The Non-Proteolytic Role of the Ubiquitin-Associated Motif and Ubiquitin Cascade Enzymes on Yeast SNF1 Kinase

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By

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ABSTRACT

The heterotrimeric AMP-dependent protein kinase (AMPK) family is activated upon low energy states, conferring a switch towards energy-conserving metabolic pathways through immediate kinase actions on enzyme targets, and delayed alterations in gene expression through its nuclear re-localization. This family has known roles in stress resistance, lifespan, and nutrient adaptation, making it a relevant target for improving human health. The budding yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) AMPK, the SNF1 kinase, is readily activated by low glucose growth conditions making it a simple model to study the activation and activity of this enzyme class. These 3-subunit enzymes contain a highly conserved ubiquitin-associated (UBA) motif in most catalytic subunits whose function we have confirmed to be inhibitory. We demonstrated that UBA motif mutations significantly enhanced SNF1 kinase activation and biological activity including oxidative stress resistance and lifespan. Significantly, the enhanced UBA-dependent longevity and oxidative stress response is dependent on the Forkhead 1 (Fkh1) and Fkh2 stress response transcription factors, which in turn influence Snf1 catalytic subunit expression. The catalytic subunits are poly-ubiquitinated in yeast and humans with negative regulatory impacts on enzyme activity, yet the ubiquitin cascade enzymes involved are unknown. *S. cerevisiae* has 13 ubiquitin conjugating enzymes (E2) and a simplified repertoire of ubiquitin ligases (E3) that were screened for their impact on SNF1 kinase activity. We discovered that a discrete cluster of stress-responsive E2s (Ubc1, Ubc4, and Ubc5) and their associated E3 enzyme, Rsp5, affected discrete aspects of SNF1 kinase regulation, none through proteolytic degradation. The deletion of Ubc1 lowered catalytic subunit abundance, due to a synchronized decrease in Fkh1/2 proteins resulting in impaired *SNF1* gene transcription. Furthermore, the Fkh1/2 cognate transcription factor, Hcm1, failed to enter the nucleus in the absence of Ubc1. This implies that Ubc1 acts indirectly through transcriptional effects to modulate SNF1 kinase activity. We also determined that yeast strains disrupted for Ubc4/5 and Rsp5 functions were markedly impaired for nuclear import of the SNF1 kinase despite its full phosphorylation and activity, all without effecting protein stability. Together, this study supports a positive role for ubiquitin enzyme cascade in SNF1 kinase activation at multiple steps and is not limited to proteolytic degradation.

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LIST OF ABBREVIATIONS

ADP	Adenosine Diphosphate
Aak2	AMP-activated protein kinase 2
AMP	Adenosine Monophosphate
AMPK	5’ AMP-activated Protein Kinase
APC	Anaphase Promoting Complex
ART	Adaptor protein
ATP	Adenosine Triphosphate
Asy	Asynchronous
Bmh1	Brain modulosignalin homolog 1
Bmh2	Brain modulosignalin homolog 2
C. elegans	*Caenorhabditis elegans*
Clb2	Cyclin B2
CLS	Chronological Lifespan
CDC	Cell Division Cycle
Cdc20	Cell division cycle 20
Cdh1	Cdc20 homolog
CHX	Cycloheximide
CIDEA	Cell Death-Inducing DNA fragmentation factor A-like effector A
CM	Complete Medium
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DM2	Type 2 Diabetes Mellitus
DO	Drop-Out media
D. melanogaster	*Drosophila melanogaster*
E1	Ubiquitin activating enzyme
E2	Ubiquitin conjugating enzyme
E3	Ubiquitin ligase enzyme
ECL	Enhanced Chemiluminescence
ER	Endoplasmic Reticulum
EGFR	Epidermal Growth Factor Receptor
ERAD	Endoplasmic Reticulum-Associated protein Degradation
FACS	Fluorescence-Activated Cell Sorting
Fhl1	Forkhead-Like 1
Fkh	Forkhead
FOXO	Forkhead Box O
G1	Gap1 phase
G2	Gap2 phase
Gly	Glycine
Golgi	Golgi apparatus
GFP	Green Fluorescent Protein
Hcm1	High-copy suppressor of calmodulin 1

HECT	Homologous to the E6-AP Carboxyl Terminus
His	Histidine
HXK2	Hexokinase 2
HXT1	Hexose Transporter 1
Ifh1	Interact with Forkhead 1
Leu	Leucine
Lkb1	Liver Kinase B1
LPP	Lambda Protein Phosphatase
Lys	Lysine
MAPK	Mitogen-Activated Protein Kinase
Met	Methionine
Mig1	Multicopy Inhibitor of GAL gene expression 1
NEDD4.1	Neuronal precursor cell-Expressed Developmentally Downregulated 4.2
NES	Nuclear Export Signal
NLS	Nuclear Localization Signal
Pds1	Precocious Dissociation of Sisters 1
PHD	Plant Homeodomain
POR1	Porin 1
POR2	Porin2
PP1	Protein Phosphatase 1
RING	Really Interesting New Gene
RLS	Replicative Lifespan
Rod1	Resistance to O-Dinitrobenzene 1
Rpb1	RNA polymerase 1
RPS22B	Ribosomal Protein S22B
rRNA	ribosomal RNA
Rsp5	Reverses Spt- Phenotype 5
RT	Reverse Transcription
S. cerevisiae	*Saccharomyces cerevisiae*
SCF	Skp1-Cullin-F-box
SD	Standard Deviation
Sip1	SNF1-Interacting Protein 1
Sip2	SNF1-Interacting Protein 2
Sirt1	Sirtuin 1
SNF1 kinase	Sucrose Non-Fermenting 1kinase
Snf1	Sucrose Non-Fermenting 1
Snf4	Sucrose Non-Fermenting 4
SnRK1	Sucrose non-fermenting Related Kinase 1
SUC2	Sucrose-proton symporter 2
Thr	Threonine
tRNA	transfer RNA
Tub1	Tubulin 1
Tyr	Tyrosine
Ura	Uracil

UBA	Ubiquitin-Associated domain
UBC	Ubiquitin-Conjugating enzyme
Ubp	Ubiquitin-specific protease
WT	Wild Type
YPD	Yeast extract, Peptone, Dextrose media

CHAPTER 1 Literature Review

1.1 Yeast is a fundamental tool to study biology

Yeast is one of the simplest eukaryotes, yet many essential cellular processes are the same in yeast and higher eukaryotic cells. Many important proteins in humans and other mammals were first discovered through their yeast orthologs, including enzymes, cell cycle proteins, and cell signaling proteins (Ohsumi *et al.* 1993). The yeast genome has been fully sequenced and can be easily manipulated. Yeast cells divide approximately every 1.5-2 hours (Jorgensen *et al.* 2002), and therefore can be easily maintained in the lab. All these advantages make yeast a very powerful and inexpensive tool to study conserved cellular mechanisms. Yeast has been widely used to study cellular and molecular biology, and used as a fundamental tool to study cell cycle (Dutcher and Hartwell 1982; Kuhne and Linder 1993; Landry *et al.* 2014), DNA (Deoxyribonucleic Acid) repair (Niu and Klein 2016), aging (Lin *et al.* 2003; Lu *et al.* 2011), gene functions (Celenza *et al.* 1989; Dubacq *et al.* 2004), and protein interactions (Schmidt *et al.* 1999). The high similarity between yeast and other eukaryotes allows us to investigate basic processes in yeast and then extend that knowledge to humans and other species. Studying lifespan in yeast, for instance, has extended our understanding of aging in humans. For example, publications indicate that yeast lacking *SCH9* show significant increase in lifespan under caloric restriction growth conditions (Liu *et al.* 2015). Similarly, Akt, the mammalian ortholog of Sch9, has been shown to negatively regulate lifespan of human cells *(*Choi *et al. 2016;* Miyauchi *et al.* 2004). In addition, many other research areas including cancer biology, are highly related to the findings of cell cycle studies in yeast (Guaragnella *et al.* 2014). Various mutations involved in cell division and DNA replication had been found in yeast before their human orthologs in cancer were realized. For example, genes that regulate cell division (*CDC* genes) were first found in yeast (Martegani *et al.* 1984; Hwang *et al.* 1998). Strains lacking these genes lose the ability to regulate normal cell division, indicating their responsibility in maintaining proper cell growth (Kuhne and Linder 1993; Zachariae and Nasmyth 1999). Findings in yeast have contributed to the universal understanding of how the cell cycle is organized and

regulated in eukaryotic cells, and have applications within a number of different fields to prevent, diagnosis, and treat cancer (Guaragnella *et al.* 2014).

1.2 AMP-activated protein kinase (AMPK) enzyme family

All cells require a constant energy supply to survive despite variations in nutrients or necessary energy expenditures. From single yeast cells to higher eukaryotes such as plants, fish, and mammals, there are nutrient and energy sensors, which constantly monitor and adjust metabolic pathways to ensure cellular homeostasis. One of the most important sensors to regulate homeostasis is the 5' adenosine monophosphate-activated protein kinase (AMPK) family of enzymes (Backhed *et al.* 2007; Hedbacker and Carlson 2008; Coughlan *et al.* 2013). AMPK acts as a metabolic switch between anabolic and catabolic metabolism through its activity as a protein kinase to switch to catabolic states (Gowans and Hardie 2014). In mammalian systems, under conditions of abundant caloric intake/sedate lifestyle, insulin control of metabolism is dominant and AMPK is inactive. In the 'feasting' conditions, higher eukaryotes store fat, glycogen, and protein due to the influence of insulin. In contrast, under low energy states, insulin levels drop and AMPK is maximally activated to trigger catabolism, burn stored fuel for ATP (adenosine triphosphate), and subsequently correct the energy deficit under conditions of 'fasting'. The AMPK complex in all eukaryotes consists of three subunits that together make a functional heterotrimeric enzyme, including one each of α (catalytic), β (regulatory), and γ (regulatory) subunits. Each of these three subunits takes on a specific role in the specificity and activity of AMPK. In mammalian cells, there are two types of α subunits, two types of β subunits, and three types of γ subunits (Gowans and Hardie 2014; Hardie 2014). Yeast provides a much simpler system to study AMPK regulation as it contains only a single α and γ subunit each, and three β subunits (Hedbacker and Carlson 2008). In yeast, the simple manipulation of glucose abundance in the growth media allows regulated activation of the yeast AMPK without the confounding influences of insulin or other hormones, or the subtle effects that tissue differences appear to have in animal models (liver versus muscle versus adipose tissue for example) (Ross *et al.* 2016). The SNF1 (Sucrose Non-Fermenting) kinase is the yeast version of AMPK, and is also found as a heterotrimeric complex containing the Snf1 catalytic subunit (α subunit), Snf4

activating subunit (γ subunit), and one of three (Sip1/Sip2/Gal83) regulatory subunits (β subunits) (Hedbacker and Carlson 2008) (note the capital "SNF1" refers to the kinase complex and "Snf1" refers to the α subunit of the kinase). The Snf1 α subunit can be phosphorylated, which is necessary for the activation of the SNF1 kinase itself. Snf4, the single γ subunit, has been shown to non-catalytically contribute to the activation of the SNF1 kinase (McCartney and Schmidt 2001). It physically associates with Snf1 through the bridge of one of the β subunits. Each of the three β subunits can independently exist within the heterotrimeric complex, depending on the cellular compartment and biological cues (Vincent *et al.* 2001). Research indicates that the enzyme complex preferentially binds different β subunits under different circumstances. Details of the SNF1 kinase subunits will be described in section 1.5.

1.3 General mechanisms of AMPK activation and regulation

The mechanisms that trigger the activation of AMPK are similar in almost all ways between yeast and mammalian systems, including the canonical and essential step of the single phosphorylation of the α subunit within the catalytic domain by upstream discrete kinases (Woods, Johnstone*, et al.* 2003; Mairet-Coello *et al.* 2013). Additionally, the γ subunit of AMPK undergoes a conformational change that enhances the physical association with the catalytic α subunit and is triggered by an increased AMP (adenosine monophosphate)/ATP ratio, a signal of depleted cellular energy stores. The excess AMP or ADP (adenosine diphosphate) binds the two "Bateman domains" (domain that composed of a beta-alpha-beta-beta-alpha secondary structure pattern) located on the γ subunit, consequently changing its protein conformation in both yeast and mammalian AMPK family members (Momcilovic *et al.* 2008; Mayer *et al.* 2011; Davis *et al.* 2013; Gowans and Hardie 2014). This allosteric change of the γ subunit further enhances the exposure of the active site threonine (Thr) on the α subunit to its upstream kinases. As a result, the upstream kinases phosphorylate the α subunit of AMPK on its activating loop at Thr172 residue (Thr210 in yeast), promoting maximum enzyme activation. In yeast, there are three well-identified upstream kinases targeting the SNF1 kinase, comprising Pak1, Elm1, and Tos3. Each alone has the ability to phosphorylate Thr210 and fully activate the SNF1 kinase (Sutherland *et al.* 2003; Kim *et al.* 2005; Liu *et al.* 2011). In

addition to the activating signal triggered by the ratio of AMP/ADP abundance, there are additional signals that influence AMPK activity. It is also activated by other cellular signals such as calcium (Mairet-Coello *et al.* 2013), heat shock (Kodiha *et al.* 2007), leptin (Minokoshi *et al.* 2002; Suzuki *et al.* 2007), and muscle contraction after exercise (McGee *et al.* 2003). Although AMPK must be phosphorylated for activation, phosphorylation has now been revealed to not be the only mechanism regulating the final activity of AMPK. New reports have determined that post-translational alterations including ubiquitination (Al-Hakim *et al.* 2008), SUMOylation (Simpson-Lavy and Johnston 2013), and acetylation (Lu *et al.* 2011), can influence the activity of AMPK enzymes, adding a subtle layer, rather than an ON/OFF switch, of regulatory complexity to this central energy sensing enzyme.

1.4 Nuclear localization of AMPK in response to its activation state

Both mammalian and yeast AMPK have been shown to undergo a rapid cytoplasmic-nuclear relocation upon stimulating conditions (Vincent *et al.* 2001; Ju *et al.* 2011). Phosphorylation and the presence of a nuclear localization signal (NLS) on the catalytic subunit are essential for mammalian AMPK (Ju *et al.* 2011). The entry and exit of AMPK from the nucleus likely utilizes the general cargo-protein nuclear import machinery at the nuclear pore (Kazgan *et al.* 2010). However, import regulation and the specific process that converts the low energy trigger to result in import are still unknown.

The nuclear heterotrimeric complex has a discrete β subunit incorporated into the complex. In yeast, the β subunit, Gal83 (galactose metabolism 83), has the unique ability to translocate between the cytoplasm and nucleus, and was long thought to direct the whole kinase complex for nuclear shuttling. In contrast, the other β subunits, Sip1 and Sip2 (Snf1-interacting protein), do not localize to the nucleus, but rather to the ER/Golgi (endoplasmic reticulum/Golgi apparatus) (Yu *et al.* 2012) or plasma membrane (Lin *et al.* 2003), respectively. Surprisingly, previous research indicated that the Snf1 catalytic subunit has the ability to move into the nucleus even without the presence of Gal83, as shown by *GAL83* deletion studies in yeast (Vincent *et al.* 2001). Also interesting was the observation that a non-phosphorylatable, yet kinase active, version of the Snf1 catalytic subunit was sufficient to tether the Snf1-Gal3-Snf4 trimeric complex in the cytosol

(Vincent *et al.* 2001). Many questions remain regarding the glucose-responsive translocation of the activated SNF1 kinase into the nucleus. Is there a cytosolic tether? Does the Snf1 α subunit contain a functional nuclear localization signal? These remain important questions to determine in order to elucidate the mechanisms that direct the nuclear import and export of the yeast SNF1 kinase/AMPK in response to environmental signals.

1.5 Cellular and biological functions of the SNF1 kinase in yeast

The SNF1/AMPK family of protein kinases is highly conserved in fungi, yeast, plants, invertebrates, and animals. In the budding yeast *Saccharomyces cerevisiae (S. cerevisiae)*, the SNF1 kinase is primarily known for its role in adaptation to growth in the presence of limited glucose, its preferred energy source, and adapt instead to growth on sucrose or other non-fermentable sugars (Carlson *et al.* 1981; Celenza and Carlson 1986; Hardie *et al.* 1998). Yeast strains with deletions of *SNF1* or *SNF4* unable to grow on sucrose (Celenza *et al.* 1989; Jiang and Carlson 1997; Momcilovic and Carlson 2011), explaining the naming of these genes.

In yeast, the Snf1 catalytic subunit is comprised of 632 amino acid residues, and contains a kinase domain near the N terminus, and independent C termini regulatory regions including domains that associate with other subunits (Fig. 1.1). Activation of the Snf1 catalytic subunit requires phosphorylation on Thr210 on the activation loop (Nath *et al.* 2003). Mutation of Thr210 results in complete inactivity of the SNF1 kinase (McCartney and Schmidt 2001). However, neither Snf4 nor the β subunits are mandatory for phosphorylation to occur on Thr210 (McCartney and Schmidt 2001; Zhang *et al.* 2011).

The Snf4 subunit is a constitutively expressed protein independent of glucose availability or the activation state of the enzyme (Jiang and Carlson 1997; Vincent *et al.* 2001). It has a relatively larger number of protein abundance (20 times more than Snf1) and simultaneously binds both Snf1 and β subunits (Celenza *et al.* 1989; Ghaemmaghami *et al*. 2003; Adams *et al.* 2004). Snf4 is required for the activity of the SNF1 kinase complex, both *in vivo* and *in vitro*, through binding to the auto-inhibitory domain within the Snf1 subunit, and preventing the allosteric exposure of the catalytic domain to allow

for full activation (Momcilovic and Carlson 2011). A classic yeast 2-hybrid method assessing Snf1-Snf4 associations offered a powerful tool to investigate the activation of the SNF1 kinase, utilizing Snf1-Snf4 association (Jiang and Carlson 1997).

The three well-characterized β subunits of the SNF1 kinase are Sip1, Sip2, and Gal83. They exhibit a large structural overlap among each other and are individually sufficient for SNF1 kinase activation even when they are present alone (Hedbacker and Carlson 2008). Overall, the abundance (molecule/cell) of Sip1 and Sip2 is about 2:1 in yeast cells, and with a greater number of Gal83 (~10 times of Sip2) (Ghaemmaghami *et al*. 2003). Therefore, yeast cells can survive under different growth conditions when SNF1 kinase activity is required. However, each β subunit has its own discrete function that is required under different circumstances. For example, Gal83 is the major component contributing to SNF1 kinase activity when grown under glucose limitations, due to its ability to shuttle with the complex to the nucleus and alter the transcription of the adaptive pathways to use non-fermentable sugar sources (Vincent *et al.* 2001). Sip2 is also required when cells grow in non-fermentable carbon sources but additionally, the acetylation on Sip2 subunit has been shown to affect aging (Lin *et al.* 2003; Lu *et al.* 2011). Sip2 appears to remain in the cytosol regardless of the activation state of the enzyme. Sip1 is unique as it contains an N-myristorylation sequence and will localize to the vacuole in response to carbon stress (Hedbacker *et al.* 2004). All three β subunit isoforms are able to independently complement for the loss of the other two, and can maintain a strong degree of the normal function of the SNF1 kinase (Vincent *et al.* 2001). The abundance of the Snf1 subunit is decreased in the absence of all three β subunits (Schmidt and McCartney 2000). However, it is not known if deletion of a single β subunit affects the Snf1 protein abundance, and whether this observation is through transcription or protein stability.

As a sensor of glucose abundance, the SNF1 kinase remains fully inactive under normal (abundant) glucose conditions and is rapidly and reversibly activated under limiting glucose conditions. Yet, this is not the only important biological pathway the SNF1 kinase is involved in. Importantly, the SNF1 kinase also functions as a central component of the kinase signaling pathways that responds to environmental stress, such as salinity (Kulik *et al.* 2012), oxygen free radicals (Li *et al.* 2005), temperature extremes

(Morano *et al.* 2012), and pH changes (Hsu *et al.* 2015). Under optimal growth conditions, when glucose and ATP are abundant, cells are anabolic and producing more ATP than is consumed. There is active regulation of gene expression to maintain this metabolic state, and although we focus on those in yeast, there is analogous regulation of gene expression in higher eukaryotes and humans. In animals, this anabolic state is also finely regulated through the anabolic actions of insulin and its signaling pathway (Reynolds *et al.* 2012; Arkun 2016). In yeast, under anabolic conditions, there is up-regulation of several genes including those encoding glycolytic enzyme hexokinase 2 (*HXK2*) (Palomino *et al.* 2006), ribosomal protein S22B (*RPS22B*) (Celton *et al.* 2012), and hexose transporter 1 (*HXT1*) (Solis-Escalante *et al.* 2015). Simultaneously, this optimal growth condition state represses the expression of many genes, including those involved in the utilization of alternative carbon sources (sucrose-proton symporter 2, *SUC2*) (Yang *et al.* 2015), gluconeogenesis (*RDS2*) (Soontorngun *et al.* 2007), and respiration (*POR1* and *POR2* that encode mitochondrial porin) (Strogolova *et al.* 2012) by a process known as glucose repression.

Under low glucose and/or high stress conditions, the yeast SNF1 kinase is activated and its kinase activity targets key cytosolic and nuclear targets to change the activity of metabolic pathways toward catabolism. Stored fuels are burned for energy, synthetic pathways are repressed, and energy homeostasis is restored (Carlson *et al.* 1981; Amodeo *et al.* 2010; Momcilovic and Carlson 2011; Hsu *et al.* 2015). Active SNF1 kinase phosphorylates metabolic pathway components, altering their ON/OFF status to switch to a catabolic environment. For instance, the SNF1 kinase phosphorylates acetyl-coenzyme A carboxylase during glucose-limiting growth conditions (Zhang *et al.* 2013), and inhibits fatty acid biosynthesis (Shirra *et al.* 2001; Nicastro *et al.* 2015). In addition, activated SNF1 kinase translocates to the nucleus and further activates downstream targets including transcription factors. For example, the SNF1 kinase phosphorylates Mig1 (multicopy inhibitor of *GAL* gene expression 1), the repressor of *SUC2* expression, subsequently releasing the glucose repression and allowing adaptation to sucrose utilization (Carlson *et al.* 1981).

The budding yeast *S. cerevisiae* contains seven unlinked genes (*SUC* genes) that encode invertase (Carlson *et al.* 1981; Carlson and Botstein 1982). Invertase is an

important enzyme that catalyzes the hydrolysis of both sucrose and raffinose, thereby supplying glucose for yeast to survive in low- or non- glucose environments (Carlson and Botstein 1982, 1983). Yeast may produce one or several invertases, however, the reference strain S288C, which is used in our and others research, only encodes and expresses *SUC2* (Carlson and Botstein 1983). Therefore, most of the studies that investigate invertase activity mainly focus on *SUC2*. *SUC2* encodes two forms of invertase in *S. cerevisiae*, external and internal. Internal invertase is localized in the cytoplasm, and has very low expression levels and has no known function in any cellular process (Gascon *et al.* 1968). External invertase is localized in the periplasmic space hydrolyzes non-fermentable carbon sources and produces monosaccharides including glucose and fructose, which are utilized by the cell (Gascon *et al.* 1968; Carlson and Botstein 1983; Schmidt and McCartney 2000). For our research purposes, we evaluate both invertase activity and *SUC2* expression to determine the SNF1 kinase activation and its nuclear activity.

1.6 Phosphorylation is an activating signal for the SNF1 kinase

Activation of the SNF1 kinase requires a single phosphorylation on the Thr210 residue of the Snf1 catalytic subunit (Hardie 1999; Nath *et al.* 2003). Snf1 consists of a catalytic domain, a ubiquitin-associated domain (UBA) domain adjacent to the auto-inhibitory domain, and the domains that can interact with β or γ subunits (Fig. 1.1) (Rudolph *et al.* 2005; Amodeo *et al.* 2007; Cho *et al.* 2014). The catalytic domain is in the N-terminal of this subunit. Phosphorylation causes an unfolding of the Snf1 catalytic subunit, releasing its autoinhibitory domain from the catalytic domain and thereby enhancing function. The UBA domain is found in many catalytic subunits of the AMPK enzyme family, and early studies in both yeast (Momcilovic and Carlson 2011) and mammalian systems (Jaleel *et al.* 2006; Cho *et al.* 2014) suggest that it plays a modest role in regulating the activity of the enzyme complex. The UBA domain was believed to affect the kinase activity and have the ability to bind to independent substrates that are mono- or poly-ubiquitinated. However, there is no proof of this sort of interaction in either mammalian or yeast AMPK to date.

When yeast cells grow in glucose-rich environments, the SNF1 kinase is maintained in an inactive dephosphorylated state and resides mainly in the cytoplasm

(Hardie 1999; Zhang *et al.* 2011). When the levels of glucose become depleted, the SNF1 kinase is phosphorylated by upstream kinases including Sak1 (also known as Pak1) (Nath *et al.* 2003), Tos3 (Kim *et al.* 2005), and Elm1 (Sutherland *et al.* 2003). Once activated, the SNF1 kinase can regulate the expression of different genes after its import into the nucleus by inhibiting transcriptional repressors (e.g. Mig1) and targeted phosphorylation of nuclear proteins (Treitel *et al.* 1998; McCartney and Schmidt 2001), either by stimulating transcriptional activators (e.g. Sip4) (Schmidt and McCartney 2000), or by activating the transcription machinery directly (Shirra *et al.* 2005; Ruiz *et al.* 2011). When glucose is added back to cells, the SNF1 kinase is rapidly inactivated by dephosphorylation, via the Reg1-Glc7 protein phosphatase 1 (PP1) complex and further exported from the nucleus (Fig. 1.2) (Sanz, Ludin*, et al.* 2000; Ruiz *et al.* 2011; Zhang *et al.* 2011).

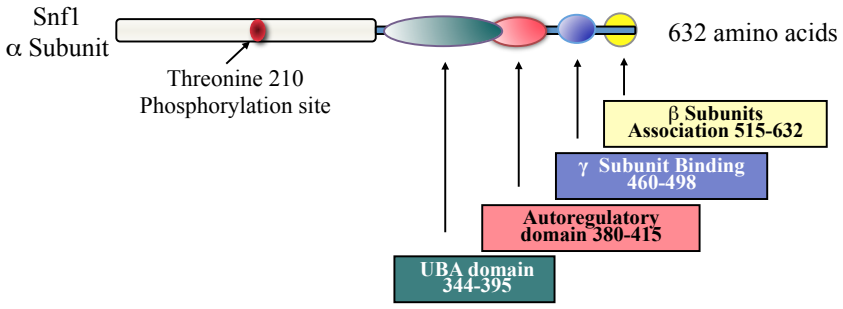

Figure 1.1 The functional domains of the Snf1 catalytic subunit. The Snf1 subunit consists of the N-terminal catalytic domain, a UBA domain, an auto-inhibitory domain, as well as documented binding sites for the associated β subunit, and γ subunit. The allosteric changes within the Snf1 subunit triggered by phosphorylation or associations with the β or γ subunits effect the conformation of catalytic domain and its enzymatic activity.

1.7 The ubiquitin system and AMPK

Ubiquitination is an important post-translational modification that has a pivotal role in numerous biological functions including cell growth, apoptosis, and innate immune responses (Chen *et al.* 1993; Muratani and Tansey 2003; Harkness *et al.* 2004; Finley *et al.* 2012; Postnikoff and Harkness 2012; Becuwe and Leon 2014). Ubiquitin is a small, abundant, and highly conserved regulatory protein that has been found in every eukaryote examined (Hoeller and Dikic 2009; Finley *et al.* 2012). It attaches to the target proteins

via a covalent bond. There are three sequential steps in this process of ubiquitin attachment. First, the E1 ubiquitin-activating enzyme activates ubiquitin. This ATP-requiring step initiates the cascade. Second, ubiquitin is transferred to an E2, the ubiquitin-conjugating enzyme (UBC). The final step of ubiquitination creates a covalent isopeptide bond between a lysine of the target protein and the C-terminal glycine of ubiquitin. Non-lysine, or non-conventional ubiquitin attachments have been found including at the N-terminal end of proteins (Varshavsky 1996), and at serines and cysteines (Kravtsova-Ivantsiv and Ciechanover 2012). In general, this step requires the activity of one of hundreds of E3 ubiquitin ligases in higher eukaryotes (Chang *et al.* 2000; Rodrigo-Brenni and Morgan 2007; Baskin and Taegtmeyer 2011; Stoll *et al.* 2011). The most well-known consequence of ubiquitination is protein degradation of the substrate protein via the proteasomal pathway. Substrates that have been poly-ubiquitinated can be recognized by the receptor of the proteasome and then undergo protein degradation (Fig. 1.3) (Finley *et al.* 2012).

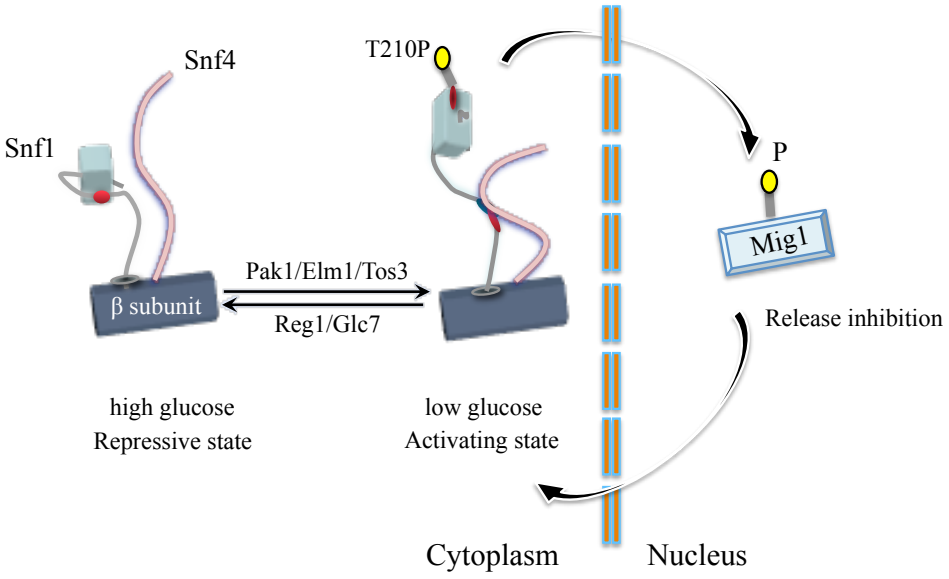

Figure 1.2 Schematic illustration of the SNF1 complex. The SNF1 kinase consists of three subunits. Growth in low glucose activates the SNF1 kinase and the catalytic α-subunit, Snf1, is phosphorylated on Thr210. The phosphorylated SNF1 kinase complex then translocates into the nucleus, and targets Mig1 for phosphorylation. Conversely, growth in high glucose is repressive, and the Thr210 residue on Snf1 is actively dephosphorylated, and the whole complex shuttles back to the cytoplasm.

1.7.1 The ubiquitin system in yeast

In yeast, the ubiquitination system has been widely investigated and has been shown to share the same enzyme cascade as in mammals, albeit with fewer E2 and E3s. There is one E1, thirteen E2s, and it is difficult to state an exact number of E3s (60-100) as they are still being discovered (Finley *et al.* 2012). Nonetheless, yeast provides a simpler system in which to study ubiquitination (Finley *et al.* 2012). Studies have shown that conversion of lysine to arginine can effectively abolish ubiquitination on that site, preventing degradation, if that was the substrates fate, and affect the normal functions of the target proteins (Al-Hakim *et al.* 2008; Hiraishi *et al.* 2009).

The cellular consequences of ubiquitin attachment are diverse and extend far beyond degradation. For example, proteins targeted with a poly-ubiquitin chain built through lysine-48 (of the ubiquitin moieties themselves) can be recognized by the proteasome and undergo degradation (Jacobson *et al.* 2009), whereas mono-ubiquitin may function as a localization signal (Hicke 2001). In addition, poly-ubiquitin chains formed through lysine-63 have been shown to participate in cell signaling events (Li and Ye 2008). Researchers indicate that ubiquitination also functions as protein trafficking or kinase/phosphatase activation signals. These processes are involved in cell survival and cancer development (Hoeller and Dikic 2009). Clarifying the purpose of Snf1 ubiquitination from among the many potential outcomes will be important to further our understanding of the role of ubiquitination in SNF1 kinase/AMPK family regulation (Jaleel *et al.* 2006; Al-Hakim *et al.* 2008; Cho *et al.* 2014).

1.7.2 The current knowledge of the role of ubiquitination in regulating SNF1 kinase and AMPK

The biological or environmental signal that triggers ubiquitination of the α subunits of AMPK is not yet known. Research in mammalian cells and tissues have shown that discrete subunits of AMPK can be ubiquitinated under experimental conditions (Baskin and Taegtmeyer 2011; Lee *et al.* 2013; Pineda *et al.* 2015). This generated much interest, as there are several potential roles that ubiquitin may play in the regulation of the AMPK. This includes potential roles for ubiquitin in targeting the enzyme for degradation (a negative influence), signaling for subcellular localization (relevant when considering the nucleo-cytoplasmic shuttling of the enzyme), and in promoting protein associations (a

potential positive role).

There are valid concerns and limitations to the previous studies of catalytic subunit ubiquitination. The experimental conditions used to detect poly-ubiquitination of the α subunit for yeast and mammalian AMPK were far from physiological, and involved manipulation of the targets, including plasmid overexpression, mutations, and strong inhibition of deubiquitination (Al-Hakim *et al.* 2008; Wilson *et al.* 2011; Lee *et al.* 2013). In both yeast and mammalian systems, the result of ubiquitination was a decrease in the catalytic subunit abundance, and a decrease in direct or indirect kinase activity on downstream targets. This led to the conclusion that ubiquitination of the AMPK family has an inhibitory function. Importantly, the nuclear import and allosteric associations were not investigated. The specific ubiquitination sites on the α subunits have not been formally identified, nor have the identity of the responsible ubiquitin cascade enzymes been determined. In yeast, large-throughput proteomic analysis has consistently identified two lysines within the catalytic subunit that are ubiquitin-attachment sites (Starita *et al.* 2012). The biological importance of these residues has not been tested. Also, ubiquitination and degradation have not been correlated to the activation state of the AMPK enzymes, and have not been tested under physiological conditions.

Different research groups have reported that AMPK function can be regulated by ubiquitination on the α subunit, the β subunits, and the upstream kinases. However, the findings vary immensely with the system being studied and the analytical methods used. For example, AMPK β subunit in mouse brown adipose tissue can be targeted for degradation by the E3 ubiquitin ligase CIDEA (cell death-inducing DNA fragmentation factor A-like effector A) (Qi *et al.* 2008). Another group tested whether Lkb1 (liver kinase B1), upstream kinase of AMPK, was poly-ubiquitinated and subsequently behaved as a functional enzyme (Lee *et al.* 2015). Research also showed that AMPK α1 subunit when targeted for poly-ubiquitination is followed by a decreased enzymatic activity of AMPK (Pineda *et al.* 2015). In yeast, the Snf1 α subunit has been shown to be poly-ubiquitinated, affecting its Thr210 phosphorylation (Wilson *et al.* 2011). Proteomic analysis using mass spectroscopy identified 2 lysine (Lys) residues (Lys80 and Lys461) within the α subunit as sites for ubiquitination (Starita *et al.* 2012). No report of ubiquitination on the Snf4 subunit has been published.

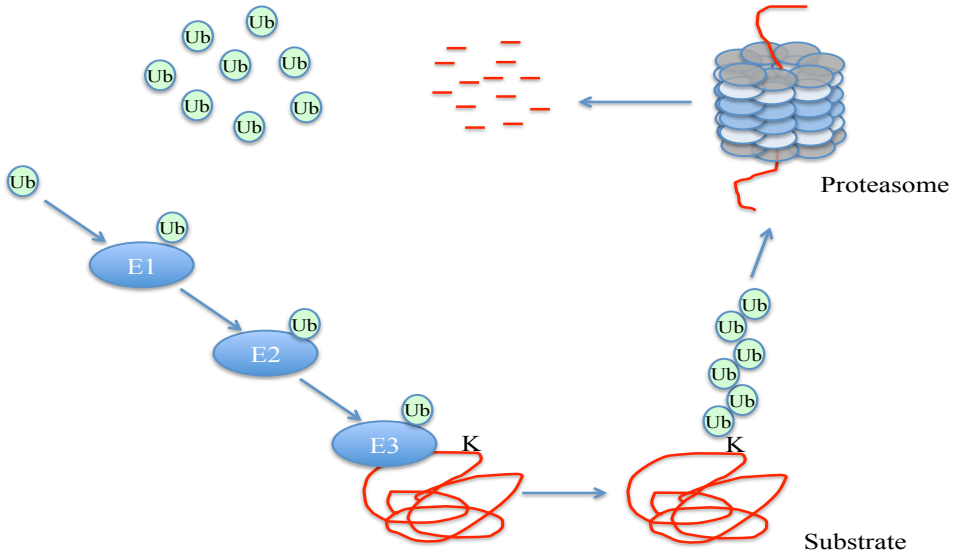

Figure 1.3 Illustration of protein ubiquitination and proteasomal degradation. Ubiquitin (Ub) can be activated by E1 ubiquitin-activating enzyme, then transferred on E2 ubiquitin conjugating enzyme, eventually attached to the target substrate by E3 ubiquitin ligase. Ubiquitin can also attached to the exciting ubiquitin on the substrate, forming a poly-ubiquitin chain. The poly-ubiquitin chain can be recognized by the proteasome, further undergo protein degradation. Adapted from (Finley *et al.* 2012).

1.7.3 The current knowledge of the role of ubiquitin-associated domains in SNF1 kinase and AMPK regulation

Ubiquitin cascade enzymes are able to bind free ubiquitin protein, and subsequently transfer the ubiquitin to selected substrates. Besides these E2/E3 enzymes, there are proteins that also non-covalently bind ubiquitin including those that contain the motif called the ubiquitin-binding domain (Hicke *et al.* 2005). Generally, ubiquitin-binding domains can interact directly with mono-ubiquitin and/or poly-ubiquitin with, or without, ubiquitin-ubiquitin linkage specificity. To date, there are 9 known such motifs that can bind either mono- or poly-ubiquitin (Hicke *et al.* 2005). One well-investigated motif is the UBA domain. The UBA is a protein motif that has been found throughout eukaryotes and in many proteins, including enzymes (Hofmann and Bucher 1996). This motif contains

relatively short ~45 amino acid residues that is thought to fold to form a hydrophobic protein face that interacts non-covalently with the globular head of ubiquitin that is independently attached covalently to another protein substrate. The covalent ubiquitin second site can be within the same protein as the UBA domain in larger proteins. The UBA motif has been identified to bind ubiquitin, multi-ubiquitin chains, and ubiquitinated proteins, and thus functions as a motif to enhance protein-protein interaction and subcellular relocation (Raasi *et al.* 2005). Most components of the ubiquitin-proteolysis pathway, including ubiquitin enzymes, and/or receptors, contain the UBA domain, suggesting that a major role of the UBA domain is in protein degradation. However, some UBA motifs are incapable of binding ubiquitin regardless of mono- or poly-ubiquitin chains or the linkage involved. For instance, the proteasome subunit, Rpn10 (proteasome regulatory particle), has an UBA domain but does not bind either mono-ubiquitin or poly-ubiquitin chains under any circumstances (Lin *et al.* 2011; Lipinszki *et al.* 2012). The UBA domain has been separated into 4 defined classes based on linkage-selective poly-ubiquitin chains. Specifically, class 1 UBA selectively binds lysine-48 ubiquitin chains whereas class 2 UBA motifs prefer to bind lysine-63 ubiquitin chains. Class 3 UBA motifs do not bind any ubiquitin, and class 4 UBA binds all kinds of ubiquitin (Raasi *et al.* 2005).

