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Investigation into Regulation of the SNF2 Gene by Alternate mRNA Isoforms in S. cerevisiae Yeast

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Investigation into Regulation of the *SNF2* Gene by Alternate mRNA Isoforms in *S. cerevisiae* Yeast

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular Biology

by

Lauren A. Thurlow

2023

ABSTRACT OF THE DISSERTATION

Investigation into Regulation of the *SNF2* Gene

by Alternate mRNA Isoforms

in *S. cerevisiae* Yeast

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Doctor of Philosophy in Molecular Biology

University of California, Los Angeles, 2023

Professor Tracy L. Johnson, Chair

Eukaryotic organisms have evolved complex gene regulatory networks to launch coordinated responses to external conditions and stimuli. Under environmental stress, such as nutrient depletion, these responses involve reallocation of cellular resources away from the products of growth and cell cycle stimulating genes and towards the products of stress-responsive genes. This occurs through several mechanisms, including changes in transcription, decreased ribosome biogenesis, altered translation initiation, and changes in mRNA features that modulate translation efficiency and transcript stability. Upon nutrient starvation in the yeast *Saccharomyces cerevisiae,* diploid cells undergo meiosis leading to sporulation. Previous studies show that the catalytic component of the Swi/Snf chromatin remodeling complex, Snf2, is

responsible for shifting gene expression away from intron-rich ribosomal protein genes (RPGs) to enhance splicing of meiotic intron-containing genes (ICGs) during sporulation, and that a similar process occurs during the transition from growth to quiescence known as the diauxic shift in yeast. During both of these complex cell-state transitions, Snf2 protein levels change dramatically while SNF2 mRNA levels remain relatively stable.

The aim of this study is to understand the mechanism by which Snf2 protein levels change in response to nutrients. Here I describe alternate transcription start sites (TSS) at the *SNF2* gene locus under batch growth conditions, which produce SNF2 transcripts with distinct 5' leaders affecting downstream translation propensity. Specifically, a long transcript isoform of SNF2 mRNA containing three upstream open reading frames (uORFs), is capable of inhibiting the translation of the downstream protein-coding ORF. I identified the previously unannotated TSS of the long SNF2 isoform and performed RNA analysis in mutant and wild-type cells under various conditions to demonstrate that the transcript isoforms undergo nutrient-responsive transcript isoform switching, which is affected by the transcriptional regulator Ume6. Parallel protein analysis via Western blotting shows that this regulation indeed affects Snf2 expression, and that there is an inverse relationship between expression of the long SNF2 isoform and Snf2 protein levels. In light of the conservation of Snf2-family proteins, investigating *SNF2* regulation in response to environmental changes in *S. cerevisiae* may carry important implications for understanding the regulation of Swi/Snf chromatin remodeling activity in higher eukaryotes.

The dissertation of Lauren A. Thurlow is approved.

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University of California, Los Angeles

2023

To Mother Nature, the original scientist,

who supports and inspires me each day.

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LIST OF COMMON ACRONYMS

DNA	deoxyribonucleic acid
RNA	ribonucleic acid
ATP	adenosine triphosphate
Swi/Snf	Switch (Swi)/Sucrose non-fermenting (Snf)
SNF2	Yeast Swi/Snf core ATPase
BRG1	Mammalian Swi/Snf core ATPase
mRNA	messenger RNA
ORF	open reading frame
uORF	upstream open reading frame
TSS	transcription start site

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Elizabeth Pérez, Trase Aguigam, and Znala Williams, all directly contributed to results shown in this dissertation and helped me grow as a mentor. My previous mentors at Loyola Marymount University, including Professor Jeremy McCallum, Professor Emily Jarvis, Professor Martina Ramirez, Professor Ed Mosteig, and Beth Marsh, all believed in me and guided the first stages of my development as a researcher. My friends and family, especially my mother, Suzanne Rodriguez, my sister, Gillian Thurlow, my grandfather, Robert Rodriguez, as well as David Goetz, Emily Gervais, Ruben Alcala, and too many others to list them all, anchored me as a human with unwavering love and stood by me in the most difficult moments. Thank you all so much!

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- Thurlow, L., Clay, K., Wene, T., Groom, M., Keyes, E., Lang, A. & McCallum, J. (2018) "G-quartet Formation from N9-Benzylguanine Derivatives." ***Letters in Organic Chemistry*** 15 (11), pp 909-914, doi: 10.2174/1570178615666180329161419

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- UCLA Eugene V. Cota-Robles Fellowship (2016-2017)

CHAPTER 1

Introduction

Life on Earth experiences cyclical fluctuations, whether it is between day and night, high and low temperatures, or abundance and lack of nutrients. From single-celled microbes to complex multicellular organisms such as humans, we are all subject to changes happening in the environment, and our survival depends on the ability to adapt and make adjustments accordingly. In times of plenty, energy goes towards growth and expansion. In times of scarcity, energy is reserved for only what is necessary for survival. At the cellular level, this manifests through changes in gene expression and modulation of processes such as the cell cycle. To carry out these changes, life has evolved gene regulatory networks to launch efficient, coordinated responses to external conditions and stimuli. The mode of control of these gene regulatory networks has been the focus of my research.

Within eukaryotic nuclei, the DNA is wound around histone proteins to form structures called nucleosomes, which are further coiled into larger chromatin fibers. This tight packaging allows up to billions of base pairs of DNA to fit into the nucleus of every cell. This enormous library of genetic information must be highly organized, and its expression highly regulated.

To illustrate this point, the genome can be compared to a massive library. If each gene is a book, it must only be taken off the shelf when it needs to be “read” and expressed, then placed back on the shelf once it is no longer needed. Importantly, many genes have detrimental effects when expressed constitutively, and the gene products are only needed at specific times, locations, or in particular amounts. Due to the energy input required, cells must be selective about which genes are expressed, just as a

human needs to be selective about which books to read amongst the myriad in the library.

How do cells make these decisions? Just as books are labeled and classified in a library, genes are marked with epigenetic modifications, which are chemical signals that alter the structure of the chromatin to change its accessibility. Certain epigenetic modifications recruit chromatin remodeling complexes to unwind and prepare regions of the genome for transcription, while others cause regions of the genome to become compact and not accessible for transcription. In the close quarters of the nucleus, these changes in chromatin structure are crucial for determining which genes are expressed.

Swi/Snf chromatin remodeling complexes are essential to eukaryotic gene regulation.

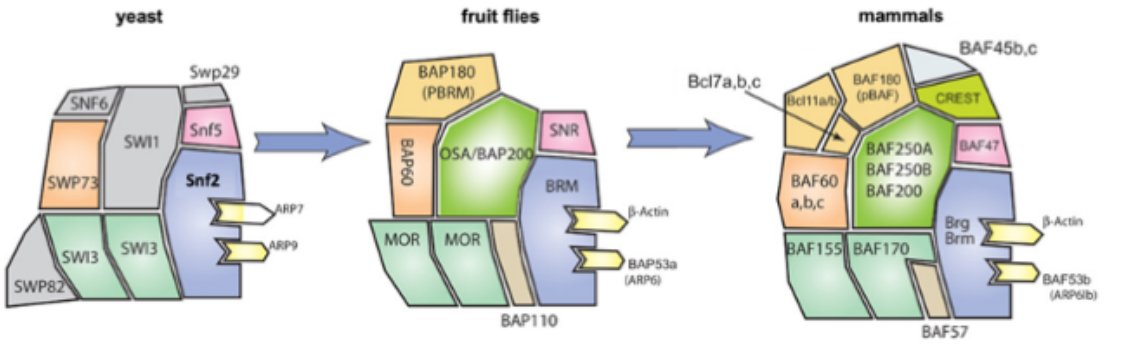
Indispensable in the process of eukaryotic gene regulation are Swi/Snf chromatin remodeling complexes. Swi/Snf is one of four families of chromatin remodeling complexes, alongside ISWI, CHD and INO80 (*1*). These complexes interact with chromatin by recognizing epigenetic modifications on histone proteins, and slide, eject, or replace nucleosomes in an ATP-dependent manner, altering the accessibility of the DNA for RNA transcription, DNA repair, and DNA replication and recombination (*1*). The complex was originally discovered through yeast genetic screens for mutations affecting mating type switching (“Switch – Swi”) and sucrose fermentation (“Sucrose non-fermenting – Snf”) (*2*). Swi/Snf regulates widespread changes in gene expression of a unique set of genes when compared to other chromatin remodeling complexes (*3*).

The core ATPase of Swi/Snf is surrounded by a number of other highly conserved subunits as well as “accessory” subunits that mediate interactions with other factors that direct and influence their chromatin remodeling activity (*4*). A comparison of the composition of Swi/Snf complexes is shown in Figure 1.1.

In the yeast *Saccharomyces cerevisiae*, there are two Swi/Snf family complexes, Swi/Snf and RSC, which regulate non-overlapping regions of the genome (*5*). For example, RSC is important for genome maintenance and mitotic division. Swi/Snf, on the other hand, regulates ribosome biogenesis, metabolism and stress responses. RSC is more abundant and broad in its function (i.e. regions occupied by all 3 RNA polymerases), whereas Swi/Snf is less abundant and more targeted (*6*). Unless otherwise indicated, I will be referring to the latter Swi/Snf complex throughout this dissertation, rather than to the entire family of complexes.

The Swi/Snf core ATPase is called Snf2. In higher eukaryotes such as humans, the Swi/Snf ATPase is most commonly known as BRG1, BRM or SMARCA4. Between yeast and humans, the average conservation at the amino acid level of homologous genes is about 30% identity, and Snf2 conservation with BRG1 is above average at 39.09% (Figure 1.2). Conservation of the ATPase domain is so high (66.87% identity, as shown in Figure 1.2) that the human BRG1 ATPase domain can be swapped into yeast SNF2 with no negative consequences on cellular growth (*7*). The ATPase domain grips double-stranded DNA on a nucleosome and translocates it in relation to the histone octamer using the energy from ATP hydrolysis, generating a loop which then propagates around the nucleosome and out the other side (*8-11*). This changes the position of the nucleosome on the DNA sequence, thereby altering chromatin structure.

Figure 1.1



Adapted from Kadoch and Crabtree, *Science Advances* (2015)

Figure 1.1. Schematic diagram of Swi/Snf complex components and their evolution across eukaryotes (12). Note the persistence of the Snf2-family core ATPase (blue subunit).

Another notable and highly conserved domain present in SNF2 family proteins is the bromodomain (39.44% amino acid identity between yeast and humans, as shown in Figure 1.2), which binds to acetyl groups on histone protein tails. Acetyl modifications of histones are often associated with active genes. This is not only because the acetyl groups neutralize the positive charge attracting histones to the negatively charged DNA backbone, loosening the coiling of the chromatin and repelling neighboring nucleosomes, but also because bromodomain-carrying transcriptional regulators and chromatin remodeling complexes, such as Swi/Snf, can recognize and perform their nucleosome-sliding function on them.

In higher eukaryotes, Swi/Snf takes on more diversified roles in addition to those that are evolutionarily conserved with yeast. It plays a role in the development and maintenance of various tissues and cell types, including but not limited to those of the cardiovascular, immune, nervous, and reproductive systems (*13-15*). Deletion of the SNF2 homolog, BRG1, in mammalian cells, causes downregulation of a significant portion of genes involved in a wide variety of processes, including systemic and anatomical structure development and cellular differentiation (*16*). In light of the importance of Swi/Snf in development and maintenance of cellular identity, it is not surprising that misregulation of this key regulator is implicated in cancer. In fact, mutations affecting BRG1 are present in over 25% of human tumors (*12, 17*).

Figure 1.2

Figure 1.2. Amino acid sequence conservation between highly conserved Snf2-family protein domains in yeast (*S. cerevisiae*) and humans (*H. sapiens*). Between the two species, the ATPase domains share 66.87% identity, while the Bromodomains share 39.44% identity.

Swi/Snf regulates the use of cellular resources by altering gene expression in response to nutrients.

In yeast, Swi/Snf regulates chromatin accessibility at the promoters of ribosome biogenesis genes, metabolic genes, meiotic genes, and stress response factors (*18-22*). Genes regulated by Swi/Snf tend to be highly transcribed when active and are enriched for canonical TATA-boxes (*3*). Swi/Snf activity is required to maintain chromatin openness around the genes it regulates, and once Swi/Snf is depleted or inhibited, the chromatin accessibility is impaired (*23*). When nutrients are plentiful, Swi/Snf opens up the chromatin at the promoters of ribosome biogenesis and metabolic genes to allow cells to take advantage of the resources available to them. Under nutrient-poor conditions or other environmental stress, Swi/Snf occupancy decreases and the chromatin at those genes closes as part of the reallocation of cellular resources away from growth and cell cycle stimulating genes and towards stress-responsive genes (*22*). This allows cells to conserve and recycle resources through shifting to oxidative phosphorylation and activation of autophagy.

While haploid yeast cells transition to stationary phase or quiescence upon nutrient starvation, prolonged or extreme nutrient starvation leads to mating and causes the resulting diploid yeast cells to undergo meiosis leading to sporulation. Sporulation is an adaptive response to stress whereby a single diploid cell divides into four haploid gametes, all carrying unique combinations of the genetic information of the mother cell and protected within an outer membrane called an ascus (*24*). The functionally dormant spores are transcriptionally and translationally inactive and require miniscule amounts of energy compared to mitotic yeast cells, which allows them to survive for much longer

and under more extreme conditions. When they reenter favorable conditions, the shuffling of the mother cell's DNA that occurred during meiosis offers phenotypic diversity and a potential survival advantage in their new surroundings.

We have previously shown that the catalytic component of the Swi/Snf chromatin remodeling complex, Snf2, is responsible for shifting gene expression away from intron-rich ribosomal protein genes (RPGs) to enhance splicing of meiotic intron-containing genes (ICGs) during sporulation (*20*). A similar process occurs during the metabolic transition known as the diauxic shift in yeast to enhance expression of genes involved in respiration (*19*). These processes occur through changes in Snf2 occupancy on the DNA, which is influenced not only by epigenetic modifications, but by changes in Snf2 protein levels.

Snf2 activity is regulated in a nutrient-responsive manner.

The chromatin remodeling activity of Snf2 is directed to specific genes not only by histone marks on the genes to be remodeled, but by post-translational modifications of Snf2 itself. Snf2 contains a histone-like structure with lysine residues that have been demonstrated to be targets of the histone acetyltransferase Gcn5 of the nutrient-responsive SAGA complex (*25*). Once this region of Snf2 is acetylated, the Snf2 bromodomain intramolecularly interacts with it, reducing the availability of the bromodomain to interact with acetylated histones, and thereby reducing the recruitment of Swi/Snf to acetylated regions of the genome. Under nutrient stress conditions, this allows Swi/Snf chromatin remodeling activity to be diverted away from the highly acetylated ribosome biogenesis and metabolic genes and towards genes with condition-

specific transcriptional activators present (*20, 22*). Dynamic, genome-wide changes in Snf2 occupancy allows cells to respond quickly to changes in nutrient availability.

We have previously reported that, in yeast, Snf2 levels drop once cellular programs have been set in motion (*19, 20*). This likely occurs so that the energetically expensive genes which Swi/Snf activates can be turned off when no longer needed, to conserve energy and promote survival under changing conditions. Remarkably, while Snf2 protein levels and chromatin occupancy change dramatically during nutrient responses, *SNF2* mRNA levels remain relatively stable, suggesting translational regulation of the SNF2 mRNA.

