awaiting re-feeding and exit from quiescence, during more favorable conditions.

Once cells are quiescent, proteins that are required for mitotic progress become dispensable, but proteins needed to recover from quiescence are available. The variations, isoforms, abundance, and post-translational modifications of these proteins at each stage will, of course, also have clear distinctions. Thus it is clearly identified that the entry into quiescence, much like the progression through any other growth phase, is a distinct program of changes in *Saccharomyces* (Brauer et al., 2008; De Virgilio, 2012;

Galdieri et al., 2010; Gray et al., 2004; Hedbacker & Carlson, 2008; Werner-Washburne et al., 1993; Werner-Washburne et al., 1996).

PKA itself responds to stationary phase in a manner that is temporally and spatially relevant. Bcy1 is increasingly phosphorylated by PKA, in a self-activating mechanism, as the cell is progressively starved for nutrients into stationary phase. The abundance of Bcy1 is also increased approximately 8-fold once shifted to post-stationary phase (De Virgilio, 2012; Werner-Washburne et al., 1991; Werner-Washburne et al., 1996), and translocates to the cytoplasm, from the nucleus, as stationary phase approaches (De Virgilio, 2012). This phosphorylation destabilizes the regulatory subunit, leaving the catalytic subunits free, while the concentration of Bcy1 ensures the cell is primed for a subsequent and coordinated decrease in TPK activity.

As growth and consumption shift from exponential phase to late stationary, the cell culture densities increase, the nutrients are progressively depleted, and eventually the number of budded cells decrease. This decrease in rate of growth has been directly associated with more of the cell's time waiting for "START", which is the transition from G1 to S of the mitotic cycle, and progressively activates stress response transcriptional activity. In order to progress to the S phase of the cell cycle, the contents must be doubled, and as a result, also the cellular mass. Among the proteins amassed are a series of cyclins required for the transition from G1 to S. Since the entry into "START" has been shown to be primarily based on size commitment, the lack of appropriate

accumulation of mass acts as a checkpoint, and can force the cell into an extended G1 period (Johnston, Pringle, & Hartwell, 1977; Zaman et al., 2008).

PKA is required for viability (in all stages of growth), including the mitotic transition from G1 to S of the cell cycle (Griffioen & Thevelein, 2002). PKA is required for entry into "START", and includes the capacity to resume growth, at the transition from G0 to G1, which is the exit from quiescence (Brauer et al., 2008). A lack of PKA has been shown to play a major role in inducing cell cycle arrest in G1 in the presence of abundant glucose, indicating the need for PKA in both the entry into quiescence, and progression into "START" (exit from quiescence) (Brauer et al., 2008; Gray et al., 2004; Johnston et al., 1977). Thus START is most sensitive to the depletion of PKA. When this halt of mitotic progression (at G1-S transition) occurs during starvation for natural nutrients, this can activate stress response proteins such as transcription factors Msn2 and Msn4 (Brauer et al., 2008; Smith et al., 1998; Zaman et al., 2008).

Hyperactivation of the PKA activity also tends to lead to enlarged cells, with appropriate accumulation of mass for mitotic progression. One approach to hyperactivation is the deletion of the gene encoding the regulatory subunit, *BCY1*; another is the deletion of the *IRA* Ras-GAP genes (Zaman et al., 2008). The *IRA1* deletion strain constructed in W303 did grow comparably to the WT in both rate of proliferation, and the CFU size. The W303 *bcy1* mutants, however, had difficulty in germination (explained above). Post-

germination cells were viable, but not as convenient to explore as the *ira1* mutants, and thus were not included in this study.

While it is well-known that the prolonged starvation for particular nutrients can lead to entry into stationary phase and eventual quiescence, less is known about the exit from quiescence. To exit quiescence, and to re-proliferate, once post-stationary, yeast would require the re-introduction of the lost nutrient(s), and specific genes must be activated, e.g. *GCS1* (and *SED1*) (Werner-Washburne et al., 1996). *gcs1* mutants are viable in quiescence, but unable to exit successfully and consistently, by failing to pass the START checkpoint, thus hindering the ability to enter a proliferative state post-quiescence (Gray et al., 2004). Like PKA, loss of *GCS1* and *SED1* causes a defective entry into the START state, exiting quiescence. To date, a clear connection between PKA or Snf1, and either of these two key START proteins has not been found.

For *Saccharomyces*, Snf1 can become dephosphorylated by Reg1-Glc7 upon addition of glucose post-stress (Werner-Washburne et al., 1996; Zaman et al., 2008). As mentioned in previous literature, it is possible that PKA may also phosphorylate Reg1-Glc7 (or Sit4, another phosphatase associated with Snf1) leading to the deactivation of these phosphatases in yeast (Barrett et al., 2012; Ruiz, Xu, & Carlson, 2011). Snf1 is known to phosphorylate Reg1-Glc7 in a positive feedback mechanism to increase activation of itself (Zaman et al., 2008). This phosphatase deactivation would inhibit dephosphorylation of Snf1 upon exit from quiescence, also assisting the primed

response to re-introduction of nutrients. This and other current literature suggests the interplay between PKA and Snf1 may offer one piece of the puzzle for this exit from quiescence.