Despite the fundamental role of the UBA domain in recognition and attachment of ubiquitin-interacting substrates, it also contributes to the stability, function, and subcellular localization of the proteins that bind to ubiquitin or ubiquitin chains (Aguilar *et al.* 2003; Jang *et al.* 2012). These functions have both proteolytic and non-proteolytic outcomes. For example, many E2s and E3s contain the UBA domain. These ubiquitin cascade enzymes have the ability to process the substrates, and following proteolysis. However, non-proteolytic outcomes also regulate the normal function of the cells. For instance, ubiquitin chains can be protected when attached to a UBA domain, and therefore cannot be disassembled by deubiquitination or ubiquitin-specific protease (Ubp) enzymes, results in protein stability. Also, research shows that the UBA motif on Ent1, a key endocytic protein in yeast, can bind the ubiquitinated cell-surface transmembrane protein, Ste2, which facilitates Ent1 subcellular localization (Aguilar *et al.* 2003). Another potential role of the UBA motif is to provide a chaperone-like function to protect specific

ribosomal proteins that have N-terminal ubiquitinations (Murata *et al.* 2009). It is unclear how they escape the detection of the ubiquitination degradation pathway, but possibly the UBA domain on ribosomal subunits bind the ubiquitin moiety on these nascent ribosomal proteins to escape the proteasome detection and eventually assemble into the mature ribosome (Finley *et al.* 1989; Madura 2002). These roles are of particular interest to us, given that the Snf1 catalytic subunit has a Class 3 UBA motif.

The UBA domain is found within the yeast Snf1 α catalytic subunit as well as on nearly all mammalian AMPK-like α subunits (Jaleel *et al.* 2006; Cho *et al.* 2014). Of significance, the yeast SNF1 kinase UBA domain is a Class 3, non-ubiquitin binding motif (Momcilovic and Carlson 2011). Thus, the function of the UBA domain remains unclear, yet early insights through systematic mutations throughout the catalytic, UBA, and regulatory domains of the yeast Snf1 α subunit suggest it may play an inhibitory role in SNF1 kinase activity. Mutations throughout these regions resulted in enhanced phosphorylation of the α subunits and enhanced targeting against *in vitro* SNF1 kinase substrates (Momcilovic and Carlson 2011).

1.7.4 Functions of the E2 ubiquitin conjugating enzyme Ubc1 in yeast

Knowing that the α subunit of the yeast AMPK is ubiquitinated, it will be of interest to use yeast to identify the E2 and/or E3 involved in targeting the Snf1 protein, and investigate details of ubiquitin-mediated AMPK regulation. The ubiquitin conjugating enzymes responsible for targeting the AMPK catalytic subunits have not yet been revealed in yeast, mammals, or other eukaryotes, to the best of our knowledge. In yeast, there is only one E1 enzyme and 13 known E2s that have been identified (Finley *et al.* 2012). There are discrete phenotypes in yeast associated with the loss of these E2 functions that have highlighted their normal biological functions in the cell. In particular, there are a cluster of E2s (Ubc1, Ubc4, and Ubc5) that are associated with the yeast stress response and their mutation slowed growth and division (Seufert and Jentsch 1990; Seufert *et al.* 1990; Stoll *et al.* 2011). Given that the SNF1 kinase functions within the stress response pathway, it will be of particular interest to determine if there is a link between these E2s and the ubiquitination of the Snf1 catalytic subunit.

Ubc1 has functions that overlap with other E2s such as Ubc4 and Ubc5. They have

been recognized as the same subfamily of E2s, due to the similarity of their stress-related functions. Ubc4 and Ubc5 are such close homologs of each other; they are frequently simultaneously disrupted in yeast, as they can independently complement for each other (Seufert and Jentsch 1990). Literature indicates that simultaneous disruption of *UBC1* in the *ubc4Δ* or *ubc5Δ* mutant strains enhances the severity of stress sensitive phenotypes to a greater degree than that of single mutation. Additionally they exhibit phenotypes such as slow growth, prolonged doubling time, and failure to survive after meiotic segregation (Seufert and Jentsch 1991; Chen *et al.* 1993). However, despite this functional overlap, Ubc1 does have discrete functions and target preferences, and it plays an important role in regulating yeast cell growth.

Of interest, Ubc1 and Ubc4 both individually interact with a multi-subunit ubiquitin E3 ligase, the Anaphase Promoting Complex (APC), which can target selected proteins for degradation in synchrony with the cell cycle, yet the E2s exhibit their own target preferences. Specifically, Ubc1 was found to catalyze rapid poly-ubiquitination of pre-ubiquitinated substrates, whereas Ubc4 prefers to initiate the first ubiquitin covalent bond onto targets (Stoll *et al.* 2011). Also, Ubc1 is essential for the lysine-48 linked poly-ubiquitin chain (Rodrigo-Brenni and Morgan 2007), whereas Ubc4 is dispensable for poly-ubiquitin chain formation and may be important in mono-ubiquitination (Stoll *et al.* 2011). In addition, *UBC1* mRNA (messenger ribonucleic acid) and protein level have been found to be elevated when yeast cells proceed into stationary phase, compared to exponentially growing cells (Rodrigo-Brenni and Morgan 2007), whereas *UBC4* is expressed in growing cells but hardly detectable in stationary phase (Seufert and Jentsch 1990).

Ubc1 appears to be involved in many discrete biological functions through its effect on modifying protein turnover, protein degradation, and vesicle production (Seufert *et al.* 1990; Horak and Wolf 2001; Rodrigo-Brenni and Morgan 2007; Xu *et al.* 2009). *UBC1* gene disruption results in yeast strains that grow slowly with enlarged cells, and tend to form aggregates. Even though an abnormal cell morphology was observed (Rodrigo-Brenni and Morgan 2007) (Seufert *et al.* 1990), the molecular explanation for the formation of the enlarged cell body and cellular aggregation is not clear. Interestingly, there is a strain dependency on the requirement of Ubc1 for cell viability. Even through

some publications suggest that cells cannot survive in the absence of Ubc1 (implying it is an essential gene) (Seufert and Jentsch 1991), others indicate that the *UBC1* deleted strain is viable, including the strain we use for our investigations. For example, our *ubc1Δ* mutant, derived from the sporulated *UBC1/ubc1Δ* diploid, survives. In fact, the haploid *ubc1Δ* has an increased growth rate and cell size after growth on yeast peptone dextrose media (YPD) plates after several generations (Seufert *et al.* 1990).

The most striking phenotype of *ubc1Δ* strains is observed in the regulation of the cell cycle. Ubc1 is specifically required for viability during early stages of cell growth after germination (Seufert *et al.* 1990). Also, a recently published paper indicated that Ubc1 can be regulated by phosphorylation. This post-translational modification on Ubc1 affects its activity, and further enhances the tolerance to high temperatures in yeast (Meena *et al.* 2011).

Orthologs of Ubc1 in other species such as human, plants, and insects have been widely investigated. For example, UbcH1, the human ortholog of Ubc1, is involved in UV damaged DNA repair (Kaiser *et al.* 1994); Ubc1 is related to the stress response in higher plants, and also affects flowering time in *Arabidopsis* (Xu *et al.* 2009); knockdown of Ubc20, the ortholog of Ubc1 in *Caenorhabditis elegans* (*C. elegans*), results in arrested larval development (Jones *et al.* 2002). Together, this indicates that Ubc1 is highly related to the stress response and to cell cycle regulation. Nowadays, more details of yeast Ubc1 and its orthologs in other species have been discovered. In this thesis, we identified Ubc1 as required for Snf1 function.

1.7.5 The role of the ubiquitin conjugating enzymes Ubc4 and Ubc5 in yeast

Two highly homologous E2s, Ubc4 and Ubc5, are essential to the yeast ubiquitin cascade, like Ubc1, and are particularly involved in selective degradation of short-lived and abnormal proteins (Seufert and Jentsch 1990, 1991; Chen *et al.* 1993). They complement each other, therefore either Ubc4 or Ubc5 could be dispensable if the other one is absent (Seufert and Jentsch 1990; Stoll *et al.* 2011). They are strongly linked to the yeast stress response. Therefore, they are also potential candidates of interest that may target SNF1 kinase and affect its function. Ubc4 and Ubc5 are usually studied together, and yeast strains harboring the double *ubc4/5Δ* are viable, but have extremely slow

growth rates and exquisite sensitivity to elevated temperatures. As mentioned above, Ubc1, Ubc4, and Ubc5 constitute a subfamily of E2s. Overexpression of Ubc1 in the *ubc4/5Δ* mutant yeast strain can partially compensate for these phenotypes (Seufert *et al.* 1990). There are, however, subtle differences between Ubc4 and Ubc5, with Ubc4 showing the dominant role in some cases, including sporulation (Seufert and Jentsch 1990). In addition, *UBC4* mRNA transpation is found in exponential growing phase, whereas *UBC5* mainly expresses in stationary phase (Seufert and Jentsch 1990). Furthermore, only the deletion of *UBC4* in combination with *UBC1* is lethal, whereas *ubc1Δ ubc5Δ* double null mutant can survive (Seufert *et al.* 1990).

In mammalian cells, the main function of the Ubc4 and Ubc5 orthologs is via protein targeting and degradation. For example, epidermal growth factor receptor (EGFR) can be ubiquitinated by Ubc4/5 after internalization, and subsequently degraded (Eden *et al.* 2012). Also, Ubc5 contributes to the regulation of the transcription factor and tumor suppressor, p53. Suppression of Ubc5 activity inhibits p53 ubiquitination and degradation (Davis *et al.* 2013). Interestingly, an E3 ubiquitin ligase NEDD4.2 (neuronal precursor cell-expressed developmentally downregulated 4.2), an ortholog of Rsp5 (Reverses Spt-Phenotype 5) in mammalian cells, works closely with Ubc4/5 through its HECT (homologous to the E6-AP carboxyl terminus) domain (Stoll *et al.* 2011). Both genetic and cellular studies in yeast indicate that Rsp5 is the main E3 ubiquitin ligase that associates with Ubc4 and Ubc5 (Stoll *et al.* 2011), and there have been reports of interactions with Ubc1 also (Hiraishi *et al.* 2006; Stoll *et al.* 2011).

1.7.6 The role of E3 ubiquitin ligases in yeast

During ubiquitination, the last step involves a large variety of ubiquitin ligases (E3s). Humans have an estimated 1000 ubiquitin ligases, which provide protein target specificity. These E3s are classified into four major families, including HECT, RING-finger (really interesting new gene), U-Box, and PHD-finger (plant homeodomain). Particularly, HECT and RING-finger families constitute the majority of E3s (Finley *et al.* 2012). The RING-finger E3 family contains subfamilies such as APC and Skp1-Cullin-F-box (SCF) subfamilies, which are highly relevant to cell cycle regulation (Harkness *et al.* 2002; Harkness *et al.* 2004; Rodrigo-Brenni and Morgan 2007). In addition, the binding domains of these E3s provide the specificity to bind the substrates (Finley *et al.* 2012).

1.7.6.1 The yeast APC ubiquitin E3 ligase

The APC is an E3 ubiquitin ligase that selects target proteins involved in cell cycle regulation for degradation. In yeast, it is a multi-subunit protein complex containing 11 core subunits, with two co-activators, Cdc20 (cell-division cycle protein 20) and Cdh1 (Cdc20 holomogy), which bind the APC in a cell-cycle dependent manner (Zachariae and Nasmyth 1999). It is highly conserved in eukaryotic cells and is represented in a similar form and function in humans and other animals. The APC localizes to the nucleus, and its major function is to trigger the transition from metaphase to anaphase, by degrading specific proteins that inhibit sister chromatin separation (Jia *et al.* 2013; de Boer *et al.* 2016). As an example, Pds1 (precocious dissociation of sisters 1) and Clb2 (Cyclin B2) are APC targets that have to be degraded in order for cells to exit metaphase and reenter the cell cycle (Hilioti *et al.* 2001; Wang *et al.* 2001; Simpson-Lavy and Brandeis 2010; Machu *et al.* 2014). In yeast, four subunits of the APC complex (including Cdc16, Cdc27, Cdc23, and Apc5) consist almost entirely of repeating protein motifs, providing scaffolding and support to mediate protein-protein interactions within this multi-subunit enzyme complex (Thornton *et al.* 2006). Mutations in these key components of the APC complex led to decreased infinity for the substrates targeted for degradation and decreased activation of APC by upstream activators. In addition, there is evidence that Apc5 and Apc10 are required for yeast lifespan and stress response, which has the potential to connect to the SNF1 kinase functions in stress and aging (Harkness *et al.* 2004).

1.7.6.2 The yeast Rsp5 ubiquitin E3 ligase

Rsp5, and its mammalian counterpart, Nedd4.2, are essential ubiquitin ligases required for ubiquitination of a wide variety of proteins and biological functions. It is a member of the HECT superfamily of E3 ubiquitin ligases (Stoll *et al.* 2011). The literature has shown multiple roles for Rsp5 including contributing to intracellular trafficking of proteins (Becuwe *et al.* 2012), regulation of lipid biogenesis (Kaliszewski *et al.* 2006), and response to a variety of stresses (Stoll *et al.* 2011). Rsp5 contains a catalytic HECT domain (Krsmanovic and Kolling 2004), a C2 domain that recognizes and binds lipids and proteins, and three WW domains that are involved in various protein-protein interactions through a PY motif (Chang *et al.* 2000). In addition, Rsp5 has been shown to contain both a NLS and a nuclear export signal (NES), which allows it to shuttle

between the nucleus and cytoplasm and select targets in both subcellular locations (Cholbinski *et al.* 2011). The variety of domains and flexibility of the Rsp5 protein structure extends the ability of Rsp5 to bind substrates and consequently makes Rsp5 a key protein implicated in various critical pathways.

Protein quality control systems in eukaryotic cells protect them from the accumulation of abnormal proteins. Rsp5/Nedd4.2 has been identified as a major constituent of this quality control system. A well-known mechanism whereby Rsp5 functions in the protein degradation pathway is to remove heat-damaged, misfolded proteins (Fang *et al.* 2014; Sommer *et al.* 2014). Rsp5 targets cytosolic misfolded proteins for proteasomal degradation in respond to heat shock, allowing for cell survival after this damage. Loss of Rsp5 function results in a massive accumulation of misfolded proteins in the cell and a loss of cell viability. Rsp5 also targets misfolded proteins, which accumulate in the plasma membrane, through the ART (adaptor protein)-Rsp5 pathway. In this pathway, ART-Rsp5 clears the misfolded proteins and protects cell surface integrity (Zhao *et al.* 2013; Becuwe and Leon 2014).

In addition to protein quality control through proteasomal pathways, Rsp5 is also involved in autophagy (Shcherbik and Pestov 2011; Belgareh-Touze *et al.* 2017), endocytosis (Becuwe *et al.* 2012; Becuwe and Leon 2014), and the lysosomal degradation pathway (Hettema *et al.* 2004). Rsp5 has also been determined to play a role in regulating biogenesis of RNA at several steps. In detail, Rsp5 binds Rpb1 (RNA polymerase 1), the largest subunit of RNA polymerase II, through its WW domain, subsequently ubiquitinating and degrading it, thereby altering the activity of the polymerase (Huibregtse *et al.* 1997; Chang *et al.* 2000). Secondly, Rsp5 is involved in pre-RNA maturation and nuclear export. Research indicates that nuclear export of the pre-RNA S60 is affected in the *rsp5* mutant, and failed to transport from the nucleus to the cytoplasm (Neumann *et al.* 2003). Also, S35 pre-mature RNA is accumulated in the Rsp5 mutant, consistent with the RNA-processing defect (Neumann *et al.* 2003). In addition, Rsp5 can also modulate transfer RNA *(tRNA*) processing and transport (Neumann *et al.* 2003). All of these aspects indicate the essential role of Rsp5 in RNA biogenesis and processing.

Another well-established mechanism of Rsp5 in yeast is to modulate plasma membrane endocytosis through the arrestin-like protein Rod1 (Becuwe *et al.* 2012;

Becuwe and Leon 2014). Rod1 (resistance to o-dinitrobenzene 1) is known to be a cytosolic target for SNF1 kinase phosphorylation under low glucose conditions. It corresponds with endocytosis and vacuolar trafficking of Hxt1 and Hxt3, which function as high-capacity glucose transporters (O'Donnell *et al.* 2015). However, upon the return of abundant glucose, Rod1 is rapidly dephosphorylated and becomes a substrate for ubiquitination by Rsp5, which is a prerequisite for glucose transporter endocytosis and membrane insertion, allowing glucose to enter the cells. This mechanism reveals a coordinated action between Rsp5 and the SNF1 kinase in response to glucose signaling within the cells (Becuwe *et al.* 2012; Becuwe and Leon 2014).

1.8 Lifespan and stress response

1.8.1 Role of AMPK and SNF1 kinase in cellular stress response and aging

The cellular stress response pathways are highly conserved throughout eukaryotic cells, and many insights into these molecular mechanisms have been elucidated in yeast (Martegani *et al.* 1984; Hwang *et al.* 1998) and proven to be conserved in human cells and tissues (Woods, Johnstone*, et al.* 2003). The stress pathways are responsive and monitor environmental conditions through multiple sensor proteins including heat shock proteins or stress proteins (Hong and Carlson 2007; Morano *et al.* 2012). They quickly react to a variety of stresses including lack of nutrients, heat shock, hypoxia, or exposure to hyperosmolarity, and initiate a variety of responses to allow for cell survival. In mammalian cells, AMPK can be activated by a high AMP:ATP ratio such as during exercise, fasting, or muscle contraction (Andersson 2003; McGee *et al.* 2003; Backhed *et al.* 2007; Chiacchiera and Simone 2010); in eukaryotes such as yeast or plants, SNF1 kinase functions as an environmental sensor, responding to reduced glucose levels, dehydration and low light availability (Ludin *et al.* 1998; Kulik *et al.* 2012; Hsu *et al.* 2015). The role of the SNF1 kinase in cellular adaptation and survival in response to limited glucose/nonfermentable carbohydrates is particularly well elucidated (Hedbacker and Carlson 2008). SNF1 kinase quickly responds to nutrient limitation, and phosphorylation of the Snf1 subunit is essential for further activation of the kinase complex. Lack of the catalytic subunit Snf1 dramatically reduces the ability to respond to glucose limitation.

1.8.2 The SNF1 kinase is required for normal lifespan

The SNF1 kinase is also required for cell survival upon exposure to several other environmental stressors and to facilitate a normal lifespan. Quantitative measure of lifespan is possible in yeast using either the chronological lifespan (CLS) or replicative lifespan (RLS) assays, which measure distinctly different aspects of aging. CLS measures the survival of stationary phase (non dividing) cells, whereas RLS measures the number of daughters that can bud off from a single mother cell (Fabrizio *et al.* 2001; Fabrizio *et al.* 2004; Sinclair 2013; Postnikoff and Harkness 2014; Sutphin *et al.* 2014). CLS measures how long a cell population remains metabolically active without doubling/budding, and RLS defines the mitotic activity of individual yeast cells. Deletion of any single subunit of the yeast SNF1 kinase decreased maximal lifespan. For instance, the Sip2 β subunit of the SNF1 kinase has been proven to be a key regulator to effect yeast lifespan (Ashrafi *et al.* 2000; Lin *et al.* 2003; Lu *et al.* 2011). Deletion of the Sip2 β subunit results in a rapid aging phenotype with the average RLS reduced by 60% compared to a wild type (WT) isogenic yeast strain. However, the *sip2Δ* mutant exhibits normal growth on nonfermentable carbohydrates (Jiang and Carlson 1997). Deletion of the Sip1 β subunit, which localizes the complex to the vacuole, causes even greater lifespan reduction, to 80% of WT. The third β subunit, Gal83, is the subunit that co-localizes with the SNF1 kinase to the nucleus. Deletion of *GAL83*, or the single γ subunit, *SNF4*, does not exert significant effects on yeast lifespan. Surprisingly, research indicates that removal of the Snf4 subunit is able to rescue the shorted lifespan of *sip2Δ* mutant (Ashrafi *et al.* 2000).

1.8.3 The yeast FOXO orthologs, Fkh1 and Fkh2, contribute to the stress response

The forkhead box O (FOXO) transcription factors are a family of well-known stress sensors. The FOXO family of proteins are strongly conserved between yeast and higher eukaryotes and carry out similar roles (Greer, Dowlatshahi*, et al.* 2007; Greer, Oskoui*, et al.* 2007; Reynolds *et al.* 2012; Tullet *et al.* 2014). The FOXO protein orthologs regulate diverse biological processes including cell cycle control, metabolic regulation to environmental cues, apoptosis, and DNA damage repair. A large number of FOXO family members have been identified in higher eukaryotes, making it difficult to precisely

determine their respective contributions to cellular stress responses. In contrast, yeast has only four FOXO members, (Fkh1, Fkh2, Hcm1, and Fhl1) (Hollenhorst *et al.* 2000; Rodriguez-Colman *et al.* 2013), which present a chance to investigate their regulation in a greater depth. Fhl1 (forkhead-like 1) regulates ribosome biogenesis. Research indicates that Fhl1 binds to Ifh1 (interact with forkhead 1), a co-activator that binds promoter regions of ribosome mRNA, and further enhances their transcription (Martin *et al.* 2004; Mallick and Whiteway 2013). Hcm1 (high-copy suppressor of calmodulin 1) has a role in regulating cell cycle progression through G2 (Gap2 phase of cell cycle). It has also been determined to be an upstream transcription nal activator of the two remaining FOXO family member in yeast, Fkh1 and Fkh2 (Pramila *et al.* 2006; Rodriguez-Colman *et al.* 2010; Landry *et al.* 2014; Negishi *et al.* 2016). Fkh1 and Fkh2 (forkhead 1 and 2) are conserved with those in higher eukaryotes, and exhibit a strong degree of homology to each other (Hollenhorst *et al.* 2000). Genetic redundancy of these two genes in yeast means that many studies have to use yeast strains harboring a combined deletion. Indeed, double deletion of *FKH1* and *FKH2* affects cell morphology, cell cycle progression, longevity, and stress response (Hollenhorst *et al.* 2000; Postnikoff *et al.* 2012; Ostrow *et al.* 2014).

1.8.4 The yeast FOXO orthologs, Fkh1 and Fkh2, have a role in aging

Many models, including yeast, flies, and worms have found that FOXO family members extend lifespan when FOXO expression is increased. For example, the FOXO protein ortholog, DAF16, in *C. elegans* enhanced worm lifespan through changes to the insulin-signaling pathway (Greer, Dowlatshahi*, et al.* 2007; Tullet *et al.* 2014). Also, dFoxO, the ortholog in *Drosophila melanogaster* (*D. melanogaster*), has been shown to be a key regulator that modulates growth and proliferation of fruit flies *(*Puig and Tjian *2006;* Alic *et al. 2014)*. Conversely, mutations or deletions of FOXO proteins in multiple model systems resulted in reduced lifespan and diminished stress responses. In addition, AMPK directly phosphorylates and activates DAF16 in *C. elegans*, subsequently increases stress resistance and extends its longevity (Greer, Dowlatshahi*, et al.* 2007; Tullet *et al.* 2014). In yeast, a double deletion of both *FKH1* and *FKH2* dramatically impaired CLS and response to caloric restriction (Postnikoff *et al.* 2012). Besides, the double deletion cells were shown to be more sensitive to oxidative stress in stationary

phase, compared to the isogenic WT or single deletions (Postnikoff and Harkness 2012; Postnikoff *et al.* 2012).

1.8.5 The yeast FOXO orthologs, Fkh1 and Fkh2, are required for stress survival

The longevity of the cell depends on its own health status. It also depends on the response to the environment in which it lives. Fast and proper responses to environmental changes including, but not limited to, heat, nutrient limitation, and free radicals, assists the organism to survive. FOXO members play an important role in stress response in many species, and AMPK has also been proven to regulate stress responses and to work with FOXOs in mammalian systems (Greer, Oskoui*, et al.* 2007). This suggests the interactions between FOXOs and AMPKs are evolutionarily conserved. Under normal stress-free conditions, yeast FOXO members, Fkh1 and Fkh2, regulate cell-cycle progression by controlling the transcription of cell cycle dependent proteins such as Clb2 (Hollenhorst *et al.* 2000; Simpson-Lavy and Brandeis 2010). Under stress conditions, FOXOs have been proven in many model systems to enhance the activity of other proteins or enzymes necessary for DNA repair (Miyauchi *et al.* 2004) and normal cellular functions (Greer, Oskoui*, et al.* 2007). In yeast, the combined deletion of *FKH1* and *FKH2* generates a yeast strain with a reduced capacity to survive oxidative stress, similar to the growth defect of *snf1Δ* mutants (Postnikoff *et al.* 2012).

1.8.6 Enhanced AMPK activity promotes longevity in many organisms

AMPK has a tight connection with cellular longevity in all eukaryotes tested including yeast and mammals (Lu *et al.* 2011; Yao *et al.* 2015). Multiple studies from independent laboratories using yeast, worms, plants, and animals suggest higher AMPK activity promotes longer lifespan. In yeast, deletion of *SNF1* results in a dramatic decrease of both CLS and RLS (Ashrafi *et al.* 2000; Lin *et al.* 2003; Harkness *et al.* 2004; Lu *et al.* 2011). Many lifespan studies employ simple model systems such as yeast, worms, or fruit flies. Ease of quantitation and faster outcomes make these models powerful tools to study aging. Indeed, many key factors that affect aging have been found in these model systems. For instance, Aak-2 (AMP-activated protein kinase 2), the *C. elegans* ortholog of AMPK, has been shown to be required for extension of lifespan under states of caloric restriction (Lee *et al.* 2008; Schwarz *et al.* 2015). In plants, AMPK is necessary to maintain the

function and production of mitochondria, which is highly relevant to aging (Hartl and Finkemeier 2012). AMPK-induced mitochondrial biogenesis is suggested to slow down the aging process. In lower eukaryotes, but also in higher animals, there is a need for activated AMPK to slow down the aging process. Research indicates that activating AMPK in human cells can stimulate production and activation of Sirt1 (Sirtuin 1), which is found to increase in correlation with longer lifespans (Salminen *et al.* 2011; Lam *et al.* 2013). In addition, Sirt1 can also be activated by caloric restriction, which as noted above is associated with stimulation of AMPK activity (Lam *et al.* 2013).

1.9 The role of AMPK in human health and disease

1.9.1 A healthy lifestyle activates AMPK activity

AMPK has a role in human health, including correlations with diabetes, cancer, and aging. For example, due to its role in maintaining energy homeostasis, dysfunction of AMPK is linked with metabolic disorders such as insulin resistance/ Type 2 diabetes mellitus (DM2) (Owen *et al.* 1979; Bergeron *et al.* 1999; Arkun 2016). It is not known if blunted AMPK activity is a result of obesity and insulin resistance, or a cause contributing to the metabolic disorders (Baur *et al.* 2006; Coughlan *et al.* 2013; Ruderman *et al.* 2013; Arkun 2016). It is known that weight loss, insulin sensitizing medications, and exercise all activate AMPK with simultaneous health benefits (Musi *et al.* 2001; McGee *et al.* 2003; Ruderman *et al.* 2013). Interestingly, genome wide screening has not found AMPK mutations to be present in higher proportion in those individuals with metabolic syndromes (obesity, insulin resistance), suggesting that the dysregulation of AMPK activity may be at the post-translational level. Extended exercise helps to enhance AMPK activation and activity, further improving mitochondrial biogenesis (Reznick and Shulman 2006), increasing the rate of cellular glucose uptake, and lowering serum glucose levels (Bergeron *et al.* 1999; McGee *et al.* 2003). Paradoxically, constitutive activation of AMPK mediates glycogen accumulation in skeletal and cardiac muscles, which is associated with cardiac dysfunctions and is detrimental to health (Hunter *et al.* 2011). Activated AMPK also inhibits further cholesterol synthesis and storage, and releases glycogen stores from the liver for energy use, ultimately reversing the abundance of hepatic fat and diminishing the appearance of fatty liver (Baur *et al.* 2006; Takekoshi *et*

al. 2006; Ross *et al.* 2016).

Lifestyle changes improve diabetic outcomes and glucose control, and are first line therapies for the management of DM2 (Lam *et al.* 2013). In addition, recent studies indicate a novel role for AMPK in increasing blood supply to exercised muscles by stimulating and stabilizing neovascularization (Nagai *et al.* 2014), and angiogenesis (Ouchi *et al.* 2005; Neurath *et al.* 2006). All of these health benefits for humans are likely the results of increased AMPK activity during exercise or long-term training (Takekoshi *et al.* 2006). Furthermore, research on human skeletal muscle biopsies from healthy males found that even during a single round of high intensity acute exercise, AMPK appears to contribute to the energy homeostasis (Hoffman *et al.* 2015). Natural activating genetic mutants of AMPK have been found in pigs (interestingly, in the γ subunit) (Andersson 2003) that are prized for their lean yet heavy muscle mass. By extrapolation, this may infer that enhanced AMPK activity could benefit human health through regulation of adipose content within metabolically active skeletal muscle.

1.9.2 AMPK and human lifespan and health span

In human life, AMPK activity contributes to health at least at the cellular level. As a sensor, AMPK promotes autophagy, which functions to clear the cell of protein aggregates and recycle the fundamental components for reuse (Kim *et al.* 2011; Inoki *et al.* 2012). Once AMPK activity drops sharply, cells lose the ability to repair damaged DNA and clear dysfunctional proteins (Kim *et al.* 2011; Pineda *et al.* 2015). It has been found that the AMPK activity drops with aging, resulting in a loss of AMPK-dependent activity associated with maintaining normal cellular functions (Hardman *et al.* 2014). Evidence for the need of functional AMPK in human health comes from studies looking at the impact of dysfunctional AMPK in animal models and cell lines. These results further extend the knowledge of AMPK dysfunction, leading to other diseases that impact human life. For example, genetic and pharmacological manipulation on *C. elegans* AMPK showed that activated AMPK protects neurons from the dysfunction induced by human exon-1 huntingtin expression (Vazquez-Manrique *et al.* 2016). AMPK also works as a metabolic tumor suppressor, thereby preventing cancer development (Rutherford *et al.* 2016; Wang *et al.* 2016; Zou *et al.* 2016).

Our modern lifestyle chronically inhibits AMPK activity due to the overabundance

of nutrients and low physical activity, both of which hold AMPK in the repressed, un-phosphorylated state. This, in combination with aging, is worse for cellular and body health, or the AMPK system, than aging alone. Investigations have indicated that in mouse models, suppressed AMPK function reduces insulin sensitivity and produces inflammation, which may contribute to metabolic syndrome and its associated insulin resistant state (Baur *et al.* 2006; Coughlan *et al.* 2013; Ruderman *et al.* 2013). Metabolic syndrome, conversely, further inhibits AMPK activity, subsequently affecting human health (Ruderman *et al.* 2013). A globally prescribed medication, metformin, is a derivative of the French Lilac plant, and has been used to treat DM2. It is an effective insulin sensitizer, lowering blood glucose levels and effectively improving the metabolic balance. It was used for years before it was discovered that it indirectly activated AMPK, as detected by its phosphorylation, presenting a mechanism that may explain its benefits to metabolic dysfunction in humans.

CHAPTER 2 Hypothesis and Objectives

2.1 Rational

In the literature, encompassing multiple model systems, the regulated and dynamic control of the activation and activity of the heterotrimeric kinase enzymes AMPK and the AMPK-like family have been shown to be very important to affect normal cell functions. This enzyme class is necessary to maintain cellular and organismal health and lifespan while enduring changes in environmental stressors and altered energy needs. Furthermore, while it is acknowledged that the correct function and regulation of AMPK/AMPK-like activities is correlated with human health and metabolic regulation, it is challenging to study the underlying regulatory mechanisms to appreciate how to optimize this to improve clinical health outcomes, as research in humans is limited. The study of AMPK in human health is limited by the complexity of tissue expression (8 subunits preferentially expressed in different tissues), higher-level hormonal influences on AMPK activity and activation, and reversible post-translational modifications, among others. Research models including mouse and *in vitro* human cell culture have been used to investigate and elucidate the fundamental aspect of AMPK control, as have worms (*C. elegans)*, plants, and zebrafish (*Danio*), all of which struggle with tissue-specificity and subunit composition. Yeast have proven to be a powerful tool to study the fundamental underlying mechanisms regulating the activation and activity of AMPK, referred to as the SNF1 kinase in *S. cerevisiae.* Using this model, there is a simplicity of subunit options (a single catalytic α subunit, single regulatory γ subunit, and three defined β regulatory subunits), no tissue-specific alterations, and no hormonal influences.

Using this as a model to study mechanisms of yeast AMPK regulation, we investigated the role of the ubiquitin system, including the specific UBA motif within the catalytic α subunit, on yeast SNF1 kinase regulation. We asked if we could identify the E2 and E3 enzymes that are involved in the previously reported ubiquitination of the Snf1 α subunit, and the fundamental regulatory role of the highly evolutionarily conserved UBA domain.

UBA domains can influence protein-protein associations (such as within the kinase complex itself) or enzyme activities in response to external ubiquitination signals. Mutations within the yeast Snf1 UBA domain had been done previously (Momcilovic and

Carlson 2011) and suggested that it may have an inhibitory role, yet mutations within the human UBA domain showed different results (Jaleel *et al.* 2006; Cho *et al.* 2014). We directly addressed this through several complementary techniques. Next, we set out to screen all yeast E2s and selected E3s for their role in regulating SNF1 kinase activity. We felt this was a particularly important goal as targeted protein ubiquitination can alter stability, nuclear location, protein associations, and enzyme activity, any of which is relevant to controlled SNF1 kinase.

2.2 Hypothesis

We hypothesize that ubiquitin binding motifs and ubiquitin-conjugating enzymes play distinct roles in the regulation of the yeast SNF1 kinase/AMPK.

2.3 Objectives

1. **Determine the regulatory role of the ubiquitin-associated (UBA) motif within the Snf1 catalytic subunit.**
2. **Screen the yeast ubiquitin conjugating enzymes for their regulatory role in SNF1 kinase activation and activity.**
3. **Determine the role of the ubiquitin ligase Rsp5 in SNF1 kinase activation and activity.**
4. **Determine the protein stability of SNF1 kinase subunits under enzymatically activating and repressive conditions and upon destruction of ubiquitin-attachment sites.**

CHAPTER 3 Material and Methods

3.1 Whole Protein Extraction

Cells were grown to logarithmic phase ($OD_{600} \leq 1$) when harvested in either YPD (1% yeast exact, 2% peptone, and dextrose media) or selective medium. To extract whole protein, cells were pelleted and mechanically broken using glass beads in the presence of radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris, 150 mM sodium chloride, 0.1% Sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1% Triton X-100), protease inhibitor, and phosphatase inhibitor. Cells were pelleted again, supernatant was collected and kept frozen in -80°C freezer (Harkness *et al.* 2002).

3.2 Western Blot Analysis

Whole protein concentration from yeast cells was measured using the commercially available Bradford assay and a standard curve. Samples with equal amounts of total protein were routinely applied to either 7.5% or 10% acrylamide gels for separation. After the proteins were sufficiently separated, they were transferred to a nitrocellulose membrane. The membrane was cut into pieces if multiple targets were being simultaneously probed for, depending on the size of target proteins of interest. These membranes were incubated in blocking buffer (3% Bovine serum albumin (BSA) or 3% skim milk, 1 hour), selected primary antibodies (4°C overnight), and secondary antibody for detection (1 hour). Tris-buffered saline (TBS) with 1% Tween-20 (TBST) was used to wash off the primary and secondary antibodies from the membrane (15 minutes for 4 times) before exposure to Enhanced Chemiluminescence reagents (ECL). The chemiluminescence signal was captured on a VersaDoc (BIO-RAD) molecular imager and quantitation performed with VersaDoc software (Quantity One 4.6.9).

3.3 Protein Stability Assay

To inhibit protein synthesis, 10 μg/mL cycloheximide in 95% ethanol (CHX) was added to dividing early-logarithmic phase cells in YPD medium. Equal cell numbers were collected every 30 minutes for cell lysate preparation and subsequent Western Blot analysis.

3.4 Fluorescence Microscopy

Fluorescence microscopy was used to detect green fluorescent protein (GFP) signal in whole living yeast cells. Selective media lacking histidine (His-) or complete medium (CM) was used for strains containing plasmid-expressed Snf1-GFP or endogenous Snf1-GFP, respectively. To determine the subcellular localization of Snf1-GFP or Mig1-GFP, logarithmically growing cultures were divided between non-activating conditions (2% glucose) versus activating conditions (5% glycerol) for 30 minutes. Live cells were stained with mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) for immediate DNA visualization. All images were captured using a 100x objective (oil). To determine the subcellular localization of Hcm1-GFP, cells were arrested in G1 (Gap1 phase of cell cycle) using α factor. Images were taken every 30 minutes from release of G1 arrest. Cells were viewed with an Olympus BX51 fluorescence microscope 100x objective equipped with an Infinity 3-1 UM camera. Images were collected using Infinity Analyse software version 5.0. A minimum of 125 cells for each strain and condition were consecutively scored for co-localization of the GFP-tagged subunits and DAPI nuclear staining.

3.5 Invertase Assay

Yeast strains were grown to early-logarithmic phase in 2% YPD. 1 x 10^6 cells were removed to ice from the 2% glucose sample, based on optical density (an OD_{600} of 1 is approximately equivalent to 2 x 10^7 cells). The remaining cells were washed and resuspended in 0.05% YPD for 2 hours. 1 x 10^6 cells were again removed as the low glucose, activated samples. Colorimetric measurement of invertase activity (glucose production from sucrose) was performed, with the following parameters. 50 µl cell suspensions had 0.5 M sucrose (12.5 µl) added for 10 minutes at 37°C, before stopping the reaction with 75 µl 0.2M dipotassium monohydrogen phosphate (K_2HPO_4). 500 µl Assay Mix (50 µl of 5000 U/mL glucose oxidase, 62.5 µl 1 mg/mL peroxidase, and 375 µl 10 mg/mL *o*-dianiside (in 95% ethanol) into 25 ml 0.1 M potassium phosphate buffered to pH 7.0) was added to start the color reaction. After 20 min at 37°C the color was developed with 500 µl 6 M hydrogen chloride (HCl). OD_{540}, blanked to a no sucrose control, gave values used to calculate invertase activity, reported as mM glucose converted/minute/10^6 cells. Activity was normalized (value of 1) to that of 2% glucose

WT in each biological repeat. Statistical analysis was done using PRISM Version 6.0b software and 2-way ANOVA (Harkness and Arnason 2014).

3.6 2-hybrid Analysis

The yeast 2-hybrid reporter strains (WT and its modified versions *ubc1Δ*, or *rsp5*^{ts}) were doubly transformed with pairs of empty vectors (negative control: pGAD-C2 and pGBD-C2), the same backbones expressing target genes, regarding to the different manuscripts. 1x10^5 cells of logarithmic cultures (in 3 µl volumes) of each transformation sets were repeatedly spotted on the glucose gradient of the slant plates, and grown at 30°C until colonies were established. The images were scanned before and after color development. The glucose gradient slant plates involved sequential stacking and cooling of 20 ml 2% glucose (bottom) and 0.05% glucose (top) media poured at ~30° slant into petri plates. Selective medium lacking leucine and uracil (Leu$^-$Ura$^-$) was used in both layers for dual plasmid maintenance. Plates were equilibrated overnight prior to use. Freshly prepared warm liquid X-Gal agarose overlay medium was layered to completely cover cells, solidified and incubated at 30°C and images scanned again after color development.