Alternate transcript isoforms control gene expression by changing translation dynamics.

Expression of genes involved in different phases of meiosis must be turned off and on in a highly controlled manner under low-nutrient conditions. Rather than shutting off and restarting transcription at the promoters of these genes, many of the genes have alternate transcription start sites that produce distinct mRNAs with extended 5' leaders that affect their downstream translational output (*26-28*). These alternate mRNA isoforms have been termed long undecoded transcript isoforms (LUTIs) (*28*).

In general, the observed discordance between mRNA and protein levels of genes regulated by alternate isoforms is due to altered translation dynamics. In 97% of cases, the extended 5' leaders contain short peptide-encoding sequences called upstream open reading frames (uORFs) (*27*). During canonical cap-dependent translation initiation, scanning ribosomes interact with and translate these uORFs into short

peptides (Figure 1.3) (*29*). This prevents translation initiation on the main ORF downstream, effectively inhibiting expression of the encoded gene, which is why these alternate isoforms are called “undecoded.” The generation of extended isoforms with altered ribosome occupancy due to the presence of uORFs may also increase the sequestration of transcripts in stress granules (*30*).

At the level of transcription, Ume6 is the key factor regulating expression of alternate isoforms (*27*). Ume6 is a binding partner of the Rpd3L histone deacetylase complex and primarily represses transcription of meiotic genes until the presence of Ime1 (inducer of meiosis 1) recruits the SAGA complex to counteract the repression and activate early meiotic genes (*31, 32*). Ume6 binds to URS1 sites, which have a consensus motif of *GGCGGC* and are typically found within 300 bp of promoters of LUTI-regulated genes (*27*). It is important to note that transcription of alternate isoforms appears to affect transcription of canonical isoforms to varying degrees. According to previous studies, the canonical isoform is only downregulated by alternate isoform transcription in about 50% of cases (*27*). Additional gene features, including changes in chromatin landscape such as H3K36 trimethylation, are correlated with stronger repression of the canonical isoform (*27, 33*).

LUTI-based regulation of Snf2 as a mechanism for modulating Snf2 protein levels

Snf2 controls expression of some of the most highly expressed genes in the cell (e.g. ribosomal protein genes or RPGs) and regulates the most energy-intensive process in a cell, namely translation (*34*). Hence, tight and robust regulation of *SNF2* is critical. We considered mechanisms of *SNF2* regulation whereby Snf2 protein levels

Figure 1.3

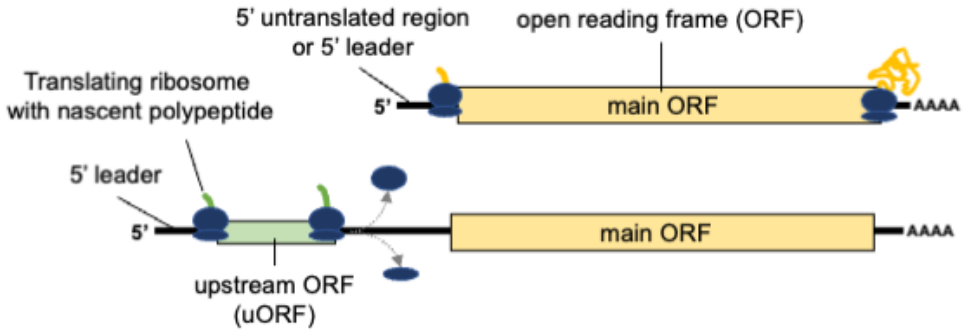


Figure 1.3. Schematic comparing translation dynamics on alternate transcript isoforms. Under canonical, cap-dependent translation conditions, ribosomes begin scanning at the 5' cap and initiate translation on the first open reading frame encountered. When an extended transcript contains uORFs, ribosomes translate short peptides and are released before reaching the main ORF.

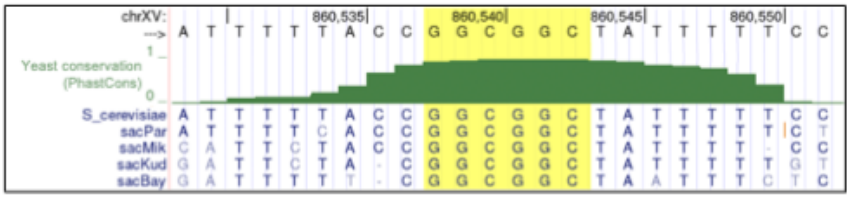
could be rapidly tuned as conditions demanded. Our analysis of published ribosome profiling and RNA sequencing studies conducted in the context of meiosis (*35*) revealed a SNF2 isoform with an extended 5' leader. Moreover, ribosome occupancy at putative uORFs was detected in this 5' leader, suggesting a mechanism whereby Snf2 protein levels could be regulated by LUTI expression. Further analysis of these sequences revealed that there are 2 conserved regions in the 5' leader of the SNF2 long isoform, one of which is a URS1 site, shown in Figure 1.4. This suggests that Ume6 is involved in *SNF2* regulation. Notably, the URS1 site is found on the negative strand, a finding that adds an intriguing twist to the potential LUTI regulation.

Summary

The aim of this study is to understand the mechanism by which Snf2 protein levels change in response to nutrients. In light of the conservation of Snf2, investigating its regulation in response to environmental changes in *S. cerevisiae* may carry important implications for Snf2 chromatin remodeling activity in higher eukaryotes.

In Chapter 2, I describe alternate transcription start sites (TSS) at the *SNF2* gene locus under batch growth conditions, which produce SNF2 transcripts with distinct 5' leaders affecting downstream translation propensity. Specifically, a long mRNA isoform of SNF2 contains three upstream open reading frames (uORFs), which inhibits the translation of the downstream protein-coding ORF. I identified the previously unannotated TSS of the SNF2 long mRNA isoform and performed RNA analysis in cells collected during batch growth to demonstrate that the transcript isoforms undergo nutrient-responsive transcript isoform switching, which is affected by the transcriptional

Figure 1.4



Adapted from UCSC Genome Browser

Figure 1.4. Analysis of the *SNF2* promoter region across *Saccharomyces* yeast species reveals a highly conserved sequence matching the URS1 consensus motif. The level of conservation at each genome position across yeast species is represented by the green histogram (Phastcons). The URS1 consensus motif is highlighted in yellow. Publicly available data accessed and analyzed on UCSC Genome Browser (genome.ucsc.edu) using the sacCer3 assembly.

regulator Ume6. Parallel protein analysis shows that this regulation indeed affects Snf2 expression, that there is an inverse relationship between expression of the SNF2 long isoform and Snf2 protein levels, and that the translational silencing conferred by this isoform is uORF-dependent.

In Chapter 3, I describe preliminary results related to my studies on SNF2 regulation, which require further experimentation. These include analyses of sequence features of *SNF2* that affect transcription of the short and long isoform. Preliminary results suggest an important role for both histone methylation and acetylation in this regulation. In Chapter 4, I discuss the implications of my findings and how they fit into the larger picture of eukaryotic gene regulation.

CHAPTER 2

Alternative mRNA isoforms regulate expression of the chromatin remodeler Snf2 in response to nutrient changes.

INTRODUCTION

Swi/Snf is a highly conserved chromatin remodeling complex that plays an important role in eukaryotic gene expression by regulating a large number of critically important genes (*3*). Many of these genes are highly transcribed once activated by Swi/Snf, and are involved in diverse processes such as cellular metabolism (*19*), ribosome biogenesis (*22*), differentiation (*36*), and meiosis (*20*).

The Swi/Snf complex interacts with chromatin by recognizing epigenetic modifications on histone proteins. The catalytic core of Swi/Snf complexes (Snf2 in yeast and Brg1 or SMARCA2/4 in higher eukaryotes) slides nucleosomes in an ATP-dependent manner, altering the accessibility of the DNA for RNA transcription, DNA repair, DNA replication, and recombination (*1*). Snf2 family proteins contain one or more bromodomains which interact with acetylated lysine residues found on histones near promoters, directing its chromatin-remodeling activity to active genes (*1*).

Despite the fundamental importance of Snf2 activity to eukaryotic cells, the regulation of Snf2 protein expression is not well understood. Previous studies in yeast have shown that Snf2 protein levels decrease in nutrient-poor conditions while SNF2 transcript levels remain steady (*19, 20*), suggesting that the downregulation of Snf2 protein in response to nutrients occurs post-transcriptionally.

This study elucidates the factors underlying regulation of the *SNF2* gene in the yeast *Saccharomyces cerevisiae*. Specifically, I describe how alternative SNF2 mRNA isoforms with different 5’ leader lengths are transcribed from two distinct transcription start sites (TSSs) at the *SNF2* locus. Interestingly, expression of the longer isoform is inversely correlated with Snf2 protein expression, consistent with translational silencing.

Here, I demonstrate that the decrease in Snf2 protein expression in response to nutrient depletion is facilitated by upstream open reading frames (uORFs) within the unique 5' leader of the long isoform, which prevent translation of the downstream Snf2 protein coding sequence. I also present evidence supporting a model whereby nutrient-responsive transcriptional regulator Ume6 directs the switch between the two SNF2 mRNA isoforms.

RESULTS

Snf2 protein levels change as cells are depleted of nutrients and transition between states.

To determine how Snf2 protein expression changes during nutrient starvation and gradual nutrient depletion, I performed Western blot analysis on cell samples from a sporulation timecourse and from batch growth in normal, nutrient-rich yeast growth media (Figure 2.1).

For sporulation experiments, I used a diploid strain of *S. cerevisiae* which undergoes sporulation in an efficient, highly synchronized manner, called SK1. Sporulation was induced by shifting exponentially growing cells from nutrient-rich growth media (GNA) to nutrient-poor sporulation media (SPM). Within the first three hours in sporulation conditions, Snf2 levels decrease dramatically and remain low throughout the sporulation process (Figure 2.1A). This is consistent with results from previous studies, where the decrease in Snf2 protein levels is associated with a decrease in expression of ribosome biogenesis factors and increase in expression and splicing of meiotic transcripts (*20*).

Figure 2.1

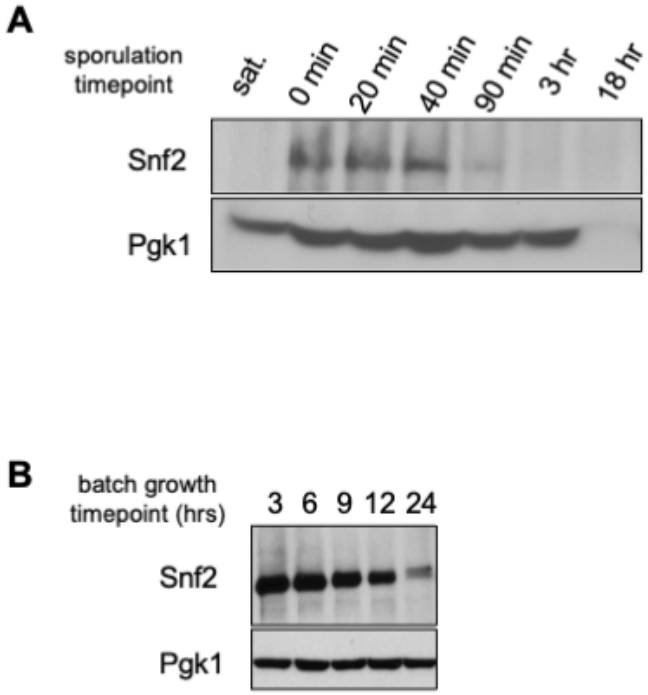


Figure 2.1. **Snf2 protein levels decrease in nutrient-poor conditions.** (A) Snf2 protein levels under sporulation conditions, analyzed via Western blot using α-Snf2 antibody (Santa Cruz Biotechnology YN-20) and Pgk1 antibody (Invitrogen 22C5D8) as loading control. (B) Snf2 protein levels during batch growth, analyzed via Western blot using α-Snf2 antibody and Pgk1 as loading control.

To evaluate Snf2 levels under batch growth conditions, I used a haploid *S. cerevisiae* strain (BY4741). Cells were grown overnight to saturation/quiescence in standard yeast growth media (YPD), then batch growth cultures were seeded at OD_{600} = 0.2 and cells were harvested at multiple timepoints. Once introduced to fresh, nutrient-rich media, previously quiescent cells gradually re-enter the cell cycle. This is associated with an increase in Snf2 protein expression, which remains high into the exponential growth phase. Around the diauxic shift, as the cells undergo the transition to respiration, then quiescence, Snf2 levels gradually decrease (Figure 2.1B) (*19*). During both of these complex cell-state transitions, Snf2 protein levels change dramatically, while total SNF2 mRNA levels remain relatively stable (data not shown).

A previously unannotated 5' extended isoform is transcribed from the *SNF2* locus

Sustained levels of SNF2 mRNA under varying conditions, even as protein levels decrease, suggest translational control of Snf2 protein expression. Previous genome-wide studies found widespread transcription of 5' extended mRNA isoforms during meiosis, which allow for dynamic translational regulation through ribosomal interactions with upstream open reading frames (uORFs) and silencing of the main protein-coding ORF (*27, 29, 35*). Analysis of published RNA sequencing and ribosome profiling data (*35*) revealed that *SNF2* was among the genes putatively regulated by transcript isoform switching. To determine whether the evidence of a 5' leader could be verified experimentally, we isolated RNA from the SK1 strain during the initial stages of meiosis and, using RT-PCR primers spanning the coding region and the predicted 5' leader, we confirmed that a continuous RNA is expressed. However, these studies were conducted