In mammalian systems, PKA phosphorylates several proteins involved in utilization of glycogen stores in liver and muscle, as well as glycolysis and chromatin remodeling. This prepares the cellular response to use “energy” (i.e. carbon), when it is re-introduced (Nelson & Cox, 2008), by priming the machinery to immediately begin to work upon re-feeding.

Additionally, the “Ras switch” is recognized as an oncogene. When constitutively activated, this also stresses the cell in a slightly different manner, a method of overworking the machinery, and thus exhausting materials in an incessant building capacity. Numerous mutations are recognized as targets to remedy this excessive activity in the cell associated with cancerous growth. The temporal balance within the PKA-Snf1 pathway is one more area that may grant new approaches to oncogenic research centered around the “Ras switch” (Aguirre-Ghiso, 2007; Allan et al., 2006; Nelson & Cox, 2008; Uhr & Pantel, 2011).

LKB1, the primary upstream kinase for AMPK, has already been identified as having a significant role in breast cancer prevention, by phosphorylating AMPK, adjusting the metabolic shift in the cell when energy runs out. This also prevents the unnecessary

growth when nutrients in the environment are unfavorable (Hardie, 2003; Hardie, 2007b; Hardie, 2007c; Hardie, 2007d; Hedbacker & Carlson, 2008). While this provides some clinical relevance, it has not resolved the numerous breast cancer complications, nor prevented cancer from exiting remission (exiting quiescence), or resurfacing post-dormancy. Very little is known about the relationships or subtle differences between cancer stem cells and dormant cancer (Uhr & Pantel, 2011). It is currently understood that cancer stem cells may be key in the decision to enter quiescence or remain proliferative (Allan et al., 2006).

Within a primary tumor, there is a heterogeneous population of cells. Dormant tumor subpopulations that have established a G0-G1 arrested state are considered to be differentiated (Aguirre-Ghiso, 2007; Allan et al., 2006). The bulk of the tumor mass is actually thought to be differentiated, non-tumorigenic cells, but some of which, stem cells, can individually break away from the mass, allowing for only a few possible fates. The options, if viable, are dormancy or proliferation, but if non-viable, or if discovered by the immune system, the only option is death.

Those individual cells, with the capacity to proliferate, can establish and initiate the new site “colonization” in the host, called micrometastases. At the micrometastases, there is a net balance between viable and nonviable cells, so there is no gain in mass overall. After evaluating the habitability of the secondary site, this becomes another mitotic checkpoint decision: 1) to continue proliferative growth in the new location, 2) enter a

state of dormancy, or 3) undergo destruction. If the secondary stage decision is to continue proliferating, another full tumor mass is formed, called a macrometastases. Very few cancer stem cells are able to reach this final secondary site of full macrometastases. At this later stage, after two checkpoint opportunities, the initiated mass will begin exchanging vascularization signals with the host environment (Allan et al., 2006). These stages and markers are much more complex than what is summarized here, but that is well beyond the scope of this text.

Regardless of these complexities, each of these two stages of mitotic cellular decision-making is an opportunity to enter or exit quiescence. In dormancy, these cells have been extremely resistant to treatments with radiation and chemotherapy (Aguirre-Ghiso, 2007; Allan et al., 2006). Due to the eukaryotic preparation for dormancy, cancer can lie dormant in the body for years. A patient can survive breast cancer, while harboring these abnormal cells in the blood and bodily tissues for decades, but remain "clinically cancer-free" for the rest of his or her life (Aguirre-Ghiso, 2007; Allan et al., 2006; Uhr & Pantel, 2011). Some of the abnormal subpopulations are not only evasive to the immune system, but also to current technological detection. Since quiescence is not well understood, the ability to find reliable markers for dormant cancer is inconsistent as well. Currently, proliferation markers are consistently identifiable in breast cancer and other solid masses. These activate the immune system and lead to degradation. Thus the entry and exit from quiescence have been identified as attractive targets for therapeutic treatments (Aguirre-Ghiso, 2007; Allan et al., 2006). Our research will,

hopefully, provide one small puzzle piece to these signaling pathways associated with quiescence entry, and the possible mitotic roles of PKA and AMPK in cancer regulation.

In the future lays the potential for determination of consistent prognostic markers. These markers would have to be reasonably divided into at least three categories correlated with cancer stem cells, the problematic immune evasion, and detachable components of heterogeneous cell populations within an established tumor mass, or within micro- and macrometastatic stages (Aguirre-Ghiso, 2007). In order to make realistic progress in the comprehension of quiescence, reliable models of the quiescent state activity would have to be established. For even this much, the PKA on-off switching and interplay with other proteins at these stages of growth still need to be deduced.