3.7 Transformation of Yeast Strains

Yeast transformation was used to generate genomic mutations either using polymerase chain reaction (PCR) products or plasmids that express proteins of interest, following the LiAc-DNA-PEG protocol. For details, cells of parental strains were pelleted from overnight YPD culture, resuspended with 100mM lithium acetate (LiAc), and incubated at 30°C for 10 minutes. Cells were pelleted again, added target DNA, 50% polyethylene glycol (PEG) and100mM LiAc, then incubated at 30°C for at least 20 minutes. After incubation, heat shock cells at 42°C for 5 minutes and quickly put on ice. Cells were pelleted and resuspended with selective media, then grew on agar plates in 30°C incubator. Only cells containing transformed plasmids or mutations can grow on selective media plates.

3.8 mRNA Expression Analysis

Total RNA was isolated (RNAeasy Kit, Qiagen) from logarithmically ($OD_{600}\leq0.4$) growing cell cultures, followed by reverse transcription (RT) (QuantiTect Reverse

Transcription Kit, Qiagen). 500 ng cDNA (complementary DNA) of each sample was used as template in amplification reactions and equal reaction volumes were retrieved. Abundance of PCR products for interested genes was normalized to signal for ribosomal RNA (*rRNA*) at 26 cycles. VersaDoc (BIO-RAD) quantitation was obtained from RedSafe nucleic acid stain signal (FroggaBio). Statistical analysis was done using PRISM Version 6.0b software and 2-way ANOVA.

3.9 Analysis of Stress Resistance

The *snf1Δ fkh1Δ fkh2Δ* strain was created by genetic crossing (*snf1Δ* [YTH1510] × *fkh1Δ fkh2Δ* [YTH2578]). Tetrads were picked and markers scored on G418 (Geneticin) and YPSuc (1% yeast extract, 2% peptone, 2% sucrose media) plates, with triple deletions confirmed by PCR. Oxidative stress was induced by exposure to hydrogen peroxide (H_20_2). The indicated strains were grown to late stationary phase in CM, with equal cell numbers left untreated, or treated with 100 mM H_20_2 for 60 minutes at 30°C. All cells were washed, then plated on YPD for 2 days at 30°C before scoring survival as a percentage compared to untreated.

3.10 Cell Cycle Arrest

To arrest cells in G1 phase, α-factor was added to asynchronous (Asy) early-logarithmic phase cell culture in YPD (pH 3.5) for 2 hours (with a final concentration of 4 μg/ml). G1 arrested cells demonstrated >90% with the expected G1 cell morphology (pear shaped) under light microscopy. Cells were released by washing away the α-factor in fresh YPD. Equal volume cell samples were collected each 30 minutes for protein extraction and/or fluorescence-activated cell sorting (FACS) analysis. Note that only strains with a mating type of Mata can be arrested by α-factor.

3.11 Flow Cytometry (FACS)

Yeast strains of interest were grown to logarithmic phase (OD_{600} of 0.4), 8 $x10^5$ cells (1 ml) were pelleted and washed with 50 mM Tris buffer (pH8.0), then resuspended in 1 mL 70% ethanol overnight at room temperature to fix. Cells were pelleted and resuspended in 500 μl of 50 mM Tris buffer and digested with 10 μl 10 mg/ml RNaseA at 37°C for 2 hours. Propidium iodide (10 μg/ml) was added to cells for ≥1 hour at room

temperature away from light. FACS was performed using the EPICS® XL and data was analyzed with Flowjo software (v10.0.7).

Statements

My contribution to the manuscript chapters in this thesis includes but not limited to 1) designing the experiments, 2) performing the experiments, and 3) collecting and interpreting the data of the following figures:

Chapter 4

Figure 4.1

Figure 4.2

Figure 4.3B

Figure 4.4C

Figure 4.5

Figure 4.6

Chapter 5

Figure 5.1A, 5.1B

Figure 5.2

Figure 5.3

Figure 5.4

Figure 5.5

Chapter 6

Figure 6.1C

Figure 6.2

Figure 6.3

Figure 6.4

Figure 6.5

Figure 6.6

Figure 6.7

Figure 6.8

Figure 6.9

Chapter 7

Figure 7.2

Figure 7.3

Figure 7.4

Figure 7.5

Figure 7.6

Figure 7.7

The results reported within each manuscript chapter support the thesis hypothesis and satisfy the objectives presented within Chapter 2. These manuscripts extend the knowledge of the ubiquitination system in the regulation of the SNF1 kinase. The manuscripts are independent investigations yet the contents are related and supportive. Chapter 4 focused on the UBA domain that potentially binds ubiquitin within the Snf1 subunit; Chapter 5 investigated the stoichiometry of the SNF1 kinase complex subunits and the potential ubiquitination-degradation pathway components that affects their stability; Chapter 6 and 7 focused on individual ubiquitin cascade enzymes and their roles in regulating SNF1 kinase activation and activity. In addition, these manuscripts document the development of the yeast 2-hybrid assay methodology using slant plates, which extends this classic technique to study protein-protein interaction on the same plate under different conditions.

CHAPTER 4 The ubiquitin-associated motif restrains SNF1 kinase activity[1]

[1]**This work has been published as**

The SNF1 Kinase Ubiquitin-associated Domain Restrains Its Activation, Activity, and the Yeast Life Span

Jiao R, Postnikoff S.D, Harkness T.A, Arnason T.G.

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4.1 Introduction

The ability to shift between feasting and fasting is a central and essential metabolic adaptation that impacts biology at every level (Owen *et al.* 1979). Abundant nutrients can trigger reproductive readiness in multi-cellular organisms, and promote cell growth and division in single-celled organisms, by switching from catabolic to anabolic metabolic pathways through the simple flipping of ON and OFF switches (Hardie 1999). One switch that is active under times of energy depletion, such as during fasting, exercise, or oxygen depletion is a non-hormonal central energy sensor and regulator known as the AMPK, and its yeast ortholog, SNF1 kinase (Carlson *et al.* 1981; Hardie 1999). All AMPK family members share distinct evolutionary homology transcending primary sequences, tertiary protein folding and quaternary protein associations of the heterotrimeric kinase complex (Rudolph *et al.* 2005; Elbing *et al.* 2006; Amodeo *et al.* 2007). The yeast SNF1 kinase contains the catalytic Snf1 (α) subunit, the regulatory Snf4 (γ) subunit, and one of three β-subunits Sip1, Sip2 or Gal83; the β-subunits bridge the α and γ subunits in the complex and individually direct the subcellular location of the kinase (Jiang and Carlson 1997; Vincent *et al.* 2001). The SNF1 kinase is important for the yeast stress response (Hardie *et al.* 1998) and facilitates adaptation to glucose limitation, regulating the activity of metabolic enzymes and the transcription of metabolic genes. In addition to metabolic effects, this family of kinases has also been found to protect against oxidative stress, and to impact the lifespan of model systems (Ashrafi *et al.* 2000; Harkness *et al.* 2004; Greer, Dowlatshahi*, et al.* 2007; Wu and Wei 2012). As an enzyme complex that must efficiently respond to metabolic signals, its activity incorporates the rapid and reversible phosphorylation of its catalytic α subunit by well defined upstream kinases (Pak1, Elm1 and Tos3) (Sutherland *et al.* 2003) and phosphatases, with a major contributor being protein phosphatase I; Reg1-Glc7 (Celenza and Carlson 1986; Ludin *et al.* 1998; Woods, Vertommen*, et al.* 2003; Momcilovic and Carlson 2011). Further layers of regulatory finesse include the reversible physical allosteric association between the α and γ regulatory subunits and the nuclear translocation of the active trimeric complex to alter gene expression that aims to preserve the energy balance of the cell (Jiang and Carlson 1997; Adams *et al.* 2004; Momcilovic and Carlson 2011). Also conserved is acetylation and ubiquitination, recently reported for

both mammalian AMPK and yeast SNF1 kinase complexes (Baskin and Taegtmeyer 2011; Lu *et al.* 2011; Wilson *et al.* 2011; Zungu *et al.* 2011), which appear to have inhibitory roles in regulation of the kinase. Similarly, SUMOylation has been shown to be a negative regulator of Snf1 (Simpson-Lavy and Johnston 2013), clearly adding to the complexity of regulation of this central energy-sensing switch.

The current epidemic of lifestyle related obesity, insulin resistance, and DM2 is overwhelming healthcare systems and budgets. The recommended lifestyle changes all stimulate AMPK; exercise, caloric restriction/weight loss, and muscle contraction all activate human AMPK with clinical metabolic benefits of improved glucose control and lowered cholesterol (Chen *et al.* 2000; Musi *et al.* 2001; Greene *et al.* 2012). In addition, a first line agent to treat DM2, metformin, indirectly results in AMPK activation (Hardie 2006; Suwa *et al.* 2006; Zhang *et al.* 2012) as well as newer evidence of direct metformin influences on AMPK (Zhang *et al.* 2012). Therefore, it is of great interest to understand how AMPK is regulated to gain insight into how this might be used to improve clinical health.

The catalytic α subunits of the AMPK and AMPK-like family share very strong domain structure between all eukaryotes, including the yeast Snf1 (Rudolph *et al.* 2005; Amodeo *et al.* 2010; Rudolph *et al.* 2010). Relevant to this report is the presence within the majority of α subunits of a consensus motif for a UBA domain (Jaleel *et al.* 2006; Momcilovic and Carlson 2011), immediately adjacent to the catalytic domain. The UBA domains are loose primary sequence motifs of ~45 amino acid resudies that are found in many protein families throughout eukaryotes that fold independently into stable surface structures (Mueller *et al.* 2004). Their role continues to be clarified, but includes the ability to bind to ubiquitin in a linkage-specific (Class 1; lysine-48 and Class 2; lysine-63) or non-selective (Class 4) manner via non-covalent associations between the hydrophobic UBA protein face and the globular head of the ubiquitin moiety. Ubiquitin-binding UBA domains contribute to target proteolysis (lysine-48 linkages) or to cell trafficking (lysine-63), yet 25% of budding yeast proteins with UBA domains, including the yeast Snf1, have not been shown to bind ubiquitin of any linkage type, defined as Class 3 (Raasi *et al.* 2005). The importance of the UBA domain to SNF1 kinase activity and activation in yeast may clarify the importance of the role of ubiquitin in regulating yeast and higher

eukaryotic AMPK. To date, complete deletion and point mutations within the yeast UBA domain resulted in a modest activating effect under both repressive and activating conditions (Momcilovic and Carlson 2011). We report here the influence of mutations within conserved residues of the Snf1 UBA domain on the activity, activation, and allosteric associations between the Snf1 and the Snf4 subunits of the yeast SNF1 kinase, in addition to its influence on lifespan and oxidative stress resistance.

4.2 Materials and Methods

4.2.1 Creation of *Snf1^{UBA}* constructs

Snf1^{UBA}-GFP: *genomic Snf1 α-subunit with UBA mutations and C-terminal GFP tag.* Two point-mutations (G357A, L367I) were introduced by PCR mutagenesis into the Snf1 α-subunit UBA motif. Genomic DNA (gDNA) from the Snf1-GFP::His3 strain (YDR477W; Life Technologies) was used as a PCR template. Two PCR products homologous to the mutated UBA sequences and extending outside the full length *SNF1* gene to include the GFP epitope and *HIS3* sequence were generated; [Snf1^{-500} 5'-TTGCGTGGCGGCGTGGATC] and [*Snf1^{UBA}* 5'-GTCTTTTTCGTA**CG**CCATGGT**G**G-ATGATAAAA] with [*Snf1^{UBA}* 5'-TTTTATCATC**C**ACCATGG**CG**TACGAAAA-AG] and Snf1^{+500} 5'- GGAACGTAAACACACCCGC] purified and co-transformed into the *snf1Δ* (YTH1510; *snf1*::G418) strain for integration and selected on His^{-} plates. Confirmation was by DNA sequencing.

Snf1-GFP: *genomic SNF1 α-subunit with WT sequence and C-terminal GFP tag.* The *SNF1-GFP-HIS3* sequence was amplified from YDR477W (*SNF1-GFP*) using non-mutagenic primers that flanked the gene locus by 500 bp (Snf1^{-500} and Snf1^{+500}) and homologously recombined into *snf1Δ* by transformation to create an isogenic Snf1-GFP.

Snf1^{UBA}-HA*: 2-micron plasmid expressing Snf1 α-subunit with UBA mutations and C-terminal HA tag.* YTH1510 was doubly transformed with both the linearized HA-tagged Snf1 plasmid (2-micron, TRP^{+}: a kind gift from M. Schmidt (Schmidt *et al.* 1999)) with an internal *SNF1* sequence deletion (BglII-NcoI digest) and the amplified linear PCR product containing full-length *SNF1^{UBA}* coding sequence. Yeast plasmid and genomic DNA was retrieved (Adams and Kamakaka 1999) from TRP^{+} colonies, and

transformed into XL-1 *E. coli* to select for re-circularized AMP^+ plasmids. *SNF1*^{UBA}*-HA* presence was confirmed by sequencing.

2 hybrid Snf1^{UBA}, Snf1^{WT}, and Reg1 plasmids: The *SNF1*^{UBA} full length sequence was amplified using *SNF1*^{UBA}*-HA* as template [forward: 5'- ATCGATGCGAATTCA-GCAGTA and reverse: 5' ATCGCTCGAGTCAATTGCTTT] digested with Pst1 and EcoRI and ligated in-frame into the pGBDU-C_2 2 hybrid plasmid (a gift from S. Fields (Fields and Song 1989)). *Reg1* sequences were amplified from genomic DNA and cloned by EcoRI-BlgII restriction digest ligation into pGAD-C_2 [Reg1 forward: 5' ATCGATGCGAATTCTCAAATCTAGCA and Reg1 reverse: 5' AAATTCTTAGATC-TAACTGCTGTCATTTC].

4.2.2 Creation of *FKH* and *SNF1* deletion strains

All strains were based on S288C. Individual cassettes for *snf1*::*kanMX6*, *fkh1*::*kanMX6* and *fkh2*::*kanMX6* were amplified with 500 bp up and downstream. Integration was into YTH4269 with selection for Kan^{res} and PCR confirmation. Combinations were created by crossing and tetrad dissection, scoring for markers, phenotypes, and confirmed by primer-specific amplification.

4.2.3 Total protein extraction and Western Blot analysis

The yeast strains used in this study are shown in Table 4.1. Protocols have been previously described in sections 3.1 and 3.2.

4.2.4 Invertase assay

Protocols have been previously described in section 3.5.

4.2.5 Fluorescence microscopy

Protocols have been previously described in section 3.4. In this study, fluorescence microscopy was used to visualize the subcellular localization of Snf1-GFP and *Snf1*^{UBA}-GFP. A minimum of 150 cells for each strain and condition were consecutively scored for co-localization of the GFP-tagged subunits and DAPI nuclear staining.

4.2.6 2-hybrid analysis

Protocols have been previously described in section 3.6. In this study, the yeast 2-hybrid reporter strain was doubly transformed with pairs of empty vectors (-ve control: pGAD-C_2 and pGBDU-C_2), the same backbones expressing unmodified Snf1 and Snf4 subunits (+ve control), or *Snf1*^{UBA} and Snf4 or Reg1.

4.2.7 Analysis of lifespan and stress resistance

Protocols have been previously described in section 3.9.

4.2.8 *SNF1* and *SNF4* expression analysis

mRNA expression analysis protocol has been previously described in section 3.8. In this study, RNA was isolated (RNAeasy, Qiagen) from logarithmically growing Snf1-GFP or *fkh1/2Δ* Snf1-GFP yeast strains that were transformed with empty vector (-, YEp24), or HA-pFkh1 or HA-pFkh2 (Hollenhorst *et al.* 2000) followed by RT. cDNA was used as template in amplification reactions and equal reaction volumes retrieved after the indicated number of cycles. Abundance was normalized to signal for *rRNA* or *TUB1* (α-Tubulin) at 26 cycles. VersaDoc quantitation was obtained from RedSafe nucleic acid stain signal (FroggaBio).

4.3 RESULTS

4.3.1 Generation of a Snf1 construct with a mutated UBA domain

To gather a greater understanding of SNF1 kinase regulation and function in yeast, we focused on the role played by the UBA domain, a loose linear sequence present in yeast and mammalian AMPK-related kinase α subunits that fold into a conserved tri-helical structure characterized by an hydrophobic face. Such UBA surfaces can associate non-covalently with the globular head of ubiquitin or poly-ubiquitin chains, yet this function has not yet been detected for the AMPK-related kinase class of human proteins (Jaleel *et al.* 2006), nor reported for the yeast α subunit, Snf1 (Raasi *et al.* 2005). It has been recently published that mutations and deletions within the yeast Snf1 UBA sequence led to modest increases in phosphorylation of the SNF1 kinase itself and of an *in vitro* target, suggesting a natural inhibitory role of the UBA domain under normal conditions (Momcilovic and Carlson 2011). The two amino acid residues selected for conservative

replacement in our studies (Gly357 and Leu361) were based on their high degree of conservation within UBA motifs in general (Raasi *et al.* 2005), as well as within the AMPK family specifically (Jaleel *et al.* 2006).

We replaced two highly conserved amino acid residues within the UBA domain of Snf1 (Snf1^{UBA}) that spans residues 348-389 (Fig. 1A). The mutated glycine residue Gly357 (*, G357A) and Leu367 (◆, L367I) (Fig. 4.1A) residues are adjacent on the hydrophobic face of the crystallized UBA motif (Jaleel *et al.* 2006) and are present in the majority of AMPK-like kinases (Fig. 4.1A). A striking exception is the absence of a convincing UBA domain consensus in human α subunits AMPKα1/α2 that lacks Gly357 yet retains the Leu367 residue. The Gly357 residue was independently mutated in the human AMPK-like study (Jaleel *et al.* 2006) and different conserved mutations were previously introduced within yeast Snf1 (Momcilovic and Carlson 2011).

4.3.2 Activation of the Snf1^{UBA} mutant is greater than wild type

Endogenous unmodified Snf1-GFP and the mutant Snf1^{UBA}-GFP were expressed at similar levels and grew equivalently on sucrose, a nonfermentable carbon source that requires SNF1 kinase function for utilization (Fig. 4.1B and C). This is a phenotype of the *snf1*Δ mutant that led to its yeast nomenclature (sucrose non-fermenting). An HA-tagged version of the same UBA mutation expressed from a high copy plasmid (Snf1^{UBA}-HA) is similarly able to complement for growth on sucrose (data not shown). The relative activation of Snf1^{UBA} was compared to WT by assessing: i) activating phosphorylation of Snf1 Thr210 (Thr(P)-210); ii) nuclear import efficacy under activating conditions; and iii) the levels of allosteric subunit associations between the Snf1 and Snf4 subunits. Activating conditions (0.05% low glucose, L) revealed a pervasive increase in the Thr(P)-210 phosphorylation of Snf1^{UBA} over Snf1 under both activating and repressive conditions for the endogenous (Fig. 4.2A) and plasmid-expressed (Fig. 4.2B) UBA versions. This trend towards activation is consistent with the outcome from unrelated mutations within the yeast UBA consensus that included both a full UBA deletion (residues 347-398) as well as the combined mutation of methionine Met356 and tyrosine Tyr358, or Leu385 alone (Momcilovic and Carlson 2011).

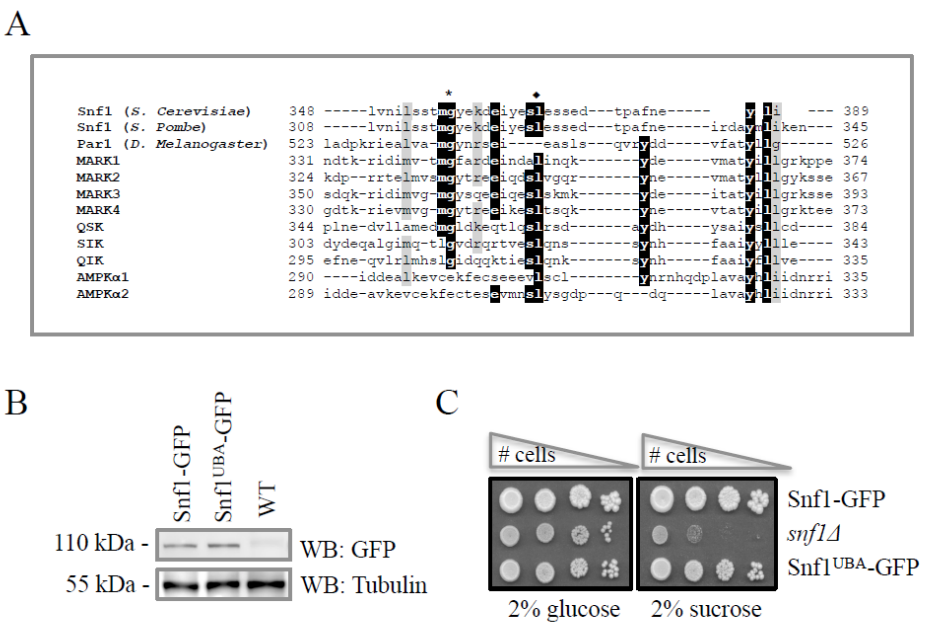

Figure 4.1 The Snf1 UBA domain mutation characteristics. (A) Amino acid sequence alignment of the α-subunit UBA domain of Snf1 to UBA domains within other AMPK family protein kinases. Single letter amino acid code. Numbering reflects amino acids within each respective UBA motif. Identical residues in black, conserved in grey. The two amino acid substitutions present in this study within the UBA domain are indicated: *G357A; ◆L367I. (B) Endogenous protein expression of genomic GFP-tagged unmodified Snf1 (Snf1-GFP), UBA mutated (Snf1^{UBA}-GFP), or untagged-parental strain (WT). (C) Spot dilutions demonstrating the growth characteristics of Snf1-GFP, Snf1^{UBA}-GFP and *snf1Δ* isogenic strains on SNF1 kinase repressive (2% glucose) and activating (no glucose, 2% sucrose) YP medium.

Activated SNF1 kinase relocates to the nucleus rapidly after stimulating conditions are introduced (Vincent *et al.* 2001). The impact of UBA motif mutations on this mechanism was tested by making use of the Snf1-GFP and Snf1^{UBA}-GFP fusion constructs, and fluorescence microscopy. The Snf1-GFP subunit was efficiently shuttled to the nucleus within 20 minutes following stimulation (82%), as was the case with Snf1^{UBA}-GFP (90%) (Fig. 4.2C and D). The greater degree of phosphorylation observed for Snf1^{UBA}-GFP under repressive conditions (2% glucose) does not result in a detectable increase in nuclear *Snf1^{UBA}* localization, demonstrating that the phosphorylated Snf1^{UBA} remains appropriately cytosolic under these conditions (Fig. 4.2D). It remains possible

that Snf1^{UBA} was shuttled at an increased rate over that of Snf1 that is beyond our capability to detect.

Activation of yeast and AMPK trimeric complexes also involves the allosteric juxtapositioning of the Snf1-Snf4 subunits, and the strength of their associations can be measured using the yeast 2-hybrid system (Fields and Song 1989); β-galactosidase production correlates with the strength of the associations between the two proteins tested. Increased 2-hybrid associations were observed between Snf4 and the Snf1^{UBA} at all glucose concentrations, and visibly exceeded the maximum associations detected between unmodified Snf1 and Snf4 (Fig. 4.2E). Together these data provides evidence that the wild type UBA domain functions to inhibit SNF1 kinase activation at least in part by hindering α-γ associations.

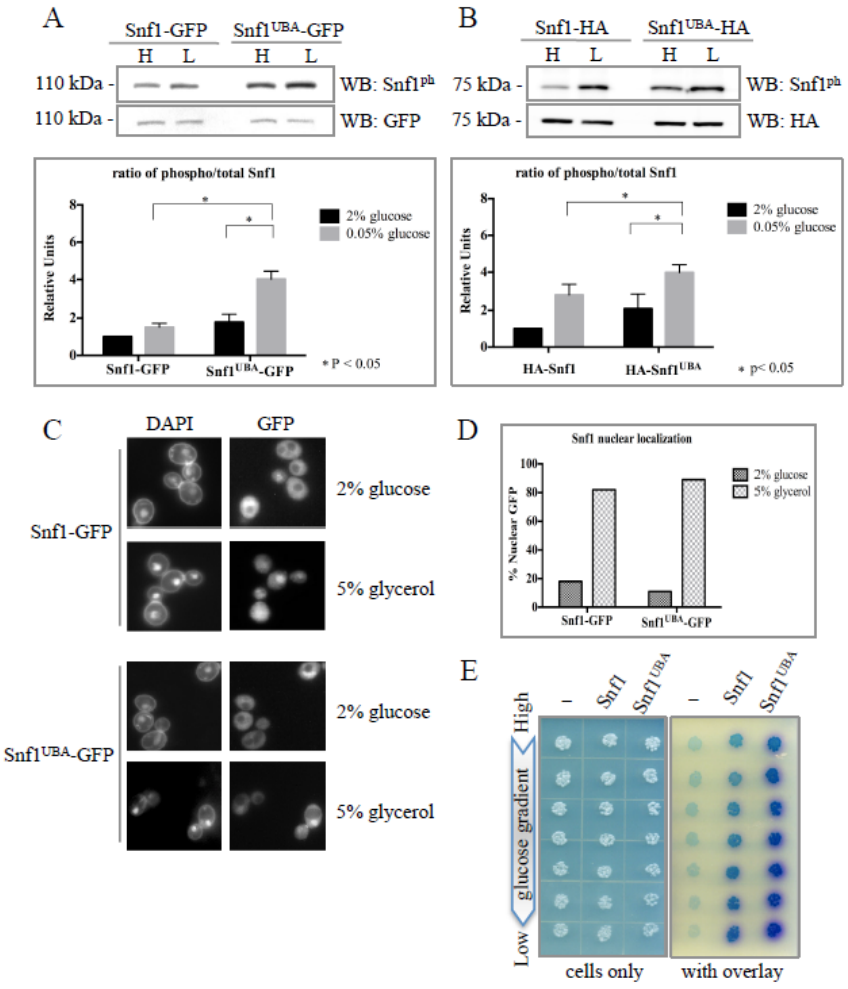

Figure 4.2 The UBA mutation increases SNF1 kinase activation. The activating Snf1 subunit Thr210 phosphorylation ($Snf1^{ph}$) is enhanced by the $Snf1^{UBA}$ mutation in both repressive high glucose (H: 2%) and activating low glucose (L: 0.05%) growth media when (A) genomically expressed under the endogenous promoter and (B) when expressed from a high copy 2-micron yeast plasmid in a *snf1Δ* strain background. The Mean ± S.D. of 4 biological repeats is shown, normalized to 2% glucose WT. 2-way ANOVA (Prism 6.0b) indicated significant differences (* $P<0.05$). (C) Subcellular fluorescent location of GFP-tagged Snf1 and $Snf1^{UBA}$ under activating (5% glycerol) and repressive (2% glucose) conditions. DAPI: DNA/nucleus location. (D) 150 cells per condition and strain, consecutively scored for co-localization of DAPI and GFP signal, expressed as % nuclear. (E) Solid support 2-hybrid associations on glucose gradient plates (H: 2%; L: 0.05% glucose) between the Snf4 subunit and either unmodified Snf1 or $Snf1^{UBA}$ before and after β-galactosidase overlay. (-); Negative control using empty 2-hybrid vectors.

4.3.3 $Snf1^{UBA}$ mutant activity is greater than wild type

Next, we analyzed the effect of the UBA mutation on SNF1 kinase enzymatic activity by indirectly measuring its ability to target and phosphorylate the transcriptional repressor Mig1 (Treitel *et al.* 1998) as compared to unmodified Snf1. Upon activation, phosphorylated and nucleus-shuttled Snf1 targets Mig1 for phosphorylation to release glucose-dependent repression of *SUC2*, which encodes the invertase enzyme. Once active, invertase catalyzes the quantifiable biochemical conversion of sucrose to glucose and fructose (Treitel *et al.* 1998). We show that $Snf1^{UBA}$-GFP cells exhibit increased invertase activity most notably under activating conditions compared to unmodified Snf1-GFP (Fig. 4.3A). This is correlated with a detectable increase in the overall intensity of the $Mig1^{ph}$ signal (Fig. 4.3B) whereas the associated $Mig1^{ph}$ "super-shift" is only seen in low glucose.

We then measured the impact of the mutated UBA motif on yeast RLS to determine if this homeostatic endpoint is also affected by SNF1 kinase activity. RLS measures the mitotic capacity of yeast cells by determining the number of daughter cells a single mother can produce (Kennedy *et al.* 1994). We previously demonstrated that Snf1 plays a role in extending yeast RLS via Mig1 inhibition (Harkness *et al.* 2004), and others have shown that RLS is reduced when the SNF1 kinase Sip1 β-subunit is deleted

(Ashrafi *et al.* 2000; Lin *et al.* 2003). Here we show that under minor nutrient stress conditions (growth on 2% glucose dropout media), *snf1*Δ cells have an obvious decrease in RLS when compared to plasmid-borne Snf1-HA (16 versus 19 generations; Fig. 4.3C). However, when Snf1^{UBA}-HA was expressed from the same high copy plasmid in *snf1*Δ cells, mean RLS was considerably increased beyond wild type levels to 27 generations.

We repeated the RLS experiment on CM media, which induces a similar nutrient stress, using strains harboring endogenous *snf1^{UBA}-GFP* and *SNF1-GFP* alleles to avoid copy number bias. Our results demonstrate that the UBA mutation, under the control of the endogenous promoter, continues to confer extended mitotic longevity (Fig. 4.4A). Thus, increased SNF1 kinase activity due to the UBA mutation increases RLS regardless of its source of expression. Our results indicate that the increased α-γ subunit associations, and nuclear transcriptional activity (invertase), play an important role in extending mitotic longevity as measured by the RLS assay.

4.3.4 Increased lifespan conferred by the UBA mutation is partially dependent on the yeast forkhead transcription proteins Fkh1 and Fkh2

In mammalian cells it is known that activated AMPK phosphorylates FOXO and results in increased FOXO-dependent transcriptional activity without altering the cellular location of FOXO (Greer, Oskoui*, et al.* 2007). The FOXO family of Fkh transcription factors promotes resistance to oxidative stress, suppresses tumor development, and enhances longevity/lifespan (Greer, Dowlatshahi*, et al.* 2007; Chiacchiera and Simone 2010). We have recently shown that the yeast FOXO orthologs, Fkh1 and Fkh2, are redundant stress responsive transcription factors that extend both RLS and yeast CLS when their activity is increased (Postnikoff *et al.* 2012). While RLS measures how long a cell can remain mitotically active, the CLS assay measures how long a population of cells can remain metabolically active once they have exited mitotic growth and entered stationary phase (Fabrizio *et al.* 2001). Furthermore, combined disruption of both *FKH1* and *FKH2* was required to decrease CLS, and the double mutants no longer responded to caloric restriction, indicating that Fkh1 and Fkh2 are critical responders to caloric restriction, a metabolic stress that also results in AMPK/SNF1 kinase activation. To date however, Snf1 has only been shown to interact with the Hcm1 (Rodriguez-Colman *et al.* 2013), and not Fkh1 or Fkh2.

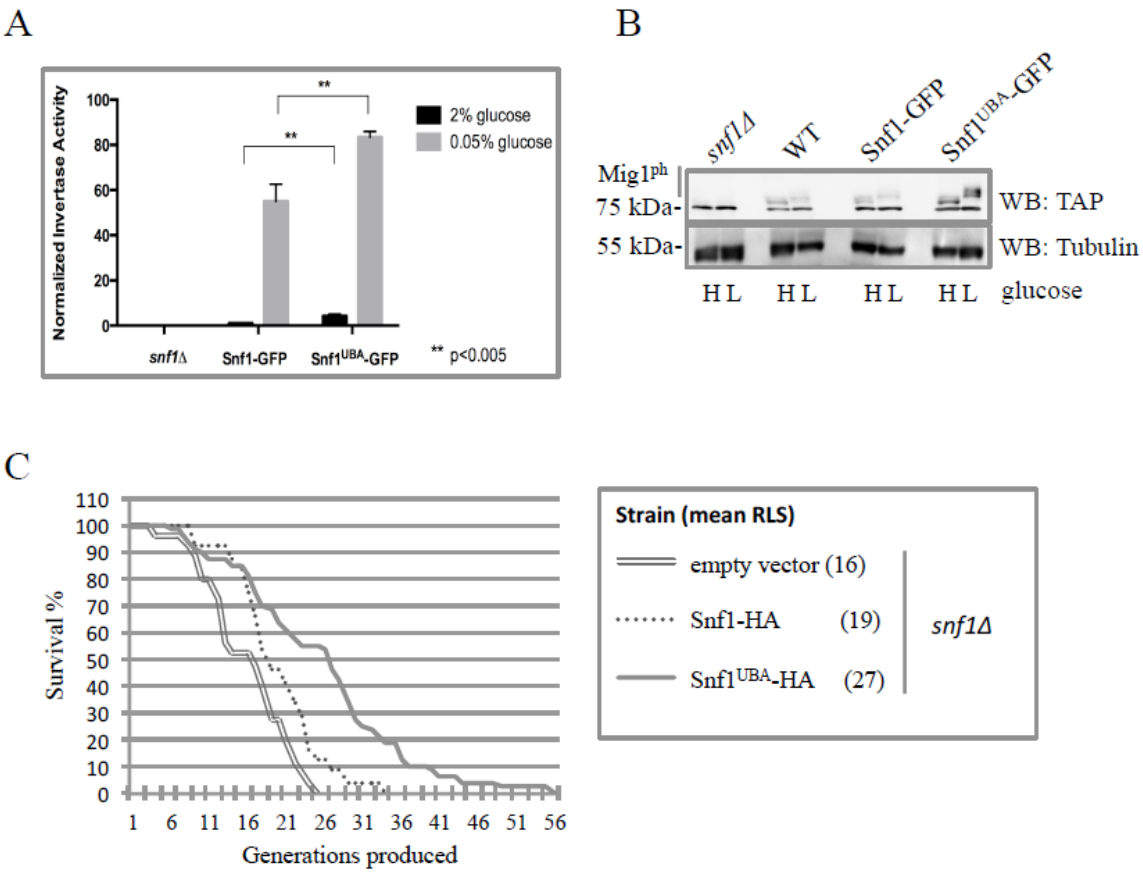

Figure 4.3 UBA mutations effect SNF1 kinase activity. (A) Comparison of invertase activity under repressive (2% glucose) and activating (0.05% glucose) conditions between isogenic yeast strains deleted for Snf1 (*snf1Δ*) or genomically expressing GFP-tagged WT (Snf1-GFP) or UBA mutated Snf1 (Snf1^{UBA}-GFP). Average of 3 biological repeats normalized to Snf1-GFP 2% glucose (value of 1), showing the mean, SD (standard deviation) and significance by 2-way ANOVA. (B) Phosphorylation shift of the SNF1 kinase nuclear target, Mig1, under activating (L, 0.05% glucose) and repressive (H, 2% glucose) conditions. Each strain has Mig1-TAP genomically expressed from the endogenous promoter (WT) in combination with the *SNF1* disruption, with the additional plasmid expression of empty vector (*snf1Δ*), Snf1-GFP, or Snf1^{UBA}-GFP. (C) Replicative lifespan assay (RLS) measuring the number of daughter cells produced by a single mother (generations) is reported for the *snf1*Δ strain expressing Snf1-HA or Snf1^{UBA}-HA. *snf1Δ* expresses empty plasmid only in the same strain (YEp24). Cells were grown on drop-out (DO) media at 30°C with 2% glucose. Values represent the average of 4 biological repeats. A was performed by Dr. T.G. Arnason. C was performed by Dr. T.A. Harkness.

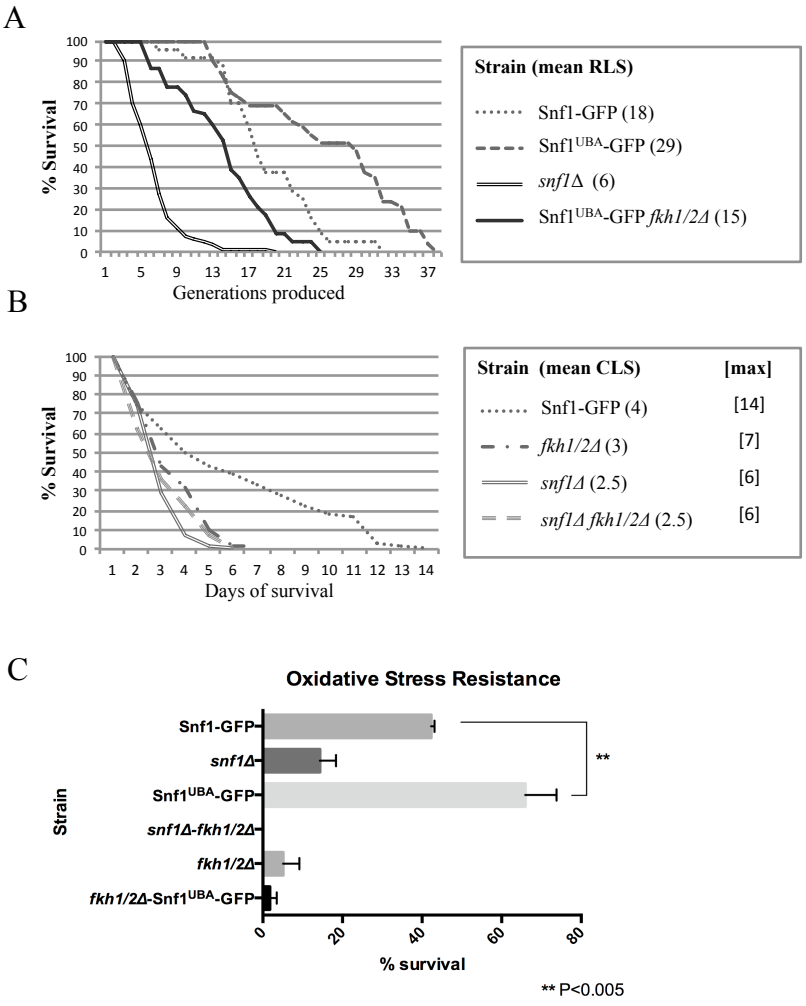

Figure 4.4 Fkh1/2 and SNF1 kinase impact lifespan and stress response. (A) Replicative lifespan of isogenic strains expressing genomic variations of the Snf1 subunit (Snf1-GFP and Snf1^{UBA}-GFP, or disrupted at the *SNF1* gene locus (*snf1Δ*)), or in combination with disruption of *FKH1* and *FKH2* grown on CM at 30°C with mean lifespan reported at 50% survival. Mean of 3 biological repeats. (B) Chronological lifespan, reporting the total length of cell survival in stationary phase cell cultures grown in water, of isogenic yeast strains with genomic disruptions of *SNF1, FKH1, FKH2,* or none, as indicated. Snf1^{UBA}-GFP was genomically integrated into the *fkh1/2Δ* and *snf1Δ* strain. (C) Oxidative stress survival of isogenic strains (as in Fig. 4A, B.) cultured to stationary phase, then plated onto complete media after 1 hour exposure to 100 µM hydrogen peroxide. Mean ± S.D. of 3 biological repeats. A was performed by Dr. T.A. Harkness, and B was performed by Dr. S.D. Postnikoff.

To test whether increased Snf1^{UBA}-dependent RLS requires an evolutionarily conserved interaction with yeast Fkh1 and Fkh2, we genomically introduced the *snf1^{UBA}-GFP* allele, which confers a long-lived phenotype, into a *fkh1*Δ *fkh2*Δ strain. Cells lacking both *FKH1* and *FKH2* cannot be used for RLS experiments because of severe flocculence (Postnikoff *et al*. 2012). The *snf1^{UBA}-GFP* allele, however, reduced flocculence in *fkh1*Δ *fkh2*Δ cells enough so that the triple mutants could be used in these RLS studies. The results show that introducing the *FKH1* and *FKH2* deletions into the Snf1^{UBA}-GFP strain reduced RLS to below WT levels (Fig. 4.4A). This provides the first evidence that Snf1 requires functional Fkh1 and/or Fkh2 proteins to regulate RLS.