Figure 2.2

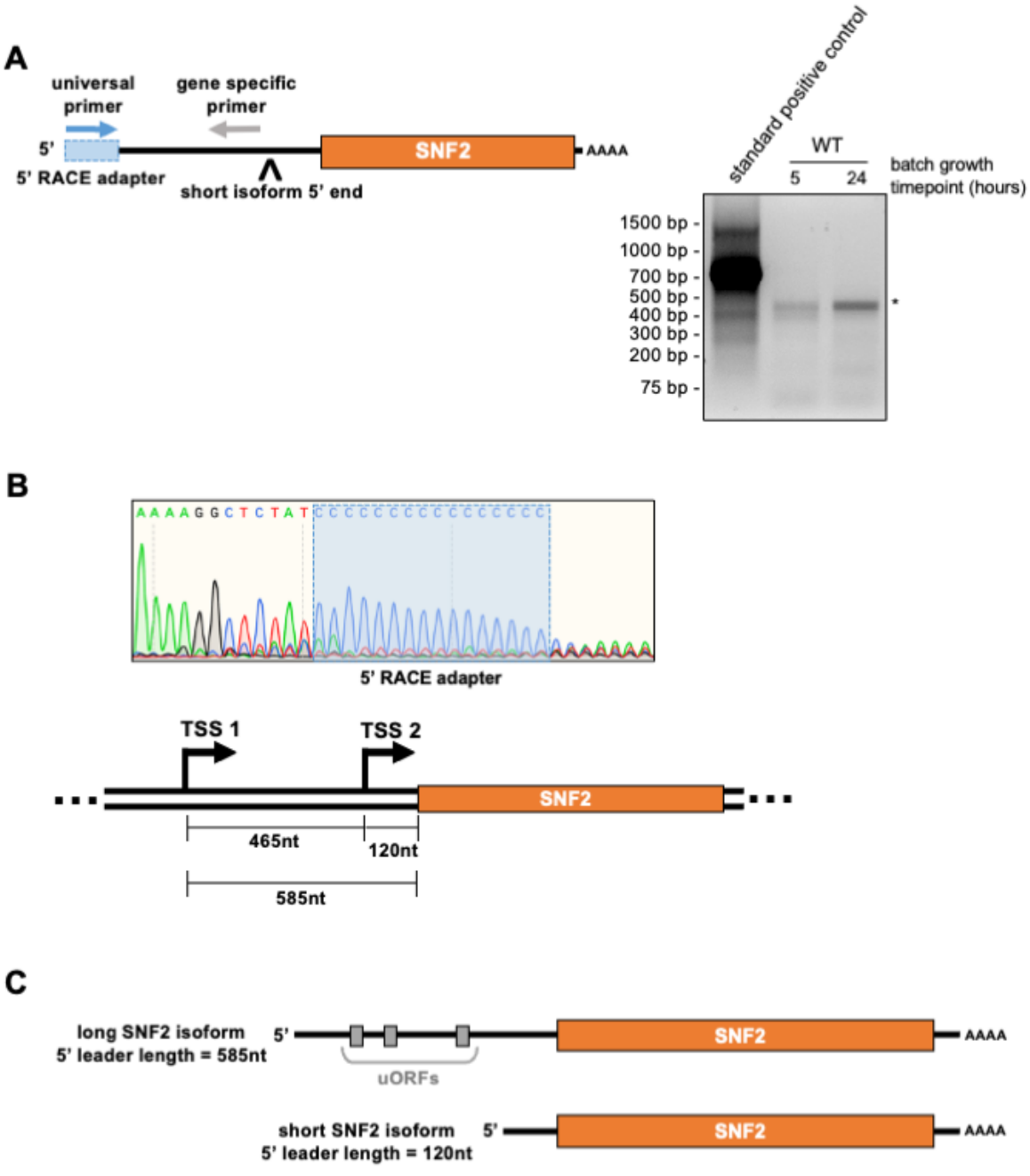

A
universal primer
gene specific primer
5'
SNF2
AAAA
5' RACE adapter
short isoform 5' end
standard positive control
WT
5
24
batch growth timepoint (hours)
1500 bp -
1000 bp -
700 bp -
500 bp -
400 bp -
300 bp -
200 bp -
75 bp -
*
B
A A A A G G C T C T A T C C C C C C C C C C C C C C C C
5' RACE adapter
TSS 1
TSS 2
SNF2
465nt
120nt
585nt
C
long SNF2 isoform
5' leader length = 585nt
5'
SNF2
AAAA
uORFs
short SNF2 isoform
5' leader length = 120nt
5'
SNF2
AAAA

Figure 2.2. *SNF2* transcription start sites mapped via 5'RACE. (A) 5'RACE was performed on equivalent RNA samples in addition to a kit-provided positive control (Invitrogen 18374058). (B) 5'RACE sequencing chromatogram and *SNF2* locus map. (C) Schematic of SNF2 mRNA isoforms characterized through the experiment shown in (A) and (B), as well as additional 5' RACE sequencing experiments (data not shown).

in the context of meiosis, and at the time it was unknown whether transcript isoform switching could occur in other contexts.

To investigate the relationship between SNF2 mRNA and protein levels during batch growth, I performed 5'RACE (rapid amplification of cDNA ends) analysis specifically designed to detect longer SNF2 mRNA isoforms on cells at different stages of growth (Figures 2.2A-B). A long isoform was detected in stationary phase cells and in exponentially growing cells. By sequencing the 5'RACE products, I determined that the long isoform has a 5' leader length of 585 nucleotides (nt), 465 nt longer than the short isoform length of 120 nt. The results of this analysis are shown in Figure 2.2C.

A 5' extended isoform is transcribed from the *SNF2* locus at levels inversely correlated with Snf2 protein levels

In order to determine the relationship between the long isoform and Snf2 protein levels, I analyzed the SNF2 RNA at different time points. I designed an RT-PCR experiment in which SNF2 long isoform levels could be determined relative to total SNF2 mRNA levels (Figure 2.3A). Equal amounts of cells were harvested during exponential growth phase (6 hour timepoint) and stationary phase (25 hour timepoint) and RT-PCR analysis was performed using random hexamers. This analysis revealed an increase in RNA upstream of the short isoform TSS at the later timepoint, consistent with the higher SNF2 long isoform levels in the later timepoint, confirming the results of the 5'RACE experiment described above, and suggesting that SNF2 long isoform expression is increased in nutrient-depleted conditions (Figure 2.3B). Furthermore, when I analyzed Snf2 protein levels, I found that the level of the protein was inversely

Figure 2.3

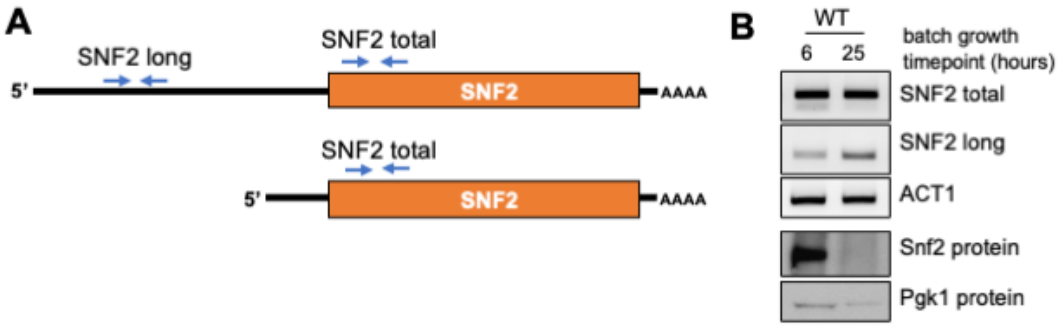


Figure 2.3. Transcription of SNF2 long isoform is inversely correlated with Snf2 protein levels. (A) Schematic of primer design for RT-PCR analysis of SNF2 transcripts. (B) Parallel RT-PCR and Western blot showing SNF2 mRNA isoforms and Snf2 protein levels during long-term batch growth timecourse. RT-PCR amplicons were separated by agarose gel electrophoresis.

correlated with levels of the 5' extended SNF2 mRNA isoform. This is consistent with the hypothesis that SNF2 long isoform expression leads to translational silencing of Snf2 protein expression.

I noticed that the SNF2 long isoform was still detectable at the shorter time point, despite high expression of Snf2 protein. I considered three non-mutually exclusive explanations. First, there may be a threshold at which the ratio of long and short isoforms either promotes or inhibits translation of Snf2 protein. For example, in nutrient-depleted conditions, the presence of the long isoform may sequester translation machinery away from the short isoform. The fact that ribosome biogenesis is shut down when nutrients are low could also contribute to the "limiting ribosomes" phenomenon. In nutrient-rich conditions, the levels of the short isoform may be high enough to escape this regulation despite the continued presence of the long isoform. A second explanation is that the results are reflecting the SNF2 mRNA levels across a population of cells which are at varying stages of the cell cycle and their own individual growth trajectories. As the overall culture is in the exponential growth phase, there may still be a small proportion of cells which are quiescent and expressing the long isoform. Likewise, as the culture depletes the available nutrients and transitions into stationary phase, perhaps a proportion of the cells take longer to upregulate the long isoform. A third possibility is that the SNF2 RNA detected through RT-PCR is a product from a cryptic transcription start site, an alternative TSS, or from the neighboring gene YPK9 which has a promoter region overlapping that of *SNF2*. As described above, early RNA analysis in the SK1 background was conducted confirming the presence of a long isoform continuous with the *SNF2* coding sequence. However, those experiments were

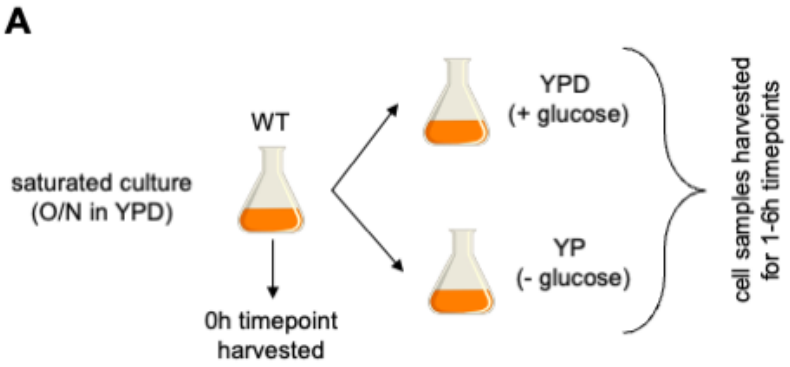
not quantitative and were done under meiosis-inducing conditions. Since current experiments cannot rule this out, this possibility will be discussed further below.

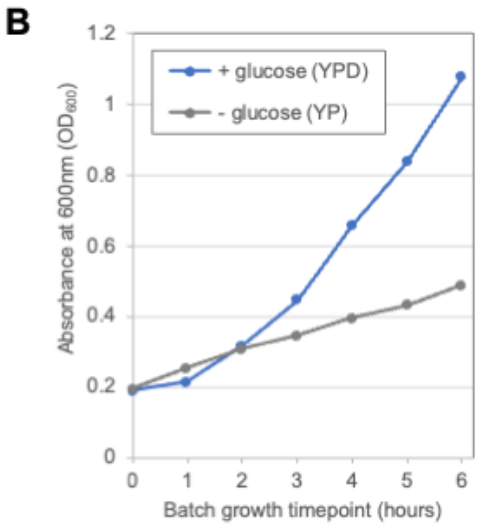
Lack of glucose leads to increased long SNF2 isoform levels and decreased Snf2 protein expression

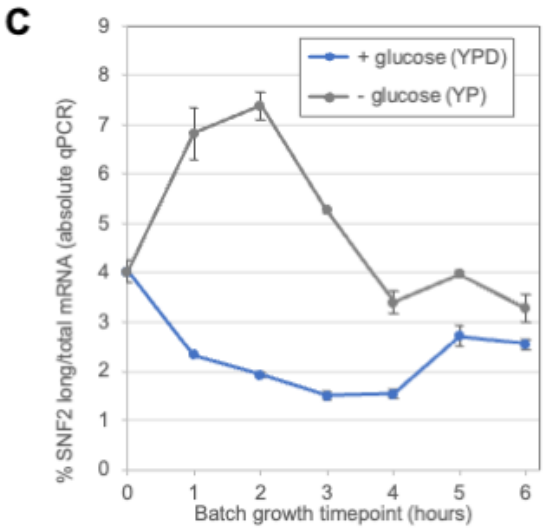
To confirm that the observed increase in SNF2 long isoform levels and decrease in Snf2 protein levels is in fact a response to nutrients, I grew WT cells as before, in either glucose-rich (YPD) or glucose-deprived (YP) media (Figure 2.4A). Cells in the glucose-deprived conditions failed to enter exponential growth (Figure 2.4B), presumably due to lack of activation of growth and ribosome biogenesis programs as a result of decreased Snf2 activity. As expected, cells exposed to glucose showed a decrease in SNF2 long isoform levels within the first 3 hours of growth, whereas cells deprived of glucose showed an increase in SNF2 long isoform levels (Figure 2.4C). The increased SNF2 long isoform expression observed in glucose-deprived samples is correlated with lower Snf2 protein levels (Figure 2.4D). These results, especially when compared between the two growth conditions, reinforce that there is an inverse relationship between the increase in the RNA product represented by the “long isoform” PCR product and Snf2 protein: cells with high levels of this RNA show lower protein levels.

Nonetheless, there are a number of more subtle observations within the growth conditions that require further elucidation. (1) In the glucose-deprived conditions, at early timepoints, there is an increase in the long/total SNF2 mRNA ratio. However, between 2-4 hours, there is a striking decrease in the long/total ratio, even while the

Figure 2.4


A
saturated culture
(O/N in YPD)
WT
0h timepoint
harvested
YPD
(+ glucose)
YP
(- glucose)
cell samples harvested
for 1-6h timepoints


B
Absorbance at 600nm (OD600)
+ glucose (YPD)
- glucose (YP)
Batch growth timepoint (hours)


C
% SNF2 long/total mRNA (absolute qPCR)
+ glucose (YPD)
- glucose (YP)
Batch growth timepoint (hours)

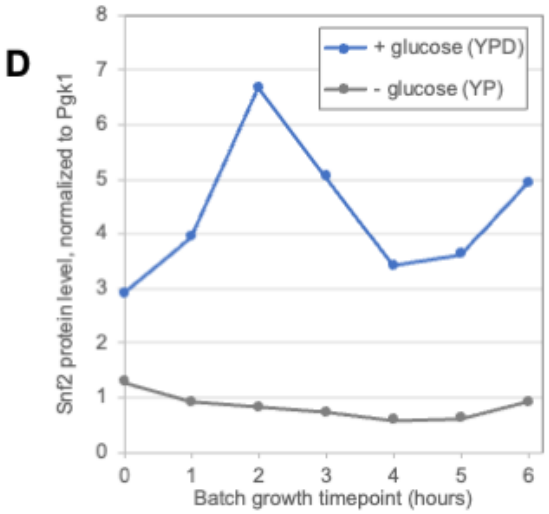

D
Snf2 protein level, normalized to Pgk1
+ glucose (YPD)
- glucose (YP)
Batch growth timepoint (hours)

Figure 2.4. The impact of glucose on SNF2 long isoform transcription and Snf2 protein levels. (A) Schematic of +/- glucose timecourse procedure. (B) Growth curves of WT cells as measured during +/- glucose timecourse. (C) RNA analysis of SNF2 long isoform levels during +/- glucose timecourse. Error bars represent standard deviation across three replicates. (D) Snf2 protein levels measured via densitometry analysis of +/- glucose timecourse immunoblots. Note that although 0 hour timepoint comes from the same protein sample, it was loaded on separate polyacrylamide gels alongside + glucose and – glucose samples.

protein levels remain low. One possibility is that the levels of the long isoform remain high enough, even at earlier time points, that the long/total mRNA threshold is never met to induce protein expression. Additionally, the decrease in the long/total mRNA levels may reflect changes in the total amount of the long isoform relative to the short, which I will further elaborate on below. When cells are shifted from saturated media conditions to glucose-available conditions, the long/total mRNA isoform ratio decreases continually up to 4 hours (Figure 2.4C). I predicted that the protein would show a concomitant increase over this same period. However, between 2-4 hours, there is a drop in the amount of protein (Figure 2.4D), which is not explained by the model of the inverse relationship between the long isoform and the protein. Similarly, in these same conditions, between 4-6 hours, the ratio of the long/mRNA total increases, as does the protein.

These results reinforce that it is essential to have a detailed quantitative analysis of each of the RNA isoforms. While detection of the “long” isoform is an exciting first step, it is critical to quantify both the total amounts and the ratios of the “long” and “short” isoforms. Northern blot analyses are underway to resolve these more subtle regulatory patterns. Additionally, these studies measure steady-state levels of RNA and protein, which may not fully capture the effect of active transcription at the promoters or active translation at the open reading frames. Experiments to measure active translation and transcription are also ongoing.