As with all research, several questions are left unanswered in this project. Does Snf1 activation in the *ira1*Δ cells lag or suddenly drop at some point in chronically starved cells? We chose to evaluate the role of PKA by increasing cAMP levels via Ras. Does this effect still exist, and to the same extent, in *bcy1*Δ mutants? What are the roles of Reg1-Glc7 phosphatase in the PKA-Snf1 pathway? How do these roles of Reg1-Glc7 adjust to the changing states of growth, and the entry or exit from quiescence? Since our previous work has identified a target for PKA within Sak1 CTD, it is also important to determine if this is the only protein that PKA interacts with to down-regulate Snf1 in this transition. All of this is centered on the mechanisms specific to our Snf1-PKA pathway.

Non-Snf1-mediated regulation must also occur, just as well, non-PKA-mediated regulation, as suggested by the current literature. These issues should also be addressed.

We identified a partial mechanism for the PKA-Snf1 interaction that leads to quiescence, and suggest a purpose for the conserved (and numerous) PKA sites in eukaryotic Snf1/AMPK pathways. Ultimately, we would like to establish a model mechanism for the PKA-mediated temporal negative regulation of Snf1, establish possible distinctions in the pathway between PKA and Ras, and to identify the roles of Reg1-Glc7 in the entry and exit of quiescence.

Materials and Methods

Most methods of handling yeast for experiments or maintenance of the culture was managed by referring to (Rose, Winston, & Heiter, 1990; Rose, Winston, & Heiter, 2005). More general and specific methods are described below.

Strains and genetic methods

All strains used in this study are listed in Table 1. All strains used here are W303 genetic background. All strains were descendants from diploid wildtype (WT) MMY25, except MKY778. MKY778 was constructed by Dr. M. Orlova. Derivatives carrying *ira1*Δ*::KanMX6* and *snf1*Δ*::KanMX6* have been previously described in (Barrett et al., 2012; Orlova et al., 2006). Yeast homologous recombination was used to generate mutants with the *KanMX6* gene cassette, the cassette was first amplified by PCR, and then diploid WT

yeast were transformed with the resultant polymerase chain reaction (PCR) product. The mutant alleles were selected on solid media containing 200µg/mL kanamycin (G-418). The mutant alleles produced by the PCR reaction were transformed into heterozygous WT diploids by standard methods (Rose et al., 1990; Rose et al., 2005). The gene knockouts were confirmed by another PCR with primers outside the initial deletion region. The mutant haploid segregants were recovered by tetrad dissection of the transformed heterozygous diploid.

Table 1: *S. cerevisiae* W303 strains

Strain	Genotype	Source
MMY25	*MAT***a**/α *ura3*Δ/ura3Δ *leu2*Δ/leu2Δ *his3*Δ/his3Δ *trp1*Δ/trp1Δ *ade2*Δ/ade2Δ	this laboratory
LDY145	*MAT*α *ura3*Δ *leu2*Δ *his3*Δ *trp1*Δ *ade2*Δ	this laboratory
LDY164	*MAT*α *ura3*Δ *leu2*Δ *his3*Δ *trp1*Δ *ade2*Δ	this laboratory
LDY8	*MAT*α *ura3*Δ *leu2*Δ *his3*Δ *trp1*Δ *ade2*Δ *ira1*Δ*::KanMX6*	this laboratory
LDY141	*MAT*α *ura3*Δ *leu2*Δ *his3*Δ *trp1*Δ *ade2*Δ *ira1*Δ*::KanMX6*	this laboratory
MKY778	*MAT*α *ura3*Δ *leu2*Δ *his3*Δ *trp1*Δ *ade2*Δ *snf1*Δ*::KanMX6*	this laboratory

Media

Yeast extract-peptone dextrose medium (YPD; 2% peptone, 1% yeast extract, 2% glucose) is considered to be a complex nutrient-rich media for yeast growth. Yeast extract-peptone without glucose (YEP; 2% peptone, 1% yeast extract, no glucose) was used for the carbon-starvation stress of cells in this study. This media is identical to YPD, except it lacks glucose. For solid media 1.7% agar was added before autoclaving. The glucose was sterile-filtered separately and added after autoclaving to prevent caramelization of the sugar. Yeast used for these experiments were grown on YPD plates prior to inoculating liquid YPD. All yeast growth was at 30℃. The glucose starvation was

achieved by washing cells in (glucose-free) YEP and then re-suspending them in YEP for the designated incubation times.