A reciprocal lifespan experiment was performed to test whether Snf1 and the Fkh1/2 proteins work together. *SNF1* was deleted within the *fkh1*Δ *fkh2*Δ strain to generate the triple deletion mutant. Since we could not perform RLS due to flocculence, we performed CLS experiments. A previous global CLS study (Powers *et al.* 2006) found that *snf1*Δ strains, as well as strains deleted for any of the individual SNF1 kinase subunits (γ subunit: *snf4*Δ; β subunits: *sip1*Δ, *sip2*Δ and *gal83*Δ), all had reduced CLS. We confirm here that *snf1*Δ cells are short-lived when measured by CLS (Fig. 4.4B). *snf1*Δ CLS was found to be similar to *fkh1*Δ *fkh2*Δ CLS. Importantly, the *snf1*Δ *fkh1*Δ *fkh2*Δ CLS is similarly short-lived. These results suggest that the SNF1 kinase works in the same pathway as Fkh1 and Fkh2 to maintain normal CLS. Taken together, Fig. 4.4A and 4.4B suggest the Fkh proteins work downstream of Snf1.

4.3.5 Enhanced stress resistance conferred by the UBA mutation is dependent on the yeast Forkhead transcription proteins Fkh1 and Fkh2

In addition to lifespan, the forkhead and Snf1 cellular functions also intersect for stress survival. An evolutionarily conserved stress response network utilizing AMPK and Fkh proteins has been described (Chiacchiera and Simone 2010; Salminen *et al.* 2011; Rodriguez-Colman *et al.* 2013). In yeast, it was shown that the Fhk protein Hcm1, but not Fkh1 or Fkh2, was phosphorylated by Snf1 under stress conditions (Rodríguez-Colman *et al.* 2013). We have recently reported that the Fkh1 and Fkh2 proteins are important for survival after oxidative stress exposure (Postnikoff *et al.* 2012) specifically in stationary growth phase. In contrast, while the yeast SNF1 kinase has been shown necessary for stress survival to numerous insults, sensitivity to oxidative stress in logarithmically

grown cells was not detected (Dubacq *et al.* 2004; Hong and Carlson 2007). To further our understanding of whether an interaction exists between Snf1 and the Fkh proteins, we asked if the endogenous expression of *Snf1^{UBA}-GFP* in the *fkh1/2Δ* strain alleviates the stress defect of the *fkh1/2Δ* strain alone, or augments the stress resistance over that of unmodified Snf1. Figure 4.4C shows the survival rates of the various mutants after transient exposure to 100 mMol H_20_2. Survival of logarithmic-phase *fkh1/2Δ* or *snf1Δ* strains did not exhibit sensitivity to hydrogen peroxide (data not shown). Given that the stress sensitive phenotype for the *fkh1/2Δ* did not become apparent until stationary phase, we tested if this impacted the stress sensitivity of the *snf1Δ* also. Significantly, repeat testing of these same strains grown to stationary phase revealed that the *snf1Δ* and *fkh1/2Δ* strains are individually very sensitive to oxidative stress, with less than 15% and 5% survival respectively, and unable to survive when combined (*snf1Δ fkh1/2Δ,* 0% survival) (Fig. 4.4C). This suggests a synergistic interaction. The Fkh proteins are likely triggered by other factors and may have SNF1 kinase-independent functions. The *snf1^{UBA}* allele conferred enhanced survival to oxidative stress over unmodified Snf1, with 66% vs 43% survival, respectively. In contrast, the introduction of the *snf1^{UBA}* allele into the *fkh1/2Δ* strain did not confer any survival advantage to oxidative stress exposure, with both strains at <5% survival. This suggests that in order for the Snf1^{UBA} strain to respond to oxidative stress, a functional Forkhead protein response system is paramount. Consistent with the findings for RLS, our data supports the idea that Snf1 activates the Forkhead transcription factors in times of stress.

4.3.6 Fkh1/2 transcription factors are required for *SNF1* expression

Loss of Fkh1/2 resulted in an inability of the overactive Snf1^{UBA} derivative to complement for oxidative stress resistance (Fig. 4.4C). The *Drosophila* Daf16/FOXO was recently reported to directly regulate the expression of an atypical AMPK-like γ-subunit (Tullet *et al.* 2014), and Fkh1 and Fkh2 affect the transcription of numerous yeast genes (Ostrow *et al.* 2014), leading us to ask if the loss of Fkh1/2 activity resulted in decreased transcription of SNF1 kinase subunits. Figure 4.5A shows that the total protein abundance of Snf1^{UBA}-GFP was decreased in the absence of Fkh1/2, and that overexpression of either Forkhead protein from a plasmid partially restored Snf1 protein levels (Fig. 4.5B). This observation was mirrored at the transcriptional level where *SNF1*

expression was decreased by the combined disruption of Fkh1/2 (Fig. 4.5C, 4.5D), and subsequent Fkh1 or Fkh2 overexpression from a high copy plasmid partially restored *SNF1* expression towards baseline, with Fkh1 showing a greater induction of *SNF1* expression than Fkh2. These results were normalized to the *rRNA* signal to account for the differences within the isogenic strains tested (Fig. 4.5E). *SNF4* expression was negligibly impacted and was not influenced by *FKH1* or *FKH2* overexpression from a plasmid (Fig. 4.5D, 4.5E).

4.3.7 Mutations to the UBA domain do not impact Reg1 interactions with Snf1

Snf1 activation was increased overall and less repressed by high glucose levels upon introduction of conserved changes to the yeast UBA sequence (Fig. 4.2A,B), and the same sequence modifications enhanced Snf1-Snf4 allosteric associations even under repressive glucose levels (Fig. 4.2E). These defects are strikingly similar to those seen by disruption of the PP1 phosphatase activity that reverses the SNF1 kinase activating Thr(P)-210 phosphorylation, specifically in *reg1Δ* strains (Ludin *et al.* 1998). The PP1 regulatory subunit, Reg1, binds directly to Snf1 in a glucose-responsive manner and *reg1Δ* strains are constitutively active for SNF1 kinase, independent of glucose abundance (Jiang and Carlson 1996; Sanz, Ludin*, et al.* 2000). To the best of our knowledge, the portion of the Snf1 subunit that Reg1 binds to has been delineated to the N-terminal catalytic domain of Snf1 and not pinpointed further (Sanz, Alms*, et al.* 2000). The Snf1 UBA motif is C-terminal to this, making a direct disruption to Reg1-Snf1 associations unlikely. However, indirect effects mediated through UBA sequence alterations affecting the binding of an intermediary (such as Sip5 which is reported to bind both Snf1 and Reg1 (Sanz, Ludin*, et al.* 2000)) or by inducing structural changes within Snf1 may be detected as defects in Reg1-Snf1 associations. 2-hybrid analysis was used to compare Snf1-Reg1 and Snf1^{UBA}-Reg1 interactions throughout a glucose gradient and demonstrated an indistinguishable intensity of color development (Fig. 4.6). The Snf1-Reg1 and Snf1^{UBA}-Reg1 interactions exhibited the expected increase in association as the glucose concentrations dropped to 0.05% and is significantly lower than the positive control (Snf1-Snf4) in this assay. Because of the weak Snf1-Reg1 interactions observed even at the lowest glucose levels, subtle disruptions may not have been detected.

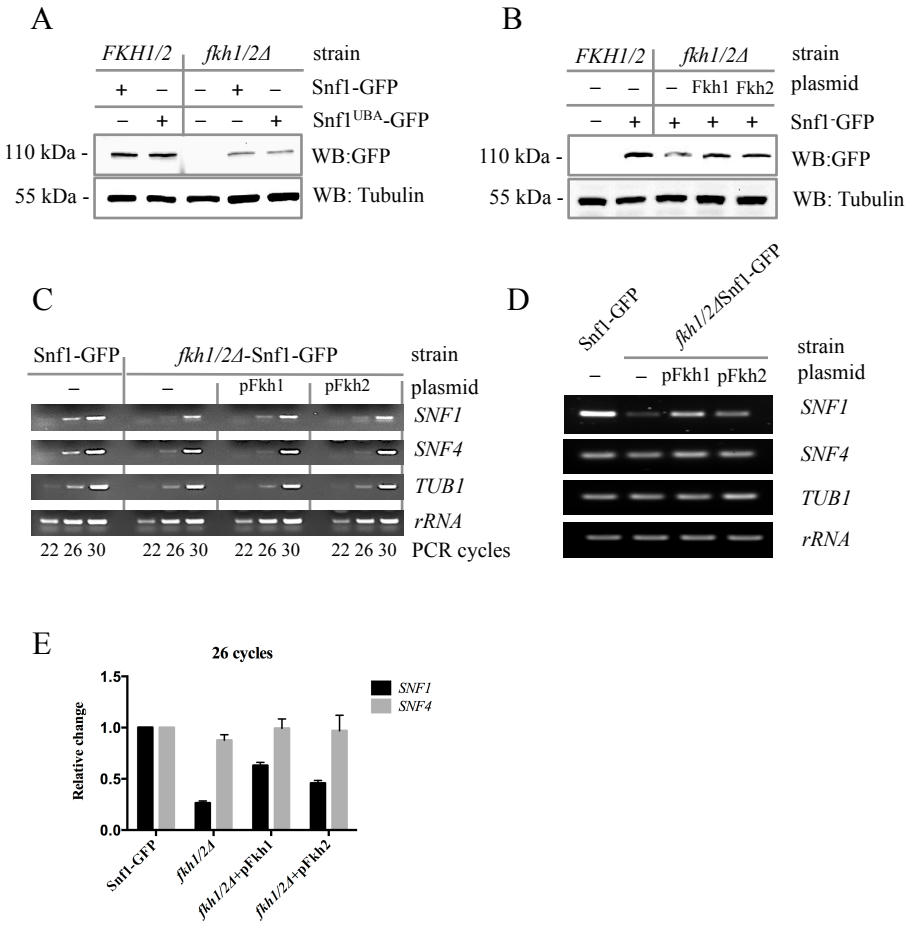

Figure 4.5 *SNF1* expression requires Fkh1 and Fkh2. (A) Western Blot of whole cell lysates from logarithmically-growing isogenic yeast strains in 2% glucose probed for GFP and tubulin protein abundance. (B) Isogenic strains with genomic Snf1 variants were transformed with high-copy HA-tagged Fkh1 or HA-Fkh2 plasmids. Cells were grown in 2% glucose drop-out media. (C) Reverse transcription PCR analysis of *SNF1*, *SNF4*, *TUB1* and *rRNA* transcript abundance of isogenic strains with, or disrupted for, *FKH1* and *FKH2* and with or without plasmid overexpression of HA-Fkh1 or Fkh2. ~200 bp products for all. (D) Representative agarose gel of RT-PCR analysis of *SNF1*, *SNF4*, *TUB1, and rRNA* at 26 cycles from the strains indicated. (E) Versadoc quantitation of DNA signal at 26 PCR cycles (RedSafe; FroggaBio), normalized to *rRNA* expression for each condition. Mean ± S.D. of 3 biological repeats.

Taken together, the results presented here indicate that the natural UBA domain functions as a negative influence on SNF1 kinase activation and activity in part by influencing steady state phosphorylation and Snf1-Snf4 subunit interactions. The critical readout of the impact of the UBA mutation is seen in increased stress resistance and replicative lifespan. Our study shows that the independently described role for Snf1 and the Fkh proteins in life span and stress response may be due to actions within the same or overlapping pathways, where Fkhs act upstream of Snf1 to promote *SNF1* gene expression, while completing a positive feedback loop by facilitating SNF1 kinase biological functions.

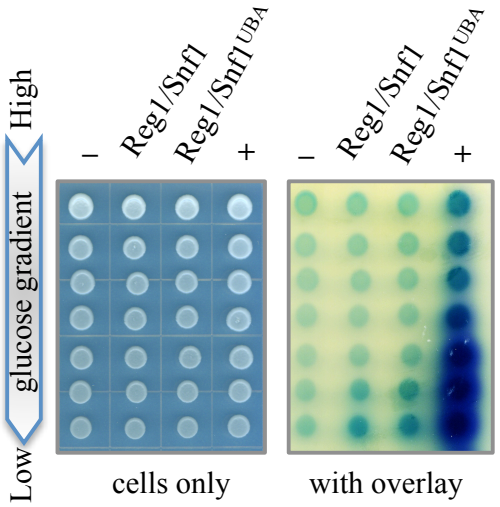

Figure 4.6 Reg1-Snf1 associations are not disrupted by UBA sequence alterations. Solid support 2-hybrid assay on glucose gradient plates (High: 2%; Low: 0.05% glucose) between the Reg1 protein and either unmodified Snf1 or *Snf1*^{UBA} before and after β-galactosidase overlay. (–); Negative control using empty 2-hybrid vectors. (+) positive control using unmodified Snf1 and Snf4 as in Fig. 4.2E.

4.4 DISCUSSION

Given the strong proclivity for regulatory mechanisms of SNF1 kinase/AMPK control to be conserved between yeast and higher eukaryotes, and the potential importance of the UBA domain in providing an additional layer of regulatory sophistication of the enzyme, we set out to further characterize the *in vivo* role of the

UBA domain in the regulation of the SNF1 kinase. Here, evidence is presented confirming an endogenous repressive role for the yeast UBA consensus in SNF1 kinase activation and activity, as well as a complex genetic and functional interaction with the Fkh proteins, Fkh1 and Fkh2.

The extensive presence of a loosely conserved UBA domain within the AMPK family catalytic α subunits opens the possibility of a regulatory role for this motif. Although UBA domains have the potential to recruit ubiquitinated proteins to their hydrophobic motif and thereby affect their activity and activation, this appears to be variable. Specifically, the yeast SNF1 kinase UBA domain, like other Class 3 yeast UBA domains, has not been shown to bind to mono- or poly-ubiquitin (Raasi *et al.* 2005), an activity also not detected for the UBA domains in human AMPK-related kinases (Jaleel *et al.* 2006). Nonetheless, mutations to the α subunit UBA domains in yeast Snf1 and human AMPK-related proteins did affect activity, albeit reportedly in opposing directions. In *S. cerevisiae* it was found that complete deletions, and selected mutations of the yeast Snf1 UBA motif did not impair phosphorylation or activation of the yeast SNF1 kinase; rather, there was a subtle increase in both (Momcilovic and Carlson 2011). In contrast, the UBA domain was necessary for full activity when studied in AMPK-related protein kinases (Jaleel *et al.* 2006). This AMPK study mutated consensus amino acids or completely deleted the UBA motif and found that this prevented phosphorylation and activation of the immune-purified α subunit fragments when analyzed *in vitro*, suggesting an activating function to this motif under normal conditions. It is possible, however, that the manipulation, truncation, and expression levels in this mammalian system were not representative of *in vivo* responses to activating signals.

4.4.1 The UBA domain dampens SNF1 kinase activation through modified allosteric associations

Replacement of two highly conserved amino acids within the Snf1 UBA sequence revealed a positive effect on the activity of the SNF1 kinase. Our investigations confirm and extend the observations that UBA sequence mutations positively affect the activation of the yeast kinase, shown specifically here to be through an enhancement of Snf1-Snf4 subunit associations, proportionately under both repressive and activating states (Fig. 4.2E) with reproducible enhancements of Thr(P)-210 and maintenance of efficient

nuclear import (Fig. 4.2A-D). Stimulatory conditions have been well documented to promote allosteric associations between these subunits (Celenza *et al.* 1989; Cheung *et al.* 2000; Sproul *et al.* 2009) and this work indicates that the inherent function of the natural UBA domain may be to regulate SNF1 kinase activation by restraining α-γ associations. The mechanism underlying this modulation of association remains to be determined, yet a glucose-responsive association of a peripheral protein(s) with the hydrophobic face of the UBA domain may introduce steric hindrance between the α-γ subunits, thereby preventing full activation. A direct test of the physical associations between the PP1 regulatory subunit, Reg1, and Snf1 did not demonstrate a disruption by the conserved mutations within the UBA domain, as could have been predicted by the Snf1^{UBA} phenotype (Fig. 4.6).

4.4.2 The UBA domain dampens SNF1 kinase nuclear activity

In addition to looking at the activation of SNF1 kinase, we also looked at UBA influences on activity. The activated SNF1 kinase targets nuclear transcriptional repressors to facilitate adaptation to non-glucose carbohydrate sources, such as the lifting of *SUC2* gene repression and subsequent production of invertase, an enzyme that cleaves sucrose into glucose and fructose (Treitel *et al.* 1998). Invertase activity was increased over wild type by the presence of the *snf1^{UBA}* allele under activating conditions, and less noticeably under repressive, supporting an inhibitory role in controlling SNF1 kinase activity. This assay is a quantitative measure of SNF1 kinase enzymatic activity on its nuclear target, the transcriptional repressor Mig1. A qualitative increase in Mig1 phosphorylation signal intensity was observed in *snf1^{UBA}* cells under activating and repressive growth, yet the phosphorylation shift was limited to activating conditions only. Whereas there is a loss of glucose repression in the activation of SNF1 kinase, its nuclear activity remains well controlled, as seen by the maintenance of regulated nuclear import (Fig. 4.2C, 4.2D). The observation that the *snf1^{UBA}* mutation did not affect normal cytosolic-nuclear partitioning is important, as it suggest that this regulatory step remains intact. This is emphasized by acknowledging that the strong allosteric association between subunits and increased phosphorylation of Snf1^{UBA}, even under repressive conditions, do not translate to enhanced nuclear import in high glucose. Subsequently,

measures of nuclear activity (invertase and $Mig1^{ph}$) under glucose repression are therefore maintained, as observed (Fig. 4.3A, 4.3B).

4.4.3 The UBA domain influences lifespan in a Forkhead dependent mechanism

SNF1 kinase is known to be necessary for normal lifespan, as deletion of any subunit resulted in shortening of CLS (Powers *et al.* 2006). A striking effect of the UBA mutation is an enhanced RLS of *S. cerevisiae* beyond that of WT. This effect was not diminished by lowering the copy number, as endogenous $Snf1^{UBA}$-GFP expression levels (Fig. 4.4A) were as capable of promoting lifespan extension as high copy plasmid expression of $Snf1^{UBA}$-HA (Fig. 4.3C). Performing the RLS assay on media with nutrient limitations revealed the need for Snf1 for RLS maintenance under stress conditions. This is in keeping with prior observations using yeast, worms, and mice systems that nutrient and caloric restriction independently promotes lifespan (Ashrafi *et al.* 2000; Baur *et al.* 2006; Greer, Dowlatshahi*, et al.* 2007; Lam *et al.* 2013).

FOXOs and AMPK have known physical associations, including the direct phosphorylation of FOXO3A by AMPK resulting in increased transcriptional activity, longevity and resistance to oxidative stress (Greer, Dowlatshahi*, et al.* 2007). It was recently reported that Snf1 phosphorylates the yeast Fkh protein, Hcm1, in response to stress, facilitating shuttling of Hcm1 from the cytosol to the nucleus (Rodriguez-Colman *et al.* 2013). Hcm1 is tightly linked to Fkh1/2 activity as it transcribes *FKH1* and *FKH2* during late S phase (Synthesis phase) (Pramila *et al.* 2006). We have shown previously that the yeast APC is a crucial player in maintaining normal lifespan and stress response, and that Snf1 and Fkh1/2 are involved in these activities (Harkness *et al.* 2004; Postnikoff *et al.* 2012). While Snf1 interacts physically with Hcm1, there is no indication in the literature that Snf1 interacts with Fkh1/2. Our work clearly shows that Fkh1/2 are required for enhanced $Snf1^{UBA}$ function, as deletion of the two genes abolishes increased stress response and lifespan afforded by the UBA mutation (Fig. 4.4A and C). It remains possible that phosphorylation of Hcm1 by Snf1 ensures the expression of Fkh1 and Fkh1, negating the need for a physical phosphorylation event on Fkh1 or Fkh2.

4.4.4 SNF1 transcription is influenced by yeast Forkhead proteins

A novel insight into one mechanism whereby Snf1 and Fkh1/2 mechanistically interact arose out of our observation that the Fkh1/2 combined deletion strain exhibited decreased abundance of the Snf1 protein. We have determined that this is due to loss of Fkh1/2-dependent *SNF1* expression. *SNF4* expression was not affected despite the analogous Daf16/FOXO transcription factor in *C. elegans* contributing to the expression of the γ-subunit of an atypical AMPK-related enzyme (Tullet *et al.* 2014). In yeast, Fkh1 alone binds to the *SNF1* promoter, consistent with its greater impact on returning *SNF1* expression towards normal as compared to Fkh2 (Fig. 4.5E). Neither Fkh1 nor Fkh2 recognize the *SNF4* promoter, and no impact on *SNF4* expression was found when these proteins were overexpressed (Fig. 4.5E) (Ostrow *et al.* 2014). However, the yeast Fkhs have complementary functions as combined deletion of *FKH1* and *FKH2* are required for aging and stress phenotypes (Postnikoff *et al.* 2012); Fkh2 could therefore partially induce *SNF1* expression by complementation. We demonstrate that while the introduction of the UBA mutations enhances both mitotic lifespan and oxidative stress resistance over unmodified Snf1, this cellular role is dependent on the presence of the Fkh1/2 proteins as $Snf1^{UBA}$ was not sufficient to overcome the *fkh1/2Δ* strain phenotypes. Taken together, a positive feedback loop can be envisioned. Initial stress recognition by the SNF1 kinase leads to the activation of the Fkhs that may be mediated by Hcm1. Once the Fkhs are active, they elicit a transcriptional stress response and longevity program including the expression of *SNF1*, thus completing the loop.

4.4.5 Enhanced AMPK activity provides health benefits

There are several activating mutations within AMPK/SNF1 kinase reported throughout the literature. A naturally occurring genetic mutation in pigs within the γ subunit yields lean muscular pigs prized by the food industry and is thought to be due to enhanced AMP binding (Andersson 2003). Dominant mutations within human AMPK α subunits have also demonstrated lowered % body fat (Yeo *et al.* 2008) and lowered blood sugar and lipid levels (Backhed *et al.* 2007). Furthermore, activation of AMPK by the oral anti-diabetic drug metformin is currently in over 200 clinical trials of cancer therapy, indicating the worldwide interest in AMPK-dependent benefits to human health. Together, there is a clear clinical benefit to enhanced AMPK activity. Exercise and

caloric restriction (with weight loss) alone are capable of transiently activating AMPK and yielding health gains, yet these treatment options are difficult to adhere to, as evidenced by the current epidemic of obesity and insulin resistance. Future identification of AMPK activators and repressors may yield new metabolic targets for many human conditions. UBA-domain binding partners may play a key role in these approaches.

Table 4.1 *Saccharomyces cerevisiae* strains used in Chapter 4

Strain (previous name)	Genotype	Source/ Reference
yTER32 (PJ69-4A)	*MAT*a *trp1-901 leu2-3 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lac*	*E. Craig*
yTER70 (YTH1510)	*MATα ade2 his3 leu2 ura3 snf1Δ::kanMX6*	This study
yTER188 (YTH4269)	*MATα ade2 his3 leu2 ura3*	This study
yTER223	YTH1510 + *SNF1-GFP::HIS3*	This study
yTER222	YTH1510 + *SNF1^{UBA}-GFP::HIS3*	This study
yTER246 (YTH3792)	*MAT(?) ade2 his3 leu2 ura3 MIG1- TAP::HIS3*	This study
yTER248 (YTH3760)	*fkh1Δ::kanMX6 fkh2Δ::kanMX6*	This study
yTER249	yTER246 + *snf1Δ::kanMX6*	This study
yTER224	yTER222 + *fkh1Δ::kanMX6 fkh2Δ::kanMX6*	This study
yTER256	YTH1510 + *fkh1Δ::kanMX6 fkh2Δ::kanMX6*	This study

CHAPTER 5 The SNF1 kinase subunit stoichiometry regulates its enzyme activity

5.1 Introduction

The AMPK family has a fundamental role in maintaining cellular energy balance during times of stress or deprivation (Inoki *et al.* 2012; Hardie 2014). Through their kinase activity, immediate metabolic shifts towards catabolic pathway use are triggered by phosphorylating existing cytosolic protein targets (Inoki *et al.* 2012), whereas sustained effects occur upon the activated kinase translocating into the nucleus to target transcription factors and alter gene expression to help survive the energy deficit (Kodiha *et al.* 2007; Gowans and Hardie 2014). These enzymes are heterotrimeric protein complexes encoding a catalytic α subunit, and regulatory β and γ subunits that are evolutionarily conserved from their primary amino acid sequences to their quaternary protein associations (Hedbacker and Carlson 2008; Lee *et al.* 2008). As a simple and genetically malleable eukaryotic model, many important discoveries about the mechanisms regulating AMPK activation and activity have been identified in yeast and confirmed to also occur in higher eukaryotes including plants, mammals and humans (Hardie and Sakamoto 2006; Hedbacker and Carlson 2008; Tullet *et al.* 2014). The yeast AMPK ortholog is the SNF1 kinase, and is readily activated by removing a ready glucose supply (nutrient deprivation). The activated enzyme, as for all in this family, is phosphorylated by well-described upstream kinases at a single site, which enhances allosteric associations between the kinase subunits and triggers nuclear import (Carlson *et al.* 1981; Hardie *et al.* 1998). Of interest to us are the more subtle controls effecting SNF1 kinase activity, such as those reported for post-translational modifications to subunits of the AMPK family including acetylation (Lu *et al.* 2011; Zhang *et al.* 2013) and SUMOylation (Simpson-Lavy and Johnston 2013). These modifications appear to contribute a mild inhibition on yeast and human AMPK activity.

In both yeast and human systems, the catalytic α subunit has been reported to be ubiquitinated *in vivo*, albeit under non-physiological conditions (Qi *et al.* 2008; Wilson *et al.* 2011). In both cases, poly-ubiquitination was only detected upon engineered loss of the de-ubiquitinating activities in the cells, enabling non-physiological accumulation of ubiquitinated protein substrates. The studies in both yeast and human systems concluded that there was an inhibitory effect on kinase function: degradation of α subunit in yeast

(Wilson *et al.* 2011) and diminished phosphorylation in human cell lines (Al-Hakim *et al.* 2008; Lee *et al.* 2015). Global mass spectroscopic analysis of the yeast proteome has identified two ubiquitin attachment sites within the yeast α subunit, Snf1, at amino acids 80 and 461 (Starita *et al.* 2012), yet their specific contribution towards poly-ubiquitination anchors or impacts on SNF1 kinase activity has not been formally studied. Lastly, the stability of the remaining SNF1 kinase protein subunits (Snf4 and the three β-subunits Gal83, Sip1 and Sip2) has not been formally studied in yeast. Here, the stability of the all SNF1 kinase subunits has been assessed under activating and repressive conditions for this enzyme. Furthermore, we have mutated the potential ubiquitin-attachment site lysines (K) to conserved arginine (R) residues and measured the effect on SNF1 kinase activation, activity, and function.

5.2 Material and Methods

5.2.1 Yeast crossing and tetrad selection

All yeast strains were included in Table 5.1. Yeast strains of *rpn10Δ*-Snf1-GFP and *sn4Δ*-Snf1-GFP were crossed to generate isogenic *rpn10Δ-snf4Δ*-Snf1-GFP, *rpn10Δ*-Snf1-GFP, *snf4Δ*-Snf1-GFP, and WT-Snf1-GFP. Tetrads were selected and strains were confirmed using selective media and PCR analysis. Isogenic strains were cultured and used to detect the Snf1-GFP protein level by Western Blot as described before.

5.2.2 Invertase assay

Protocols have been previously described in section 3.5.

5.2.3 Creation of Snf1 lysine to arginine (K/R) mutants

Genomic Snf1 α subunit with C-terminal GFP tag (*SNF1-GFP::HIS3*, yTER223) was used as the DNA template. Conservative mutation of lysine to arginine (K/R) within the Snf1 subunit at amino acid positions 80 and 461 (K80R, K461R) were introduced by PCR mutagenesis. For the independent mutations, two PCR products were generated and annealed at one end through homologous regions containing the mutated K/R sequences, with the remaining sequences extending outside the full length *SNF1* gene, including the *GFP* epitope and *HIS3* sequence (primer sequences see Table 5.2); PCR products were purified and co-transformed into the *snf1Δ* (yTH1510; *snf1*Δ*::G418*) strain for genomic

integration. Integrants were selected on His- plates and mutations were confirmed by DNA sequencing.

5.2.4 Total protein extract and Western Blot analysis

Protocols have been previously described in section 3.1 and 3.2. Anti-GFP (Covance, MMS-118P-500), -Actin (Sigma, A4700, Lot005134), -HA (Roche, 11867423001), -TAP (Open Biosystems, CAB-1001), and -Tubulin (Sigma, 051M4771) antibodies were used in Western Blot analysis and the chemiluminescent signal captured on a VersaDoc (BIO-RAD) molecular imager (Quantity One 4.6.9).

5.2.5 Cycloheximide protein stability experiment

The yeast strains used in this study are shown in Table 5.1 Protocols have been previously described in section 3.3, followed Western Blot analysis.

5.2.6 mRNA expression analysis

Protocols have been previously described in section 3.8.

5.3 Results and Discussion

The inherent stability of the α, β and γ subunits of the yeast SNF1 kinase was determined *in vivo,* to clarify the role of ubiquitination in the regulation of the yeast SNF1 kinase under physiological conditions. Prior reports of α subunit ubiquitination (Pineda *et al.* 2015), γ subunit instability (Jackson *et al.* 2000), and β subunit ubiquitin-targeting in orthologous systems (Qi *et al.* 2008) raised the possibility of multifactorial regulatory controls through ubiquitin-mediated degradation. Endogenously expressed and genomically epitope-tagged versions of the individual SNF1 kinase subunits were assessed individually in isogenic yeast strains for their stability up to 3 hours (Table 5.1). Regardless of whether the SNF1 kinase complex was in its repressive (2% glucose) or activated (0.05%) state, each of the Snf1, Snf4 and three β-subunits were stable up to three hours (Fig. 5.1 A and B). Furthermore, there was no evidence of additional higher molecular weight bands to suggest mono- or poly-ubiquitination.

An assessment of yeast SNF1 kinase enzyme activity includes the quantitative measure of the enzyme invertase, encoded by the *SUC2* gene (Carlson *et al.* 1981; Carlson and Botstein 1983). *SUC2* is normally held repressed under optimal growth

conditions, yet is rapidly induced upon low glucose conditions by the active SNF1 kinase targeting the *SUC2* repressor, Mig1, for phosphorylation and subsequent translated (Treitel *et al.* 1998). The measure of invertase activity upon individual deletion of each of the SNF1 kinase subunits is shown in Figure 5.1C; an absolute (Snf1), and partial (Snf4 and Gal83) requirement for specific subunits is evident for the nuclear function of SNF1 kinase. It is well known that the nuclear SNF1 kinase heterotrimer is composed of Snf1-Snf4-Gal83, implying that even the non-catalytic Snf4 and Gal83 subunits are required for nuclear activity.

We had observed that strains deleted for *SNF4* exhibited a decrease in detectable Snf1 protein that was unaffected by glucose abundance. This held true whether *SNF1* was endogenously expressed (Fig. 5.2A) or highly expressed from a plasmid (Fig. 5.2B), implying an independence of promoter transcription. We asked if this might be a stoichiometric effect functioning to maintain heterotrimer subunit abundance. In support of this, expression of *SNF4* from a high copy plasmid was able to effectively increase Snf1 protein levels back to that of WT in a *snf4Δ* strain (Fig. 5.2C). We next queried if the decreased Snf1 abundance was due to its degradation, and formally assessed the protein stability of endogenous Snf1 in the absence of the Snf4 subunit (Fig. 5.2D). Semi-quantitative measurement of the Snf1-GFP signal revealed that there was a slow (T1/2~1.5-2.0 hours) decline in Snf1 protein abundance in the absence of Snf4, which was independent of the activation state of the SNF1 kinase (Fig. 5.2D). Interestingly, the reciprocal experiment revealed that Snf4 remained stable up to 3 hours in the absence of the Snf1 subunit (Fig. 5.2F), indicating that the α and γ subunits are regulated differently.

Of the three β-subunits, the individual loss of Gal83 was alone in affecting Snf1 protein abundance (Fig. 5.3A). The decrease in Snf1 protein was not related to a loss of its transcription, as *SNF1* mRNA levels were unaffected in either the Snf4 or Gal83 deletion strains (Fig. 5.3B).

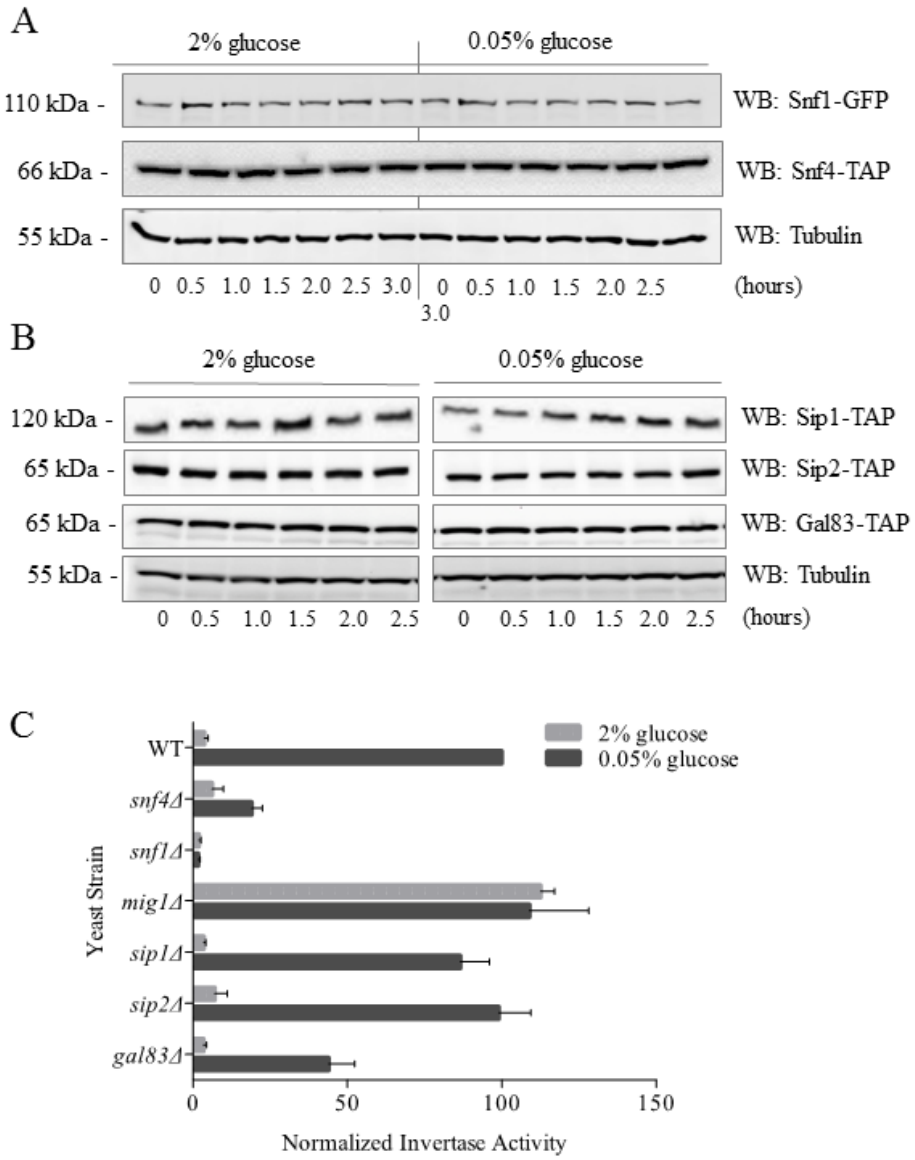

Figure 5.1 SNF1 kinase activity is dependent on inherent subunit abundance. ***A*** and ***B*****,** Protein level of endogenous tagged Snf1 α subunit, Snf4 γ subunit, and all 3 β subunits (Sip1, Sip2, Gal83) in WT strains grown under activating (0.05% glucose) and repressive (2% glucose) conditions. Cycloheximide was added at time "0" and cell lysates were collected every half hour up to 3 hours at the times indicated. ***C***, Comparison of invertase activity under repressive (2% glucose) and activating (0.05% glucose) conditions between isogenic yeast strains with the indicated SNF1 kinase subunit deletions. Reported values are normalized to WT in 0.05% glucose (value of 100). C was performed by Dr. T.G. Arnason.

To investigate the importance of the reported ubiquitin-attachment site lysines in SNF1 kinase function, we created K/R versions of Snf1 with K80R, K461R and the combination K80/461R, and integrated them genomically under the endogenous promoter. Steady state levels of Snf1 and these K/R derivatives were assessed in isogenic strains with, or without, Snf4 protein (Fig. 5.4A). In the WT background, Snf1 and its derivatives were readily detectable, yet in all cases the deletion of *SNF4* resulted in similar declines of Snf1 protein without a stabilizing effect with lysine mutations (Fig. 5.4A). Therefore, the mutation of Lys80, Lys461 or the combination did not stabilize Snf1 such as would be expected if these were the site of ubiquitin-mediated degradation signals. Formal degradation assays of $Snf1^{K80R}$ and $Snf1^{K461R}$, in the absence of Snf4 were performed and confirmed that there was no stabilization of the Snf1 protein upon lysine mutation.

The significance of $Snf1^{K80R}$ and $Snf1^{K461R}$ on SNF1 kinase function was next determined through analysis of *SUC2* transcription under activating conditions in unmodified and K/R derivatives of Snf1. RT-PCR of *SUC2* mRNA did not reveal obvious deficiencies in *SUC2* expression as compared to WT (Fig. 5.4E).