It is important to recognize that the measurements presented here between 0 to 2 hours are readouts of expression—RNA and protein—comparing cells which are prevented from exiting stationary phase to cells which are allowed to exit stationary

phase. As such, there may be more subtle requirements for Snf2 regulation under these specific conditions that are not fully captured by my model.

The long SNF2 5' leader represses translation of a main ORF in an uORF-dependent manner

The mechanism of long isoform-mediated gene repression has been previously shown to be dependent on upstream open reading frames (uORFs), which inhibit translation of the main protein-coding open reading frame (ORF) (*27-29, 35*). Upon examination of genome-wide RNA sequencing and ribosome footprinting datasets (*35*), I found RNA reads corresponding to a long isoform as well as ribosome occupancy at putative uORFs in the *SNF2* promoter region, hinting at such a mechanism. To test the role of the uORFs within the SNF2 long isoform leader without the confounding issue of impacting Snf2 protein function, including cellular growth, I employed a β-estradiol-inducible reporter system in cells expressing the estrogen receptor/activator domain construct Lex-ER-AD. The W303-based strain expressing the Lex-ER-AD construct as well as the plasmid used for cloning were generously provided by the Elçin Ünal Lab (UC Berkeley) (*27*). I cloned the 585 nt SNF2 long isoform 5' leader sequence upstream of the coding sequence of a ubiquitinylated-GFP (ubi-GFP) fusion protein, then transformed the linearized construct into W303 cells expressing Lex-ER-AD. The GFP serves as an easily-detectable readout for translation propensity of the 5' leader, and the ubiquitin group causes rapid turnover to allow for detection of changes in translation output (Figure 2.5A).

Figure 2.5 (part 1)

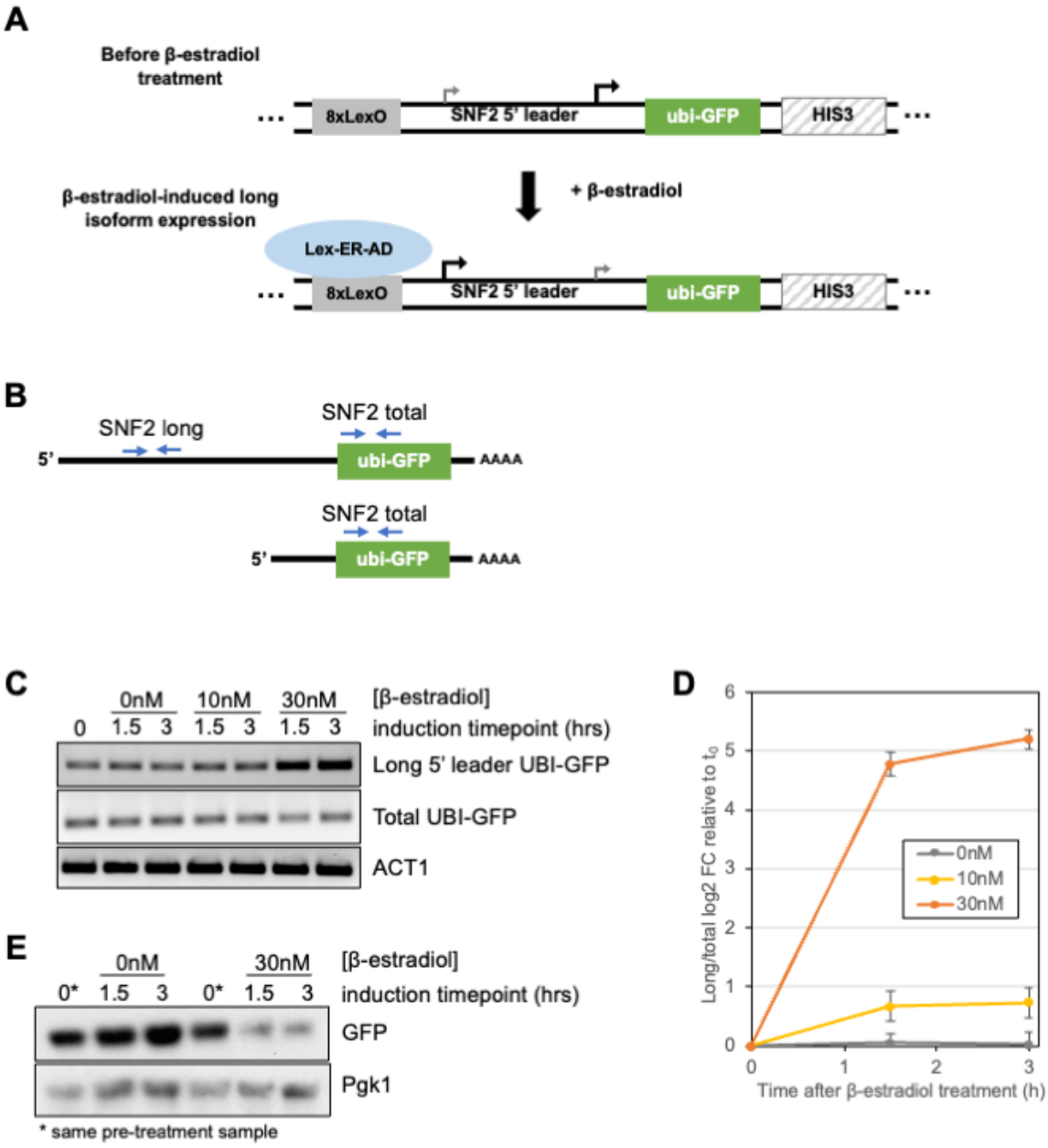


Figure 2.5 (part 2)

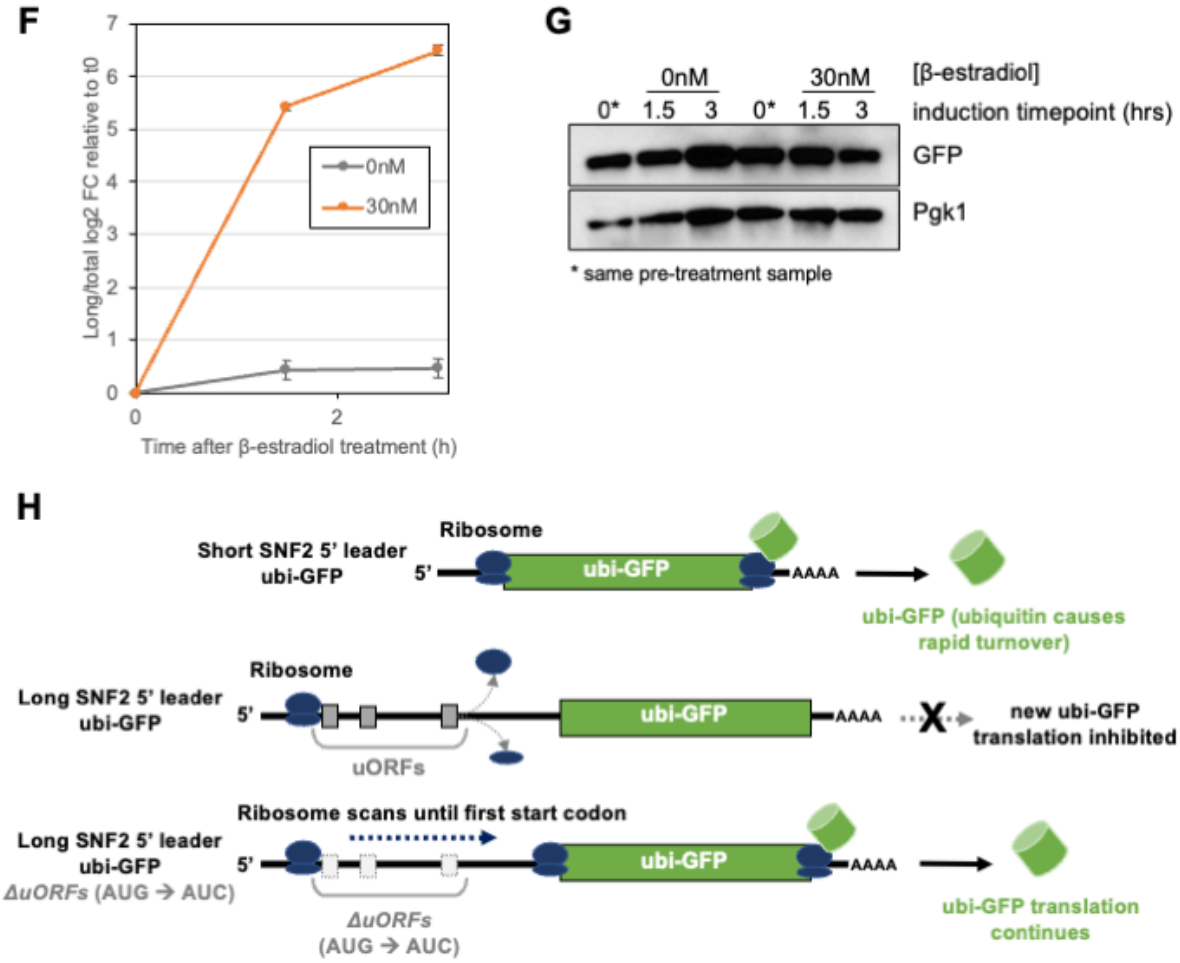


Figure 2.5. Inducible reporter system provides insight into translation of alternate isoforms. (A) Schematic of SNF2 leader-ubi-GFP inducible reporter system, where addition of β-estradiol causes an increase in long isoform transcription. (B) Schematic of mRNA isoforms produced by reporter system and RT-PCR primer positioning for RNA analysis of reporter experiments. (C) Results of RNA analysis described in (B). (D) WT 5' leader RT-qPCR results. (E) WT 5' leader Western blot results. (F) *ΔuORFs* 5' leader RT-qPCR results. (G) *ΔuORFs* 5' leader Western blot results. (H) Model of uORF-dependent long SNF2 5' leader regulation of main ORF translation.

Exponentially growing cells were back-diluted to OD_{600} = 0.2, then treated with 0nM, 10nM or 30nM β-estradiol, and timepoints were taken pre-treatment, 1.5 hours post-treatment, and 3 hours post-treatment. RNA analysis was performed through RT-PCR and RT-qPCR using primer pairs that detect either the SNF2 long isoform ubi-GFP mRNA isoform or the total SNF2 ubi-GFP mRNA.

Induction with 30 nM β-estradiol resulted in a dramatic increase in RNA expressed from the long isoform promoter, while total ubi-GFP mRNA levels remained relatively stable, mirroring the regulation observed at the endogenous *SNF2* locus (Figure 2.5B). The induction of the long isoform was quantitatively confirmed through RT-qPCR (Figure 2.5C).

Parallel protein analysis was performed via Western blotting (Figure 2.5D). In untreated cells, ubi-GFP levels increased during the 3 hour span of the experiment, which is similar to the pattern of Snf2 protein expression expected in the first 3 hours of batch growth. The cells treated with 30nM β-estradiol, on the other hand, displayed a striking decrease in ubi-GFP level at the 1.5 hour and 3 hour timepoints. This provides strong evidence that the sequence contained within the 5' extended region of the SNF2 long isoform is sufficient for inhibiting translation of the downstream ORF.

To determine whether the regulation conferred by the long isoform is dependent on uORF translation, a mutant construct was designed in which the start codon of each of the three uORFs in the SNF2 long isoform 5' leader was changed from AUG to AUC, which was predicted to prevent ribosome-uORF interactions. Upon β-estradiol induction of the *ΔuORFs* long isoform (Figure 2.5E), ubi-GFP expression continued (Figure 2.5F). This suggests that, in the *ΔuORFs* mutant, the ribosome is no longer able to initiate

translation of the uORFs, and continues scanning until it reaches the main ORF, thereby allowing translation of ubi-GFP post-β-estradiol induction (Figure 2.5G).

A nutrient-responsive transcriptional regulator is involved in regulation of alternative SNF2 mRNA isoforms

Ume6 binds to a consensus motif commonly known as the upstream repressive sequence 1 (URS1). Previous studies showed that URS1-bound Ume6 regulates long isoform expression in response to nutrients. At the *SNF2* locus, there is a highly conserved region 120 bp upstream from TSS1 and 300 bp downstream from TSS2, which contains the URS1 core consensus motif (Figure 2.6A). However, this sequence is found on the opposite strand from the *SNF2* gene, so it is unclear in which direction its recruitment of Ume6 may have an impact.

Using the same RT-PCR approach shown in Figure 2.3A, I analyzed SNF2 long isoform levels in WT and *ume6Δ* cells during pre-diauxic-shift batch growth (Figure 2.6B). Importantly, the RT-PCR assay I deployed is agnostic to directionality due to the use of random hexamers, so I simply confirmed that *UME6* deletion activates expression of an RNA containing the sequence amplified by the primer pair used in the assay. This product was detected in all but one time point (3 hours) in WT cells.

There are a number of intriguing implications of this data. These results suggest that there may be a “burst” in short isoform SNF2 mRNA transcription that drives the associated increase in Snf2 protein levels (Figure 2.6C). Notably, the peak in Snf2 expression is reminiscent of the protein peak observed in Figure 2.4D.

Figure 2.6

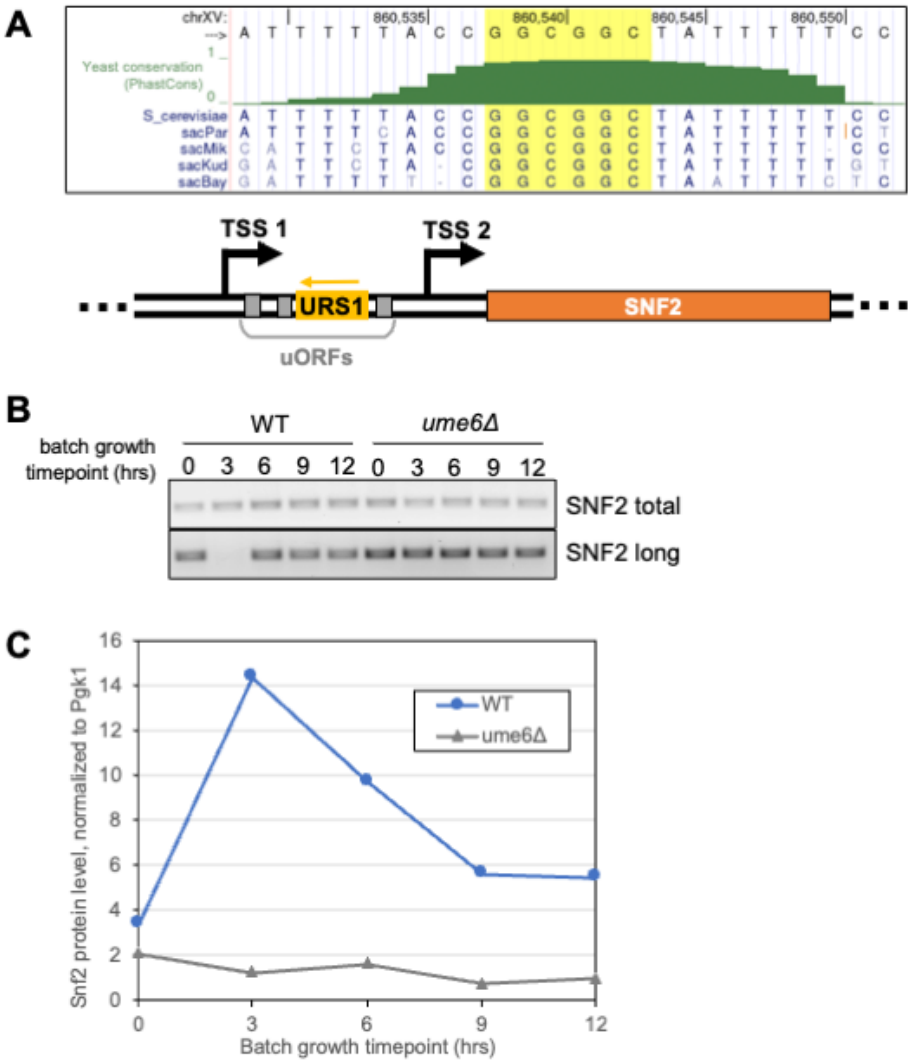


Figure 2.6. Deletion of *UME6* causes derepression of long SNF2 isoform and corresponding decrease in Snf2 protein expression. (A) A consensus URS1 site is located within the SNF2 promoter region, between the two SNF2 TSSs observed in this study. Data and PhastCons yeast conservation alignment obtained from UCSC Genome Browser. (B) RT-PCR results of WT vs. *ume6Δ* timecourse. (C) Quantified Western blot results of WT vs. *ume6Δ* timecourse.