DNA fragment transformation in yeast

A standard lithium acetate transformation was done, in the presence of polyethylene glycol (PEG). Primers (~60 nt) were designed for the *IRA1* gene knock-out, and replacement with *KanMX6* cassette (Table 2). The fragment was amplified by PCR. Gene knock-out was confirmed with PCR and new primers on agarose gel. WT yeast were transformed with the resultant PCR fragment (containing mutant alleles) by standard methods (Rose et al., 1990; Rose et al., 2005). PCR was performed by Gene AmpPCR System 9700 thermocycler (Applied Biosystems) using Accuprime High Fideldity DNA polymerase (Invitrogen) according to the manufacturer's instructions. Transformants were selected by colony purification on solid media containing Kanamycin described above.

Table 2: Primers for *IRA1* deletion and confirmation

Primer #	Description	Sequence
151F	forward k/o	5' GAGCTTCAAACTTAACATTCTTCTTCAGCATATAACATAC 3'
152R	reverse k/o	5' GCAAGAATGTCGTGCTCCTAATTTTAAATTAGAGTATTGC 3'
LD1F	forward confirmation	5' GTTAAGCTATTTAACGAAAGCG 3'
LD2R	reverse confirmation, internal to gene	5' TATACTTTACACAAATCTCTACG 3'
LD3R	reverse confirmation	5' GTGTGTGATATCAATAAAGGTGC 3'
k/o = knock out primer, combined with (~20nt) of either 5' or 3' of *KANMX6* cassette respectively		

Preparation of cell extracts and Immunoblot assays of T210 phosphorylation of Snf1

Cells were grown and incubated in conditions specified in the text. Protein extracts were prepared as described in Orlova et al. (2008). Briefly, a 3-4mL culture was boiled in a

water bath for 5 min to arrest Snf1 in its phosphorylation state. This was followed by 10min at room temperature to cool, followed by harvesting, and alkaline treatment with 0.1 N NaOH up to 5 min. Finally cultures were boiled in 30µL 2xSDS-PAGE loading buffer/1.0 OD_{600} of cells for protein extraction. (SDS-PAGE loading buffer was supplemented with β-mercaptoethanol (BME) at 1:20 to reduce disulfide bonds.) Extracts were separated on SDS-PAGE. Proteins were analyzed by immunoblotting using anti-phospho-Thr172-AMPK antibody 2531L (rabbit, Cell Signaling Technologies), which strongly recognizes phospho-Thr210-Snf1. To remove previous antibodies, membranes were incubated in 0.2M glycine pH 2.5 for 10 min. Membranes were reprobed with anti-polyhistidine antibody H1029 (mouse, Sigma-Aldrich) to detect the total Snf1. This binds to an endogenous stretch of 13 consecutive histidines at the N-terminus of the Snf1 protein (Barrett et al., 2012; Orlova, Barrett, & Kuchin, 2008). The signals were detected by enhanced chemiluminescence using ECL plus (Thermo Scientific) or HyGlo (Denville Scientific Inc.).

Thermotolerance spotting assay

For repressed (stress-free) conditions, cells were grown in nutrient-rich media YPD, and were incubated until mid-log phase as previously described in Orlova et al. (2008). For derepressed (stress) conditions, unstressed cells were collected by centrifugation, and shifted to no glucose conditions in YEP, after being washed in yeast extract-peptone (YEP) without any glucose. The stressed cells were re-suspended in YEP for further incubation. These were incubated for the indicated times, and subsequently heat shocked at 50°C for the indicated times. Serial dilutions of cells suspensions were

spotted onto plates, and then incubated at 30℃ for 1-2 days. Strains used here were LDY145 (WT), LDY141 (*ira1*), and MKY778 (*snf1*).

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Appendix: PKA recognition sites within the yeast and mammalian proteins of PKA-Snf1 pathway

Yellow = primary preference for phosphorylation: (Arg-Arg-X-Ser)

Green = secondary preference: (Arg/Lys-X-Ser/Thr) or (Arg/Lys-X-X-Ser/Thr)

Purple = T-loop phosphorylation site: Thr172 (& adjacent Ser173) in AMPK, or Thr210 (& adjacent Ser211) in Snf1