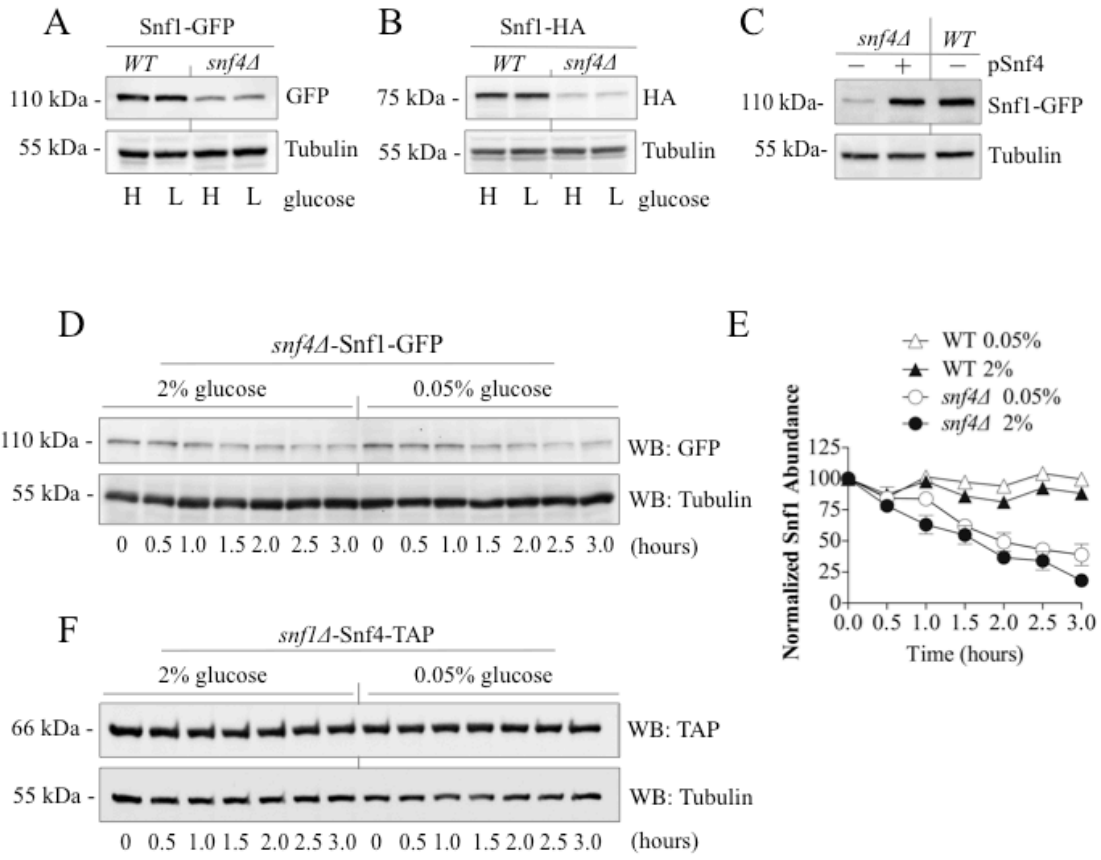

Figure 5.2 The Snf1 catalytic subunit is unstable in the absence of the Snf4 γ subunit. ***A,*** Comparison of protein abundance of endogenous Snf1-GFP in the *snf4Δ* mutant as compared to its isogenic WT strain. ***B,*** Comparison of the protein abundance of plasmid-expressed Snf1-HA in the *snf4Δ* mutant as compared to its isogenic WT strain. ***C***, Western Blot analysis of the Snf1-GFP protein abundance in the *snf4Δ* strain, with and without, plasmid-expressed Snf4 subunit. ***D*** and ***E***, Western analysis and its quantitation of Snf1-GFP protein stability in the *snf4Δ* strain after CHX addition. F. Protein stability of endogenous Snf4-TAP protein in the *snf1Δ* strain under activating and repressive growth conditions. H: high glucose (2%), repressive. L: low glucose (0.05%), activating conditions.

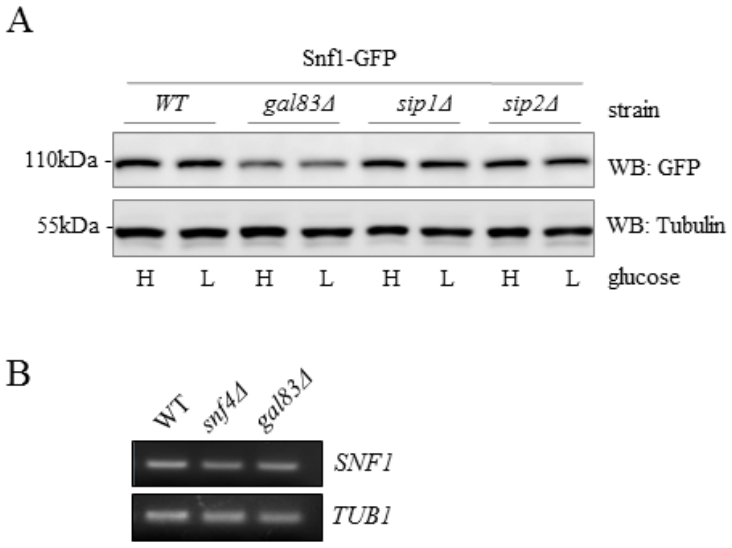

Figure 5.3 Steady state Snf1 protein abundance is dependent on the Gal83 subunit. ***A*****,** Endogenous Snf1-GFP protein abundance in isogenic WT strains, or individually deleted for *SIP1*, *SIP2,* or *GAL83* grown under activating (L) and repressive (H) conditions. ***B,*** RT-PCR of *SNF1* mRNA expression in WT, *snf4Δ*, and *gal83Δ* strains. Samples were taken after 26 cycles.

To directly assess the involvement of ubiquitin-mediated protein degradation via the proteasome, we made use of the *rpn10Δ* strain, which lacks a critical yeast proteasome subunit rendering it nonfunctional. In such mutants, poly-ubiquitinated proteins destined for degradation accumulate (Lin *et al.* 2011; Lipinszki *et al.* 2012). Snf1 abundance was noted in strains deleted for *SNF4*, *RPN10*, or the combination of the two. The *rpn10Δ* mutation did not result in Snf1 protein accumulation when combined with *snf4Δ* (Fig. 5.5A). From this, we conclude that the proteasome was not involved in the Snf1 protein degradation under these conditions.

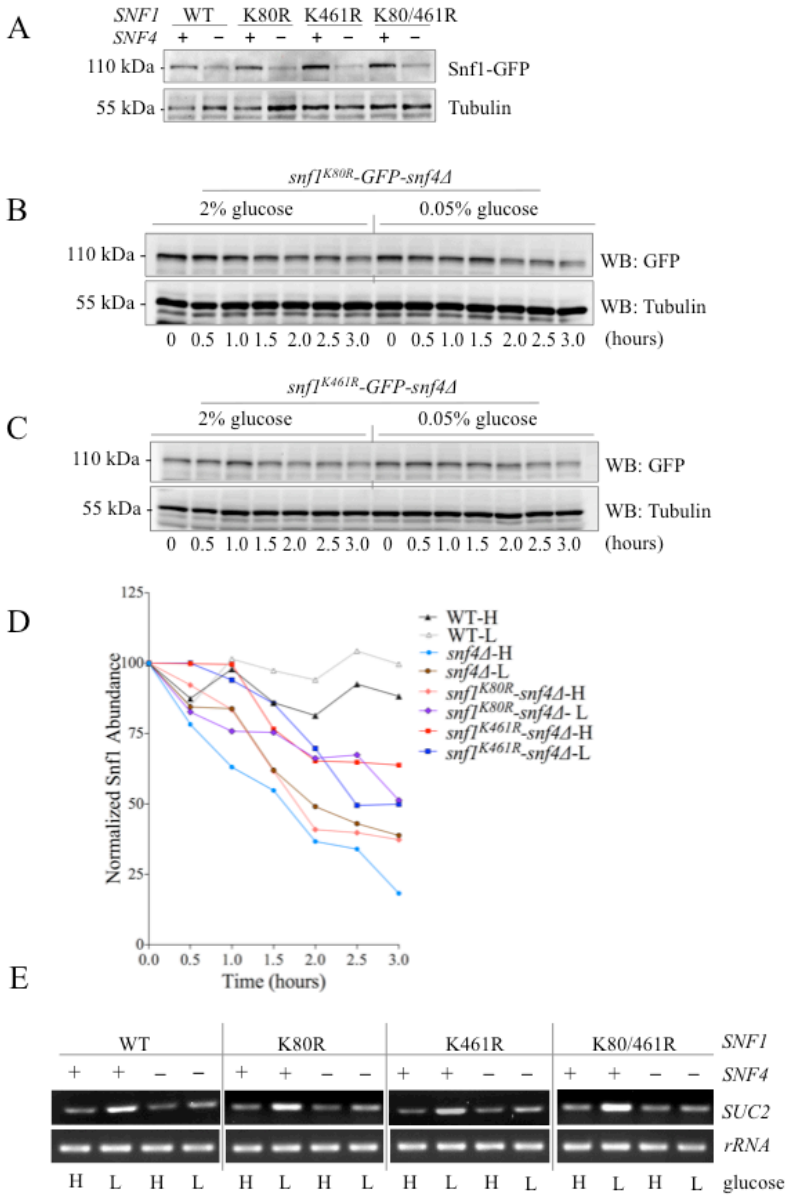

Figure 5.4 Lysine to arginine mutations within Snf1 do not alter Snf1 protein stability. ***A*****,** Steady state Western analysis of endogenous Snf1-GFP protein levels in early logarithmic phase in the absence of Snf4 subunit with K81R, K461R and combined mutation K80/461R. ***B,*** and ***C***, Extended Western Blot analysis of Snf1$^{K/R}$ in *snf4* mutant strains over a 3 hours time-course in 2% and 0.05% glucose conditions. ***D***, quantitation of Snf2-GFP protein levels from **C**, with time "0" were normalized as 100. ***E*****,** RT-PCR of *SUC2* mRNA expression after 26 cycles in WT and the individual K/R strain with, and without the presence of *SNF4*. H: high glucose 2%, repressive). L: low glucose 0.05%, activating conditions. It is impaired in *snf4Δ* mutants, regardless of the lysine to arginine mutations on Snf1 subunit.

A supportive experiment was performed adding MG132 as a potent chemical inhibitor of the 20S proteasome (Han *et al.* 2009) while assessing for Snf1 abundance. As shown in Figure 5.5B, the addition of MG132 did not alter the amount of Snf1 protein in the absence of Snf4. For neither the *rpn10Δ* nor the MG132 experiments did we notice additional higher molecular weight bands associated with Snf1, to suggest the presence of abundant poly-ubiquitin chain attachments.

Despite the previous detection by others of Snf1 poly-ubiquitination, and its correlation with decreased protein levels (Wilson *et al.* 2011), our investigations conclusively demonstrated that the subunits of the yeast SNF1 kinase are stable in both the active and inactive enzyme complex. We have determined that the relative abundance of the Snf1 catalytic subunit depends on the presence of the Snf4 and/or Gal83 subunits, and not on a functional proteasome. We suggest that this is a physiological correction for subunit stoichiometry rather than a regulatory ubiquitin-mediated clearance of excess protein via the proteasome. Lastly, although Lys80 and Lys461 within the Snf1 subunit have been reported to be ubiquitin-attachment sites (Starita *et al.* 2012), our mutational analysis failed to detect a role for these positions in stability or function. Future experiments in multicellular organisms or higher eukaryotes, may determine discrete roles for the ubiquitin-α-subunit conjugates.

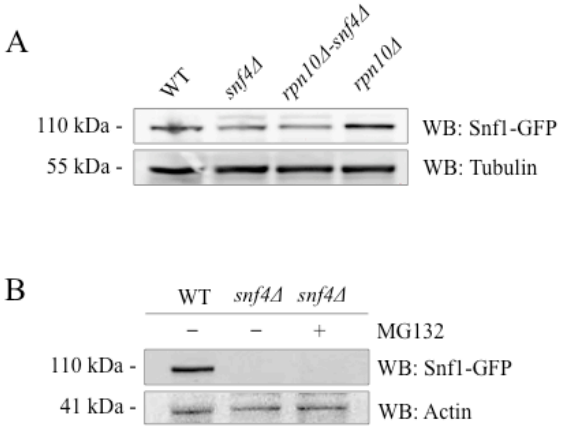

Figure 5.5 Inhibition of 20S proteasome activity does not impact SNF1 protein abundance. ***A***, Western analysis of endogenous Snf1-GFP steady state abundance in isogenic WT, *snf4Δ, rpn10Δ,* and *snf4Δ-rpn10Δ* strains. ***B***, Western analysis of Snf1-GFP abundance comparing levels in WT and *snf4Δ* strains with and without the addition of the proteasome inhibitor, MG132.

Table 5.1 *Saccharomyces cerevisiae* strains used in Chapter 5

Strain	Genotype	Source/Reference
yTER65	*MATa ade2 his3Δ200 leu2-3 lys2Δ201 ura3-52*	T. Harkness
yTER70	*MATa ade2 his3Δ200 leu2-3 lys2Δ201 ura3-52 snf1Δ::kanMX6*	W. Xiao
yTH1611	*MATa ade2 his3Δ200 leu2-3 lys2Δ201 ura3-52 snf4Δ::kanMX6*	W. Xiao
yTER5	*MATa ade2 his3Δ200 leu2-3 lys2Δ201 ura3-52 mig1Δ::kanMX6*	W. Xiao
yTER29	*MATa ade2 his3Δ200 leu2-3 lys2Δ201 ura3-52 sip1Δ::kanMX6*	W. Xiao
yTER41	*MATa ade2 his3Δ200 leu2-3 lys2Δ201 ura3-52 sip2Δ::kanMX6*	W. Xiao
yTER30	*MATa ade2 his3Δ200 leu2-3 lys2Δ201 ura3-52 gal83Δ::kanMX6*	W. Xiao
yTER223	*MATa ade2 his3 leu2 ura3 SNF1-GFP::HIS3*	R. Jiao *et al.* (2015)
yTER1	*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 SNF4-TAP::HIS3*	Open Biosystems
yTER118	*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 SIP1-TAP::HIS3*	Open Biosystems
yTER119	*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 SIP2-TAP::HIS3*	Open Biosystems
yTER117	*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 GAL83-TAP::HIS3*	Open Biosystems
yTER250	yTER223 + *snf4Δ::kanMX6*	This study
yTER8	yTER1+ *snf1Δ::kanMX6*	This study
yTER275	yTER223 + *sip1Δ::kanMX6*	This study
yTER276	yTER223 + *sip2Δ::kanMX6*	This study
yTER274	yTER223 + *gal83Δ::kanMX6*	This study
yTER235	yTER223 + $snf1^{K80R}$	This study
yTER236	yTER223 + $snf1^{K461R}$	This study
yTER237	yTER223 + $snf1^{K80R\text{-}K461R}$	This study
yTER244	yTER235 + *snf4Δ::kanMX6*	This study
yTER251	yTER236 + *snf4Δ::kanMX6*	This study
yTER230	yTER237 + *snf4Δ::kanMX6*	This study
yTH3638	*MATa ade2 his3Δ200 leu2-3 lys2Δ201 ura3-52 rpn10Δ::kanMX6*	W. Xiao
yTH4269	*MATα ade2 his3 leu2 ura3*	H. Harkness
yTER342	yTH4269 + *rpn10Δ::kanMX6*	This study
yTER325	yTER250 × yTER342 WT	This study
yTER326	yTER250 × yTER342 *snf4Δ*	This study
yTER327	yTER250 × yTER342 *snf4-rpn10Δ*	This study
yTER328	yTER250 × yTER342 *rpn10Δ*	This study

Table 5.2 Primers used in Chapter 5

Primer	Sequences
SNF1 F	5' AAAGGATGGGCGTGATGGGACTC 3'
SNF1 R	5' GTCGGAGTCACCTTGCTCGGTATTAGTC 3'
K80R F	5' ATACCACTACCGGCCAAAGAGTTGCTCTAAAAATC 3'
K80R R	5' GATTTTTAGAGCAACTCTTTGGCCGGTAGTGGTATG 3'
K461R F	5' GGATCAGTATCGAGAAGAGGACTCTACAG 3'
K461R R	5' ACTGTAGAGTCCTCTTCTCGATACTGATCCATGAAGGGTGATT 3'

CHAPTER 6 The E2 enzyme Ubc1 affects SNF1 kinase activity[2]

[2]**This work has been published as**

The ubiquitin-conjugating enzyme, Ubc1, indirectly regulates SNF1 kinase activity via Forkhead-dependent transcription

Jiao R, Lobanova L, Waldner A, Fu A, Xiao L, Harkness T.A, Arnason T.G

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6.1 Introduction

The SNF1 kinase class of serine/threonine kinases, which includes the AMPK in other systems, are of widespread interest because of their important roles in glucose homeostasis, stress resistance, and aging (Owen *et al*. 1979; Hardie 1999; Lu *et al*. 2011). These enzymes are inactive under optimal conditions, yet are rapidly activated in response to a wide variety of nutritional and stress cues. The active kinases, in turn, exert their activity to alter cellular pathways at the protein and transcriptional level to maintain homeostasis or to direct adaptive mechanisms for stress resistance. Simply, low glucose growth conditions will activate SNF1 kinase in yeast, whereas muscle contraction or fasting will do the same in animals (Celenza and Carlson 1986; Bergeron *et al*. 1999; Hardie 1999). The dominant, essential, and finely responsive regulatory step is the phosphorylation of Snf1 by upstream kinases, balanced by its controlled dephosphorylation (Celenza and Carlson 1986; Sutherland *et al*. 2003). This governing event is conserved between yeast and all higher eukaryotes. In yeast, SNF1 kinase directly phosphorylates a variety of downstream targets, including the nuclear target Mig1 (Treitel *et al*. 1998) and cytosolic target Rod1 (Becuwe *et al*. 2012). Mig1 functions as a transcriptional inhibitor that prevents *SUC2* expression, whereas Rod1 is involved in glucose transporter endocytosis (Becuwe *et al*. 2012). Several other described events act to regulate SNF1 kinase including allosteric tightening of the Snf1 (α) and Snf4 (γ) subunits within the heterotrimeric complex (Jiang and Carlson 1997), and the nuclear accumulation of the Snf1-Gal83 (β subunit)-Snf4 enzyme within the nucleus, necessary for transcriptional changes (Vincent *et al*. 2001). In addition, our recent work reported that the yeast orthologs of mammalian FOXO transcription factors, Fkh1 and Fkh2, regulate *SNF1* gene transcription, impacting the protein abundance of the Snf1 subunit (Jiao *et al*. 2015). The Snf1 subunit contains autoinhibitory and UBA domains that act as restraining functions on activity (Hedbacker and Carlson 2008; Jiao *et al*. 2015). More recently, additional negative influences have been shown to occur through Snf1 subunit post-translational modifications, specifically ubiquitination and SUMOylation, which effectively decreased Snf1 protein abundance through degradation, with resulting reductions in SNF1 kinase activity (Wilson *et al*. 2011; Zungu *et al*. 2011; Simpson-Lavy and Johnston 2013). Ubiquitin becomes covalently attached to target

proteins through the sequential action of ubiquitin activating (E1), conjugating (E2), and ligase (E3) activities: In yeast there is a single E1, a finite well-described group of thirteen E2s, and an ever-expanding recognition of E3 ligase activities.

Our goal was to identify discrete E2s that are involved in SNF1 kinase regulation in response to glucose levels and anticipated revealing those which are involved in Snf1-ubiquitin attachment. Here, we report that the cell cycle and stress-related E2, Ubc1, indirectly affects SNF1 kinase activity not through stability, but through upstream events affecting the yeast FOXO orthologs Fkh1/2 that provide transcriptional control of the Snf1 subunit. Ubc1 is known to act, along with Ubc4, with the APC to target and poly-ubiquitinate substrates for cell-cycle related degradation to enable exit from metaphase (M phase), and entry to G1 (Rodrigo-Brenni and Morgan 2007), and the *ubc1Δ* mitotic arrest point is consistent with a disruption of APC-dependent exit from mitosis and failure to enter G1 (Seufert *et al*. 1990). Our data suggests Ubc1 acts on a third Fkh, Hcm1, in an APC-independent manner to impact the SNF1 kinase via upstream events. Hcm1 appears to require Ubc1 to facilitate its nuclear shuttling, and activation of the Fkh/Snf1 stress response pathway.

6.2 Materials and Methods

6.2.1 Creation of *UBC1* deletion strains

All strains utilized in this manuscript are listed within Table 6.1. The *ubc1*Δ::*KanMX6* cassette was amplified using primers 500 bp up and downstream of *UBC1* and genomic DNA isolated from the *UBC1*/*ubc1*Δ::*KanMX6* diploid strain (yTER301) as template. The entire cassette was individually integrated into the Fkh1-TAP, Fkh2-TAP, Mig1-TAP, Rod1-TAP, Snf4-TAP, Gal83-TAP, Hcm1-GFP, Hcm1-TAP, and 2-hybrid reporter strains, with primary selection for successful integrants being Kan^{Res} and final confirmation by PCR. Snf1-GFP *ubc1Δ* was created by crossing (*SNF1*-GFP::*HIS3* x *ubc1*Δ::*HIS3*), tetrad dissection, scoring for markers, phenotypes, and confirmation by primer-specific PCR amplification.

6.2.2 Total protein extract and Western Blot analysis

Protocols have been previously described in section 3.1 and 3.2. Anti phospho-AMPK (Cell Signaling, 2535L), GFP (Covance, MMS-118P-500), Actin (Sigma, A4700,

Lot005134), Clb2 (Santa Cruz, y-180), TAP (Open Biosystems, CAB-1001), and Tubulin (Sigma, 051M4771) antibodies were purchased and the chemiluminescent signal captured on a VersaDoc (BIO-RAD) molecular imager (Quantity One 4.6.9).

6.2.3 Invertase assay

Protocols have been previously described in section 3.5.

6.2.4 Fluorescence microscopy

Protocols have been previously described in section 3.3. In detail, fluorescence microscopy was used to determine the subcellular localization of Snf1-GFP and Mig1-GFP, A minimum of 125 cells for each strain and condition were consecutively scored for co-localization of the GFP-tagged subunits and DAPI nuclear staining.

6.2.5 2-hybrid analysis

Protocols have been previously described in section 3.6. In this study, the yeast WT 2-hybrid reporter strain and the modified 2-hybrid strain *ubc1Δ* (*ubc1*Δ::*KanMX6* cassette integrated into PJ69-4 α) were doubly transformed with pairs of empty vectors (-ve control: pGAD-C2 and pGBD-C2), or the same backbones expressing unmodified Snf1 and Snf4 subunits (+ve).

6.2.6 mRNA expression analysis

Protocols have been previously described in section 3.8. In detail, RNA was isolated (RNAeasy Kit, Qiagen) from logarithmically (OD_{600} ≤0.4) growing WT Snf1-GFP or *ubc1Δ* Snf1-GFP yeast strains that were transformed with pFkh1-GFP or pFkh2-HA plasmids followed by RT (QuantiTect Reverse Transcription Kit, Qiagen).

6.2.7 Cell cycle arrest

Protocols have been previously described in section 3.10. WT and Hcm1-GFP were used in this experiment. Equal volume cell samples were collected each 30 minutes for protein and fluorescent activated cell sorting (FACS) analysis. The same Hcm1-GFP strain was used in Fig. 6.6C and D and Fig. 6.7A and B.

6.2.8 Flow cytometry

Protocols have been previously described in section 3.11.

6.2.9 Cycloheximide experiment

Protocols have been previously described in section 3.3.3. To stop protein synthesis, 10μg/ml CHX was added to live logarithmic phase cells ($OD_{600} \leq 0.8$) in YPD media. Equal cell numbers were collected every 30 minutes for cell lysate preparation and subsequent Western Blot analysis.

6.3 Results

6.3.1 Deletion of the E2 enzyme, Ubc1, impairs SNF1 kinase-dependent invertase activity

When glucose is limiting, yeast adapts to using alternative carbon sources. A key function of the SNF1 kinase is to adapt metabolic pathways to non-glucose carbohydrate sources, and the mechanism is particularly well documented for sucrose. Sucrose utilization requires the expression of invertase, an enzyme that cleaves the disaccharide sucrose molecule into glucose and fructose, which is encoded by the *SUC2* gene (Treitel *et al*. 1998). When glucose is abundant, *SUC2* expression is repressed by the binding of the transcriptional repressor, Mig1, at the *SUC2* promoter. Under limiting glucose conditions, activated SNF1 kinase enters the nucleus, phosphorylates Mig1 protein via its inherent kinase activity and releases Mig1 from the *SUC2* promoter. The subsequent translation of *SUC2* can be quantitatively determined by colorimetric Invertase assay (Harkness and Arnason 2014) or directly through RT-PCR, as an indirect measure of SNF1 kinase activity.

Figure 6.1A demonstrates the expected rise in invertase activity after 2 hours of growth in low (0.05%) glucose in a WT yeast strain, and the complete dependence of this on the Snf1 α catalytic subunit (*snf1Δ*). In contrast, disruption of the *SUC2* transcriptional repressor Mig1 (*mig1Δ*) demonstrates high activity regardless of glucose levels, as expected for loss of regulated repression. Deletion of the *UBC1* gene (*ubc1Δ*) resulted in a significant decrease in invertase activity as compared to the WT. This defect was also observed in WT and *ubc1Δ* strains carrying the endogenous Snf1-GFP fusion into either strain (WT-Snf1-GFP or *ubc1Δ*-Snf1-GFP*)*. To expand on this, we also tested invertase activity at short intervals leading up to the 2-hour time point (Fig. 6.1B) and report the presence of early (1 hour) and sustained impairment of invertase activity. The

corresponding changes to *SUC2* mRNA level in the WT and *ubc1Δ* strains, under activating and repressive conditions, paralleled that of the invertase activity (Fig. 6.1C).

6.3.2 Loss of Ubc1 function does not impair SNF1 kinase nuclear accumulation, allosteric associations or substrate targeting

Invertase defects (or decreases in maximal *SUC2* expression) can arise from disruption of any one of multiple steps in SNF1 kinase activation, including protein abundance, activating phosphorylation, allosteric associations, nuclear import, or phosphorylation of Mig1. These stages can be isolated and independently assessed to pinpoint where the Ubc1 protein is affecting SNF1 kinase activity. First, we asked if Ubc1 was required for movement of the Snf1-Gal83-Snf4 kinase complex into the nucleus under activating conditions, as a failure to efficiently accumulate in the nucleus would explain the impairment in transcriptional release of *SUC2* expression. We initially expressed Snf1-GFP constitutively from a 2μ yeast plasmid transformed into WT and *ubc1Δ* strains and used live fluorescent microscopy of these isogenic strains to localize Snf1-GFP to the nucleus (identified by DAPI staining) (Fig. 6.2A) and observed that the plasmid-expressed Snf1 subunit rapidly relocates to the nucleus after stimulating conditions in a manner indistinguishable from WT (Vincent *et al.* 2001). Furthermore, endogenous expression of a genomic version of GFP-tagged Snf1 subunit did not alter the efficiency of nuclear accumulation in WT or *ubc1Δ* isogenic strains (Fig. 6.2B). Multiple biological repeats of these experiments allowed us to score the relative efficiency of nuclear import of Snf1-GFP in these WT and *ubc1Δ* strains, demonstrating that there is no impairment of nuclear accumulation in the absence of the functional Ubc1 protein.

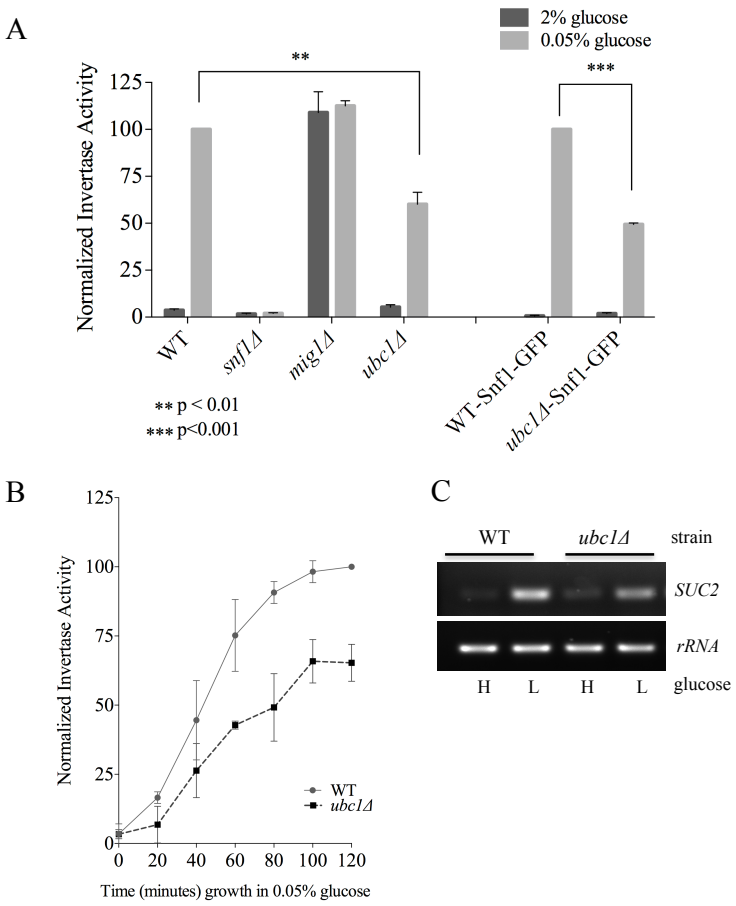
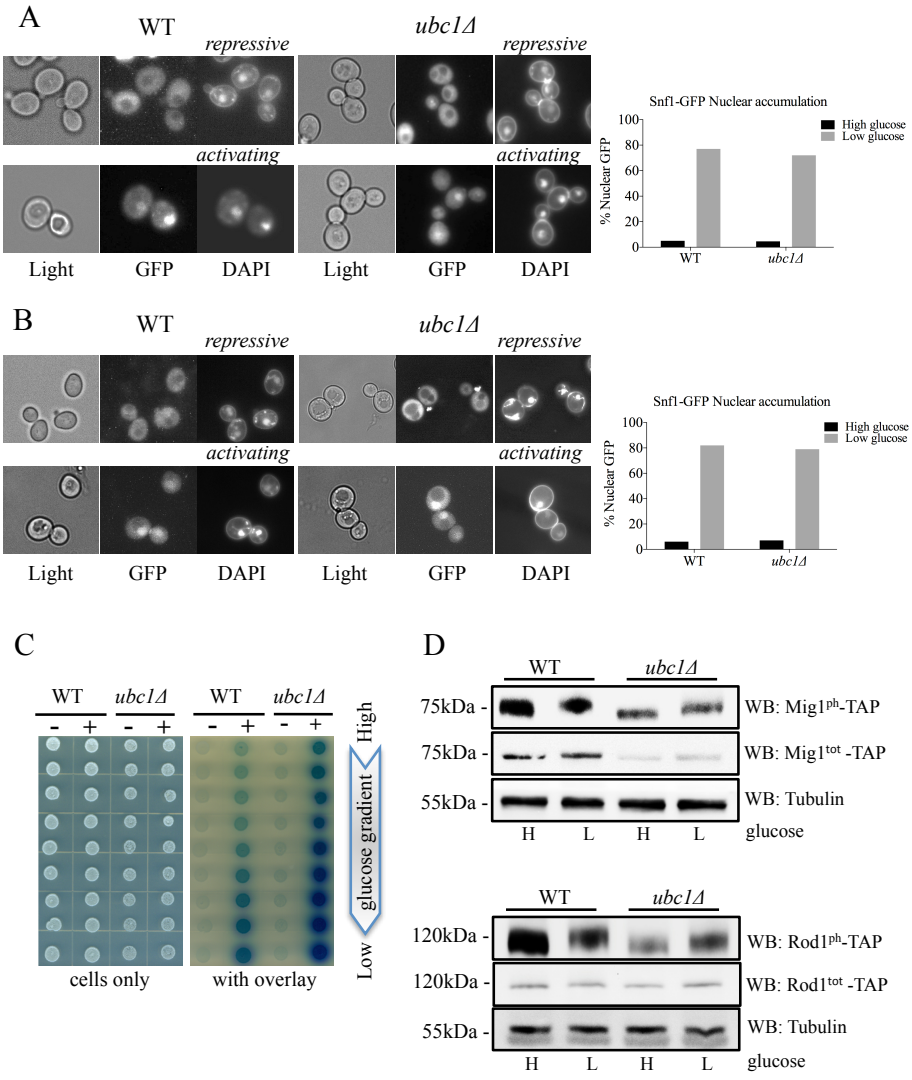


Figure 6.1 Yeast strains deleted for the ubiquitin conjugating enzyme, Ubc1, are impaired for SNF1 kinase-dependent invertase activity expressed from the *SUC2* gene. Comparison of invertase activity under repressive (2% glucose) and activating (0.05% glucose) conditions between isogenic yeast strains normalized to WT in 0.05% glucose (value of 100), showing the mean and SD. WT-Snf1-GFP and *ubc1Δ*-Snf1-GFP have a genomic *GFP* sequence integrated in-frame with the endogenous Snf1 sequence. ***A,*** Invertase activity after 2 hours of activating growth conditions. Statistical significance based on 3 biological repeats using t-test (Prism 6.0). ***B,*** Chronological invertase activity of WT and *ubc1Δ* strains, sampled intermittently over 2 hours following shift to low glucose media. Average of four biological repeats with SD are indicated for each time point. ***C,*** Agarose gel of RT-PCR products (26 cycles) using primers against *SUC2* and *rRNA* loci from RNA isolated from WT and *ubc1Δ* strains grown in 2% (H: high) and 0.05% (L: low, 2 hours) glucose. A and B were performed by Dr. T.G. Arnason.

Next, we assessed if the *UBC1* disruption interfered with the allosteric associations between the α and γ subunits of the kinase upon activation. 2-hybrid analysis was used to compare Snf1-Snf4 interactions throughout a glucose gradient; β-galactosidase production results in a visible blue color and correlates with the strength of the associations between these two proteins (Jiao *et al.* 2015). In WT yeast, we observed increased Snf1-Snf4 associations within the 2-hybrid assay as the glucose concentration drops, which is also seen in the isogenic *ubc1Δ* strain, at even greater levels than WT (Fig. 6.2C). Clearly, the invertase defect linked with *UBC1* disruption is not related to allosteric hindrance.

We next asked if the *UBC1* deletion affected the ability of SNF1 kinase to phosphorylate known protein targets. De-repression of *SUC2* requires SNF1 kinase-dependent phosphorylation of the nuclear transcriptional repressor protein, Mig1. Non-nuclear targets for SNF1 kinase phosphorylation include the Rod1 protein that resides at the plasma membrane (Becuwe *et al*. 2012). Glucose responsive phosphorylation of both nuclear Mig1 and cytosolic Rod1 can be directly assessed by Western analysis by their visible phospho-shift to higher molecular weights (Treitel *et al*. 1998; Becuwe *et al*. 2012). We find a noticeable upwards phospho-shift of each target in low glucose in both WT and *ubc1Δ* strains (Fig. 6.2D). Although there is an apparent decrease in Mig1 protein abundance in the *ubc1Δ* strain, the phospho-shift is not impaired, nor is there a defect in the expected Mig1 nuclear export under activating conditions (Fig. 6.9C). Therefore, Ubc1 deletion does not impair the enzymatic activity of SNF1 kinase.


A
WT
ubc1Δ
repressive
repressive
activating
activating
Light
GFP
DAPI
Light
GFP
DAPI
Snf1-GFP Nuclear accumulation
% Nuclear GFP
High glucose
Low glucose
WT
ubc1Δ
B
WT
ubc1Δ
repressive
repressive
activating
activating
Light
GFP
DAPI
Light
GFP
DAPI
Snf1-GFP Nuclear accumulation
% Nuclear GFP
High glucose
Low glucose
WT
ubc1Δ
C
WT
ubc1Δ
WT
ubc1Δ
High
glucose gradient
Low
cells only
with overlay
D
WT
ubc1Δ
75kDa
WB: Mig1ph-TAP
75kDa
WB: Mig1tot -TAP
55kDa
WB: Tubulin
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WT
ubc1Δ
120kDa
WB: Rod1ph-TAP
120kDa
WB: Rod1tot -TAP
55kDa
WB: Tubulin
H
L
H
L
glucose

Figure 6.2 Loss of Ubc1 function does not impair SNF1 kinase nuclear accumulation, allosteric associations or substrate targeting. ***A,*** Fluorescent microscopy of GFP-tagged yeast Snf1 constitutively expressed from a high copy 2μ plasmid or ***B,*** expressed from the endogenous Snf1 promoter in isogenic WT and *ubc1Δ* strains grown under repressive (2% glucose) or shifted to activating (5% glycerol for 30 minutes) conditions. The percent of cells with Snf1-GFP nuclear accumulation was quantitated from four biological repeats. 125 consecutive cells were scored for co-localization of GFP and DAPI signal in high and low glucose in the isogenic WT and *ubc1Δ* strains. ***C,*** 2-hybrid associations between empty vectors (-) and Snf1-Snf4 pairs (+) in WT and *ubc1Δ* strains are shown. Equal cell numbers were spotted down a glucose gradient (0.05% to 2% glucose) before and after β-galactosidase color development from overlay. ***D,*** Isogenic WT and *ubc1Δ* strains harboring endogenous TAP-epitope tags to Mig1 and Rod1 were divided into high (H: 2%) and low (L: 0.05%) glucose media for 30 minutes prior to cell lysis. Duplicate sample were run in parallel on 10% acrylamide gels (for total TAP-protein; $Mig1^{tot}$ and $Rod1^{tot}$, upper panels) and 7.5% (to enhance the phospho-shift; $Mig1^{ph}$ and $Rod1^{ph}$, lower panels). WB: Western Blot primary antibody/target. Light: non fluorescent 100 x objective. GFP: green fluorescent protein epitope tag. DAPI: (4',6-Diamidino-2-Phenylindole, Dihydrochloride) a fluorescent DNA interchelator.

6.3.3 Snf1 protein abundance, but neither stability nor phosphorylation, is decreased by Ubc1 disruption

An obvious question to ask was whether the role of Ubc1 in SNF1 kinase regulation was to target Snf1 for ubiquitination, and ultimately degradation. It is known that the catalytic Snf1 α-subunit can be poly-ubiquitinated and its abundance affected (Wilson *et al*. 2011). We compared the steady-state abundance of endogenous Snf1-GFP protein in logarithmically growing WT and *ubc1Δ* strains (Fig. 6.3A) and observed a clear decrease in Snf1 abundance, irrespective of the activation state of the enzyme, limited to the *ubc1Δ* strain. This is not consistent with Ubc1-dependent ubiquitination and subsequent degradation of Snf1, as this would instead manifest as an increase in Snf1 protein in the *UBC1* deletion. Activating Snf1 phosphorylation was maintained at near-WT levels despite the decrease in total Snf1 protein (Fig. 6.3A). In addition to Snf1, protein levels of both the endogenous Snf4 γ and Gal83 β subunits were likewise decreased in the *ubc1Δ* strain, compared to WT (Fig. 6.3B). To directly assess if there

was enhanced degradation of Snf1 in the absence of Ubc1 function, we performed CHX protein degradation assays of Snf1 over a 3-hour period (Fig. 6.3C) in WT and *ubc1Δ* strains. Snf1 was stable over the three-hour period in the WT strain and also appeared stable in *ubc1Δ*. To confirm the stability of Snf1 in the *ubc1Δ* strain, a biological repeat was performed with a greater protein load (80 µg/lane versus 40 µg/lane) (Fig. 6.3D). These results suggest that Ubc1 does not play a role in Snf1 protein stability.

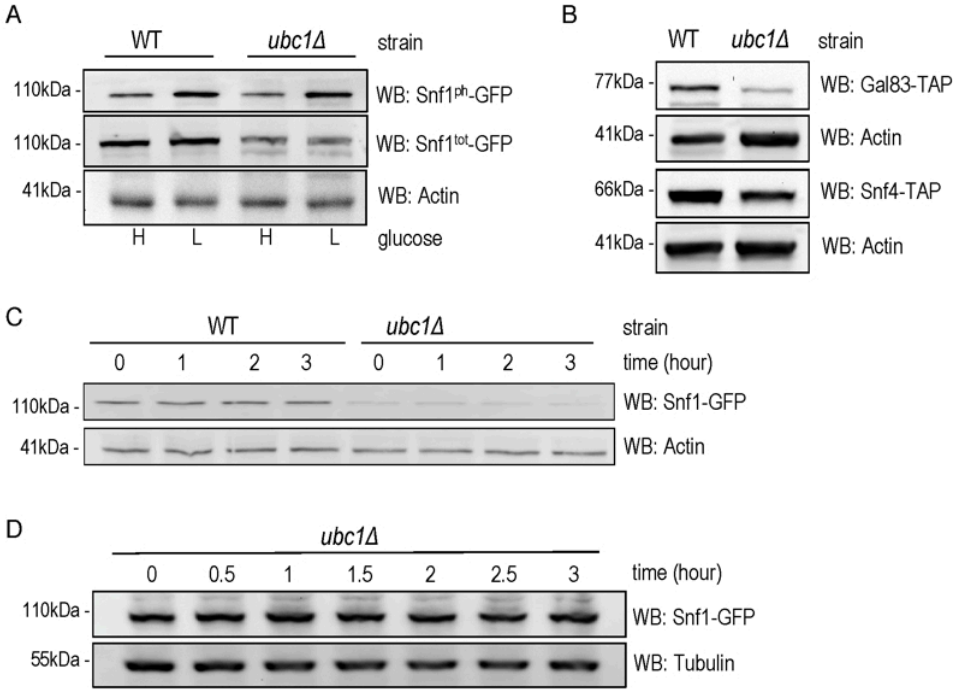

Figure 6.3 Snf1 protein abundance, but neither stability nor phosphorylation, is decreased by Ubc1 disruption. ***A,*** Early logarithmic phase WT and *ubc1Δ* strains harboring genomic Snf1-GFP were grown in 2% (H) and 0.05% (L) glucose for 1 hour prior to cell lysis, and equal total protein loaded in duplicate for Western analysis of total Snf1 ($Snf1^{tot}$-GFP) and phosphorylated Snf1 ($Snf1^{ph}$-GFP). ***B,*** WT and *ubc1Δ* strains harboring genomic Gal83-TAP or Snf4-TAP were treated as in (***A***) and Western analysis for TAP abundance is shown. ***C,*** Assessment of Snf1 protein stability over 3 hours in WT and *ubc1Δ* strains in the presence of cycloheximide, added at time (0), in 2% glucose. Equal cell numbers were removed at the indicated time points with 40 µg loaded. ***D,*** Biological repeat of Snf1-GFP stability (as in ***B***) is shown with 80 µg protein loaded per lane, and additional time-points.