Furthermore, in *ume6Δ* cells, SNF2 long isoform was present at higher levels across the timecourse, suggesting that Ume6 represses long isoform expression when nutrients are present. This increase in long isoform was associated with lower overall Snf2 levels and a lack of the peak of Snf2 expression observed at the 6 hour timepoint in WT cells (Figures 2.6C).

While it is clear that *UME6* deletion leads to an increase in transcription of the long SNF2 isoform and a decrease in Snf2 protein expression, the mechanism by which Ume6 represses TSS1 in WT cells is unclear. Since Ume6 recruits Rpd3L, a histone deacetylase complex, under non-meiotic conditions, it is assumed to be a repressor (*31, 32, 37*). Deletion of *UME6* may simply open the chromatin downstream of the URS1 site, increasing the expression of the long transcript with a concomitant decrease in the Snf2 protein. Remarkably, Ume6 can also recruit activators, especially in the context of meiosis (*31*), with some evidence of positive regulation by Ume6 happening in the phospholipid biosynthetic pathway as well (*38*). So, if Ume6 acts as an activator, then the presence of Ume6 could lead to expression of a transcript in the *antisense* direction that disrupts expression of the long isoform; loss of Ume6 would reverse this inhibition. Hence, the effect of *UME6* deletion would be the same: expression of the long transcript from TSS1 and a concomitant decrease in the Snf2 protein.

Either of these mechanisms would be a novel mode of regulation. Nonetheless, distinguishing between these mechanisms is critical for establishing how *SNF2* and other highly-regulated genes are controlled. Moreover, clarifying whether an antisense transcript is produced, and the timing of its expression, could help to explain some of the more confounding aspects of the RNA results I have observed. Nonetheless, these

results provide new insights into the nutrient-dependent regulation of a critical chromatin remodeler, Snf2, by the transcriptionally regulated expression of a 5' leader containing sequences that confer translational control.

DISCUSSION

Alternative transcript isoforms play an important role in *SNF2* regulation in response to nutrients. I have characterized the expression of a 5' extended, long SNF2 mRNA isoform containing three uORFs, which are capable of silencing translation of the downstream Snf2-coding ORF. The transcriptional regulator Ume6 influences the relative amounts of the long and short isoform transcription in accordance with nutrient conditions and stages of growth, allowing Snf2 protein levels to change and thereby promoting growth and survival (Figure 2.7).

Using cDNA-based assays, I have demonstrated that the levels of the long SNF2 isoform change in relation to total SNF2 mRNA levels, however, there are limitations to this method. Due to the nature of the overlap between the long and short isoforms, it is not possible to detect changes in short isoform levels via RT-PCR since there is no unique short isoform sequence to amplify. This presents complications when drawing conclusions about changes in transcription at the short isoform TSS. To bypass this issue entirely and quantitatively measure absolute amounts of each transcript, I will use Northern blotting, where RNA is directly loaded into an agarose gel and separated by size through electrophoresis, then detected via hybridization with ^{32}P-labeled probes. This important evidence will clarify how each SNF2 isoform changes throughout phases of growth and under various conditions.

Figure 2.7

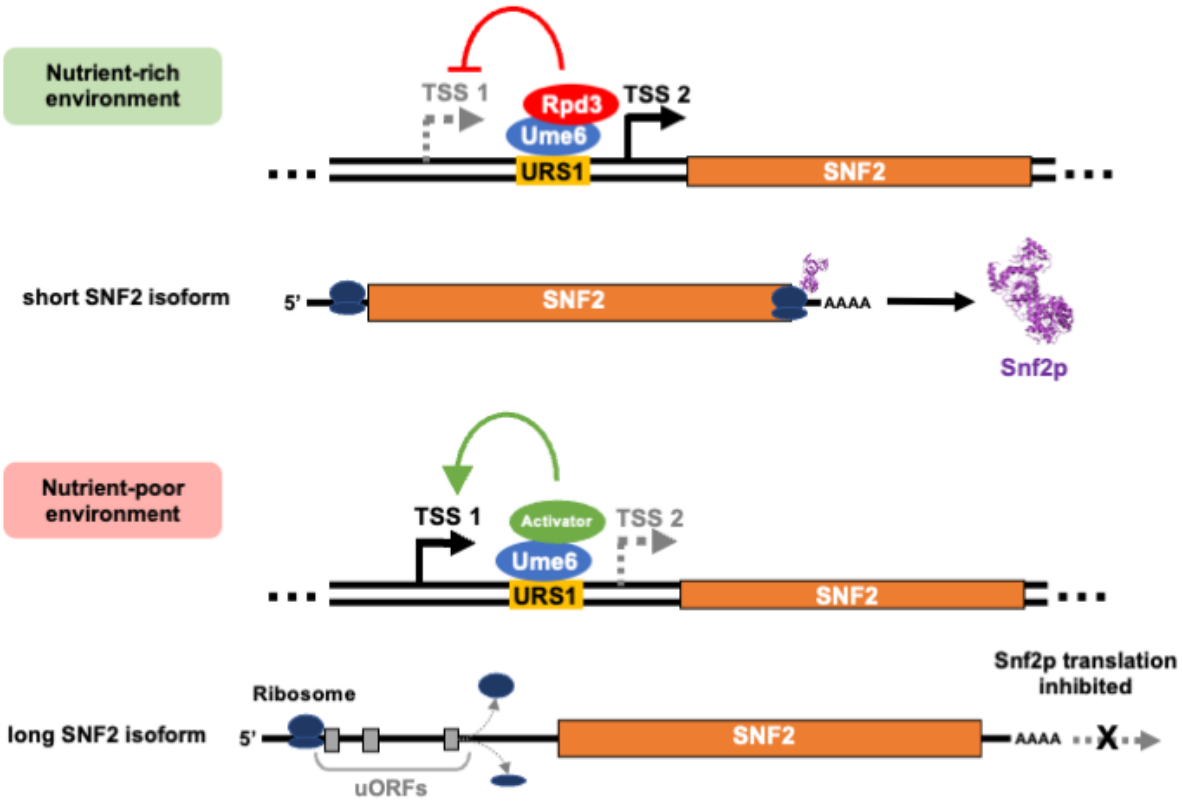


Figure 2.7. Model for regulation of SNF2 by alternate transcript isoforms.

Although the data provided here shows a relationship between Ume6 and *SNF2* expression, the mechanism behind this interaction remains unclear. The consensus Ume6 binding motif, URS1, at the *SNF2* locus is found on the plus strand of chromosome 15, while the *SNF2* gene is found on the minus strand, raising questions about the impact of Ume6 binding on transcription dynamics and directionality of its effects. The URS1 site is 120 bp upstream from TSS1 and 300 bp downstream from TSS2, situated between the second and third uORF of the long isoform 5' extended sequence. Could Ume6 binding at URS1 be impacting *SNF2* expression through canonical Rpd3L-mediated repression of transcription of the long isoform? Could a non-coding antisense RNA be interfering in long isoform transcription in a Ume6-dependent manner? These questions open the door to an exciting set of follow up experiments, including further RNA analysis via Northern blotting with parallel ChIP experiments to measure Ume6 binding.

The inducible reporter system, which allowed me to demonstrate the impact of uORFs in the SNF2 long 5' leader on downstream translation, is a powerful tool. Here I have used it to show that mutation of the start codons of the uORFs in the long SNF2 5' leader is sufficient to derepress expression of the protein from the downstream promoter, indicating that translation initiation occurring at those uORFs silences downstream translation. With this tool in hand, it is possible to answer a number of important questions. What is the relative contribution of each of the uORFs? Is the presence of an uORF sufficient to render a transcript nutrient sensitive? Importantly, the URS1 binding site is part of the leader sequence that was placed upstream of the GFP reporter. It would be interesting to see whether induction of the long isoform is

abrogated when the gene encoding Ume6, which binds to URS1 and appears to repress TSS1 as described above, is overexpressed. This could provide further evidence to elucidate the mechanism of Ume6 regulation at the SNF2 locus.

In summary, the dynamic transcription of alternate isoforms at the *SNF2* locus provides rich opportunities for multi-faceted regulation of this key chromatin remodeler. Given the conservation of Snf2-family proteins among eukaryotes, from yeast to humans, and the complex involvement of Snf2 homologs in diseases such as cancer, a deeper understanding of the mechanistic pathways governing Snf2 expression in yeast could carry important implications for human health and disease interventions.

MATERIALS AND METHODS

Table 2.1. Strains used in this study.

Strain background	Strain code	Genotype	Source
S. cerevisiae SK1 K8409	TJY6917	*MATa/MAT_HO URA3::tetO224 LEU2::tetR-GFP REC8-HA3::URA3 lys2 his3 trp1*	Manuel Ares/Angelika Amon? or Venkataramanan, *et al., NAR* 2017
S. cerevisiae BY	TJY6724	*MATa his3Δ leu2Δ LYS2 met15Δ ura3Δ*	Venkataramanan, *et al., NAR* 2017
S. cerevisiae W303	LT22	*MATa trp1::pGPD1-LexA-ER-HA-B112::TRP1*	This study (derived from UB8374, Tresenrider, *et al., Mol. Cell* 2021)

		his3::8xLexO-SNF2leader-ubiGFP::HIS3	
S. cerevisiae W303	LT27	*MATa trp1::pGPD1-LexA-ER-HA-B112::TRP1 his3::8xLexO-SNF2leaderΔuORFs-ubiGFP::HIS3*	This study (derived from UB8374, Tresenrider, *et al., Mol. Cell* 2021)
S. cerevisiae BY	LT21	*his3Δ leu2Δ ura3Δ ume6Δ::KanMX*	This study (derived from Horizon Discovery UME6+/- YSC6274-201926134)
S. cerevisiae BY		*his3Δ leu2Δ ura3Δ cbf1Δ::KanMX*	This study (derived from Horizon Discovery CBF1+/- YSC6274-201929139)

Table 2.2. Primers used in this study.

Primer name	Sequence (5'->3')
SNF2-5RACE-GSP2	GCC CCG TTT CGT CAA TCA ATT TG
ACT1-F	GTA CCA CCA TGT TCC CAG GTA TT
ACT1-R	AGA TGG ACC ACT TTC GTC GT
SNF2-210920-1-qF (SNF2 long)	TTG GAA TTT TGC AGG TAG CC
SNF2-210920-1-qR (SNF2 long)	CCC GTT TCG TCA ATC AAT TTG T
SNF2_ORF1-qF (SNF2 total)	CCA CAG CGT CAA TTT AGC AAC
SNF2_ORF1-qR (SNF2 total)	CTG TTG GCG TTG CAT TTG TAA TTC
SNF2-short-F	gta tat aaa tca tcg gga agg tca gc
UBI-R	GGT CAA AGT CTT GAC GAA AAT CTG

Strain construction

The SNF2leader-ubi-GFP inducible reporter system was constructed via DNA assembly (NEBuilder HiFi DNA Assembly, New England BioLabs) of the 585 bp SNF2 leader sequence (gBlocks Gene Fragments of SNF2 leader, WT and ΔuORF versions, Integrated DNA Technologies) into a *HIS3* integration plasmid containing 8xLexO-pCYC1 and ubi-GFP which was engineered to accept the SNF2 leader gene fragments (derived from Ünal Lab plasmid pUB1562). The resulting plasmids were digested via PmeI (New England BioLabs) for integration at the *HIS3* locus in *S. cerevisiae* W303 cells (UB8374) containing a β-estradiol-responsive transcriptional activator (LexA-ER-

HA-B112). Positive clones were confirmed by colony PCR and Sanger sequencing of the insertion.

The Ume6 knockout strain, *ume6Δ,* was obtained through sporulation and dissection of heterozygous diploid yeast knockout strains from Horizon Discovery (see Table 1).

Yeast cultures

For yeast sporulation, diploid *S. cerevisiae* SK1 (TJY6917) cells were grown to saturation in standard yeast growth media (YPD) at 30°C, then used to inoculate cultures in ultra-rich media (GNA) at OD_{600} = 0.3. GNA cultures were grown at 30°C for 4 hours, then washed and resuspended in an equal volume of sporulation media (SPM). Sporulation cultures were incubated at 25°C and samples were collected periodically by cell number.

For batch growth experiments, *S. cerevisiae* budding yeast (BY, TJY6724) were grown to saturation in standard yeast growth media (YPD) at 30°C, then used to inoculate fresh YPD cultures at OD_{600} = 0.2. Batch growth cultures were grown at 30°C and samples were collected periodically by cell number.

For β-estradiol induction experiments, exponentially growing *S. cerevisiae* W303 cells containing SNF2leader-ubi-GFP inducible reporter system (LT22 and LT27) were back-diluted to OD_{600} = 0.2, then treated with either 10nM or 30nM β-estradiol in DMSO (Sigma Aldrich) alongside a 0nM/DMSO control lacking β-estradiol. Samples were collected prior to induction and at 1.5 hours and 3 hours post-induction.

RNA isolation

For analysis of mRNA isoforms, total RNA was first isolated via phenol:chloroform isoamyl alcohol extraction and EtOH precipitation, then treated with DNase I to remove any contaminating DNA particles before a final purification via acid phenol:chloroform extraction and EtOH precipitation. RNA purity and concentrations were measured using NanoDrop spectrophotometer.

5'RACE

Samples for 5'RACE were prepared using equal amounts of total RNA using the 5' RACE System for Rapid Amplification of cDNA Ends kit (Invitrogen), then amplified via PCR using the universal primer provided in the kit and a SNF2-specific primer. Amplicons were analyzed by gel electrophoresis and Sanger sequencing to determine the precise 5' end of the long SNF2 isoform.

RT-PCR and RT-qPCR

For transcript detection and quantification, cDNA was reverse transcribed from equal amounts of RNA using the Maxima First Strand cDNA Synthesis kit (Thermo Scientific). RT-PCR and RT-qPCR were performed using gene-specific primers. Purity of RNA samples was confirmed by RT-null controls. For preliminary, non-quantitative detection of transcripts, RT-PCR amplicons were analyzed by agarose gel electrophoresis. All RT-qPCR experiments were conducted using iTaq Universal SYBR Green Supermix (Bio-Rad) in a Bio-Rad CFX96 thermocycler. Serially diluted gDNA

and/or plasmid standards were used to create standard curves for absolute quantification of transcript levels.