A. AMPK: (Arg/Lys-X-Ser/Thr) or (Arg/Lys-X-X-Ser/Thr)

NCBI – FASTA

>gi|786491|gb|AAB32732.1| AMP-activated protein kinase, AMPK [human, skeletal muscle, Peptide, 552 aa]
MAEKQKHDGRVKIGHYVLGDTLGVGTFGKVKIGEHQLTGHKVAVKILNRQKIRSLDVVGKIKREIQNLK
LFRHPHIIKLYQVISTPTDFFMVMEYVSGGELFDYICKHGRVEEMEARRLFQQILSAVDYCHRHMVVH
RDLKPENVLLDAHMNAKIADFGLSNMMSDGEFLRTSCGSPNYAAPEVISGRLYAGPEVDIWSCGVILY
ALLCGTLPFDDEHVPTLFKKIRGGVFYIPEYLNRSVATLLMHMLQVDPLKRATIKDIREHEWFKQDLPSY
LFPEDPSYDANVIDDEAVKEVCEKFECTESEVMNSLYSGDPQDQLAVAYHLIIDNRRIMNQASEFYLAS
SPPSGSFMDDSAMHIPPGLKPHPERMPPLIADSPKARCPLDALNTTKPKSLAVKKAKWHLGIRSQSKP
YDIMAEVYRAMKQLDFEWKVVNAYHLRVRRKNPVTGNYVKMSLQLYLVDNRSYLLDFKSIDDEVVEQ
RSGSSTPQRSCSAAGLHRPRSSFDSTTAESHSLSGSLTGSLTGSTLSSVSPRLGSHTMDFFEMCASLITTL
AR
(http://www.ncbi.nlm.nih.gov/protein/AAB32732.1
http://www.ncbi.nlm.nih.gov/protein/786491?report=fasta)

B. SNF1: (Arg/Lys-X-Ser/Thr) or (Arg/Lys-X-X-Ser/Thr)

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MSSNNNTNTAPANANSSHHHHHHHHHHHHHGHGGSNSTLNNPKSSLADGAHIGNYQIVKTLGEGS
FGKVKLAYHTTTGQKVALKIINKKVLAKSDMQGRIEREISYLRLLRHPHIIKLYDVIKSKDEIIMVIEYAGNE
LFDYIVQRDKMSEQEARRFFQQIISAVEYCHRHKIVHRDLKPENLLLDEHLNVKIADFGLSNIMTDGNFL
KTSCGSPNYAAPEVISGKLYAGPEVDVWSCGVILYVMLCRRLPFDDESIPVLFKNISNGVYTLPKFLSPG
AAGLIKRMLIVNPLNRISIHEIMQDDWFKVDLPEYLLPPDLKPHPEEENENNDSKKDGSSPDNDEIDDN
LVNILSSTMGYEKDEIYESLESSEDTPAFNEIRDAYMLIKENKSLIKDMKANKSVSDELDTFLSQSPPTFQ
QQSKSHQKSQVDHETAKQHARRMASAITQQRTYHQSPFMDQYKEEDSTVSILPTSLPQIHRANMLA
QGSPAASKISPLVTKKSKTRWHFGIRSRSYPLDVMGEIYIALKNLGAEWAKPSEEDLWTIKLRWKYDIG
NKTNTNEKIPDLMKMVIQLFQIETNNYLVDFKFDGWESSYGDDTTVSNISEDEMSTFSAYPFLHLTTKL
IMELAVNSQSN
(http://www.yeastgenome.org/cgi-bin/locus.fpl?locus=snf1)

C. LKB1: (Arg-Arg-X-Ser) or (Arg/Lys-X-Ser/Thr) or (Arg/Lys-X-X-Ser/Thr)

NCBI-FASTA

>gi|3024670|sp|Q15831.1|STK11_HUMAN RecName: Full=Serine/threonine-protein kinase STK11; AltName: Full=Liver kinase B1; Short=LKB1; Short=hLKB1; AltName: Full=Renal carcinoma antigen NY-REN-19
MEVVDPQQLGMFTEGELMSVGMDTFIHRIDSTEVIYQPRRKRAKLIGKYLMGDLLGEGSYGKVKEVL
DSETLCRRAVKILKKKKLRRIPNGEANVKKEIQLLRRLRHKNVIQLVDVLYNEEKQKMYMVMEYCVCG
MQEMLDSVPEKRFPVCQAHGYFCQLIDGLEYLHSQGIVHKDIKPGNLLLTTGGTLKISDLGVAEALHPF
AADDTCRTSQGSPAFQPPEIANGLDTFSGFKVDIWSAGVTLYNITTGLYPFEGDNIYKLFENIGKGSYAI
PGDCGPPLSDLLKGMLEYEPAKRFSIRQIRQHSWFRKKHPPAEAPVPIPPSPDTKDRWRSMTVVPYLE
DLHGADEDEDLFDIEDDIIYTQDFTVPGQVPEEEASHNGQRRGLPKAVCMNGTEAAQLSTKSRAEGR
APNPARKACSASSKIRRLSACKQQ
(http://www.ncbi.nlm.nih.gov/protein/Q15831.1;
http://www.ncbi.nlm.nih.gov/protein/3024670?report=fasta)