6.3.4 SNF1 expression and Forkhead protein abundance are decreased upon Ubc1 deletion

We addressed the possibility that *SNF1* transcription was decreased in the *ubc1Δ* strain, as an explanation for the decreased abundance of the Snf1 protein. RT-PCR of the *SNF1* gene revealed a clear decrease in mRNA level in the *ubc1Δ* strain (Fig. 6.4A), being approximately 50% that of the isogenic WT (Fig. 6.4B). We had previously found the yeast FOXO orthologs, Fkh 1 and Fkh2, to be involved in *SNF1* gene expression (Jiao *et al*. 2015) and asked if the absence of Ubc1 function was affecting Fkh1/2 activity, upstream of Snf1. In asynchronous cells, we observed that disruption of *UBC1* noticeably diminished Fkh1 protein abundance, while Fkh2 protein was essentially absent in the same strain even when significantly more protein (lysate) was tested (80 μg/lane for Fkh2 versus 15 μg/lane for Fkh1) (Fig. 6.4C). Fkh1 and Fkh2 are known to be transcriptionally regulated in synchrony with the cell cycle under the influence of a third forkhead member, Hcm1 (Pramila *et al*. 2006). Yeast strains deleted for *UBC1* have been found to exhibit with cell cycle defects and to accumulate with large buds in late G2/M phase (Seufert *et al*. 1990). Indeed, we found that the protein abundance of endogenous Clb2 was markedly elevated in the *ubc1Δ* strain as compared to WT, consistent with an accumulation of cells residing in G2/M phase when Clb2 levels are highest (Hollenhorst *et al*. 2000). To confirm this, FACS of early logarithmic (OD_{600} of 0.4) asynchronous yeast cells from *ubc1Δ* strains demonstrated a significant inherent accumulation of cells with fully replicated DNA (2n) in G2/M (Fig. 6.4D). Light microscopy of cells representative of those undergoing FACS show a heterogeneous population in WT with various bud sizes, yet a clear accumulation of large budded yeast cells in *ubc1Δ*, consistent with previous reports of G2/M delay (Seufert *et al*. 1990). The published report noting that *FKH1* and *FKH2* transcript levels are highest in G2/M phase and lowest in G1 (Pramila *et al*. 2006) was inconsistent with the decreased Fkh protein levels we observed in the *ubc1Δ* strain partially stalled at G2/M, leading us to investigate this further.

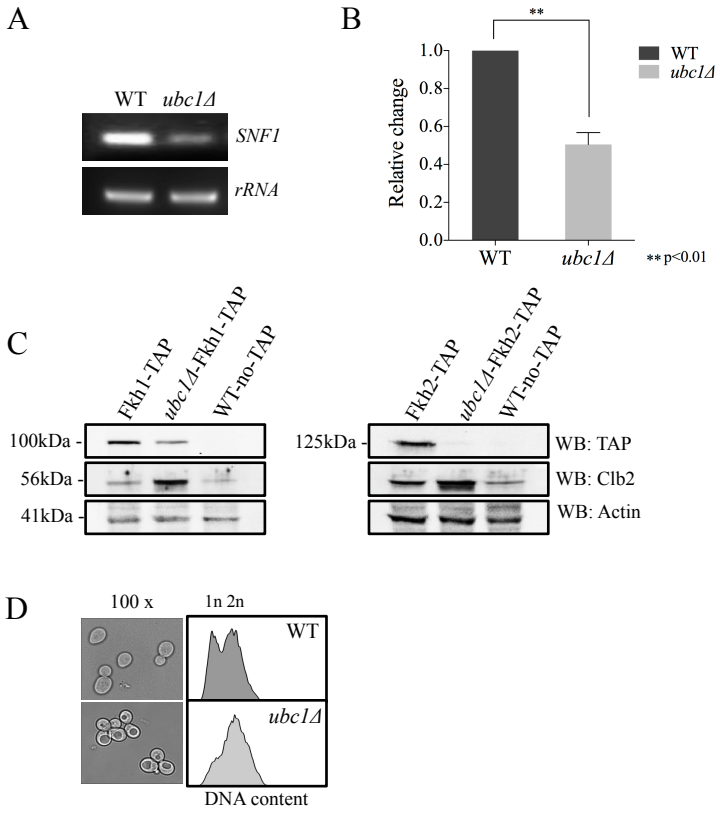

Figure 6.4 *SNF1* expression and Forkhead protein abundance are decreased by Ubc1 deletion. *A,* *SNF1* and *rRNA* products from RT-PCR (26 cycles) were run on an agarose gel. RNA was isolated from isogenic WT and *ubc1Δ* strains that were grown to early log phase. ***B,*** Quantitation of three biological repeats of *SNF1* and *rRNA* abundance from (A) is shown with mean, SEM, and t-tailed significance (Prism 6.0). ***C,*** The transcription factors Fkh1 and Fkh2 were endogenously TAP-tagged within the WT and *ubc1Δ* strains, and steady-state protein levels in 2% glucose (Fkh1-TAP left panel, 15 µg/lane, and Fkh2-TAP right panel 80 µg/lane) assessed by Western analysis. Clb2 is detected endogenously. **D,** Light microscopy (100x objective) of an early logarithmic asynchronous culture showed the proportion of large-budded cells, with corresponding FACS identifying the relative population of cells with replicated DNA (2n) in *ubc1Δ* compared to the WT strain.

6.3.5 Snf1 protein and glucose-responsive *SUC2* expression levels are nearly reestablished by Fkh1 or Fkh2 overexpression in the *ubc1Δ* strain

Given that Fkh1 and Fkh2 can drive the expression of *SNF1* (Jiao *et al*. 2015), and that Fkh1/2 gene expression fluctuates with the cell cycle, we asked if Snf1 protein levels fluctuate in synchrony with the cell cycle. There have been no reports of SNF1 kinase being regulated in a cell cycle dependent manner to the best of our knowledge. A G1 arrest-release experiment was performed in WT yeast to determine the inherent abundance of the Snf1 subunit protein level throughout the cell cycle. Fig. 6.5A reveals that Snf1 protein levels do not change as synchronized cells exit G1 and pass through the cell cycle, with fluctuations of Clb2 used as a surrogate marker for passage through the cell cycle (Kuhne and Linder 1993). Further confirmation of successful synchronization comes from the FACS analysis of these cells, showing the gradual shift of 1n to 2n (replicated) DNA (Fig. 5B). To ascertain if the decreased Snf1 abundance was a simple result of limited forkhead proteins, Fkh1 and Fkh2 were constitutively expressed from plasmids in the *ubc1Δ* strain. The resulting Snf1 protein (Fig. 6.5C) and *SNF1* transcripts (Fig. 6.5D) are increased with Fkh1 expression, which is not as apparent with Fkh2. Advancing one step further, we similarly investigated if Fkh1 or Fkh2 expression in the *ubc1Δ* strain restored the low-glucose-activated *SUC2* expression to that of WT levels. Figure 6.5E shows that low glucose-induced expression of *SUC2* is at, or higher than, WT levels in the presence of the Fkh1 plasmid.

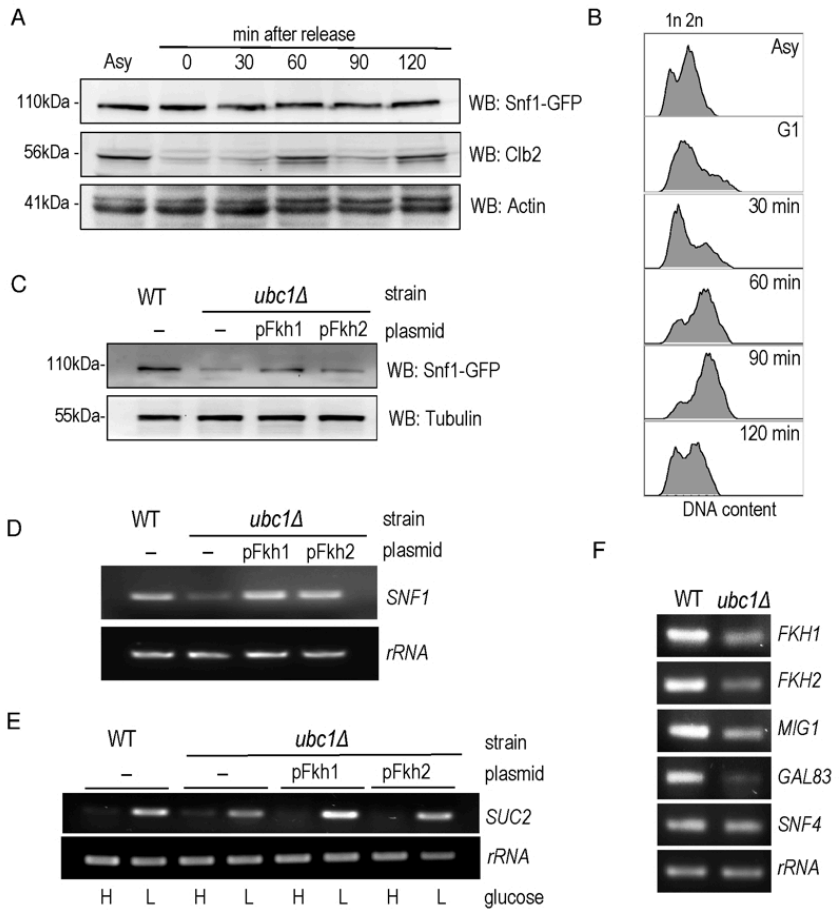

Figure 6.5 Snf1 protein and glucose-responsive *SUC2* expression levels are reestablished by Fkh1 or Fkh2 overexpression in the *ubc1Δ* strain. ***A,*** α-factor arrest release of the WT yeast strain harboring an endogenous Snf1-GFP tag, with detection of Snf1-GFP and endogenous Clb2. Equal cell numbers were taken after release from G1 phase at the time points indicated, and Western analysis of the cell lysates as shown. ***B,*** Corresponding FACS of samples in (***A***) at the indicated time points, with un-replicated (1n) and replicated (2n) DNA abundance indicated. ***C,*** Western analysis of steady-state Snf1-GFP protein levels in WT and *ubc1Δ* strains with, or without, 2μ plasmid expression of Fkh1 (pFkh1) and Fkh2 (pFkh2) in 2% glucose. ***D,*** RT-PCR (26 cycles) of *SNF1* and *rRNA* expression in the *ubc1Δ* strain with, and without, independent 2μ plasmid expression of Fkh1 (pFkh1) and Fkh2 (pFkh2), with comparison to isogenic WT. ***E***, RT-PCR (26 cycles) of *SUC2* expression under repressive (H: 2%) and activating (L:0.05%) glucose levels with strains and plasmids as described in (***D***). ***F,*** RT-PCR of genes encoding SNF1 kinase γ subunit (*SNF4*), β subunit (*GAL83*), *SUC2* repressor, *MIG1*, and the *FKH*s (*FKH1, FKH2*), comparing the *ubc1Δ* and WT strains. Asy: asynchronous. G1: α-factor arrest in G1.

To distinguish between etiologies underlying the decreased protein abundance of Fkh1 and Fkh2 (Fig. 6.4C), Snf4 and Gal83 (Fig. 6.3B) and Mig1 (Fig. 6.2D), we asked if the *ubc1Δ* deletion also affected their transcription. With the exception of Snf4, all RT-PCR products were of lower abundance in the *ubc1Δ* strain (Fig. 6.5F). Our data suggests that Fkh1/2 transcription of *SNF1* does not fully control Snf1 protein abundance in the *ubc1Δ* strain; despite compensated *SNF1* mRNA levels (Fig. 6.5D), Snf1 protein levels did not reach that of WT (Fig. 6.5C). Furthermore, constitutive plasmid expression of Snf1-HA within the *ubc1Δ* strain did not result in WT levels of Snf1 protein (Fig. 6.9A), suggesting that Snf1-HA is decreased in the *ubc1Δ* strain regardless of its endogenous transcription. Finally, we tested whether over-expression of the Snf4 γ subunit would enhance Snf1 proteins levels, and concluded that it did not (Fig. 6.9B).

6.3.6 Hcm1 is impaired in its cell cycle-dependent nuclear import, upon deletion of Ubc1

We sought a more detailed explanation for the decrease in Fkh1 and Fhk2 expression within the *ubc1Δ* strain, and focused on Hcm1, the Fkh family member known to regulate Fkh1 and Fkh2 expression in a cell cycle dependent manner (Pramila *et al*. 2006). We tested both Hcm1 protein abundance, and its ability to shuttle between the cytosol and nucleus. We first found that the steady-state protein abundance of Hcm1 was modestly decreased upon disruption of the *UBC1* gene (Fig. 6.6A), although the protein appeared stable over an extended three-hour period (Fig. 6.6B). Hcm1 normally exhibits regulated nuclear import during G1 of the cell cycle, and we asked if the expected nuclear accumulation of Hcm1 in G1 (Rodriguez-Colman *et al*. 2013) is, in fact, disrupted by deletion of the *UBC1* gene. Fluorescent microscopy was used to determine endogenous Hcm1-GFP location within cells arrested in G1 and then released. We observed a clear nuclear accumulation in arrested WT cells, in direct contrast to a lack of nuclear-GFP signal in the *ubc1Δ* strain arrested in G1 (Fig. 6.6C). The morphology of the yeast cells, combined with the flow analysis (Fig. 6.6D) confirms that G1 arrest was successful in WT and *ubc1Δ* strains.

To further analyze Hcm1 protein abundance differences between the WT and *ubc1Δ* strains, we compared the Hcm1 protein level throughout the cell cycle after G1 arrest and release in WT. Figure 6.7 showed the cell-cycle fluctuations in Hcm1 protein

levels, fully consistent with Hcm1 levels reported by others (Landry *et al*. 2014). 90 minutes after release, Hcm1 protein level reached maximum, followed by a decline at 120 minutes at which time Clb2 levels peaked. Supporting this, FACS analysis and microscopy images collected at the 120 minute time-point are consistent with late mitosis/telophase; fully replicated (2n) and late mitosis (double budded, nuclei separated) (Fig. 6.7B). The doublet signal observed for the Hcm1-GFP Western Blot analysis is not present in the Hcm1-TAP western blots, and is thus a non-specific artifact.

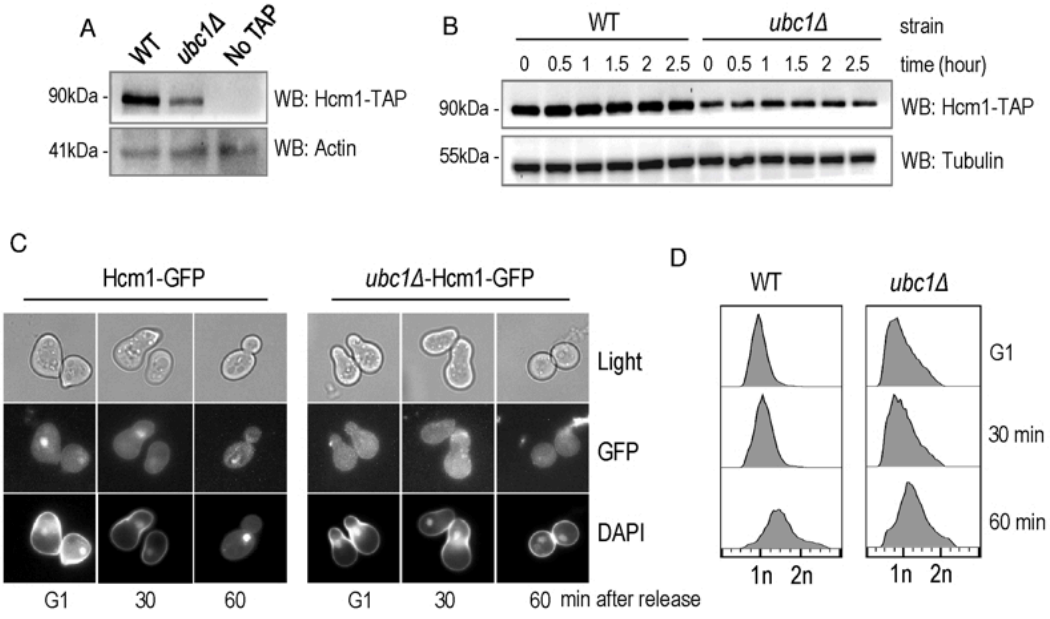

Figure 6.6 Hcm1 is impaired in its cell cycle dependent nuclear import upon deletion of Ubc1. ***A,*** Equal numbers of early logarithmic phase cells from WT and *ubc1Δ* strains with, or without, an endogenous Hcm1-TAP tag were lysed followed by TAP Western analysis. ***B***, Assessment of Hcm1 protein stability over 3 hours in WT and *ubc1Δ* strains in the presence of cycloheximide, added at time (0), in 2% glucose. Equal cell numbers were removed at the indicated time points with 30 µg loaded. ***C,*** Fluorescent microscopy of genomically integrated GFP-tagged Hcm1 expressed from its endogenous promoter in isogenic WT and *ubc1Δ* strains. Both strains were arrested in G1 followed by release, with cells collected at the indicated time-points. ***D,*** FACS analysis of cells collected at the time-points in (*A*), highlighting the relative proportion of replicated DNA (2n) upon release from G1.

6.3.7 Ubc1 impacts *SNF1* gene expression in an APC-independent manner

Ubc1 is known to act with the APC to facilitate the metaphase-to-anaphase transition, and we were interested in determining whether Ubc1's impact on transcription was APC-dependent, or –independent. We made use of yeast cells harboring the Apc5 subunit mutation ($apc5^{CA}$), to test this. Figure 6.7C indicates that *SNF1* gene expression was not affected in the $apc5^{CA}$ temperature sensitive strain, and was decreased only in *ubc1Δ* cells. We next compared if the cell cycle arrest position and cell morphology between the $apc5^{CA}$ and *ubc1Δ* strains were similar. Early logarithmic, asynchronous, yeast cells from *ubc1Δ* and $apc5^{CA}$ strains were analyzed using FACS and cells were imaged using propidium iodide staining of nucleic acid (Fig. 6.7D). Interestingly, the inherent arrest point of the *ubc1Δ* strain exhibited a distinctly different 1n/2n content, and nuclear positioning to that of $apc5^{CA}$ cells (Fig 6.7D). Together, these results clearly indicate that the role of Ubc1 on *SNF1* expression is independent of the APC.

6.3.8 Schematic of Ubc1-dependent mechanisms and potential targets impacting SNF1 kinase activity

Figure 6.8 graphically summarizes the observations made regarding the role of Ubc1 in SNF1 kinase function. In general, deletion of Ubc1 function resulted in decreased protein abundance of all three SNF1 kinase subunits tested, with transcriptional declines limited to Snf1 and Gal83 subunits, as Snf4 expression was not affected. The maintenance of WT-levels of Snf1 phosphorylation suggests a rebalancing of upstream kinase and phosphatase activities. The impaired *SNF1* expression is due to a simultaneous decrease in *FKH1* and *FKH2* expression and protein levels, ultimately appearing to arise from a failure of Hcm1 to accumulate in the nucleus to facilitate their transcription. The Ubc1 protein target involved in Hcm1 shuttling is not known. Enhanced allosterism within the SNF1 kinase complex may arise from the relative hyper-phosphorylation of Snf1, or from the loss of a *Factor X* that contributes to steric hindrance of subunit associations.

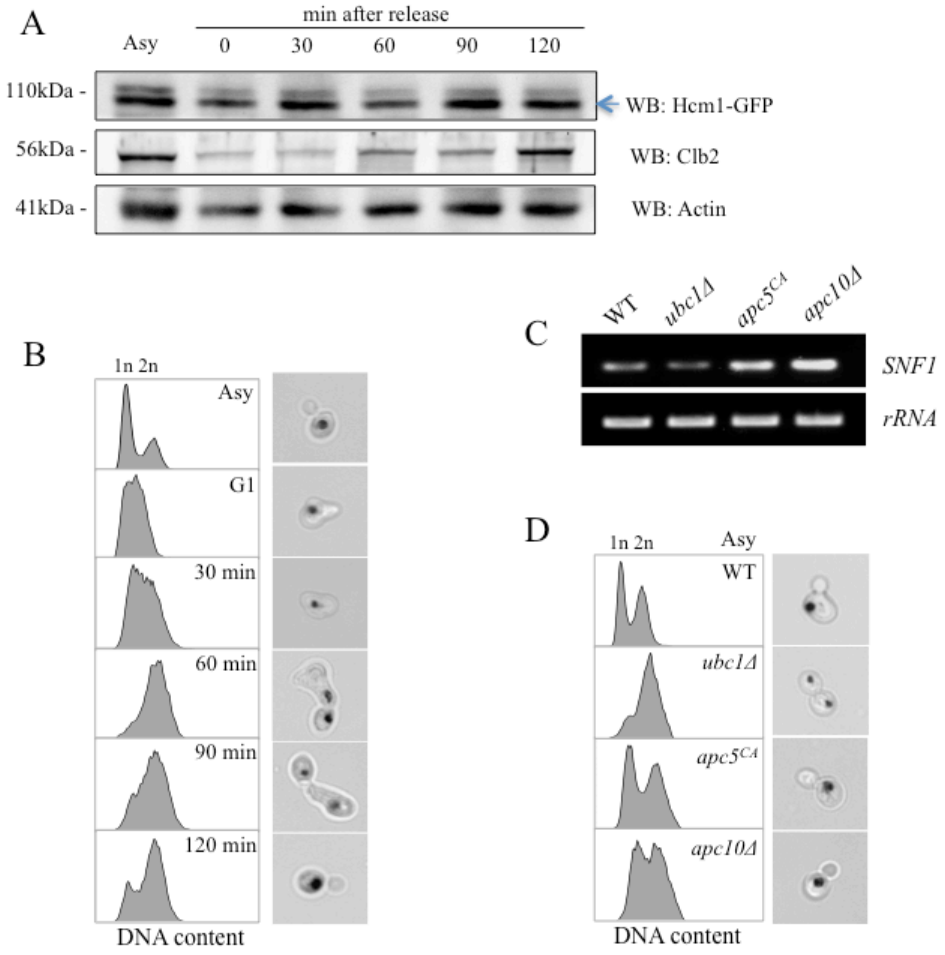

Figure 6.7 Hcm1 abundance is cell cycle dependent. Cells arrest at late M phase upon Ubc1 deletion. ***A***, α-factor arrest release of the WT yeast strain harboring an endogenous Hcm1-GFP tag, with detection of Hcm1-GFP and endogenous Clb2. Equal cell numbers were taken after release from G1 phase at the time points indicated, and Western analysis of the cell lysates as shown. ***B,*** Corresponding FACS analysis of WT samples in (***A***) at the indicated time-points, with un-replicated (1n) and replicated (2n) DNA content indicated. Light images (100x objective) indicate the cell morphology and nuclear position (propidium stained nucleic acid). ***C***, RT-PCR (26 cycles) of *SNF1* and *rRNA* expression in the *ubc1Δ apc5*^{CA} strains, with comparison to isogenic WT. ***D,*** FACS of early logarithmic asynchronous cultures demonstrating the relative population of cells with replicated DNA (2n) in *ubc1Δ* and *apc5*^{CA} strains, compared to isogenic WT, with representative images of cell morphology and nuclear position. Asy: asynchronous.

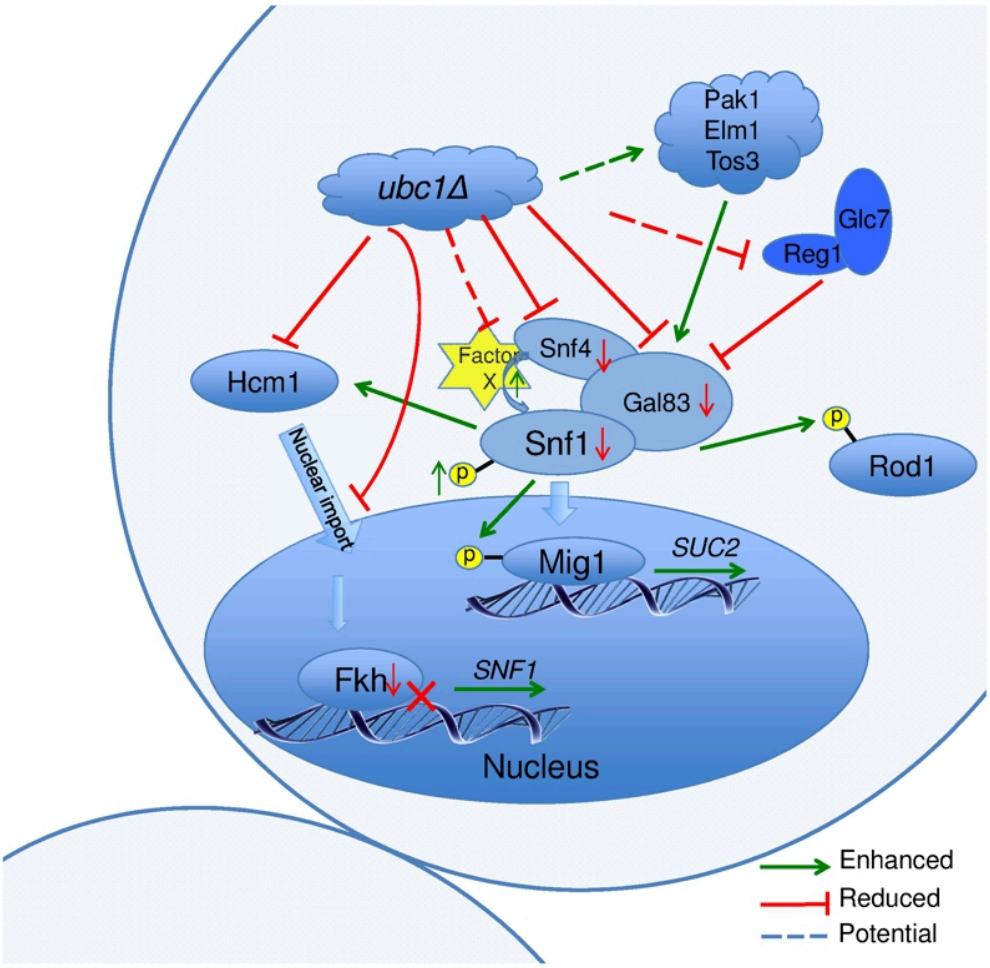

Figure 6.8 Schematic models of Ubc1-dependent mechanisms and their potential targets impacting SNF1 kinase activity. In the *ubc1Δ* strain, the Hcm1 protein fails to shuttle to the nucleus in a cell cycle dependent manner. The mechanisms is unknown, yet may include the failure of Ubc1 to degrade a cytosolic Hcm1 tether, or to provide an ubiquitin-mediated import signal. The lack of nuclear Hcm1 results in impaired expression of *FKH1/2* genes, which required Hcm1 for expression. The decrease in Fkh1/2 protein in turn impedes the expression, and subsequent protein abundance, of Snf1. The Snf1 protein present, however, retained its functional ability to target cytosolic (Rod1) and nuclear (Mig1) proteins for phosphorylation, and itself be phosphorylated and trans-located in response to activating conditions. There are enhanced allosteric associations between Snf1 and the regulatory Snf4 subunit in the absence of Ubc1 function, again by an unknown mechanism that may involve the removal of a moiety causing steric hindrance, *Factor X*. No obvious candidate protein is known that associates with the activated complex that would be stabilized by a loss of E2 activity.

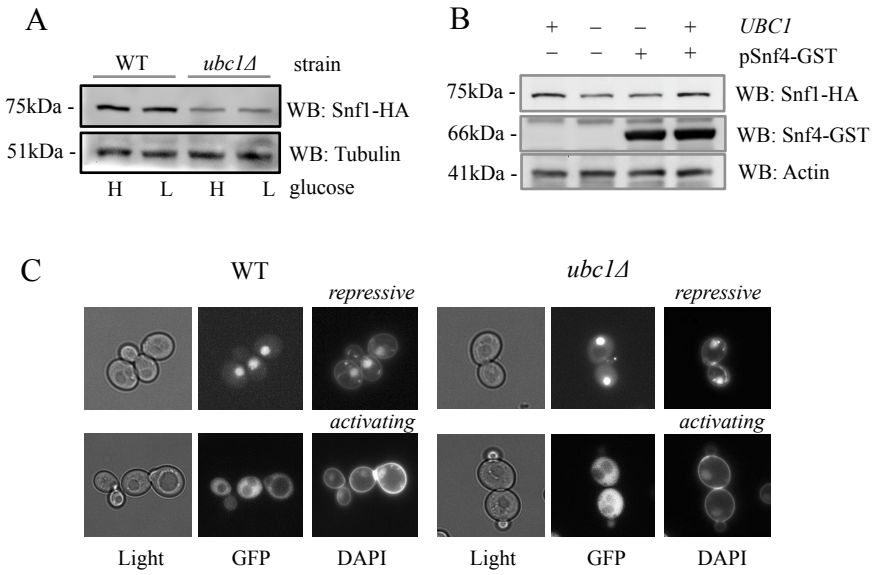

Figure 6.9 Snf1 protein levels are decreased in the *ubc1Δ* strain, regardless of its transcription level. A, HA tagged Snf1 subunit was constitutively expressed from a high copy 2μ plasmid in WT and *ubc1Δ* strains. Transformed strains were divided, grown under activating (L) and repressive (H) glucose concentrations, and protein lysates taken for anti-HA Western analysis. **B,** plasmid expression of constitutively expressed Snf4-GST in the *ubc1Δ* strain (*UBC1*−) does not compensate for WT (*UBC1*+) protein levels of Snf1-HA (also plasmid expressed). Total protein was tested using anti-HA or anti-GST Western Blot. Anti-Tubulin and Actin western analysis was used as loading control. L: 0.05% low glucose; H: 2% high glucose. **C**, Fluorescent microscopy of GFP-tagged Mig1, a *SUC2* transcriptional repressor constitutively expressed from a high copy 2μ plasmid in WT and *ubc1Δ* strains grown under repressive (2% glucose) or shifted to activating (5% glycerol for 30 minutes) conditions. Light: non-fluorescent 100 x objective. GFP: green fluorescent protein epitope tag. DAPI: (4',6-Diamidino-2-Phenylindole, Dihydrochloride) a fluorescent DNA interchelator.

6.4 Discussion

The SNF1 kinase/AMPK family of enzymes are tightly regulated, non-hormonal, sensors of stress and nutrient availability that facilitate adaptation of cellular pathways to maintain homeostasis. The yeast SNF1 kinase is a heterotrimeric protein kinase complex that is activated, in part, by energy and nutrient limitations via an essential phosphorylation step. Understanding the intricacies of the subtle regulatory mechanisms

controlling their activation and activity is also of great interest. Many fields, not the least of which is the potential benefit to human health, will advance with greater knowledge of regulatory targets to enhance the activity of these enzymes.

Through its kinase function, activated SNF1 kinase shifts the utilization of specific metabolic pathways in order to maintain cellular ATP levels (Hardie *et al*. 1998). The SNF1 kinase is strongly evolutionarily conserved from yeast to humans, and fundamental mechanisms regulating SNF1 kinase activity in yeast have been proven to be likewise used in higher eukaryotes, including the essential phosphorylation on its α subunit, regulated dephosphorylation, allosteric subunit associations, and nuclear shuttling (Hedbacker and Carlson 2008). In addition to these discrete steps in SNF1 kinase activation, it has also been reported that the catalytic α subunit of the yeast SNF1 kinase, Snf1 is poly-ubiquitinated and degraded (Wilson *et al.* 2011), similar to an earlier report of this mechanism acting on the human ortholog, AMPK (Al-Hakim *et al.* 2008). Of note, we do not find evidence for inherent Snf1 protein instability and degradation in these experiments, irrespective of the kinase activation state or glucose availability (Fig 6.3A).

The covalent attachment of ubiquitin to a target protein requires the concerted action of the ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin-ligase (E3) activities (Finley *et al.* 2012), and specific protein targets are recognized and selected by specific E2/E3 combinations (Rodrigo-Brenni and Morgan 2007). The identity of the E2(s) required for the reported ubiquitin conjugation to Snf1 has not been reported in yeast or in other eukaryotes to our knowledge, and as an approach to identify these factors, we systematically screened E2 deletion strains for effects on invertase activity, a SNF1 kinase-dependent event. Given the well-known consequence of protein stability changes upon ubiquitination, we had predicted that deletion of important E2 enzymes in Snf1-ubiquitin targeting would create a stabilizing effect on Snf1, with the possibility of enhanced SNF1 activity due to Snf1 accumulation.

Here, we do not report the E2 involved in targeting Snf1 for ubiquitination. Instead, we report that deletion of the gene encoding the E2 Ubc1 resulted in a ~50% reduction of SNF1 kinase activity that did not arise from deficiencies in glucose-regulated nuclear import or subunit associations, or in its ability to target cytosolic or

nuclear targets for phosphorylation. Rather, the explanation resides in the noticeable decrease of Snf1 protein in the *ubc1Δ* strain, regardless of whether the growth conditions are activating or repressive for SNF1 kinase activity (Fig. 6.3A). It is interesting to note that, despite lower total Snf1 protein, a conspicuous and consistent enhancement of Snf1-Snf4 interactions exists throughout the 2-hybrid glucose gradient (Fig. 6.2C) and the relative degree of Snf1 phosphorylation is maintained at near WT levels (Fig. 6.3A), yet was not capable of complementing for SNF1 kinase invertase activity. A simultaneous decrease of the β (Gal83) and γ (Snf4) subunits of the SNF1 kinase heterotrimer in this strain very likely contributes to this decrease in nuclear activity. This phenomenon of Snf1 hyper-phosphorylation has been noticed previously (Hsu *et al.* 2015) and has been proposed to be a compensatory mechanism to preserve SNF1 kinase function, with our results suggesting that it may also extend to allosterism. Decrease in the level of one subunit within SNF1 kinase has also been shown to correspond to decreases in the remaining (Elbing *et al.* 2006). Accordingly, we observed decreases in the γ subunit (Snf4) and the β subunit (Gal83) in the *ubc1Δ* strain (Figure 6.3B), perhaps as a means of preserving normal stoichiometry within the enzyme complex.

One of the major functions of ubiquitin conjugation is to target proteins for degradation by the proteasome (Seufert *et al.* 1990). In contrast to protein degradation resulting from poly-ubiquitination through lysine-48 chains, mono-ubiquitination of proteins has been shown to contribute to protein stability (Torres *et al.* 2009). Furthermore, ubiquitin conjugation can also have other important functions unrelated to protein degradation such as subcellular trafficking and protein associations (Wiebel and Kunau 1992). Ubc1 is known to contribute to lysine-48 poly-ubiquitin chain formation (Rodrigo-Brenni and Morgan 2007), whereas *in vivo* reports of intrinsic mono-ubiquitination activity were not found. Interestingly, we found the Snf1 subunit to be stable for hours in both the WT and *ubc1Δ* strains (Fig. 6.3C), without detection of higher molecular weight bands under these physiological conditions to suggest Snf1 is poly-ubiquitinated. While the stability of Snf1 in the WT strain was initially unexpected, it is clear that the previously reported affects of Snf1 instability in response to SUMOylation and ubiquitination required the forced accumulation of poly-ubiquitin chains attached to Snf1. This was enabled by genetic manipulation of the yeast strains, through disruption of

the gene encoding the ubiquitin-removal function, *UBP8,* and the resulting poly-ubiquitinated proteins that are targeted for proteasomal degradation (Wilson *et al.* 2011; Simpson-Lavy and Johnston 2013). Otherwise Snf1 was not noticeably degraded.

Unexpectedly, the deletion of *UBC1* resulted in a substantial decrease in *SNF1* expression (Fig. 6.4A and 6.4B), and is likely a strong contributing cause for the low Snf1 protein levels in this strain. This correlates with a simultaneous decrease in Fkh1 and Fkh2 protein and transcript levels (Fig. 6.4C and 6.5F). We had previously demonstrated that Fkh1, and to a lesser extent Fkh2, are necessary for *SNF1* expression (Jiao *et al.* 2015). Constitutive expression of either Fkh1 or Fkh2 in the *ubc1Δ* strain returned *SNF1* transcript levels towards WT levels; Fkh1 was able to restore Snf1 protein levels and *SUC2* induction to a greater extent than Fkh2 alone (Fig. 6.5C and 6.5E).

The expression of *FKH1* and *FKH2* fluctuates with the cell cycle, and we asked if the inherent cell cycle defect in *ubc1Δ* might be affecting the low Fkh1/2 levels. The *ubc1Δ* strain is reported to arrest as large budded cells, which we also observed (Fig. 6.4D). Our FACS analysis confirmed the high proportion of cells with replicated DNA indicating G2/M (Fig. 6.4D), and our propidium iodide staining for nuclei acid shows that the nuclei have clearly separated (Fig. 6.2A & B, Fig. 6.7E), altogether consistent with a late mitotic arrest. Lastly, the clear accumulation of Clb2 in asynchronous *ubc1Δ* cells is known to be a result of deficient E2 activity for the APC-dependent targeting of Clb2 for degradation, which is required for cell cycle progression through M into G1 (Rodrigo-Brenni and Morgan 2007), explaining the arrest point of the *ubc1Δ* strain. The discrete differences between the $apc5^{CA}$ mutant and *ubc1Δ* cell cycle arrest points suggests that the Ubc1 cell cycle defect may be APC^{Cdh1} mediated. APC^{Cdh1} controls the M/G1 transition, whereas APC^{Cdc20} controls the metaphase/anaphase transition. General defects in APC function result in large budded cells with the nucleus aligned at the bud neck, as observed in Fig. 6.7D. Nonetheless, our observation indicates the cell cycle arrest in *ubc1Δ* cells is consistent with an isolated impairment of APC activity. Furthermore, the expression of *FKH1/2* is normally high in G2/M in WT cells (Pramila *et al.* 2006), yet we note an unexpected clear decrease of *FKH1/2* expression in the *ubc1Δ* strain that is enriched for G2/M phase cells. The answer appears to be due to Ubc1 affecting Hcm1, a third member of the Fkh transcription factor family. Hcm1 is known to drive *FKH1/2* expression

(Pramila *et al.* 2006). A decline in Hcm1 protein abundance may explain the low Fkh1/2 protein levels in the absence of Ubc1, as Hcm1 was present at lower levels in the *ubc1Δ* strain, even compared to WT M-phase cells (peak expression of Hcm1) (Fig. 6.6A, Fig. 6.7A). What also became evident, however, was a specific defect in Hcm1 nuclear entry: Hcm1 is known to shuttle between the cytosol and the nucleus in a cell-cycle manner, which controls its availability to the promoter regions of target genes, including *FKH1/2* (Rodriguez-Colman *et al.* 2010). It is interesting to find that deletion of *UBC1* severely impairs the nuclear accumulation of Hcm1 in G1 arrested cells (Fig. 6.6B). The role of ubiquitin in regulated nuclear import of target proteins has been well established (Muratani and Tansey 2003; Hoeller and Dikic 2009). Accordingly, Ubc1 may normally target Hcm1 directly (or indirectly through an associated chaperone protein) for ubiquitination to promote import of Hcm1, or alternatively target a cytosolic tether for degradation. The identity of such a putative Ubc1-target controlling Hcm1 nuclear import was not revealed in this study, and we were unsuccessful in identifying Hcm1-ubiquitin conjugates through co-immunoprecipitation experiments (not shown).