Immunoblotting/Western blotting

All Western blots were prepared using cell number-equivalent samples. For Snf2 blots in Figures 2.1, 2.3B, and 2.6D, 6% SDS polyacrylamide gels were used. For all other blots, 10% SDS polyacrylamide gels were used. Following transfer to PVDF membranes and incubation in blocking buffer (2% non-fat milk in TBS for Snf2 blots and 5% non-fat milk in TBS for all other blots), proteins of interest were tracked by incubation with specific antibodies.

Snf2 protein was detected using goat α-Snf2 primary antibody (Santa Cruz Biotechnology YN-20, 1:200 dilution) and donkey α-goat HRP secondary antibody (Santa Cruz Biotechnology SC-2020, 1:2,857 dilution). Pgk1 was detected using α-Pgk1 monoclonal primary antibody (Invitrogen 22C5D8, variable from 1:2,000 to 1:10,000 dilution) and α-mouse HRP secondary antibody (either Santa Cruz Biotechnology SC-2005, 1:2,857 dilution, or Abcam ab131368, 1:10,000 dilution). Ubi-GFP was detected using mouse α-GFP monoclonal primary antibody (Takara Bio 632381, 1:5,000 dilution) and rat α-mouse HRP secondary antibody (Abcam ab131368, 1:10,000 dilution).

Chemiluminescent substrate (Prometheus ProSignal Dura ECL) was applied to blots before X-ray film exposure. Developed film images were scanned into digital files. Quantification, where applicable, was done via densitometric analysis in ImageJ software.

CHAPTER 3

Deciphering transcriptional regulation of *SNF2*

INTRODUCTION

Although there are many nuances to *SNF2* regulation by alternate isoforms, it can be simplified into four modes of transcriptional control: (1) positive regulation of expression of the short SNF2 mRNA, (2) positive regulation of expression of the long SNF2 mRNA, (3) negative regulation of expression of the long SNF2 mRNA, and (4) negative regulation of the expression of the short SNF2 mRNA. In Chapter 2, I demonstrated that these short and long isoforms exist, and that at least one previously characterized regulator, Ume6, imposes negative regulation of the long isoform. This leaves a number of outstanding questions to be addressed, namely, what factors are contributing to the other modes of *SNF2* regulation?

RESULTS

Point mutations in the *SNF2* promoter region impair Snf2 protein expression, growth, and sporulation.

While my studies showed a clear role for sequences downstream of the *SNF2* TSS1 in repressing this start site, I wanted to also understand the sequences upstream that could affect its use. So, I deployed a site directed mutagenesis strategy, starting with SK1 cells, where we first detected the long isoform. A CRISPR/Cas9 system designed for use in *S. cerevisiae* (a generous gift from the Manny Ares laboratory) was used to generate two point mutations in a region upstream of *SNF2* TSS1, as shown in Figure 3.1A, hereafter referred to as *SNF2 ΔREG.* In collaboration with summer undergraduate researcher Elizabeth Pérez, haploid mutants were independently generated in MATa and MATα cells, which both displayed impaired growth (Figure

3.1B). The two mutant strains were then crossed to yield a homozygous diploid *SNF2 ΔREG/ΔREG* mutant, which was used for further analysis. Western blotting revealed that, during exponential growth, when Snf2 protein levels were high in WT cells, Snf2 could not be detected in the *SNF2 ΔREG/ΔREG* mutant (Figure 3.1C). Since cells lacking Snf2 cannot undergo meiosis, we next attempted to sporulate *SNF2 ΔREG/ΔREG* cells alongside WT cells to determine if the point mutants abrogate meiosis; no tetrads were seen in the *SNF2 ΔREG/ΔREG* mutants (Figure 3.1D), indicating that they were unable to undergo meiosis. Previous studies have shown that Snf2 is necessary for the onset of meiosis (*20*), so it was not surprising that the mutants were unable to sporulate.

How do the point mutations abrogate Snf2 protein expression? One model is that the mutations prevent binding of a transcriptional repressor such that the inhibitory long RNA isoform is expressed. A second possibility is that the mutations might somehow increase binding of a *positive regulator*, thereby increasing expression of the long RNA. To address these possibilities, I examined the sequences in the vicinity of the mutations to look for evidence of binding of a transcriptional repressor or activator. Upon closer examination of the CRISPR-mutated region, I found a putative E-box motif, which presented a new candidate regulatory factor, Cbf1. Our previous experiments suggested that meiosis can uncover fundamental mechanisms about how cells respond to changes in nutrient availability. After all, sporulation is induced by nutrient deprivation as a mechanism for long-term survival. Hence, I set out to assess the role of Cbf1 in *SNF2* expression in cells growing in batch culture.

Figure 3.1

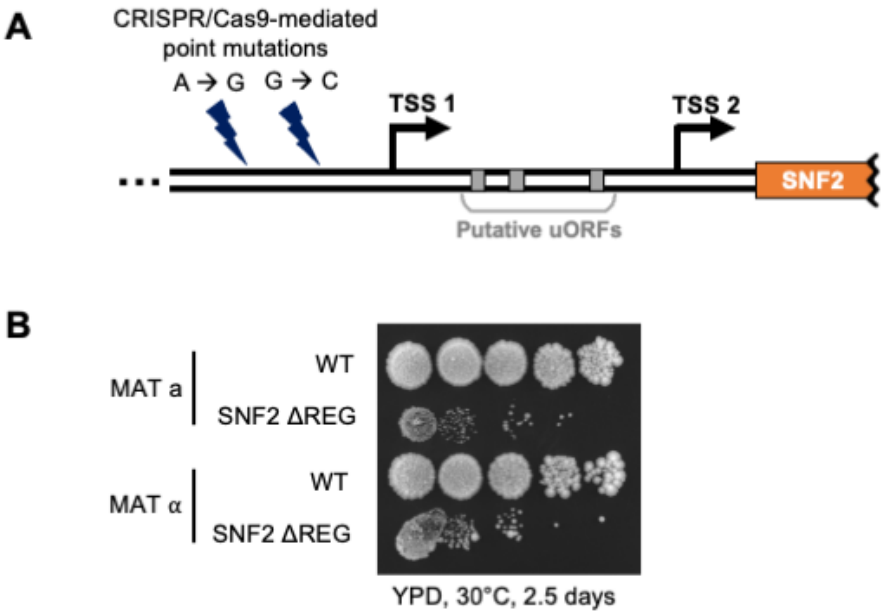

A
CRISPR/Cas9-mediated
point mutations
A → G G → C
TSS 1
TSS 2
SNF2
Putative uORFs
B
MAT a
WT
SNF2 ΔREG
MAT α
WT
SNF2 ΔREG
YPD, 30°C, 2.5 days
C
timepoint (hrs in YPD)
WT
ΔREG/ΔREG
2 4 6 8 2 4 6 8
Snf2
Pgk1
D
WT
ΔREG/ΔREG

Figure 3.1. CRISPR/Cas9-meditated point mutations in the *SNF2* promoter region impair cell growth and sporulation, likely due to decreased Snf2 protein expression. (A) Schematic depicting approximate locations of point mutations relative to the *SNF2* promoter region. (B) Yeast serial dilution growth assay comparing each mutant to the wild-type haploid SK1 strain it originated from. Results pictured after 2.5 days of growth at 30°C. (C) Snf2 protein levels in diploid SK1 cells during batch growth, showing reduction of Snf2 protein levels in the mutant cross, analyzed via Western blot using α-Snf2 antibody (Santa Cruz Biotechnology YN-20) and Pgk1 antibody (Invitrogen 22C5D8) as loading control**.** (D) Phase contrast microscopy of sporulation culture samples after 5 days in sporulation media at 25°C. White arrows indicate complete spore tetrads.

Cbf1 as a regulator of *SNF2* expression

Cbf1 (centromere binding factor 1) is a bHLH transcription factor which binds to E-box motifs in a nutrient- and cell-cycle-dependent manner (*39-42*). Its name is derived from its dual role in binding to centromeres and aiding in chromosome stabilization during cell division (*39*). Upon binding, Cbf1 recruits the ISWI (Imitation Swi/Snf) complex to remodel chromatin and thereby activate transcription (*43*). Intriguingly, there is evidence supporting interactions between *CBF1* and other Swi/Snf complex components (*44*). The consensus E-box motif is CACGTG (*45*), although there appears to be some flexibility and variation in Cbf1-bound sequences. The region flanking the E-box motif is also important for binding specificity by affecting DNA structure (*42, 46*).

At the *SNF2* locus, there is a putative E-box motif (CACAAG, which is different from the consensus sequence, but still represented in the E-box motif sequence logo (*42*)) upstream of the long isoform TSS, in close proximity to the CRISPR point mutations discussed above, which may allow Cbf1 to activate transcription of the long isoform (Figure 3.2A). This would promote downregulation of Snf2 protein expression as nutrients are depleted and cells exit the cell cycle. To test this hypothesis, I measured Snf2 protein levels in WT and *cbf1Δ* cells during a batch growth timecourse. In WT cells, Snf2 protein levels decreased post-diauxic-shift, but in *cbf1Δ* cells, Snf2 protein expression decreased only slightly and was sustained through 24 hours of growth, suggesting that Cbf1 plays a role in the timely downregulation of Snf2 protein levels as cells enter quiescence. Hence, Cbf1 is indeed a candidate for regulation of the Snf2 protein. My model suggests that this is through positive regulation of long isoform expression post-diauxic shift. While more evidence is needed to test this hypothesis,

Figure 3.2

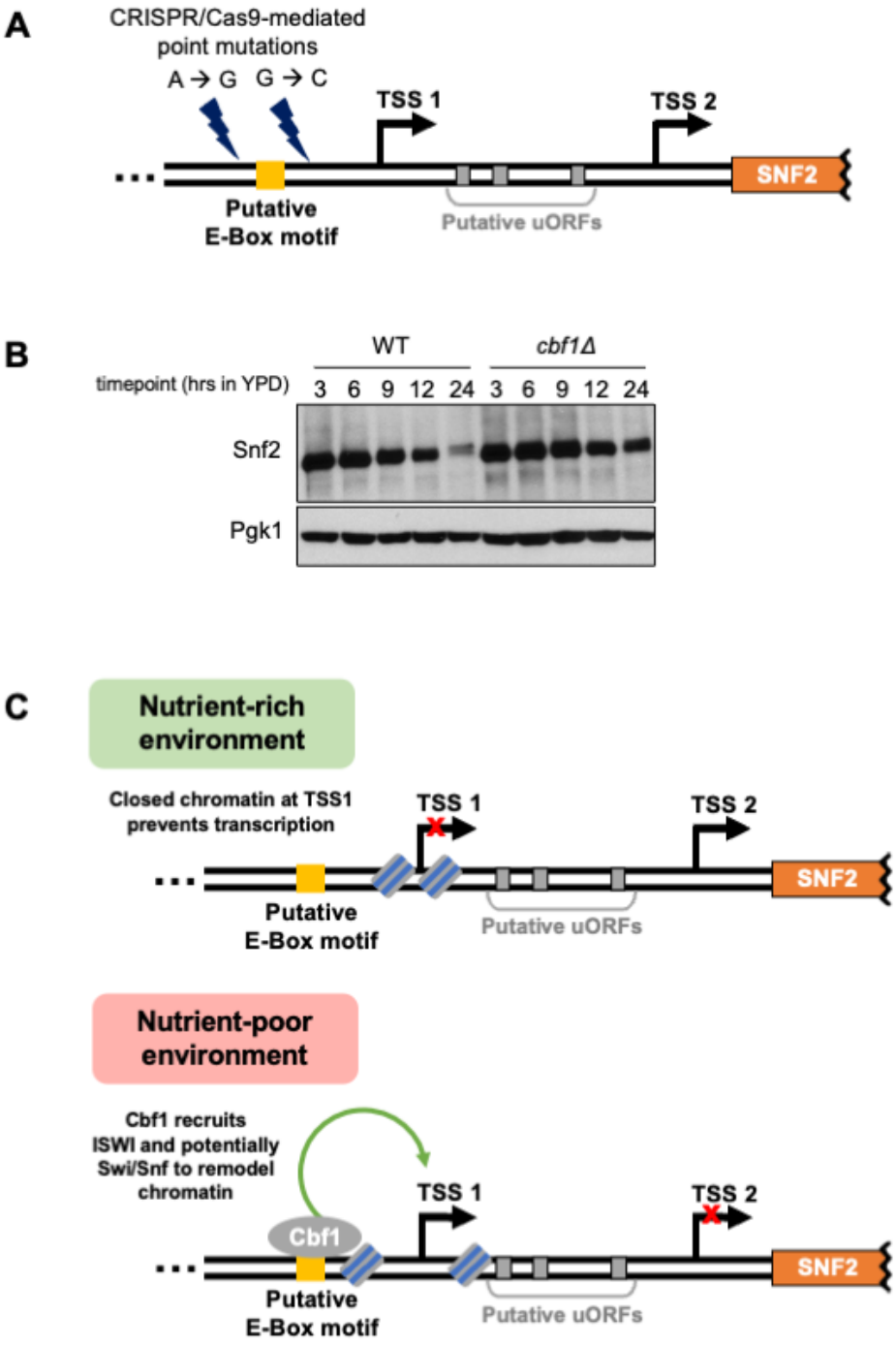

A
CRISPR/Cas9-mediated
point mutations
A → G G → C
TSS 1
TSS 2
SNF2
Putative
E-Box motif
Putative uORFs
B
WT
cbf1Δ
timepoint (hrs in YPD)
3 6 9 12 24 3 6 9 12 24
Snf2
Pgk1
C
Nutrient-rich
environment
Closed chromatin at TSS1
prevents transcription
TSS 1
TSS 2
SNF2
Putative
E-Box motif
Putative uORFs
Nutrient-poor
environment
Cbf1 recruits
ISWI and potentially
Swi/Snf to remodel
chromatin
Cbf1
TSS 1
TSS 2
SNF2
Putative
E-Box motif
Putative uORFs

Figure 3.2. Evidence supporting Cbf1 involvement in *SNF2* regulation. (A) Schematic of putative E-Box motif approximate location in relation to the *SNF2* promoter region and the CRISPR/Cas9-mediated point mutations described earlier in this chapter. (B) Snf2 protein levels in *cbf1Δ* and wild-type (WT) BY cells across a 24-hour batch growth timecourse, analyzed via Western blot using α-Snf2 antibody (Santa Cruz Biotechnology YN-20) and Pgk1 antibody (Invitrogen 22C5D8) as loading control. (C) Model of hypothesized role of Cbf1 in the activation of SNF2 long isoform transcription under nutrient deprivation.

the preliminary data suggests that Cbf1 does indeed affect expression of this transcript.

Putting the CRISPR mutant data together with the Cbf1 findings, I propose that the mutations increase Cbf1 binding to the E-box (Figure 3.2C). To test this, it is crucial to analyze the RNA and protein in strains harboring a combination of Δ*REG* and *CBF1* mutations, as described in the Discussion. Important experiments to follow-up on these findings include RNA analysis of SNF2 isoforms in *cbf1Δ* cells and ChIP-PCR to assess changes in Cbf1 occupancy associated with Snf2 downregulation.