D. Sak1: (Arg-Arg-X-Ser) or (Arg/Lys-X-Ser/Thr) or (Arg/Lys-X-X-Ser/Thr)

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MDRSDKKVNVEEVNVPSNLQIELEKSGTSSSVSLRSPTKSSATNLAGMAEGARDNASIASSSVDSLNM
LLERQRVRQLNHPQHQQHISSSLAKTPTTTSSFCSSGSSKNKVKETNRISLTYDPVSKRKVLNTYEIIKELG
HGQHGKVKLARDILSKQLVAIKIVDRHEKKQRKFFTFIKSSKISENDKIKREIAIMKKCHHKHVVQLIEVL
DDLKSRKIYLVLEYCSRGEVKWCPPDCMESDAKGPSLLSFQETREILRGVVLGLEYLHYQGIIHRDIKPAN
LLISGDGTVKISDFGVSLAASSTNSSDSSESLDELELAKTVGTPAFFAPEMCLGEDAFTRYNLTKENLFRG
SCISFMIDIWAVGVTLYCLLFGMLPFFSDFELKLFEKIVNDPLKFPTFKEIQSNKVSKVSCEEEYEMAKDLL
LKLLEKNPQKRMTIPAIKKHPFVSWDFDHVPENDEKLLSSVLEQKLRFQCNQTDQFEPISISKHELKNAV
SGVGKKIKESVLKSIPLKDPSDLSNKNYLHPTETTRGRGDANVIVSEGSVLSNIKELSANDGCLNTDSDT
NININDDDHYSGDDNDGHLTKRELERELNKFDDKHEAGNMVNLPINSSFASLDSFYIDNFAMARMG
MSSPEAGDSVSSVPNLPSAPSSTRLGRSPVFSGVTNQPSPIRPVLPQQKSSFCATGRYDKSHNSLLRNS
SSHLTSYNSGRPSSRTGRMNSRNQNLPKIPNSLSKISTTKLTELRVPKDSEIPSPAKNPNADRLRRFPVKK
NTKTPAIKDPPRININSSDKSGSKNSPIKSLYQRMKQSKDNSKTFEVRRGNFFSHFNGDDDDSSSQSSV
TSSGSESDSELSSTSSSCTSGTQSRNSSNNNAYSETESLPFEFGVDSEDGSGVLLRDLPNEDQIRPFLDIQ
PCRRMKVKSSLNLEPPSVSSSSSSSSDEDELILNVGTAGHRRRHNSSKLSELSNSPQKGSNNFMYSNGS
VHDSETTITPQNMDDLTLHQALSRSQPISKPGPLVLPKRLDQKKATTETSNLTDIVEFNGNNDHRKDK
NFDKVLYSRDLLKDALSSTNAGRRRSIPSNKIRGRKDASITMSTNVGNDEHARNTSCHGDKGQENGAI
KQRTHERSRSLTVAELNEEKRRSALP
(http://www.yeastgenome.org/cgi-bin/locus.fpl?locus=sak1)

E. TAK1: (Arg-Arg-X-Ser) or (Arg/Lys-X-Ser/Thr) or (Arg/Lys-X-X-Ser/Thr)

Uniprot

MSTASAASSSSSSSAGEMIEAPSQVLNFEEIDYKEIEVEEVVGRGAFGVVCKAKWRAKDVAIKQIESESE
RKAFIVELRQLSRVNHPNIVKLYGACLNPVCLVMEYAEGGSLYNVLHGAEPLPYYTAAHAMSWCLQCS
QGVAYLHSMQPKALIHRDLKPPNLLLVAGGTVLKICDFGTACDIQTHMTNNKGSAAWMAPEVFEGS
NYSEKCDVFSWGIILWEVITRRKPFDEIGGPAFRIMWAVHNGTRPPLIKNLPKPIESLMTRCWSKDPSQ
RPSMEEIVKIMTHLMRYFPGADEPLQYPCQYSDEGQSNSATSTGSFMDIASTNTSNKSDTNMEQVPA
TNDTIKRLESKLLKNQAKQQSESGRLSLGASRGSSVESLPPTSEGKRMSADMSEIEARIAATTAYSKPKR
GHRKTASFGNILDVPEIVISGNGQPRRRSIQDLTVTGTEPGQVSSRSSSPSVRMITTSGPTSEKPTRSHP
WTPDDSTDTNGSDNSIPMAYLTLDHQLQPLAPCPNSKESMAVFEQHCKMAQEYMKVQTEIALLLQR
KQELVAELDQDEKDQQNTSRLVQEHKKLLDENKSLSTYYQQCKKQLEVIRSQQQ KRQGTS
(http://omim.org/entry/602614; http://www.uniprot.org/uniprot/O43318;
http://www.uniprot.org/blast/?about=O43318)