It is also intriguing that a search of the literature revealed evidence that activated Snf1 is, in fact, a requirement for Hcm1 nuclear import (Rodriguez-Colman *et al.* 2013). It is possible, therefore, that the decreased Snf1 protein abundance in *ubc1Δ* cells contributes to a negative feed-forward loop, impairing Hcm1 import and ultimately decreasing *Fkh1/2* expression and protein abundance. This possibility deserves further investigation, as despite decreased Snf1 abundance, the relative activation state, as measured by its Thr210 phosphorylation, remains at WT levels.

We have discovered that the ubiquitin-conjugating enzyme, Ubc1, is indirectly required for SNF1 kinase activity at the level of transcription. Ubc1 function was first associated with the cellular stress response (Seufert and Jentsch 1991), and later with vesicle biogenesis, endoplasmic reticulum associated degradation (ERAD) (Meusser *et al.* 2005), and APC-dependent mechanisms for regulated cell cycle progression (Rodrigo-Brenni and Morgan 2007). What we describe here appears to be a novel function for Ubc1 that is independent of the APC ubiquitin ligase complex.

The schematic cartoon in Figure 6.8 highlights our observations and several questions that surround the regulation of SNF1 kinase by Ubc1 activity. Despite WT

levels of phosphorylation, nuclear import, and kinase activity on cytosolic and nuclear targets, there remains a deficiency in this activated complex to provide full *SUC2* expression. A simple explanation is that this may relate to the relatively low abundance of each of Snf1-Snf4-Gal83 protein subunits found in this heterotrimeric enzyme complex in the Ubc1 deletion strain. This decrease in Snf1 protein levels appears to be indirectly affected by Ubc1 through transcription via the Fkhs and Hcm1 and not through ubiquitin-mediated protein degradation. However, there appears to remain an overriding regulation of Snf1 protein levels that ultimately serves to maintain a stoichiometric balance between the three SNF1 kinase subunits. Secondly, the mechanism underlying the Ubc1-dependent failure of Hmc1 nuclear import is not known. Potential Ubc1 protein targets involved in Hcm1 shuttling are not reported, but may include a cytosolic tether that normally requires Ubc1 for degradation and subsequent release of Hcm1. Alternatively, ubiquitin can serve as a nuclear import signal and this would be a novel role for Ubc1. Next, the enhanced allosterism noted within the SNF1 kinase complex in the *ubc1Δ* strain may arise from the relative hyper-phosphorylation of Snf1 at Thr210, or from the loss of a *Factor X* that contributes to steric hindrance of subunit associations. Disruption of the UBA domain in Snf1 also resulted in enhances allosteric associations (Jiao *et al.* 2015), opening the possibility that this domain or protein face may have protein-binding partners that physically regulate SNF1 kinase activity through binding.

Table 6.1 *Saccharomyces cerevisiae* strains used in Chapter 6

Strain	Genotype	Reference
yTER32	*MATa trp1-901 leu2-3 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lac*	E. Craig
yTER301	*UBC1/ubc1Δ::KanMX6*	W. Xiao
yTER305	yTER32 + *ubc1::KanMX6*	This study
yTER206	*MATa ade2 his3 leu2 ura3 SNF1-GFP::HIS3*	Life Technologies
yTER279	*MATα his3-Δ200 leu2-3 112 ura3-52 lys2-801 trp1-1*	(Seufert and Jentsch 1991)
yTER277	*MATα his3-Δ200 leu2-3 112 ura3-52 lys2-801 trp1-1 ubc1Δ::HIS3*	(Seufert and Jentsch 1991)
yTER299	yTER206 × yTER277	This study
yTH3926	*MATa ade2 his3Δ200 leu2-3 lys2Δ201 ura3-52 FKH1-TAP::HIS3*	T. Harkness
yTH3929	*MATa ade2 his3Δ200 leu2-3 lys2Δ201 ura3-52 FKH2-TAP::HIS3*	T. Harkness
yTER303	yTH3926 + *ubc1Δ::kanMX6*	This study
yTER304	yTH3929 + *ubc1Δ::kanMX6*	This study
yTER246	*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 MIG1-TAP::HIS3*	Open Biosystems
yTER297	*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 ROD1-TAP::HIS3*	Open Biosystems
yTER306	yTER246+ *ubc1Δ::kanMX6*	This study
yTER307	yTER297 + *ubc1Δ::kanMX6*	This study
yTER311	*MATa ade2 his3 leu2 ura3 HCM1-GFP::HIS3*	Life Technologies
yTER312	yTER311+ *ubc1Δ::kanMX6*	This study
yTER117	*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 GAL83-TAP::HIS3*	Open Biosystems
yTER313	yTER117+ *ubc1Δ::kanMX6*	This study
yTER1	*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 SNF4-TAP::HIS3*	Open Biosystems
yTER314	yTER1+ *ubc1Δ::kanMX6*	This study
yTER315	*MATα ade2 his3Δ200 leu2-3,112 lys2Δ201 ura3-52 apc5^{CA}-PA::His5+*	T. Harkness
yTER316	*MATa ade2 his3Δ200 leu2-3,112 lys2Δ201 ura3-52 apc10::KanMX6*	T. Harkness
yTER187	*MATa ade2 his3Δ200 leu2-3,112 lys2Δ201 ura3-52*	This study
yTER70	*MATa ade2 his3Δ200 leu2-3,112 lys2Δ201 ura3-52 snf1Δ::KanMX6*	(Jiao *et al.* 2015)
yTH1482	*MATα ade2 his3Δ200 leu2-3,112 lys2Δ201 ura3-52 mig1Δ::LEU2*	M. Carlson
yTER344	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 HCM1-TAP::HIS3*	GE Dharmacon
yTER346	yTER344+ *ubc1Δ::KanMX6*	This study

CHAPTER 7 The E3 ubiquitin ligase Rsp5 is required for SNF1 kinase activity

7.1 Introduction

The SNF1 kinase is required for adaptation to non-glucose carbohydrate growth, its preferred carbohydrate source. Disruption of SNF1 kinase activity or activation renders yeast unable to efficiently grow on alternative carbohydrate sources such as sucrose, giving a clear phenotype for which it is named (Carlson *et al.* 1981). Under conditions of abundant glucose, the kinase is inactive, cytosolic, and non-phosphorylated, yet is rapidly activated when grown under limiting glucose conditions or on non-glucose carbon sources (Carlson *et al.* 1981; Hardie *et al.* 1998). Activation of the SNF1 kinase trimeric complex is not limited to the essential phosphorylation of the catalytic α subunit, Snf1, at Thr210 (McCartney and Schmidt 2001), but also includes the allosteric juxtapositioning of the α and γ subunits within the heterotrimeric complex (Jiang and Carlson 1996), and relocation to the nucleus (Hedbacker *et al.* 2004), vacuole (Yu *et al.* 2012), or plasma membrane (Schmidt and McCartney 2000), a movement dependent on the respective presence of three β subunits Gal83, Sip1, or Sip2 (Vincent *et al.* 2001). Activated SNF1 kinase rapidly targets other proteins for phosphorylation, thereby rebalancing metabolic pathways to maintain homeostasis. At the same time, transcription factors are nuclear targets of active SNF1 kinase, resulting in alterations of gene expression that establish sustained shifts in cellular metabolism. There is striking evolutionary conservation of the structure, function, and regulation of this kinase family extending to plants, animals, and humans where it is often referred to as AMPK (Crozet *et al.* 2014). Many fundamental mechanisms found to control this AMPK enzyme class have arisen from discoveries in *S. cerevisiae*, an extremely valuable model because of the simplified number of SNF1 kinase subunits available, ease of manipulating enzyme activity, and genetic malleability of the organism (Hardie 1999; Nath *et al.* 2003; Woods, Johnstone*, et al.* 2003; Hedbacker and Carlson 2008).

Additional layers of SNF1 kinase regulatory control are also known, and include additional post-translational modifications to the catalytic and regulatory subunits such as SUMOylation (Simpson-Lavy and Johnston 2013), acetylation (Lu *et al.* 2011), and ubiquitination (Wilson *et al.* 2011), the latter two of which have been shown to also occur in higher eukaryotes (Al-Hakim *et al.* 2008; Zungu *et al.* 2011; Eden *et al.* 2012). In

general, the consequences of ubiquitination are diverse and encompass proteasomal degradation (Hoeller and Dikic 2009), subcellular localization (Eden *et al.* 2012; Davis *et al.* 2013), protein associations and enzyme activation (Zungu *et al.* 2011). This rapidly reversible modification is enabled through an exquisitely regulated cascade of ubiquitin-conjugating (E2s) and ligase (E3) enzymes that direct target selection, balanced by the de-ubiquitinating enzymes that in concert modify the resulting linkages between ubiquitin moieties (Pickart and Raasi 2005). To date, the result of direct ubiquitin attachment to Snf1 appears to be inhibitory as measured both by degradation of the α subunit and decreased activity upon downstream targets (Wilson *et al.* 2011).

The ubiquitin system and its regulatory pathways is one of the most highly conserved biological processes, and it is reasonable to anticipate that an elucidation of the role of ubiquitin in yeast SNF1 kinase regulation will translate to aspects of regulation of AMPK-like kinases in other eukaryotes. To identify the E2 and E3 enzymes important in SNF1 kinase activity, we screened for defects in SNF1 kinase adaptation to growth in sucrose when their individual activities were disrupted. *S. cerevisiae* has thirteen E2 enzymes that exhibit a variety of distinct phenotypes and cellular defects when their individual E2 activity is disrupted (Finley *et al.* 2012). We identified the stress-related E2s (Ubc4 and Ubc5) and Rsp5, the cognate E3, as being robustly involved in regulated SNF1 kinase activity. Here we present evidence that the ubiquitin system plays a role in SNF1 kinase activity at multiple steps including allosteric associations and nuclear accumulation.

7.2 Materials and Methods

7.2.1 Strains and media

The yeast strains used in this study are shown in Table 7.1. Yeast were grown at 30°C in rich media (YPD: 10 g/l yeast extract, 20 g/l bactopeptone, 20 g/l glucose) or in synthetic complete drop-out media (DO: 20 g/l glucose, 1.7 g/l yeast nitrogen base, 5 g/l ammonium sulfate) lacking the appropriate nutrient for plasmid selection. The concentration of glucose was decreased from 2% to 0.05% as indicated.

7.2.2 Invertase assay

Protocols have been previously described in section 3.5.

7.2.3 Total protein extract, protein stability assays, and Western Blot analysis

Protocols have been previously described in section 3.1 and 3.2. Anti phospho-AMPK (Cell Signaling, detecting Snf1-Thr210), GFP (Covance), TAP (Open Biosystems), Actin (Sigma), and α-tubulin (Sigma) antibodies were used in this study. Protein stability assays (time course) have been previously described in section 3.3. Strains harboring integrated TAP- or GFP-epitope tags were expressed under their endogenous promoters. CHX (10 μg/ml) was added to prevent further protein synthesis at the time of culture division into 2% and 0.05% glucose media (0 min). Actin or tubulin signal is from the same nitrocellulose blot as GFP or TAP in every case.

7.2.4 Construction of a temperature-sensitive *rsp5^{ts}* 2-hybrid reporter strain

A two-step PCR-based mutagenesis protocol was used to introduce the *rsp5^{ts}* ($P_{772}S$) (Harkness *et al.* 2002) temperature-sensitive (*ts*) mutation directly into the PJ69-4α 2-hybrid strain. *PCR fragment 1*: 5' primer to the C-terminal 500 bp of *RSP5*; 3′ primer fused with 60 nucleotides of DNA 100 bps upstream of *URA3*. Genomic DNA isolated from the *rsp5^{ts}* strain is used as template. *PCR fragment 2:* 5' primer 100 bp upstream of the *URA3* gene start codon; 3′ primer fused with 60 nucleotides immediately downstream of the *RSP5* gene. Purified PCR fragments 1 and 2 were co-transformed into the PJ69-4α strain, which encodes the *ura3-52* mutant allele. Homologous recombination of the two PCR fragments introduced the recombined PCR fragment into the genome precisely at the 3′ end of *RSP5* with incorporation of the $P_{772}S$ mutation. Selection of recombinants was through growth on plates lacking uracil (*URA*-), temperature sensitivity, and sequence confirmation, based on methods described previously (Storici and Resnick 2006).

7.2.5 2-hybrid analysis

Protocols have been previously described in section 3.6. The yeast 2-hybrid reporter strain (WT) and the temperature-sensitive *rsp5^{ts}* 2-hybrid reporter strain were doubly transformed with pairs of empty vectors: empty vectors pGAD-C_2 and pGBDU-C_2 (−), or the same backbones expressing unmodified Snf1 and Snf4 subunits (+) in this study.

7.2.6 Fluorescence microscopy

Protocols have been previously described in section 3.4. Fluorescence microscopy was used to determine the subcellular localization of Snf1-GFP in live yeast cells. Each strain was transformed with a 2μ *HIS*+ plasmid expressing *SNF1-GFP* (a kind gift from M. Carlson). Logarithmic phase growth cultures were divided between non-activating DO media (Vincent *et al.* 2001) lacking histidine with 2% glucose versus activating (3% glycerol, no glucose) conditions for no more than 30 minutes. A minimum of 150 cells for each strain was scored for co-localization of the GFP-tagged Snf1 signal and DAPI nuclear staining. If the GFP signal did not accumulate in the nucleus (nuclear), nor was it excluded from the nucleus (cytoplasmic), the GFP signal was scored as homogeneous.

7.2.7 Statistical analysis

Statistical analyses used PRISM Version 6.0b software, showing mean and SD, with significance calculated by t-test.

7.3 Results

7.3.1 Invertase activity is impaired in strains disrupted for Ubc4 and Ubc5 E2 activity and Rsp5 E3 activity

Activated SNF1 kinase targets the nuclear transcriptional repressor, Mig1, for phosphorylation, thereby releasing the inhibition of the *SUC2* gene that encodes for invertase (Treitel *et al.* 1998; McCartney and Schmidt 2001). This enzyme converts sucrose to glucose and fructose, and facilitates the adaptation of yeast metabolism to this alternative sugar source (Carlson *et al.* 1981; Carlson and Botstein 1982). A quantitative colorimetric assay for glucose production from sucrose (the invertase assay) was utilized to screen yeast strains disrupted for yeast E2s for their involvement in this activity (Harkness and Arnason 2014). The screen is particularly useful as it reveals both loss of nuclear SNF1 kinase activity (in low glucose) and loss of glucose repression (in high glucose). Figure 7.1A demonstrates the expected rise in invertase activity in WT yeast after 2 hours of growth in low (0.05%) glucose, at either 30°C or 37°C. *UBC4* and *UBC5* were disrupted in combination as they exhibit strong homology and can effectively compensate for each other; phenotypes are evident upon their combined disruption in the deletion strain used (*ubc4/5Δ*) (Seufert and Jentsch 1990). Our data shows that the

combined deletion of these E2s result in significantly decreased invertase activity (Fig. 7.1A and 7.1B). There are many E3 enzymes in yeast and an exhaustive screen of all was beyond the scope of our investigation, yet several have links to SNF1 kinase that were of interest to test. Elc1 (Elongin C 1), an E3 ubiquitin ligase, had previously been reported to affect the stability of Snf4 (Hyman *et al.* 2002), yet we found invertase was not impacted by disruption of the *ELC1* gene (Fig. 7.1A). The APC is a multi-subunit E3 and has been associated with SNF1 kinase function in longevity pathways (Harkness *et al.* 2004). Mutation of the Apc5 subunit, which is essential for APC activity, did not significantly alter invertase maximums (Fig. 7.1A). The ubiquitin ligase Rsp5 and SNF1 kinase both target the arrestin-like protein, Rod1, for ubiquitin and phosphorylation, respectively (Becuwe *et al.* 2012). Rsp5 also targets glucose and galactose transporters, among others, in response to glucose levels (Roy *et al.* 2014) (Horak and Wolf 2001). Notably, Rsp5 is known to act as the ubiquitin-ligase for Ubc4/5 and Ubc1 (Stoll *et al.* 2011), and to share overlap with their stress-resistance phenotypes (Hiraishi *et al.* 2006). Mutations to Rsp5 severely impaired invertase activity in a temperature dependent manner (Fig. 7.1A and 7.1B). As shown, invertase activity is significantly decreased to less than 15% of WT in the *rsp5*^{ts} mutant at non-permissive temperatures (37°C), and defects appear evident even at 30°C, a semi-permissive temperature for this mutant.

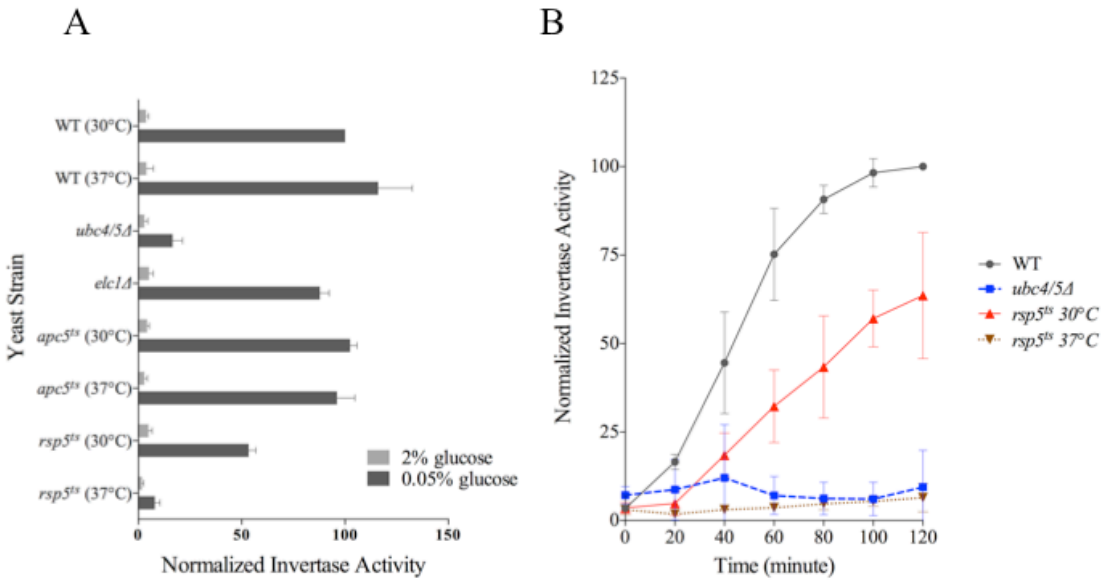

Figure 7.1 Yeast strains lacking ubiquitin enzymes Ubc4/5 and Rsp5 are impaired for SNF1 kinase-dependent invertase activity. Comparison of invertase activity under repressive (2% glucose) and activating (0.05% glucose) conditions between isogenic yeast strains normalized to WT in 0.05% glucose (value of 100), showing the mean and SD. ***A,*** Invertase activity of WT, *ubc4/5Δ*, *elc1Δ*, *apc5*^{ts}, and *rsp5*^{ts} after 2 hours of activating growth conditions. For *ts* mutants, permissive (30°C) or non-permissive (37°C) temperatures were used to impair function of the target proteins. Statistical significance was based on 3 biological repeats using a t-test (Prism 6.0). ***B,*** A time-course for invertase activity of WT, *ubc4/5Δ*, and *rsp5*^{ts} strains, sampled intermittently over 2 hours following shift to low glucose media. Average of four biological repeats with SD are indicated for each time point. A and B were performed by Dr. T.G. Arnason.

A time course invertase assay was performed on strains mutated for Ubc4/5 or Rsp5 to better appreciate the differences from wild type activity (Fig. 7.1B). The WT strain rapidly increased invertase activity. The *ubc4/5Δ* mutant strain failed to generate any meaningful invertase activity, similar to that observed at the non-permissive temperature in the *rsp5*^{ts} strain. Again, growth at 30°C appeared only semi-permissive for the *rsp5*^{ts} activity, as it never reached that of WT (Fig. 7.1A).

7.3.2 The Snf1 α subunit is stable under activating and repressive growth conditions, independent of Rsp5 activity

Maximal invertase activity requires that all preceding steps of SNF1 kinase activation occur efficiently, including phosphorylation of Snf1-Thr210, allosteric associations between Snf1 (α) and Snf4 (γ), and translocation of the trimeric complex to the nucleus (β, Gal83). However, it was possible that the role of ubiquitin in SNF1 kinase regulation was through alterations in subunit stability due to E2 and E3 targeting activity. Time course stability studies of the endogenous Snf1 protein (Fig. 7.2A) revealed that Snf1-GFP is stable up to 2 hours in the WT strain. Neither repressive nor activating glucose levels affected its stability. Next, the protein stability of endogenously expressed Snf1-GFP in the *rsp5*^{ts} strain was assessed by a protein stability time-course that included CHX addition and tested over 3 hours (Fig. 7.2B and 7.2C). This was done at semi- (30°C) and non-permissive (37°C) temperatures for the *rsp5*^{ts} conditional mutant strain. Figure 7.2C clearly revealed complete stability of the Snf1 catalytic subunit in the absence of full Rsp5 function.

7.3.3 Phosphorylation of Snf1 does not require Ubc4/5 or Rsp5

The profound decrease in invertase activity in the *ubc4/5Δ* and *rsp5*^{ts} strains may be due to a failure of Snf1 activation by phosphorylation at Thr210 (Snf1^{ph}). When tested, Snf1^{ph} was induced to WT levels upon a shift to low glucose growth conditions in the *ubc4/5Δ* strain and the *rsp5*^{ts} strain even at non-permissive growth temperatures (Fig. 7.3A and 7.3B). A ratio of Snf1^{ph}/Snf1-GFP from pooled data of repeat experiments highlighted this trend (Fig. 7.3C), and established that neither Rsp5 nor Ubc4/5 are required for efficient glucose-responsive phosphorylation of the Snf1 subunit.

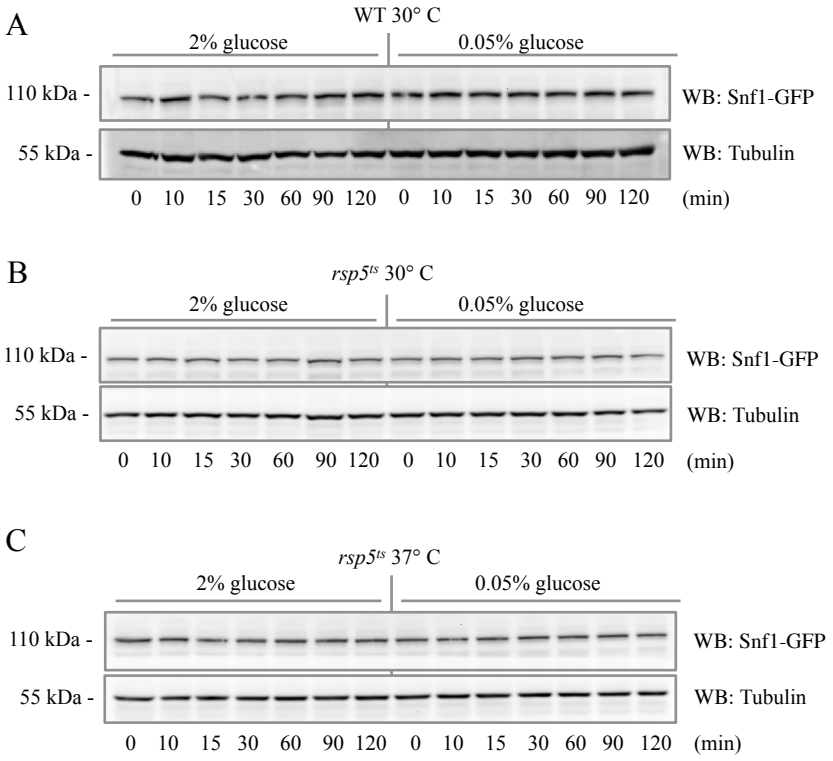

Figure 7.2 Snf1 protein abundance is independent of Rsp5 activity. Early logarithmic phase WT and *rsp5*^{ts} strains harboring genomic Snf1-GFP were grown in 2% and 0.05% glucose prior to harvest. Cycloheximide was added at time 0 to prevent new protein synthesis. Equal amount of total protein was loaded for Western blot analysis of GFP (Snf1-GFP) and Tubulin as an internal loading control. ***A,*** Assessment of Snf1-GFP protein stability over 2 hours in the WT strain. ***B***, Assessment of Snf1-GFP protein stability over 2 hours in the *rsp5*^{ts} mutant strain under semi-permissive temperature. ***C***, Assessment of Snf1-GFP protein stability over 2 hours in the *rsp5*^{ts} mutant strain under non-permissive temperature.

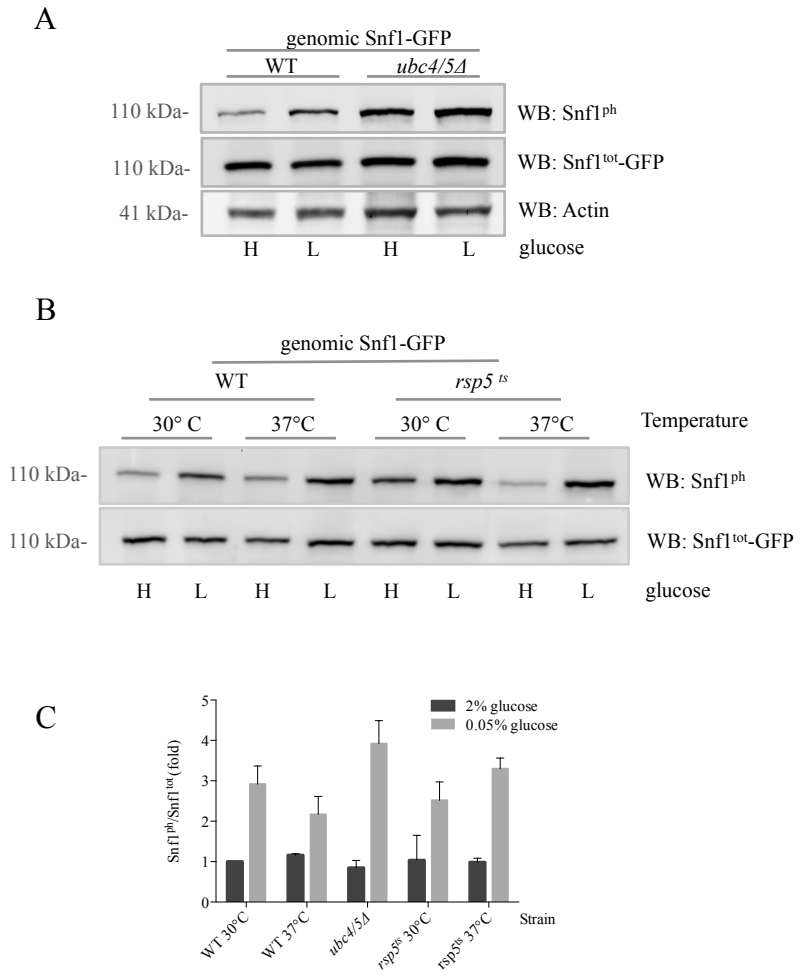

Figure 7.3 Efficient Thr210 phosphorylation of the Snf1 subunit is retained in the *ubc4/5Δ* and *rsp5^{ts}* strains. Early logarithmic phase WT, *ubc4/5Δ* and *rsp5^{ts}* strains expressing genomic Snf1-GFP were grown in 2% (H) and 0.05% (L) glucose for 30 minutes prior to cell lysis, and equal total protein loaded in duplicate for Western blot analysis of total Snf1 (Snf1^{tot}-GFP) and phosphorylated Snf1 (Snf1^{ph}). ***A***, Comparison of Snf1^{ph} versus Snf1^{tot}-GFP in WT and *ubc4/5Δ* strains. ***B***, Comparison of Snf1^{ph} versus Snf1^{tot}-GFP in WT and *rsp5^{ts}* strains under either permissive (30°C) or non-permissive (37°C) temperature. And ***C***, Statistical quantitation of three biological repeats of ***A*** and ***B***, with mean and SD.

7.3.4 Rsp5 contributes to glucose-responsive allosteric subunit associations within the SNF1 kinase complex

The preceding experiments did not identify the step at which the invertase activity was impacted by *ubc4/5Δ* or *rsp5*^{ts}, as Snf1 stability and Snf1^{ph} appeared unaffected. We next asked if allosteric associations between the α and γ subunits required an intact ubiquitin targeting cascade. The relative strength of associations between Snf1 and Snf4 can be observed using the 2-hybrid colorimetric system. To directly test the role of the ubiquitin system in SNF1 kinase allosterism, we introduced the conditional *rsp5*^{ts} mutation into a standard 2-hybrid reporter strain and measured the impact on Snf1-Snf4 protein associations. Figure 7.4 shows the results of this experiment, comparing the WT and *rsp5*^{ts} versions of the reporter strain, each transformed with pairs of plasmids, either empty vectors (-) or the Snf1-Snf4 pair (+). As seen in Figure 7.4, the relative color intensity increased down the concentration gradient for the WT strain grown at both 30°C and 34°C experiments. Introduction of the *rsp5*^{ts} conditional mutation diminished Snf1-Snf4 associations throughout the glucose gradient under semi-permissive temperatures, with an augmented effect at non-permissive temperatures. Cells were transferred to 30°C or 34°C for 2 hours prior to overlay and likewise incubated for another 16 hours before final color images were captured; the *rsp5*^{ts} strain can grow at 34°C but will not survive under extended exposure to 37°C.

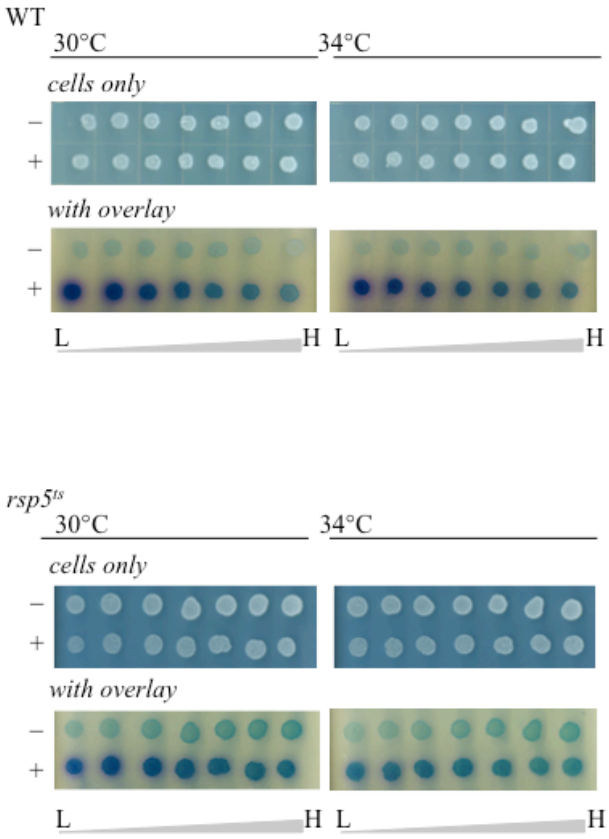

Figure 7.4 Rsp5 contributes to the allosteric subunit associations within the SNF1 kinase. 2-hybrid associations between empty vectors (-) and Snf1-Snf4 pairs (+) in WT and *rsp5*^{ts} strains are shown under permissive (30°C) and semi-permissive (34°C) temperatures. Equal cell numbers were spotted down a glucose gradient (L: 0.05% to H: 2% glucose) before and after β-galactosidase color development from overlay.

7.3.5 Compromised Rsp5 function impairs nuclear Mig1 phosphorylation by the SNF1 kinase

A defect in Snf1-Snf4 physical associations under activating conditions may partially explain the decreased invertase production in the *rsp5*^{ts} strain. Knowing that invertase activity also requires that the *SUC2* transcriptional repressor, Mig1, is phosphorylated by nuclear SNF1 kinase in order to lift repression and permit invertase to be produced led us to isolate and analyze this step in activity (Goldstein and Lampen

1975). First, an extended analysis of Snf1^{ph} was done in the *rsp5^{ts}* strain at 30°C and 37°C over a 2 hour time-course in high and low glucose (Fig. 7.5A and 7.5B). A significant and sustained phosphorylation of Snf1 was noted in low glucose at both 30°C and 37°C. To test the requirement for Rsp5 in Mig1 phosphorylation, a similar experiment was done except that the shift to 37°C was introduced at the same time as the shift to low glucose. In Figure 7.5C, a Mig1-TAP signal was observed at time zero in 0.05% glucose that shifts towards a lower band over time, consistent with a gradual loss of the Mig1 phospho-shift (Jiao *et al.* 2015). Our data indicates that the loss of Mig1 phosphorylation is neither due to its stability in *rsp5^{ts}* (Fig. 7.6A), nor the non-permissive temperature, as cells cultured at 37°C exhibit proper Mig1 phosphorylation (Fig. 7.6B). It suggests that, although SNF1 kinase remains phosphorylated and presumably active, its ability to phosphorylate Mig1 is specifically lost; the release of Mig1 inhibition on *SUC2* repression is impaired and may contribute towards the explanation for impaired invertase activity in the *rsp5^{ts}* strain. Similarly, our data indicates that Mig1 phosphorylation is lost in the absence of *UBC4/5*, whereas Rod1, the cytosolic target of SNF1 kinase, continues to be phosphorylated in this double deletion strain under low glucose growth conditions (Fig. 7.5D, lower panel). These results suggest that SNF1 kinase is selectively impaired in its nuclear activity.

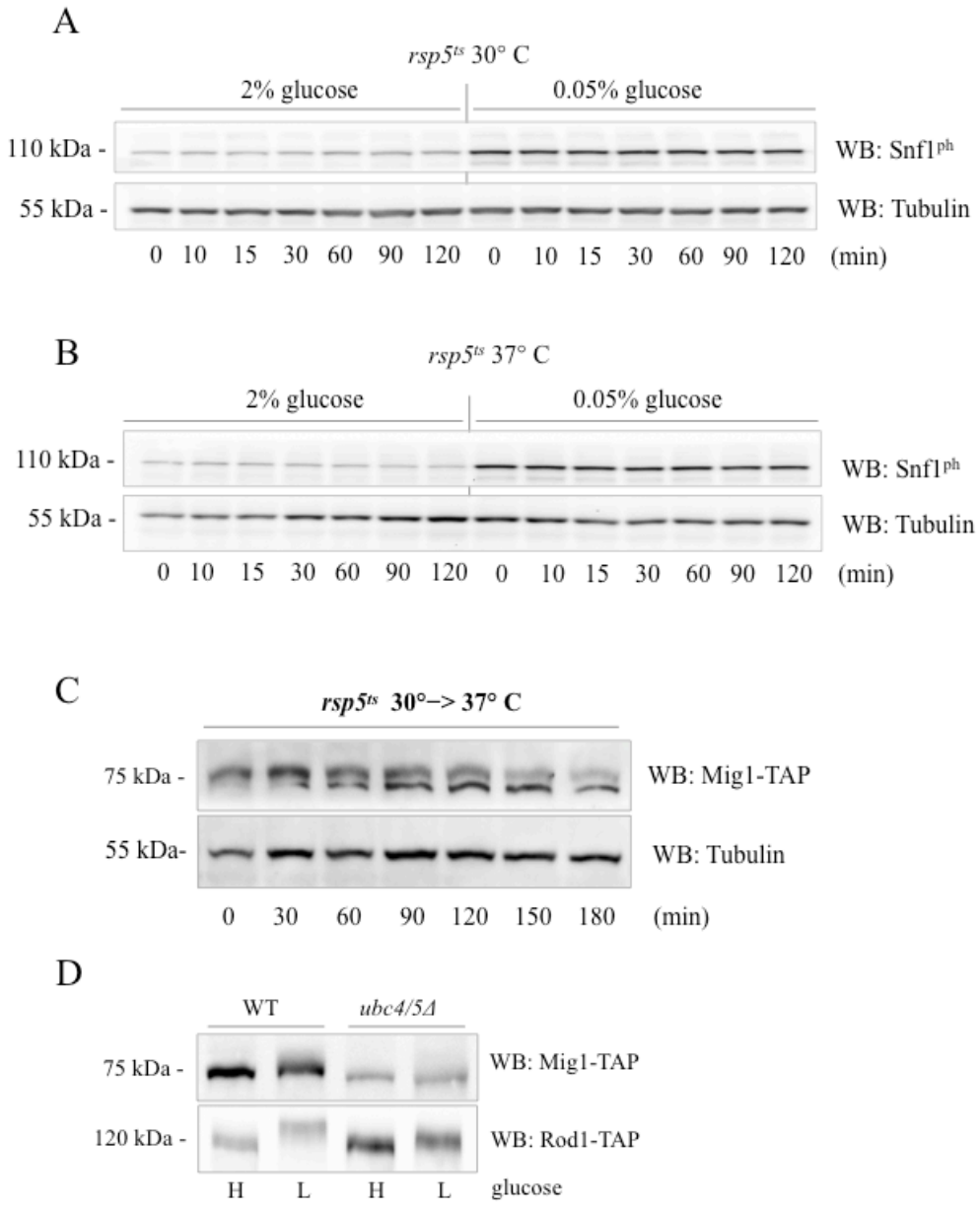

Figure 7.5 SNF1 kinase nuclear activity is impaired in *ubc4/5Δ* and *rsp5^{ts}* strains. ***A*** and ***B***, Western Blot analysis of Snf1^{ph} in *rsp5^{ts}* strain at 30°C and 37°C over a 2 hour time-course in SNF1 Kinase repressive (2% high glucose, H) and activating (0.05% low glucose, L) growth conditions. ***C***, Mig1 phosphorylation under low glucose growth conditions in the *rsp5^{ts}* strain upon a shift to non-permissive temperature (37°C) for 3 hours, ***D***, SNF1 kinase–dependent phosphorylation of Mig1 (nuclear target) and Rod1 (cytosolic target) in WT and *ubc4/5Δ* strains.