A role for Gcn5 in *SNF2* regulation

Histone lysine acetylation is a common epigenetic modification associated with active gene expression. The Swi/Snf complex is recruited to acetylated chromatin through the bromodomain on Snf2 (*47*), which directs its chromatin remodeling activity and is crucial to activation of transcriptional programs. This often happens in a hand-off between the histone acetyltransferase complex SAGA and the Swi/Snf complex (*48*).

The histone acetyltransferase of SAGA, Gcn5, has several non-histone targets as well, including lysine residues on Snf2 (*25*). Gcn5 acetylates Snf2 under stress conditions, which causes intramolecular interactions within Snf2 and reduces the affinity of its bromodomain to acetylated histones, allowing its chromatin remodeling activity to be diverted towards genes with condition-dependent transcriptional activators present (*22, 25*). Acetylation of Snf2 by Gcn5 also increases its turnover (*25*). Since both Gcn5 and Snf2 are involved in dynamic, genome-wide changes in the transcriptional landscape in response to nutrients and stress, and are known to directly interact, it

seems likely that they would be involved in the changes in transcription I observe at the *SNF2* locus, presenting the possibility of Snf2 auto-regulation.

To investigate this, I performed preliminary analysis of Snf2 protein levels in *gcn5Δ* and *H3Δ9-16* cells (Figure 3.3A). While WT cells displayed the expected down-regulation of Snf2 protein levels post-diauxic shift, *gcn5Δ* and *H3Δ9-16* cells had strikingly different results. *H3Δ9-16* cells lack the portion of the yeast histone H3 tail where lysine residues are typically acetylated, and in those cells, Snf2 protein expression was absent throughout the timecourse, indicating that lysine acetylation of that portion of the H3 histone tail is necessary for expression of at least the short *SNF2* mRNA isoform, and therefore Snf2 protein expression. In *gcn5Δ* cells, Snf2 expression continued throughout the timecourse, similar to the results seen in *cbf1Δ* cells, suggesting that the switch to the long isoform and subsequent Snf2 translational repression does not occur in the absence of Gcn5.

If the role of Gcn5 in *SNF2* regulation was mediated exclusively through changes in histone acetylation, I would expect to see similarities between *gcn5Δ* and *H3Δ9-16* cells, but that is not the case. A key takeaway from this experiment is that Gcn5 appears to be necessary for the timely downregulation of Snf2 protein levels, likely through facilitating the transcriptional switch to the long SNF2 mRNA isoform.

How might Gcn5 facilitate changes in *SNF2* transcription? There are two, non-mutually-exclusive means through which Gcn5 could be regulating the switch between *SNF2* mRNA isoforms. First, Gcn5 could be acetylating histones in the promoter region of the long isoform, thereby handing nucleosomes off to Swi/Snf for remodeling and

Figure 3.3

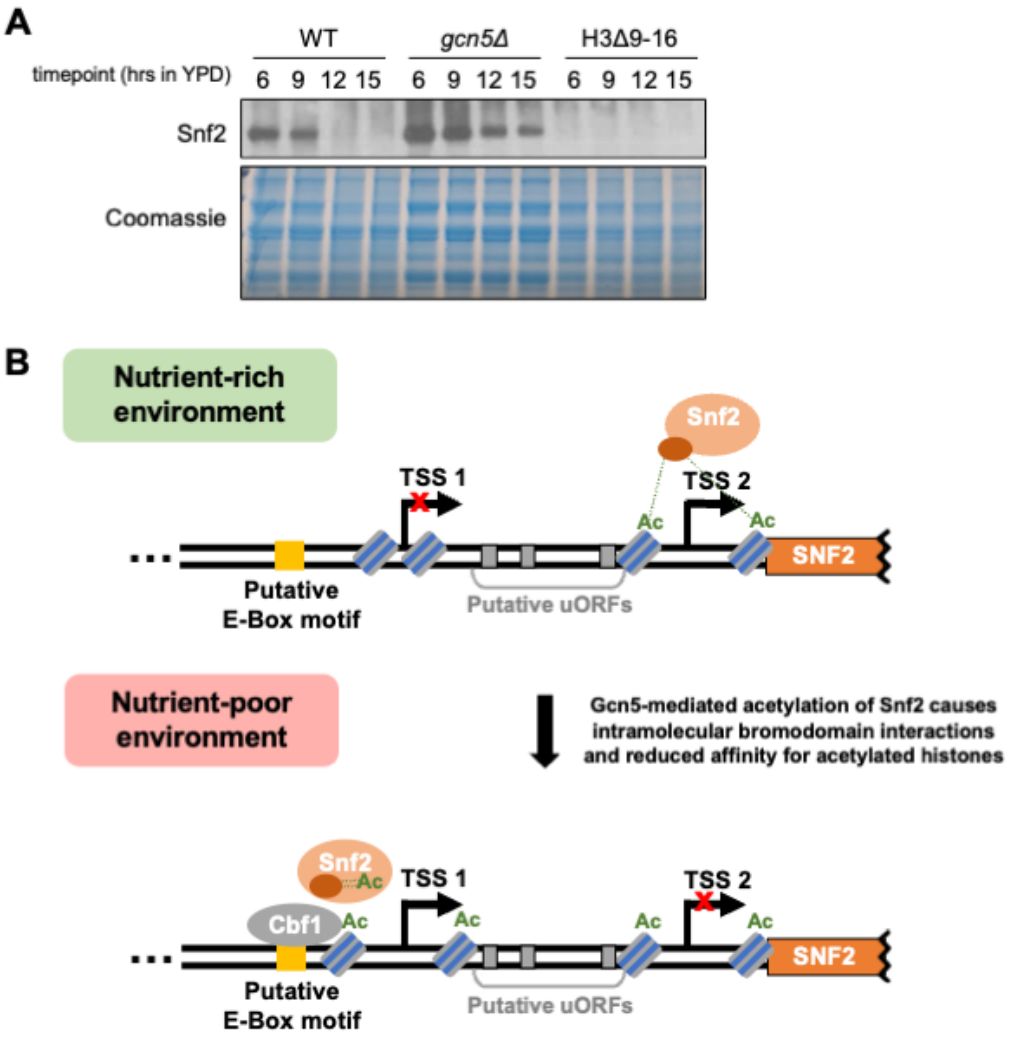

A
WT
gcn5Δ
H3Δ9-16
timepoint (hrs in YPD)
6 9 12 15 6 9 12 15 6 9 12 15
Snf2
Coomassie
B
Nutrient-rich environment
Snf2
TSS 1
TSS 2
Ac
Ac
SNF2
Putative E-Box motif
Putative uORFs
Nutrient-poor environment
Gcn5-mediated acetylation of Snf2 causes intramolecular bromodomain interactions and reduced affinity for acetylated histones
Snf2
Ac
TSS 1
TSS 2
Cbf1
Ac
Ac
Ac
Ac
SNF2
Putative E-Box motif
Putative uORFs

Figure 3.3. Evidence supporting Gcn5 involvement in *SNF2* regulation. (A) Snf2 protein levels in *gcn5Δ,* H3Δ9-16 and wild-type (WT) BY cells across a 15-hour batch growth timecourse, analyzed via Western blot using α-Snf2 antibody (Santa Cruz Biotechnology YN-20) with Coomassie staining as loading control. (B) Model for hypothesized role of Gcn5 in the activation of SNF2 long isoform transcription under nutrient deprivation.

transcriptional activation. Second, and perhaps more likely under post-diauxic shift conditions, is Gcn5-mediated acetylation of Snf2 protein, allowing Swi/Snf complexes that were previously activating transcription at the short isoform promoter to be released from the chromatin and be recruited to the long isoform promoter by whichever transcriptional activators are present, which may include Cbf1, as discussed above and depicted in the model shown in Figure 3.3B.

In the absence of Gcn5, lack of Snf2 acetylation (and therefore lack of intramolecular bromodomain interactions) may lead to Swi/Snf complexes persisting at the short isoform promoter. This would cause a delay in switching to the long isoform, and extended translation of Snf2 past the diauxic shift. Further evidence from RNA analysis and ChIP-PCR is necessary to confirm the mechanism through which these phenomena are occurring. Specifically, ChIP-PCR of tagged Snf2 in WT, *gcn5Δ*, and *cbf1Δ* cells would allow us to track the movement of Swi/Snf complexes between the *SNF2* promoters in different stages of growth. If Gcn5 and Cbf1 are indeed involved in the transition between SNF2 transcription start sites, I would expect to see the shift to the long isoform promoter disrupted in both *gcn5Δ* and *cbf1Δ* strains, supporting the preliminary evidence presented in this chapter. Additionally, it would be interesting to perform a similar experiment with a Snf2 mutant where the lysine residues targeted by Gcn5 are mutated to arginine (Snf2 K1493R and K1497R(*22, 25*)). If Snf2 acetylation is necessary for the timely release of Swi/Snf complexes from the short isoform promoter and redirection to the long isoform promoter, I would expect the Snf2 K>R mutants to have a similar impairment in isoform switching and extended Snf2 protein expression as seen in *gcn5Δ*.

DISCUSSION

The preliminary evidence highlighted above provides numerous opportunities for exploration of the intersection between Cbf1, Gcn5-mediated acetylation of Snf2, histone acetylation, Snf2 intramolecular interactions, and Swi/Snf chromatin remodeling at the SNF2 gene locus itself.

Dynamic regulation of Snf2 expression is crucial for growth, nutrient stress response and sporulation, and it is difficult to make meaningful conclusions when working with cells that do not survive under normal growth conditions. This is one of the main reasons why I implemented the inducible reporter system described in Chapter 2, where GFP expression served as a readout for translation of transcripts with different SNF2 5' leaders. A similar system could be designed to help us better understand how the CRISPR-mediated mutations are affecting SNF2 expression. This system would ideally consist of an integration vector with an easily detectable reporter such as GFP as the readout. Where it would differ from the reporter used previously is that I would retain the promoter region upstream of SNF2 TSS1 instead of the inducible promoter. In this way, the promoter region, and any transcription factor binding sites I wish to test, could be mutated freely without impacting cell growth and survival, and GFP levels could be monitored throughout normal growth conditions and sporulation, ensuring that the transcriptional and translational changes observed are not an artifact of the loss of Snf2 expression. Additionally, RNA analysis of the cells described in this chapter, both the SK1 and BY backgrounds and relevant mutants, will be conducted via RT-PCR and Northern blotting to reveal how transcription of SNF2 isoforms changes under different conditions.

Chromatin immunoprecipitation coupled with PCR (ChIP-PCR) is another valuable method that could elucidate the patterns of regulator binding and histone modifications at the *SNF2* promoter throughout timecourses and across strains. Immunoprecipitation of Snf2-bound gDNA fragments could provide evidence to support or dismiss the possibility of Snf2 auto-regulation. Similar analysis of Cbf1-bound gDNA fragments could shed light on the relationship between Cbf1 and the results seen in the *SNF2 ΔREG/ΔREG* mutant strain. I could also look at gDNA fragments containing acetylated H3 to determine how H3 lysine acetylation impacts *SNF2* expression and binding of other factors.

MATERIALS AND METHODS

Mutation of *SNF2* promoter region via CRISPR/Cas9

A plasmid containing a CRISPR/Cas9 system designed for use in *S. cerevisiae* was obtained as a gift from the Manuel Ares Lab. The guide RNA to direct the Cas9-sgRNA complex to the *SNF2* promoter region was designed by selecting a PAM sequence upstream of TSS1 and annealing synthetic oligos corresponding to that region, which when annealed, have overhangs compatible with a BaeI restriction site for directional ligation into the CRISPR/Cas9 plasmid. A double-stranded DNA rescue fragment with two point mutations was also designed. One mutation was predicted to disrupt *SNF2* regulation (the original intention of this experiment was to mutate the start codon of an additional uORF, but later 5'RACE results showed that the mutated region is in fact upstream of the long isoform TSS), and one point mutation generated an EcoRI restriction site to facilitate screening for mutant clones.

Following transformation of the plasmid and rescue fragment into HO-negative haploid SK1 cells, cells were plated on SC-URA to select for transformants with the CRISPR/Cas9 plasmid, then counter-selected on 5-FOA plates to shuffle out the plasmid and prevent further mutations from arising. Colonies were then screened via PCR amplification of the targeted region and EcoRI restriction digest. Due to the inherently low efficiency of CRISPR/Cas9 mutations, two rounds of screening were conducted: first on pooled DNA from 8 colonies within divided plate regions, then on DNA from individual colonies within plate regions that were positive in the first round. Colonies of various sizes were collected to account for potential growth defects caused by the mutation. The screening process yielded two MATa mutants and one MATα mutant, which were confirmed by Sanger sequencing and hereafter referred to as *SNF2 ΔREG*.

SNF2 ΔREG mutants growth assay

One MATa and one MATα *SNF2 ΔREG* mutant strain were selected for further analysis. Serial dilution growth assays on YPD plates at 30°C for 2.5 days revealed an impairment in both *SNF2 ΔREG* strains. The two strains were then crossed to generate a homozygous diploid *SNF2 ΔREG/ΔREG* mutant.

Yeast sporulation

For yeast sporulation, diploid *S. cerevisiae* SK1 (TJY6917) cells were grown to saturation in standard yeast growth media (YPD) at 30°C, then used to inoculate cultures in ultra-rich media (GNA) at OD_{600} = 0.3. GNA cultures were grown at 30°C for

4 hours, then washed and resuspended in an equal volume of sporulation media (SPM). Sporulation cultures were incubated at 25°C and allowed to sporulate for at least 5 days before examination with phase separation microscopy.

Immunoblotting/Western blotting

All Western blots were prepared using cell number-equivalent samples. For Snf2 blots, 6% SDS polyacrylamide gels were used. For Pgk1 blots, 10% SDS polyacrylamide gels were used. Following transfer to PVDF membranes and incubation in blocking buffer (2% non-fat milk in TBS for Snf2 blots and 5% non-fat milk in TBS for Pgk1 blots), proteins of interest were tracked by incubation with specific antibodies.

Snf2 protein was detected using goat α-Snf2 primary antibody (Santa Cruz Biotechnology YN-20, 1:200 dilution) and donkey α-goat HRP secondary antibody (Santa Cruz Biotechnology SC-2020, 1:2,857 dilution). Pgk1 was detected using α-Pgk1 monoclonal primary antibody (Invitrogen 22C5D8, variable from 1:2,000 to 1:10,000 dilution) and α-mouse HRP secondary antibody (either Santa Cruz Biotechnology SC-2005, 1:2,857 dilution, or Abcam ab131368, 1:10,000 dilution).

Chemiluminescent substrate (Prometheus ProSignal Dura ECL) was applied to blots before X-ray film exposure. Developed film images were scanned into digital files. Coomassie staining was performed on identically-loaded gels for experiment shown in Figure 3.3A.