F. TOS3: (Arg-Arg-X-Ser) or (Arg/Lys-X-Ser/Thr) or (Arg/Lys-X-X-Ser/Thr)

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MVLLKEPVQPLPRSSLLYNNASNSSSRIKETRKVKLLYNPLTKRQILNNFEILATLGNGQYGKVKLARDLG
TGALVAIKILNRFEKRSGYSLQLKVENPRVNQEIEVMKRCHHENVVELYEILNDPESTKVYLVLEYCSRG
PVKWCPENKMEIKAVGPSILTFQQSRKVVLDVVSGLEYLHSQGITHRDIKPSNLLISSNGTVKISDFGVA
MSTATGSTNIQSSHEQLLKSRALGTPAFFAPELCSTEKEYSCSSAIDIWSLGVTIYCLLFGKLPFNANSGLE
LFDSIINKPLEFPSYEEMLNGATSGITMEEYTDAKDLLKKLLQKDPDKRIKLADIKVHPFMCHYGKSDAA
SVLTNLETFHELKVSPPSSCKRVELVSLPVNSSFASLDSVYMENFDHNNLRTGADRNSTYSPSIYDANTL
SPSAYHNIGSRESSYSSFSSFTSSTAFASQISIQDAPAIGDQQCLIGESGSSLRVNSCEFPQYTTMSPVGE
YPFESTEASLSSTLTPVGNVPQRIKAHLVEGKSNSKDDLRIEADASLVFEASDAQRTRRRMSLYKL
(http://www.yeastgenome.org/cgi-bin/locus.fpl?locus=tos3)

G. CaMKK: (Arg/Lys-X-Ser/Thr) or (Arg/Lys-X-X-Ser/Thr)

NCBI – FASTA

>gi|133922603|ref|NP_061371.2| calcium/calmodulin-dependent protein kinase kinase 1 [Mus musculus]
MESGPAVCCQDPRAELVDRVAAINVAHLEEADEGPEPARNGVDPPPRARAASVIPGSASRPTPVRPS
LSARKFSLQERPAGSCLGAQVGPYSTGPASHISPRSWRRPTIESHRVAISDTEDCVQLNQYKLQSEIGKG
AYGVVRLAYNESEDRHYAMKVLSKKKLLKQYGFPRRPPPRGSQATQGGPAKQLLPLERVYQEIAILKKL
DHVNVVKLIEVLDDPAEDNLYLVFDLLRKGPVMEVPCDKPFPEEQARLYLRDIILGLEYLHCQKIVHRDI
KPSNLLLGDDGHVKIADFGVSNQFEGNDAQLSSTAGTPAFMAPEAISDSGQSFSGKALDVWATGVTL
YCFVYGKCPFIDDYILTLHRKIKNEAVVFPEEPEVSEDLKDLILRMLDKNPETRIGVSDIKLHPWVTKHGE
EPLPSEEEHCSVVEVTEEEVKNSVRLIPSWTTVILVKSMLRKRSFGNPFEPQARREERSMSAPGSLLMK
EGCGEGCKSPELPGVQEDEAAS
(http://www.ncbi.nlm.nih.gov/protein/133922603?report=genbank&log$=protalign&blast_rank=95&RID=EVS59G28014;
http://www.ncbi.nlm.nih.gov/protein/133922603?report=fasta)

H. Elm1: (Arg/Lys-X-Ser/Thr) or (Arg/Lys-X-X-Ser/Thr)

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MSPRQLIPTLIPEWAPLSQQSCIREDELDSPPITPTSQTSSFGSSFSQQKPTYSTIIGENIHTILDEIRPYVK
KITVSDQDKKTINQYTLGVSAGSGQFGYVRKAYSSTLGKVVAVKIIPKKPWNAQQYSVNQVMRQIQL
WKSKGKITTNMSGNEAMRLMNIEKCRWEIFAASRLRNNVHIVRLIECLDSPFSESIWIVTNWCSLGEL
QWKRDDDEDILPQWKKIVISNCSVSTFAKKILEDMTKGLEYLHSQGCIHRDIKPSNILLDEEEKVAKLSD
FGSCIFTPQSLPFSDANFEDCFQRELNKIVGTPAFIAPELCHLGNSKRDFVTDGFKLDIWSLGVTLYCLLY
NELPFFGENEFETYHKIIEVSLSSKINGNTLNDLVIKRLLEKDVTLRISIQDLVKVLSRDQPIDSRNHSQISSS
SVNPVRNEGPVRRFFGRLLTKKGKKKTSGKGKDKVLVSATSKVTPSIHIDEEPDKECFSTTVLRSSPDSSD
YCSSLGEEAIQVTDFLDTFCRSNESLPNLTVNNDKQNSDMKTDRSESSSHSSLKIPTPIKAMIRLKSSPKE
NGNRTHINCSQDKPSSPLMDRTVGKRTVNNSGARKLAHSSNILNFKAYINSEDSDIRETVEDVKTYLNF
ADNGQI
(http://www.yeastgenome.org/cgi-bin/locus.fpl?locus=elm1)