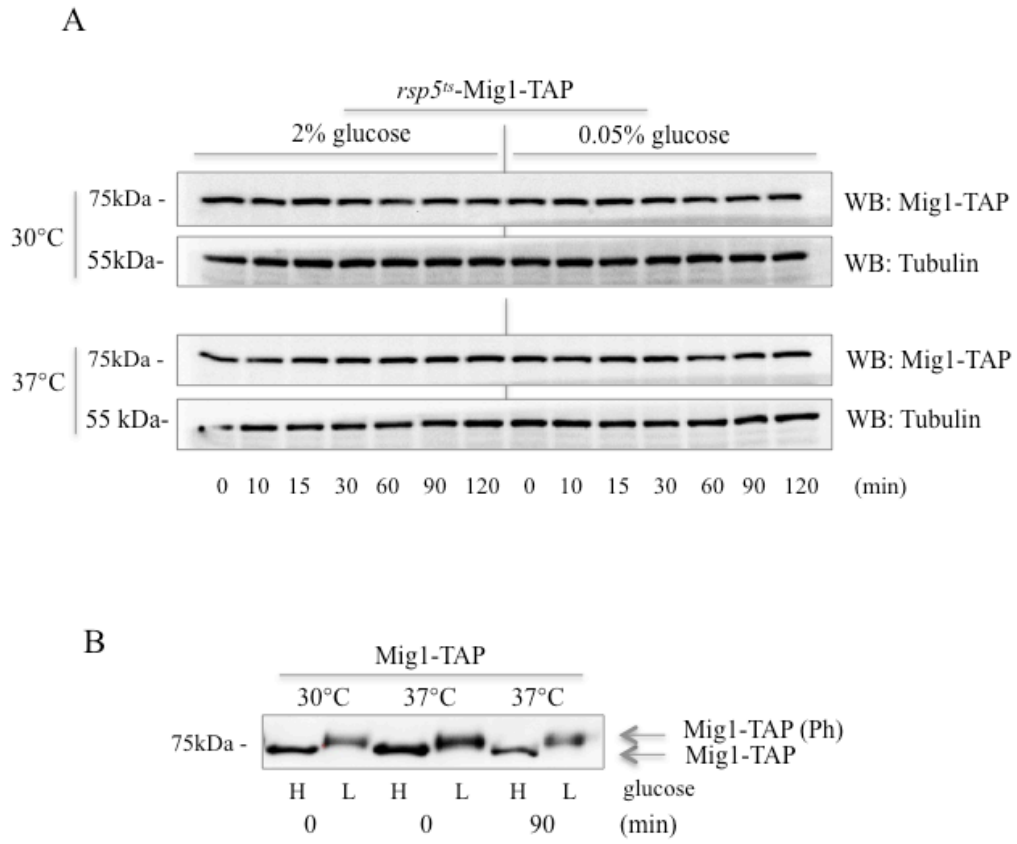

Figure 7.6 Rsp5 activity affects Mig1 phosphorylation, independent of the temperature. Early logarithmic phase *rsp5*^{ts} strain harboring genomic Mig1-TAP were grown in 2% and 0.05% glucose prior to cell lysis. Equal amount of total protein was loaded for Western blot analysis of TAP (Mig1-TAP). ***A,*** Western analysis of Mig1-TAP protein stability over 2 hours in the *rsp5*^{ts} strain, under permissive (30°C) or non-permissive (37°C) growth temperatures. ***B,*** Western analysis of Mig1 phosphorylation in WT strain under 2% glucose (H) repressive conditions versus 0.05% glucose (L) activating conditions. Cells were cultured at 30°C and 37°C prior to sample retrieval.

7.3.6 Snf1 is mislocalized under activating conditions when either Rsp5 or Ubc4/5 functions are impaired

The maintenance of Snf1^{ph} with a gradual loss of Mig1 phosphorylation, concurrent with the introduction of Rsp5 dysfunction (Fig 7.5C), next led us to ask if there was a specific defect in the nuclear import of Snf1 in the *rsp5*^{ts} strain. It was

possible that the *RSP5* and/or *UBC4/5* mutations resulted in SNF1 kinase complexes that are either mislocalized or defective in nuclear accumulation, resulting in low invertase activity. Fluorescent microscopy was used to visualize the cellular location of the GFP-tagged Snf1 subunit in WT yeast, and in the presence of mutant alleles of *UBC4/5* and *RSP5*. Figure 7.7A-D shows representative images of the subcellular location of Snf1-GFP in repressive (high glucose) and activating (low glucose) conditions, as compared to the DAPI signal identifying the yeast nucleus (and mitochondrial DNA to a lesser extent). Yeast frequently harbor relatively large vacuoles, seen as voids on both DAPI and GFP, particularly visible here for the *rsp5*^{ts} and *ubc4/5Δ* strains. The WT strain (Fig. 7.7A) demonstrated clear Snf1-GFP nuclear exclusion in high glucose (as previously reported), and strong nuclear accumulation in low glucose. In contrast, there was a noticeable lack of nuclear accumulation in low glucose in the *ubc4/5Δ* strain, while retaining appropriate cytosolic location (with obvious nuclear exclusion) of Snf1-GFP in high glucose (Fig. 7.7B). In some cells, the GFP signal appears excluded from the nucleus under activating conditions, as indicated by DAPI staining. In the *rsp5*^{ts} strain at 30°, Snf1-GFP is able to accumulate in the nucleus when activated (Fig. 7.7C), yet this is clearly lost upon shifting to the non-permissive 37°C conditions (Fig. 7.7D). In this strain, we observe that under activating conditions, the Snf1-GFP signal is widely dispersed and was excluded from the nucleus in some cases. In both *ubc4/5Δ* and *rsp5*^{ts} strains as compared to WT, we unexpectedly observed that the Snf1-GFP signal was occasionally enhanced within the vacuole to varying intensities, and in other cases was fully dispersed. To quantitate these differences in subcellular location under activating conditions, at least 150 cells per strain were scored for nuclear accumulation (% Nuclear), nuclear exclusion (% cytosolic), vacuolar accumulation (% vacuolar), and undifferentiated (% Homogeneous) (Fig. 7.7E).

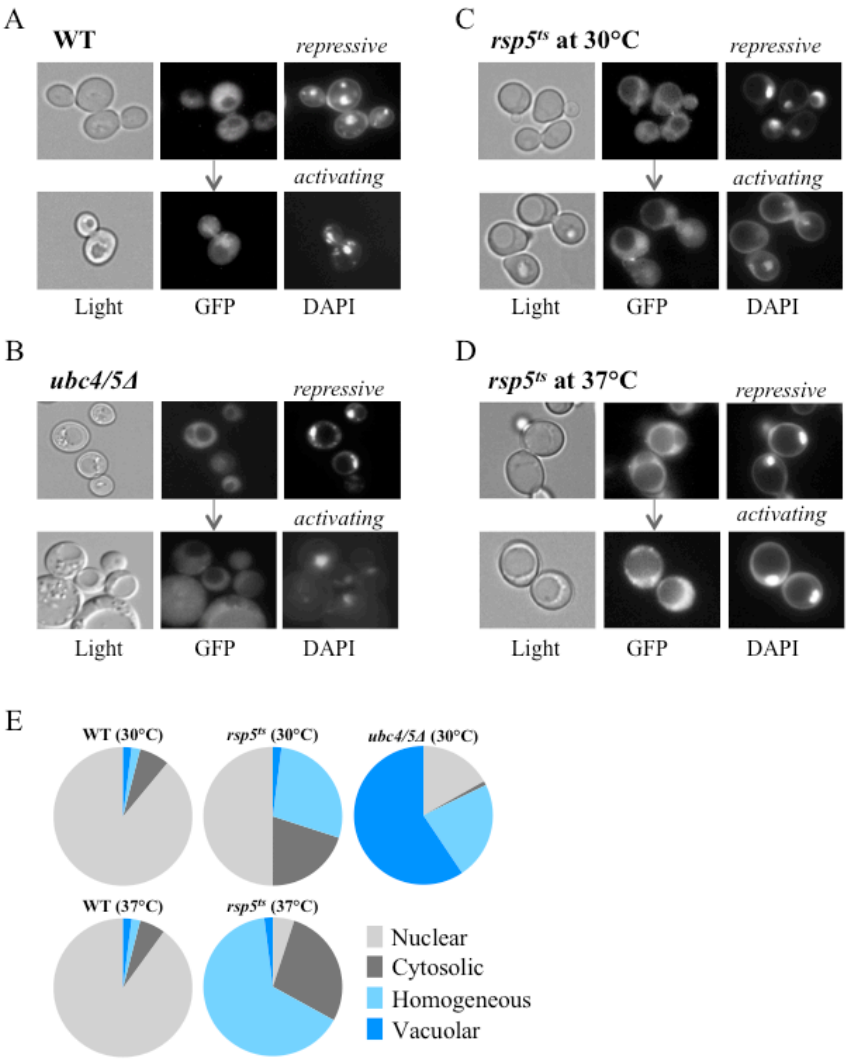

Figure 7.7 Snf1-GFP is mislocalized under activating conditions when either Rsp5 or Ubc4/5 functions are impaired. ***A-D*****,** Fluorescent microscopy of GFP-tagged Snf1 constitutively expressed from a high copy 2μ plasmid (His^+) in WT, *ubc4/5Δ*, and $rsp5^{ts}$ strains grown under repressive (2% glucose) or shifted to activating (3% glycerol for 30 minutes) conditions. 2-hour pre-exposure of the $rsp5^{ts}$ strain to non-permissive temperature (37°C) was used to disable the Rsp5 function prior to microscopy. ***E***, Quantitation of the subcellular location of Snf1-GFP under activating conditions correlated to the strains/temperature in A-D. 150 consecutive cells per condition were scored for nuclear (majority accumulated in the nucleus), cytosolic (majority in the cytoplasm with nuclear exclusion), vacuolar (accumulated in the vacuole), and homogeneous (undifferentiated between nucleus and cytoplasm).

7.4 Discussion

The SNF1 kinase is a non-hormonal regulator of metabolism that is activated under times of nutrient limitation or stress to enable changes to biological pathways that permit survival and adaptation to changing growth conditions. The yeast *S. cerevisiae* is a powerful model system to study this kinase class, which has highly conserved orthologs throughout eukaryotes, collectively referred to as the AMPK family. Conservation from yeast to humans is not only at the structural and functional level, but also found in many upstream regulatory events and downstream results (Hong and Carlson 2007).

Ubiquitination has been reported to modify the catalytic subunits in both yeast and human AMPK-related enzymes, and their poly-ubiquitin results in degradation and decreased kinase activity (Al-Hakim *et al.* 2008; Wilson *et al.* 2011). A negative regulatory role was therefore attributed to the direct attachment of ubiquitin to SNF1 kinase complex and its human orthologs. While the existence of ubiquitin attachment directly onto Snf1 (and AMPK α subunits) is known, the specific enzymes within the ubiquitin targeting cascade affecting SNF1 kinase have not been identified. We screened yeast strains mutated for ubiquitin-conjugation (E2) and ubiquitin-ligase (E3) activities to identify those components involved in regulating SNF1 kinase through ubiquitin action. Unexpectedly, we find that specific ubiquitin cascade enzymes at both the ubiquitin-conjugating (Ubc4, Ubc5) and ubiquitin-ligase (Rsp5) steps were required for positive, not negative, regulation of SNF1 kinase activity, without affecting Snf1 stability.

The yeast strain harboring combined deletions of *UBC4* and *UBC5* exhibited significant defects in invertase activity. We have previously reported that Ubc1, also a stress-related E2, is required for SNF1 kinase activity, yet this requirement was at the level of transcriptional regulation of the *SNF1* gene itself (Jiao *et al.* 2016). The Ubc1, Ubc4 and Ubc5 proteins have overlapping cellular functions in a general sense, all having been linked to the turn-over of short-lived and abnormal proteins and being necessary for survival under cellular stress (Seufert and Jentsch 1990, 1991).

Selected E3s were also analyzed and most did not show a direct effect on SNF1 kinase activity. Elc1 had previously been reported to affect the stability of Snf4 (Hyman *et al.* 2002) and due the potential impact of this on SNF1 kinase protein stoichiometry we felt it was possible that the overall enzyme activity may have been altered, yet changes in

SNF1 kinase activity were not detected. We also tested the APC, a multi-subunit E3 that has been associated with SNF1 kinase function in longevity pathways (Harkness *et al.* 2004). Mutation of the Apc5 subunit, essential for APC activity, did not significantly alter invertase maximums. We did, however, observe significant defects when the E3, Rsp5, was mutated.

The ubiquitin cascade is an orderly cascade of ubiquitin-attachment to selected targets and assembly of poly-ubiquitin chains, involving both E2s and E3s in the construction of the final chain formation. The E2s Ubc1, Ubc4 and Ubc5 have been reported to have functional associations with the cognate E3, Rsp5 (Arnason *et al.* 2005; Hiraishi *et al.* 2006). Functionally, Rsp5 can act as the ubiquitin-ligase for Ubc4/5 and Ubc1 (Nuber and Scheffner 1999), and shares overlap with their stress-resistance phenotypes (Hiraishi *et al.* 2006). Also, Rsp5 and SNF1 kinase both target the arrestin-like protein, Rod1, for ubiquitination and phosphorylation, respectively (Becuwe *et al.* 2012) and Rsp5 targets glucose and galactose transporters, among others, in response to fluctuating glucose levels (Horak and Wolf 2001; Roy *et al.* 2014). When directly tested, Rsp5 activity was found to be critical in facilitating maximum invertase activity (Fig. 7.1). Given that SNF1 kinase functions in part through stress resistance pathways, identification of this cluster of E2 and E3 enzymes was particularly relevant.

Given the well-known consequence of protein stability changes following ubiquitin attachment, we first tested if the stability of the Snf1 catalytic subunit was affected, which would provide an explanation for the loss of invertase activity. In fact, it was stable for hours in WT yeast. While initially unexpected, it became clear that the previously reported effects of Snf1 instability in response to SUMOylation (Simpson-Lavy and Johnston 2013) and ubiquitination (Wilson *et al.* 2011) required the forced accumulation of poly-ubiquitin chains attached to Snf1. In addition, it has been reported that the AMPK-like β subunits specific to mouse brown adipose tissue were unstable with half-lives of less than one hour (Qi *et al.* 2008). It appears that this mechanism is not universally applicable as all yeast β subunits are stable regardless of activating or repressing conditions (Fig. 5.1B).

In contrast to protein degradation resulting from poly-ubiquitination through lysine-48 chains, mono-ubiquitination of proteins has been shown to contribute to protein

stability (Torres *et al.* 2009). We asked if the result of removing key ubiquitin cascade component activity resulted in a change in Snf1 protein stability through absent mono-ubiquitin placement. Rsp5 is known to be involved in the mono-ubiquitin of multiple targets (Stamenova *et al.* 2004; Torres *et al.* 2009) and we directly assessed Snf1 stability over time in the absence of Rsp5 function. We observed that Snf1 remains fully stable for at least three hours in this strain, similar to WT yeast, essentially ruling out a stabilizing mono-ubiquitin event under Rsp5 control. Therefore, using our approach and strains, we do not find that Rsp5 affects the protein stability of Snf1.

The activating phosphorylation of Snf1 at Thr210 residue is essential for its cellular functions and nuclear import (Hedbacker *et al.* 2004), and therefore is a key regulatory step to assess to explain changes in invertase activity. Each strain tested was capable of augmenting the $Snf1^{ph}$ signal in response to activating conditions at levels similar, or better, than in WT yeast. In contrast to the previously explored decrease in Snf1 subunit abundance unique to the *ubc1Δ* strain (Jiao *et al.* 2016), the amount of Snf1 protein remained unaffected in the *ubc4/5Δ* and $rsp5^{ts}$ strains as compared to WT (Fig. 7.3A and 7.3B). Quantitation of the relative $Snf1^{ph}$ signal to total Snf1 confirmed full $Snf1^{ph}$ is retained in *ubc4/5Δ* and $rsp5^{ts}$ strains and an explanation for loss of invertase function is not, therefore, through defects in the activating phosphorylation step.

The increase in physical binding between the Snf1 and Snf4 subunits are well documented to occur in a glucose-responsive manner, and can be measured using the yeast 2-hybrid assay. In our study, the yeast 2-hybrid reporter strain was altered to include the temperature sensitive $rsp5^{ts}$ mutation (Harkness *et al.* 2002). Comparing the Snf1-Snf4 2-hybrid associations within the WT and $rsp5^{ts}$ strains reveals evidence that Rsp5 function is required for a normal degree of Snf1-Snf4 interactions. At 30°C, a partial interaction remains between Snf1 and Snf4, as well as a weak response to glucose levels, which may reflect the semi-permissive temperature used. The sub-lethal temperature of 34°C had to be used to permit survival of the $rsp5^{ts}$ strain throughout the duration of the experiment, and a more significant defect in Snf1-Snf4 associations are evident at 34°C compared to 30°C, presumably as Rsp5 function is lost. A complete disruption of detectable binding of Snf1 and Snf4 may occur at non-permissive temperatures (37°) but the temperature sensitive strain would fail to grow or form

colonies making it an unviable approach for this analysis. Because of the constant cross-tethering of Snf1 and Snf4 to the β-subunit regardless of the activation state (Jiang and Carlson 1997), direct tests by co-immunoprecipitation studies, for example, is also not possible. It may be that Rsp5 normally targets a repressive SNF1 kinase-binding protein for ubiquitination and degradation.

The disruption of Snf1-Snf4 allosteric associations in the *rsp5*^{ts} strain could have provided sufficient explanation for the loss of invertase activity at non-permissive temperatures, as Snf4 is necessary for normal invertase activity and it drops essentially to zero in a *snf4Δ* strain (Hubbard *et al.* 1992). However, our observation that the phosphorylation of the SNF1 kinase nuclear target, Mig1, is lost over time and while Rsp5 function is gradually blocked, suggested that it may be the nuclear function of Snf1 kinase that is specifically affected. In addition, Mig1 fails to be phosphorylated in the *ubc4/5Δ* strain, despite the cytosolic target, Rod1, remaining phosphorylated under the same conditions (Fig. 7.5D). This efficient phosphorylation of Rod1 in a glucose-responsive manner in the *ubc4/5Δ* strain clearly indicates that the *ubc4/5Δ* mutation has retained the inherent enzymatic activities of SNF1 kinase, and is not the underlying cause for loss of invertase activity.

Ubiquitination can affect target protein subcellular relocation (Eden *et al*. 2012; Davis *et al*. 2013) and ubiquitin-mediated control of nuclear import is well established for other proteins (Nie *et al*. 2007; Trotman *et al*. 2007). This led us to directly test if the nuclear import of Snf1 is impaired in the Rsp5 or Ubc4/5 mutant strains, using fluorescent microscopy. While the mechanisms regulating nuclear-cytosolic partitioning of SNF1 kinase in response to activating signals are unknown, we do know that the β subunit, Gal83, directs Snf1 to the nucleus. Snf1-Gal83-Snf4 protein complexes shuttle across the nuclear membrane depending on the Snf1 activation state; the inactive complex is nuclear-excluded in ideal growth conditions, but rapidly accumulates in response to low glucose. Interestingly, Gal83 will shuttle in a glucose-responsive manner in the absence of the Snf1 subunit, whereas Snf1 is nuclear-excluded in the absence of Gal83 (Vincent *et al*. 2001; Hedbacker and Carlson 2008). This suggests that Snf1 'piggy-backs' into the nucleus with Gal83, relying on the nuclear localization/export signals within the latter. Unexpectedly, a non-phosphorylatable Snf1 mutant protein

restrains Gal83 in the cytosol and excludes it from the nucleus, suggesting that a cytosolic Snf1 tether exists.

Our analysis of Snf1-GFP nuclear import within *ubc4/5Δ* and *rsp5*^{ts} strains show obvious defects in the nuclear accumulation of Snf1-GFP under activating conditions (Fig. 7.7). Our work shows that a fully phosphorylated, active and stable kinase fails to efficiently accumulate in the nucleus, clearly implicating Ubc4/5 and Rsp5 activity in regulated Snf1 nuclear import. Furthermore, the defects appear to be subtly distinct between the two strains. To clarify what we were consistently observing through fluorescent microscopy under activating conditions, we quantitated several deviations from the import pattern in WT yeast. First, we noted that the clear accumulation of Snf1-GFP in the nucleus decreased from ~90% in WT to less than 20% in *ubc4/5Δ* upon activation, and the *rsp5*^{ts} strain showed a temperature-dependent decline, being profoundly effected at 37°C (Fig. 7.7E). What was clarified upon quantitation in the *ubc4/5Δ* and *rsp5*^{ts} strains was the final location of Snf1-GFP upon activation. In the *ubc4/5Δ* strain, more than half of the Snf1-GFP exhibited an enhancement of vacuolar location, in contrast to the WT and *rsp5*^{ts} strains that have negligible vacuolar signal. We also note that, as increasing temperature compromises Rsp5 function, the abundance of Snf1-GFP in the cytosol increases. Lastly, both the *ubc4/5Δ* and *rsp5*^{ts} strains differ significantly from WT in that a significant proportion of Snf1-GFP signal becomes homogeneous, being diffusely localized throughout the nucleus and cytosol. We do not know the underlying mechanisms affected by the *ubc4/5Δ* and *rsp5*^{ts} mutations that impair nuclear accumulation. Several explanations come to mind and will be tested in future studies. It is possible that this mislocalization might be expected if Gal83 function was lost, and only Sip1 (Hedbacker *et al.* 2004) or Sip2 (Vincent *et al.* 2001) β-subunit localization activity remained, respectively. Sip1 is the SNF1 kinase β subunit that directs Snf1 to the vacuole and Sip2 signals cytosolic localization under activating conditions (Vincent *et al.* 2001).

Alternatively, it may be that Rsp5 is required for facilitated nuclear import, perhaps by ubiquitin-mediated degradation of a cytosolic tether under its control. We correlate the loss of Rsp5 activity with a failure of Snf1 to enter the nucleus as evidence supporting the concept of a cytosolic tether. It is also clear that removal of Ubc4 and

Ubc5 activities effects Snf1 subcellular location in a subtly different manner, due to the widespread lack of a discernably preferred location. The homogenous distribution of Snf1-GFP in this strain, in combination with the apparent mislocalization to the vacuole, implies that all three known sites (nucleus, cytosol and vacuole) are equally possible and unregulated in this strain.

Given the great evolutionary conservation of both the AMPK family and the ubiquitin system, it is highly possible that these novel roles for ubiquitination in Snf1 kinase regulation will hold true in high eukaryotes. Given the important role that AMPK plays in human metabolism, particularly when considering the current epidemic of obesity and DM2, we wonder if these insulin resistant states result in defects in nuclear localization of AMPK. An intriguing report in the non-metabolic human disease of Huntington's Chorea noted the subcellular mislocalization of AMPK (Ju *et al.* 2011).

Table 7.1 *Saccharomyces cerevisiae* strains used in Chapter 7

Strain	Genotype	Source/Reference
yTER32 (PJ69-4A)	*MATa trp1-901 leu2-3 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lac*	E. Craig
yTER23	*MATα ade2 his3Δ200 leu2-3,112 ura3-52 lys2 rsp5^{P-S}*	T. Harkness
yTER86	*Rsp5^{P-S}* in yTER32	This study
yTER223	*MATa ade2 his3 leu2 ura3 SNF1-GFP::HIS3*	R. Jiao *et al.* (2015)
yTER279 (MHY501)	*MATα his3-Δ200 leu2-3 112 ura3-52 lys2-801 trp1-1*	S. Jentsch
yTER278 (MHY508)	*MATα his3-Δ200 leu2-3 112 ura3-52 lys2-801 trp1-1 ubc4Δ::HIS3 ubc5Δ::LEU2*	S. Jentsch
yTER232	*SNF1-GFP::HIS3* into yTER23	This study
yTH3560	*MATa ade2 his3Δ200 leu2-3 lys2Δ201 ura3-52 elc1Δ::kanM*	W. Xiao
yTH1155	*MATα ade2 his3Δ200 leu2-3,112 lys2Δ201 ura3-52 apc5^{CA-PA}::His5+*	T. Harkness *et al.* (2002
yTER246	*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 MIG1-TAP::HIS3*	Open Biosystems
yTER297	*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 ROD1-TAP::HIS3*	Open Biosystems
yTER166	yTER246×yTER23	This study
yTER300	yTER278×yTER223	This study
yTER323	yTER278×yTER246	This study
yTER307	yTER278×yTER297	This study
yTER187	*MATa ade2 his3Δ200 leu2-3,112 lys2Δ201 ura3-52*	This study
yTER70	*MATa ade2 his3Δ200 leu2-3,112 lys2Δ201 ura3-52 snf1Δ::KanMX6*	R. Jiao *et al.* (2015)
yTH1482	*MATα ade2 his3Δ200 leu2-3,112 lys2Δ201 ura3-52 mig1Δ::LEU2*	M. Carlson
yTER317	yTER315+ *HCM1-GFP::HIS3*	This study
yTER318	yTER316+ *HCM1-GFP::HIS3*	This study
yTER319	yTER187+ *HCM1-GFP::HIS3*	This study

CHAPTER 8 General Discussion

8.1 Conclusions

As a key sensor of cellular energy levels, the AMPK family of kinase is ubiquitous and highly conserved in all eukaryotes, from yeast to humans. Its activation enables cells to respond rapidly to changes in environmental stress or nutrient limitations, through facilitating metabolic adaptations, namely a switch to catabolic pathways to preserve and release energy and nutrients and promote cell/organismal survival. In addition to survival, a functional AMPK is necessary to maintain homeostasis of the cells or body organs, thus it is highly relevant to organismal health. This is particularly relevant in human health, as AMPK activity is responsive to health issues including obesity, insulin resistance (DM2), cancers, and aging (Musi *et al.* 2001; Minokoshi *et al.* 2002; McGee *et al.* 2003). Exercise and weight loss activate AMPK, resulting in a net negative energy balance. Model systems have supported the notion that pharmacological activation of AMPK can provide similar benefits to health as regular exercise and normal body weight. With an inability to effectively 'prescribe' weight loss and exercise as a therapy to promote health, there has been great interest in obtaining a better understanding of how AMPK and its family members are regulated and activated to ultimately use this information to improve health outcomes. In this thesis, the regulation of the yeast AMPK ortholog, SNF1 kinase, has been elucidated to reveal that fine-tuning of its activity and activation occurs at both the transcriptional and post-translational levels.

We first demonstrated that the conserved UBA motif found in the catalytic subunit of the SNF1 kinase has an inherent inhibitory role, and functions to restrain SNF1 kinase activity. UBA motifs in general have the potential to non-covalently bind the globular head of ubiquitin attached to an unrelated protein and thereby affect enzyme activity and/or protein associations (Mueller and Feigon 2002; Varadan *et al.* 2005). However, this is not the role for the UBA domain in yeast, as previous work had elegantly determined that the UBA domain of the Snf1 does not bind mono- or poly-ubiquitin, as is true for all Class 3 UBA domains (Raasi *et al.* 2005). Therefore, the precise role of the UBA domain within the SNF1 kinase was undetermined. Our work demonstrated that conserved UBA mutations significantly alter SNF1 kinase activation

and biological activity including enhanced allosteric subunit associations within the heterotrimeric complex itself, and increased oxidative stress resistance and lifespan (Chapter 4). Significantly, the enhanced UBA-dependent longevity and oxidative stress response is at least partially dependent on the FOXO orthologs, Fkh1 and Fkh2, which are stress response transcription factors. Significantly, Fkh1/2 activity was demonstrated to influence *SNF1* gene expression.

In addition, we developed a novel 2-hybrid method to semi-quantitatively measure the allosteric associations between the SNF1 kinase subunits throughout a range of glucose concentrations representative of fully repressive (high glucose) to fully activating (low glucose) conditions. This method is based on the traditional β-galactosidase colorimetric assay and the use of slant plate growth medium, which allow us to distinguish the intensity of protein-protein associations by color development. This technique supplies a direct way to compare the trend of protein associations under variable activating intensities. We observed that the UBA domain plays an inherent role in inhibiting the SNF1 kinase allosterism between the Snf1-Snf4 subunits, as there were enhanced associations in our $Snf1^{UBA}$ mutant. The precise mechanism to explain the UBA effects on subunit associations remains unclear, yet a glucose-responsive association of a peripheral protein with the hydrophobic face of the UBA domain may introduce steric hindrance (factor X) between Snf1-Snf4 proteins, thereby preventing full activation. This would enable a very subtle level of regulatory control, with much finer tuning than the simple 'ON/OFF' switch of SNF1 kinase activity attributed to its phosphorylation at Thr210. We consider the possibility that the expression level of the SNF1 kinase subunits within the 2-hybrid vector system may be altered within *ubc1Δ* and $rsp5^{ts}$ mutant, compared to the WT, and therefore alter the 2-hybrid association strength. This will require a formal test of the Snf1 and Snf4 protein levels, which can be accomplished using commercially available antibodies.

We next determined that the SNF1 kinase is regulated by its subunit stoichiometry, and that under physiological conditions the Snf1, Snf4, and three β subunits are stable proteins regardless of the activation state of the enzyme complex (Chapter 5). This is in direct conflict with that reported in yeast (Wilson *et al.* 2011) and human (Al-Hakim *et al.* 2008), where it was observed that the catalytic α subunit was

poly-ubiquitinated, which, in yeast alone, resulted in its degradation. In contrast to these prior studies, our experiments were performed under physiological conditions, without manipulation of the ubiquitin-proteasome system, and at endogenous protein levels.

As part of our investigations, we aimed to identify the ubiquitin cascade enzymes involved in the formation of the poly-ubiquitin chains formed on the α subunits, and the influence on SNF1 kinase activity when we prevented their formation by mutating the ubiquitin-attachment site lysines. Yeast proteomics had previously identified lysines K80 and K461 (Starita *et al.* 2012) within the catalytic subunit, Snf1, as ubiquitin attachment sites. In yeast, Snf1 poly-ubiquitination had been associated with its degradation, whereas in human cell line studies the stability of AMPK-related kinases was not affected by poly-ubiquitination. We have clarified this point by demonstrating that under physiological conditions, all endogenous subunits of the yeast SNF1 kinase complex are stable regardless of the enzyme activation state and poly-ubiquitination of Snf1 is not readily detectable in WT yeast. Deletion of either the β (Gal83) or γ (Snf4) subunits reduced Snf1 protein abundance independent of proteasome activity or K80 or K461 availability, suggesting a stoichiometric correction amongst subunits, rather than ubiquitin-dependent degradation. Lastly, neither mutation at K80, K461, nor their combination, significantly impaired SNF1 kinase phosphorylation, its activity on transcriptional targets, or its stability, leaving the physiological importance of Snf1 ubiquitination in question, as well as raising the possibility of additional as-yet unknown ubiquitin-attachment sites.

We have searched for the E2 activity in yeast responsible for the previously reported poly-ubiquitination of the Snf1 subunit (Wilson *et al.* 2011). We screened yeast strains deleted for individual E2 activities and measured *SUC2* expression as an indirect marker of SNF1 kinase activation and activity (Treitel *et al.* 1998; Sutherland *et al.* 2003). Efficient *SUC2* expression of invertase is dependent on the combined steps of phosphorylation, allosteric associations, nuclear import, and kinase activity, such that a defect in invertase enzyme activity may reflect a disruption at any of these steps. If a specific E2 was responsible for poly-ubiquitination of the SNF1 kinase and its subsequent degradation, we predicted that its disruption would enhance *SUC2* expression through

enhanced protein stability. However, this was not observed for any E2, and instead several were coupled with significant decreases in SNF1 kinase activity/activation.

Chapter 6 describes our analysis of the role of the E2, Ubc1, in SNF1 kinase regulation after noting an intermediate defect in *SUC2* expression in its absence. We identified Ubc1 as an important regulator of SNF1 kinase activity specifically at the level of transcription. The protein abundance of the Snf1 and Snf4 subunits is decreased in the *ubc1Δ* strain, yet their physical association is surprisingly enhanced when assayed using 2-hybrid analysis. Furthermore, the Thr210 phosphorylation of Snf1 is relatively intensified in the *ubc1Δ* strain. Such hyper-phosphorylation has been noted previously in the literature and has been proposed to act as a compensatory mechanism to preserve SNF1 kinase activity despite decreased abundance (Hsu *et al.* 2015). Our results extend this compensatory mechanism to intensified allosterism. Our data suggests that the *SUC2* expression defect observed in the *ubc1Δ* strain comes in part from the reduced enzyme subunit stoichiometry, but not kinase activation, allosteric association, and nuclear localization as these necessary steps are fully preserved or enhanced. It is unclear how the Ubc1 E2 enzyme is involved in allosteric hindrance between Snf1 and Snf4 (factor X) and how it diminishes their 2-hybrid association.

Our data also suggests that the *SUC2* expression defect observed in the *ubc1Δ* strain comes from an impairment of *SNF1* expression. The protein abundance of Fkh1, Fkh2 and the third Fkh member, Hcm1, is significantly lower in *ubc1Δ* cells as compared to that of WT cells. Hcm1 is required for *Fkh1/2* expression, likely explaining the latters' decreased abundance. Significantly, Hcm1 fails to shuttle into the nucleus in the expected cell cycle-dependent manner when Ubc1 is not present, and is therefore unable to induce the expression of Fkh1/2. The details of how Ubc1 is involved in Hcm1 protein regulation and nuclear import are still unknown. It remains to be determined if Ubc1-dependent ubiquitination of Hcm1 directly (or a tethering protein, indirectly) could regulate the Hcm1 protein nuclear import.

Chapter 7 describes our analysis of the role of the E2s, Ubc4 and Ubc5, in SNF1 kinase regulation after noting a profound defect in *SUC2* expression in their absence. We also reported on the impact that their cognate ubiquitin ligase, Rsp5, has on SNF1 kinase activation and activity. We determined that yeast strains disrupted for Ubc4/5 or Rsp5

function were markedly impaired for nuclear accumulation of the SNF1 kinase despite its full phosphorylation and activity, all without affecting Snf1 protein stability. These results supplement the knowledge that ubiquitination and the ubiquitin cascade are involved in protein trafficking and nuclear import/export. The result supports a positive role for ubiquitin in SNF1 kinase activation at multiple steps and is not limited to ubiquitin-dependent proteolysis.

8.2 Future Directions

Future investigations of the UBA domain of AMPK within other species including plants, fish, or mammalian cell lines would be beneficial. It will be of great interest to determine if equivalent UBA mutations in plants, fish, or mammals similarly increase the activity of the AMPK or AMPK-like enzymes, and if this translates into an enhanced survival response to environmental stresses (temperature, oxidative stress, poor quality nutrients) or an increase in lifespan. The point mutations within the UBA motif from our study (Jiao *et al.* 2015) and unrelated mutations in a previous publication (Momcilovic and Carlson 2011) confirmed the inhibitory role of the UBA domain in yeast. However, little is known in higher eukaryotes. Applications can be readily considered to that of increased crop yields or even aquaculture biomass. Fish are emerging as a vertebrate model to study aging, evolution, and stress response. Interestingly, the fish genome also encodes a conserved UBA motif within the AMPK α subunit, as in other species. The genetic manipulations on the UBA sequence within the fish genome has the potential to increase its survival from aquaculture stressors with enhanced population survival and, ultimately, protein yield. Another example would be plants where there is constantly a search to attain higher crop yields. This could potentially be done through UBA mutations, should this translate into greater crop survival under adverse temperatures, moisture, soil quality, or time to maturation. The plant genomes can be effectively manipulated to allow UBA mutations using available technology such as CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats). Sucrose non-fermenting related kinase 1 (SnRK1), the plant AMPK, was proven to act as a metabolic sensor, and respond to environmental stress (Crozet *et al.* 2014). It would be supportive to show the increased stress responses when the UBA domain is mutated in plants.

We discovered that the E2 enzyme, Ubc1, regulates SNF1 kinase subunit abundance. We also determined that the subunits stoichiometry of the SNF1 kinase affects its enzymatic activity. Significantly, the decrease in Snf1 subunit abundance is due to the diminished transcriptional factors Fkh1/2, regardless of its own protein stability. The detailed mechanisms of the Hcm1 nuclear import dysfunction remains unsolved. One possible factor is ubiquitination of Hcm1 under the influence of Ubc1 activity. The role of ubiquitin in protein subcellular localization has been widely investigated (Davis *et al.* 2013) and our observed dysfunction of Hcm1 nuclear localization in the Ubc1 mutant (Jiao *et al.* 2016) makes this a testable mechanism. It may be that ubiquitination of Hcm1 controls its nuclear-cytosolic shuttling. In addition, research indicated that Hcm1 can be degraded in an E3 SCF-dependent manner (Landry *et al.* 2014), further supporting the likelihood that Hcm1 is ubiquitinated. However, we did not detect an Hcm1-ubiquitin conjugate using co-immunoprecipitation. The identification of ubiquitination sites on Hcm1 has not yet been reported, yet mass spectrometry would be a sensitive means to determine the ubiquitination sites on Hcm1. Subsequent single mutations or combined mutations of ubiquitin attachment sites (i.e. lysine to arginine) would help us to understand if translocation of Hcm1 is directly regulated through ubiquitination.

We were also very interested to observe that Snf1 subcellular localization is regulated by the ubiquitin enzyme cascade (Chapter 7) with subtle variations among the E2s and E3s identified. Surprisingly, even though Ubc4/5 and Rsp5 cooperate and associate under certain circumstances (Hiraishi *et al.* 2009; Stoll *et al.* 2011), the outcomes of Snf1 mislocalization upon mutation/deletion of these ubiquitin enzymes are quite different. Besides, a confocal microscopy could be used to visualize the Snf1-GFP distribution in yeast cells on a monolayer. This technique will further confirm the subcellular localization of Snf1 subunit.

We observed that the disruption of Ubc4/5 activity resulted in an extremely large cell size, with slow growth, and possibly a stressed cell phenotype. Within these strains, we observed a significant mislocalization of the Snf1-GFP from the nucleus to the vacuole, under activating conditions. This could due to the stress of the cell, and it remains unclear if Snf1-GFP is localized within, versus on the membrane, of the vacuole.

This can be confirmed through confocal microscopy. It may also be possible that the vacuolar-targeting β subunit Sip1 may be preferentially associated with the SNF1 kinase hetero-complex in this strain. This idea can be examined using co-immunoprecipitation, 2-hybrid assay or proximity ligation assays. We also observed that the Snf1 subunit was unable to accumulate in the nucleus without Rsp5 activity and instead dispersed homogenously throughout the cell. It remains unknown if Rsp5 directly binds to Snf1, if it ubiquitinates Snf1 directly, or if this is required for nuclear re-localization. The co-immunoprecipitation of Rsp5 and the SNF1 kinase would help to show the potential physical interaction between the two. It is possible that Rsp5 targets Snf1 nuclear import indirectly, by targeting a potential cytosolic tether (Factor X) for degradation. Such a tether associated with Snf1 may also affect the Snf1-Snf4 associations that were altered upon loss of Rsp5 activity (Chapter 7). Specifically, Snf1-Snf4 associations remained weak under activating conditions, in the absence of Rsp5 activity. It is a testable possibility that this 2-hybrid inhibition is due to the steric hindrance of a non-degraded cytosolic tether. The identification of such a tether protein is unknown, but a possibility includes Bmh1 and Bmh2 (brain modulosignalin homolog), orthologs of 14-3-3 in mammalian systems. 14-3-3 has been proven to bind the UBA domain in fruit flies (Benton *et al.* 2002) and to associate with mitogen-activated protein kinase (MAPK), an AMPK-like kinase in mammalian systems (Goransson *et al.* 2006). Bmh2 has been found to physically associate with both Snf1 (Elbing *et al.* 2006) and Rsp5 (O'Connor *et al.* 2015). Since the UBA motif within SNF1 kinase is inhibitory (Chapter 4) (Jiao *et al.* 2015), the mutant UBA domain possibly associates with one or both of Bmh1 and Bmh2. In addition, Rsp5 has been proven to cooperate with 14-3-3 in yeast, supporting the possibility that Bmh1/2 may be a potential tether (Becuwe *et al.* 2012). It would be interesting to look at the phenotype of *bmh1/2Δ* mutant strains, as we would predict that nuclear import might be unregulated, or perhaps constitutive, in the 14-3-3 deletion strain. This may be observed in the *bmh1/2Δ* strain by testing Snf1 phosphorylation, *SUC2* expression, lifespan and survival under stress conditions. Co-immnoprecipitation of the SNF1 kinase subunits and Bmh1/2 could also show if there were direct/indirect bindings.

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Appendix II

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