I. TPK1: (Arg/Lys-X-Ser/Thr) or (Arg/Lys-X-X-Ser/Thr)

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MSTEEQNGGGQKSLDDRQGEESQKGETSERETTATESGNESKSVEKEGGETQEKPKQPHVTYYNEEQ
YKQFIAQARVTVGSIVYKNFQILRTLGTGSFGRVHLIRSRHNGRYYAMKVLKKEIVVRLKQVEHTNDERL
MLSIVTHPFIIRMWGTFQDAQQIFMIMDYIEGGELFSLLRKSQRFPNPVAKFYAAEVCLALEYLHSKDII
YRDLKPENILLDKNGHIKITDFGFAKYVPDVTYTLCGTPDYIAPEVVSTKPYNKSIDWWSFGILIYEMLAG
YTPFYDSNTMKTYEKILNAELRFPPFFNEDVKDLLSRLITRDLSQRLGNLQNGTEDVKNHPWFKEVVW
EKLLSRNIETPYEPPIQQGQGDTSQFDKYPEEDINYGVQGEDPYADLFRDF
(http://www.yeastgenome.org/cgi-bin/locus.fpl?locus=tpk1)

J. BCY1: (Arg-Arg-X-Ser) or (Arg/Lys-X-Ser/Thr) or (Arg/Lys-X-X-Ser/Thr)

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MVSSLPKESQAELQLFQNEINAANPSDFLQFSANYFNKRLEQQRAFLKAREPEFKAKNIVLFPEPEESFS
RPQSAQSQSRSRSSVMFKSPFVNEDPHSNVFKSGFNLDPHEQDTHQQAQEEQQHTREKTSTPPLPM
HFNAQRRTSVSGETLQPNNFDDWTPDHYKEKSEQQLQRLEKSIRNNFLFNKLDSDSKRLVINCLEEKSV
PKGATIIKQGDQGDYFYVVEKGTVDFYVNDNKVNSSGPGSSFGELALMYNSPRAATVVATSDCLLWA
LDRLTFRKILLGSSFKKRLMYDDLLKSMPVLKSLTTYDRAKLADALDTKIYQPGETIIREGDQGENFYLIEY
GAVDVSKKGQGVINKLKDHDYFGEVALLNDLPRQATVTATKRTKVATLGKSGFQRLLGPAVDVLKLND
PTRH
(http://www.yeastgenome.org/cgi-bin/locus.fpl?locus=bcy1)

K. Reg1: (Arg/Lys-X-Ser/Thr) or (Arg/Lys-X-X-Ser/Thr)

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MSTNLANYFAGKKDIENEHVNRNASHESNSKSDVKISGNDNDNDEDMGPSVSMAVQAKNDDDFHK
STFNLKRTRSMGLLDEYIDPTKKLLGRSDDLYDNDNEYYDNSSNNSSSNSSDDDYDDGYQEHSTSVSPP
PADNDSYLIPQDDNDVVVEPERHVDYLSHEWKESEISNSWKYIILKKKKRDVDLVNAARLENASWRT
WAKARNNLKTVSPEVVNWSKDSDVTWLYGPIVRDSEGNAQSEEEHDLERGYGSDDENSKRISMPTK
NSKSIAAAPKPILKKRTVTEIIEDNALWKLNEARKHMTEMKHASVIMDPNGNKNVHDDFDALAAQVN
AQYYHYPKESNSSVSLKSQHSDKKDNSTIPNPVGENSNGGGDKGEEDLHLKSALHVQNNRSTAQSNK
SILENSTNDRKANLDQNLNSPDNNRFPSSTSSSNRDNENNSMGLSSILTSNPSEKSNKPTKNRHIHFND
RVEQCMALRYPASQSEDDESDDENKQYVDVNNNANVTTINNNRTPLLAIQHKSIPINSATEHLNKNTS
DDDTSSQSSSSSHSDDEEHGGLYINARFSRRSDSGVHSPITDNSSVASSTTSRAHVRPIIKLLPDTTLNYG
SDEESDNGEFNGYGNAVSHNVNTSRGYDYIYDYNSVYTGDTSSFLPVDSCDIVDVPEGMDLQTAIAD
DNASNYEFNNAVESKEKHVPQLHKASANNTTRQHGSHMLLYDDDNYSSSSDSEQQFIEDSQYNSSDD
EEEEDDDDQEVDDNHDEGLSLRRTLSLGKSGSTNSLYDLAQPSLSSATPQQKNPTNFTGGKTDVDKD
AQLAVRPYPLKRNSSSGNFIFNSDSEEESSSEEEQRPLPANSQLVNRSVLKGSVTPANISSQKKKALPKQ
PKASDSSQSFRIVNNTPSPAEVGASDVAIEGYFSPRNESIKSVVSGGNMMDHQDHSEMDTLAKGFEN
CHINNASKLKDKKVDSVQTTRKEASLTDSSNESLHKVVQNARGMASKYLHSWKKSDVKPQENGNDSS
(http://www.yeastgenome.org/cgi-bin/locus.fpl?locus=reg1)