Table 3.1. Oligos used for mutation of *SNF2* promoter region via CRISPR/Cas9

Oligo name	Sequence (5'->3')
guide-uORF1-1-top (guideRNA-coding oligo for insertion in p416_TEF1p_Cas9_CYC1t_crRNA_BaeI)	GTC CAA CAC CTC CAA CTC TAG TTT T
guide-uORF1-1-bottom (guideRNA-coding oligo for insertion in p416_TEF1p_Cas9_CYC1t_crRNA_BaeI)	TAG AGT TGG AGG TGT TGG ACG ATC A
RF_uORF1-1_top (rescue fragment containing mutation)	TTT TCA ATC AAA AAT gAA TTC ACA AGT CCA ACA CCT CCA ACT CTA TcG TAT TGC G
RF_uORF1-1_bottom (rescue fragment containing mutation)	AAA AAG AAA AAA AGG AAA AAA GGG CAA AAA GAG AAC GCA ATA CgA TAG AGT TGG A

CHAPTER 4

Concluding Remarks

Swi/Snf integrates extracellular signals for exquisite control of gene expression

Swi/Snf is one of the master regulators of eukaryotic gene expression. From the powerful ATP-driven nucleosome sliding activity of Snf2 at its core, to the versatility of its many subunits, the highly-conserved Swi/Snf complex performs the crucial task of altering gene expression in response to environmental and/or developmental cues. With a role in almost every cellular process, from ribosome biogenesis to differentiation, activity of the Swi/Snf complex is influenced by a large array of context-dependent regulatory factors, some of which have been addressed in earlier chapters. Upstream of those factors, beyond the scope of the present study, Swi/Snf is influenced by kinase signaling cascades. Not only does it transform the signal into action by sliding nucleosomes and activating transcription at nutrient- and stress-responsive genes, but also likely participates in the regulation of its own expression through alternate mRNA transcripts. All of this results in coordinated cell fate decisions that maintain the energetic balance between anabolic growth and catabolic autophagy.

One of the most well-known nutrient signaling pathways, the target of rapamycin (TOR) pathway, is highly implicated in Swi/Snf regulation. TOR kinases transmit signals concerning nutrient conditions to the nucleus to regulate expression of metabolic and ribosome biogenesis factors (*49*), a process for which Swi/Snf chromatin remodeling is critical. TOR also directly regulates translation initiation in the cytoplasm (*50*) through translational remodeling (*51*). Hence, it is likely that TOR signaling plays an integral role in determining the level of translation of the long and short SNF2 isoforms described here, in a condition-dependent manner.

Another major signaling cascade that conveys signals to Swi/Snf is the SNF1/AMPK pathway (*52*). SNF1 refers to the yeast version of the system, which

shares its etymology with other Sucrose Non-Fermenting (SNF) genes, and AMPK to the mammalian version. SNF1/AMPK is activated by glucose limitation (*53, 54*) and various environmental stresses (*55*). Once activated, SNF1/AMPK regulates a large set of genes at the transcriptional level, through interactions with transcriptional regulators and RNA Pol II (*56*), and also directly modulates metabolic enzymes via phosphorylation (*52*). Notably, SNF1 is also involved in the regulation of sporulation in yeast (*57*), likely through mechanisms involving *SNF2* regulation, including those described in this dissertation. In fact, SNF1 is known to interact with Gcn5 of the SAGA complex (*58*), which is also implicated in *SNF2* regulation, as described in Chapter 3.

SNF1 and TOR both influence Msn2/4 binding to stress response elements (STREs) (*59*), one of which can be found in the *SNF2* promoter region, upstream of the long isoform TSS. Swi/Snf itself is required for Msn2/4-driven transcriptional activation under stress (*21*). Putting this all together, I hypothesize that Msn2/4 (activated by both SNF1 and TOR signaling) collaborates with the Swi/Snf complex at the SNF2 locus to activate transcription of the long isoform under nutrient depletion and sporulation-inducing conditions. In other words, this is another potential mode through which Snf2 protein at the core of the Swi/Snf complex downregulates its own expression via transcriptional activation at the long isoform promoter. I began preliminary work to examine the effect of a Msn2/4 double mutant on Snf2 expression, but these studies have not yet yielded conclusive results. Nevertheless, this is a topic worthy of further investigation.

Insights from studying the detailed mechanism of *SNF2* regulation

While genome-wide transcriptome studies have increased our understanding of the presence and possible outcomes of alternate mRNA isoforms, the specific ways in which their synthesis, processing, and translation are controlled has remained largely enigmatic. This is due, in part, to the fact that regulation at the RNA level does not occur in a linear manner; it is more comparable to a complex web of overlapping possibilities. Genome-wide studies can provide a snapshot of the RNA species that are present at a particular point in time but cannot capture the full depth of interactions and their timing. This is because each gene undergoes specific, context-dependent regulation in individual cells. Gene-specific studies are needed to fill in the gaps and create a richer understanding of what is happening at the molecular level. While the majority of genes in a data set may follow a certain pattern, outliers and special cases often make for the most interesting stories.

For example, the phenomenon of mRNA:protein discordance has been described in yeast (*27, 35*) and mammalian cells (*60*), which has opened up many important areas of investigation. By focusing on the special case of *SNF2,* I not only gleaned important insights into missing pieces of the puzzle of *SNF2* regulation, but the work also provides a discrete case-study characterizing alternate mRNA products, their translational outcomes, and the transcriptional regulators involved in changes at the *SNF2* promoter. These studies also uncovered evidence for unexpected regulatory mechanisms that are almost certainly present throughout eukaryotic regulation such as (1) long distance effects of transcriptional activators and repressors, and (2) proteins that are both activators and repressors, depending on their orientation. Alternate TSSs can be found

throughout the yeast and mammalian genomes (*60, 61*), so many other genes could be undergoing similarly complex and tunable regulation. Some of the modes of SNF2 regulation covered in earlier chapters are listed below.

SNF2 modes of regulation:

- Activation of transcription of SNF2 short isoform leading to Snf2 protein expression
- Activation of transcription of SNF2 long isoform leading to decrease in Snf2 protein expression due to transcriptional interference with short isoform
- Antisense transcript leading to transcriptional interference with either activating or repressive outcome depending on affected transcript (long vs. short)
- Transcriptional interference and changes in histone modifications in the *SNF2* promoter region, which may influence and/or be a result of Snf2 activity at its own gene locus
- SNF2 long isoform leading to decrease in Snf2 protein translation due to uORF-ribosome interactions
- SNF2 long isoform leading to increase in SNF2 mRNA sequestration or otherwise influencing translation/longevity of SNF2 transcripts
- SNF2 isoform switching influenced by transcriptional regulators such as Ume6, Cbf1, Gcn5, Msn2/4, etc., dependent on their orientation, and many of which Swi/Snf is known to interact with
- Swi/Snf auto-regulation at the *SNF2* locus

All of these modes of regulation are integrated: the translational regulation imparted by the uORFs in the 5' leader of the long SNF2 mRNA is only possible because of the transcriptional regulatory events that cause activation of the long isoform promoter. Moreover, this is only possible due to signaling cascades influencing transcriptional regulators at the *SNF2* locus, including Ume6, Cbf1, Gcn5, Msn2/4 and Snf2 itself.

Meiosis as a Swi/Snf-mediated nutrient response, from yeast to mammals

Meiosis, the specialized cell division which allows for sexual reproduction, is common throughout eukaryotes. In fungi such as *S. cerevisiae* yeast, sexual reproduction is also a survival mechanism. When nutrients are depleted, diploid yeast undergo the process of sporulation and divide meiotically to generate four haploid spores, surrounded by a protective ascus and poised to reactivate with a recombined genome once nutrients are available again (*24*). We have previously shown that the catalytic component of the Swi/Snf chromatin remodeling complex, Snf2, is responsible for shifting gene expression away from intron-rich ribosomal protein genes (RPGs) to enhance splicing of meiotic intron-containing genes (ICGs) during sporulation (*20*). Along with evidence available in the literature, it is evident that meiosis in yeast is a nutrient response that confers the survival advantages of decreased metabolic activity and increased genetic diversity among the resulting spores, and that Swi/Snf plays an integral role.

Interestingly, similar cellular states are associated with regulation of meiosis during gametogenesis in higher eukaryotes. In the ovarian niche, developing

mammalian oocytes undergoing meiosis are surrounded by cumulus cells, which have been shown to starve the oocyte of glucose and instead feed it pyruvate, an alternate carbon source that channels into oxidative phosphorylation, in a regulated manner (*62*). In a separate study, when levels of BRG1, the mammalian *SNF2* homolog, were tracked in developing mouse spermatocytes (*15*), they showed the same pattern of rising and falling that Snf2 shows in SK1 yeast cells (see Figure 2.1A). There are many conserved factors and parallels between yeast and mammals, and while we don't yet have all the answers, it seems that higher eukaryotes have evolved methods of intercellular communication which still "speak the language" of nutrient signaling and utilize the power of Swi/Snf chromatin remodeling to accomplish the necessary changes in transcriptional programs.

Swi/Snf is not only essential for successful completion of meiosis, but also for subsequent reentry of the resultant cells into the cell cycle. Maternal BRG1 transcripts have been shown to play an essential role in mammalian zygotic genome activation, with their absence causing a stall at the 2-cell stage (*63*). This means that BRG1 transcripts produced during meiosis are held in the oocyte, reserved for the appropriate moment to jump-start genome activation in the zygote. Alternate transcripts and repressive uORFs are present in mammals (*60, 64-66*), but the above study did not analyze BRG1 mRNAs beyond detection, so it would be interesting to pursue characterization of the 5' end(s) of those BRG1 transcripts in oocytes and zygotes to determine whether they contain translation control elements such as uORFs. If that were the case, it would follow that both yeast and mammalian cells use alternate transcripts to regulate *SNF2/BRG1,* possibly through conserved mechanisms. Even if

BRG1 5’ leaders do not contain the regulatory elements described in yeast, this does not rule out the possibility of other modes of translational control happening in mammals. Either way, regulation of *SNF2/BRG1* at the mRNA level during meiosis is critical for eukaryotic life as we know it.

The potential for cap-independent translation of Snf2 protein from long SNF2 transcripts

As mentioned in Chapter 1 (see Figure 1.3), canonical translation initiation involves interactions between the 5’ cap of a mRNA transcript and the translation machinery, which is necessary for uORF-mediated repression. However, under certain conditions, even when TOR signaling has shut down cap-dependent translation, cap-independent translation initiation can occur (*67, 68*). This allows cells to initiate protein production even in nutrient-depleted environments, by relying on regulatory features within mRNA 5' leaders and/or spatial regulation to recruit translation machinery.

I have found evidence in the literature of two non-mutually exclusive instances where long SNF2 transcripts may be undergoing cap-independent translation. First, SNF2 mRNA has been shown to be enriched in cytoplasmic ribonucleoprotein (RNP) compartments called stress granules under arsenite stress in yeast (*30*). These compartments, while typically associated with translational silencing, are capable of transitioning into centers of cap-independent translation initiation during metabolic reactivation and reentry into the cell cycle (*69*). I speculate that the long SNF2 isoform could be sequestered in these translationally repressed RNP granules during nutrient deprivation and meiosis, then could undergo cap-independent translation, bypassing

uORF repression, once the cell encounters nutrients. This could allow Snf2 to be one of the first proteins synthesized, thus giving the cell a head start towards growth.

Whether or not it is in the context of cytoplasmic RNP granules, cap-independent translation is a nutrient-responsive phenomenon that is dependent on regulatory features in mRNA 5' leaders (*29*). Among those regulatory features are N6-methyladenosine (m6A) RNA modifications, which have been shown to serve as a landing pad for translation machinery under non-canonical translation initiation conditions (*70*). The long SNF2 isoform contains a m6A site in its 5' leader, located just downstream of the third uORF (*71*). In yeast, m6A was previously assumed to be present only during meiosis and sporulation, since the only known yeast m6A methyltransferase is Ime4 (Inducer of Meiosis 4), which is implicated in the onset of meiosis as its name implies. However, it was shown that expression and methyltransferase activity of Ime4 extends outside of the context of meiosis (*72*). This sets the stage for long SNF2 mRNAs to undergo cap-independent translation initiation as cells are transitioning from quiescence to growth.

Why has *SNF2* evolved to be so tunable?

In an ever-changing environment, life has evolved to ride the waves of scarcity and abundance in cycles of cell growth and quiescence. While a state of nutrient deficiency is often viewed in a negative light, cells are actually well-adapted for such conditions, and derive important benefits from being in a state of deficiency. This is exemplified by autophagy, a fundamental cellular process through which cells engulf and break down damaged or unnecessary components, contributing to overall cell health and functioning (*73, 74*). In higher eukaryotes, this is why caloric restriction

prevents disease and leads to increased lifespan (*75*). The clean-up and recycling of resources that happens during autophagy plays a critical role in maintaining cellular integrity, regulating energy levels, and promoting survival during periods of nutrient scarcity or stress. These important benefits can only be accessed when autophagy is activated, which only happens when signaling pathways such as TOR and SNF1/AMPK pass along signals of nutrient deprivation and stress (*76*). On the other hand, when those signaling pathways instead pass along signals of nutrient availability, quick activation of growth programs gives cells a head start to ride the wave of abundance.

These coordinated responses to the environment happen at the level of gene regulation, and as described in this dissertation and in previous studies (*19, 20*), *SNF2* is indispensable in transitioning between cell states. Accordingly, Snf2 allows cells to be poised to respond quickly to changes, which may be why its expression is not turned off completely. In a competitive environment, when nutrients are suddenly available after a period of scarcity and dormancy, the first cells to come out of the quiescent state and to start using the resources will likely be more successful. Consider this: if you knew you were going to need to use your computer at a moment's notice, but you still wanted to save the battery, you probably wouldn't shut it down completely, but would instead put it in sleep mode. Given the example of maternal BRG1 transcripts being necessary for mammalian zygotic genome activation discussed above (*63*), it seems likely that the SNF2 long isoform can serve as a similar standby to reactivate Snf2 protein expression following quiescence and/or sporulation in yeast, like the cellular equivalent of sleep mode. Rather than completely shutting down the *SNF2* gene locus, the cell switches to an isoform of SNF2 mRNA that conserves energy by decreasing Snf2 protein levels and

thereby ribosome biogenesis and the most energetically expensive process in the cell – translation. By keeping the transcriptional machinery at the *SNF2* locus, the switch is easily reversible.

Conclusion

Snf2 sensitizes the cell to its environment through its role as an integrator of signals, which is critical to proper cellular function. In yeast, it has the monumental task of controlling metabolism and cell fate. In higher eukaryotes, it is also responsible for the development and maintenance of a wide range of tissue types, including but not limited to neuronal cells, cardiomyocytes and gametes (*13-15, 77*).

Cells encounter an infinite number of overlapping conditions that need to be integrated into one coordinated response. When functioning properly, TOR and SNF1/AMPK signaling ensure that nutrient conditions are inseparable from cell fate decisions, and in higher eukaryotes, cells communicate with each other to develop properly and maintain homeostasis (*13, 14, 78*). When things go wrong, such as in the case of cancer and metabolic dysfunction, the energetic balance of the cell is disrupted (*79*), with deadly consequences unless treated. Metabolic dysfunction underlies the top two causes of death in the United States, heart disease and cancer (National Center for Health Statistics; Mortality Data for 1999-2021), and Swi/Snf is implicated in both of them. *BRG1* is mutated or otherwise misregulated in over 25% of human tumors (*12, 17*), and is necessary for maintaining cardiac tissue homeostasis (*14*). When Swi/Snf is unable to integrate signals properly, the cell's behaviors become disconnected from its environment. Unable to respond to signals from neighboring cells or fine-tune its

metabolism, the affected cell loses its identity, and becomes energetically imbalanced and malignant (*79*).

Swi/Snf, and the catalytic Snf2 ATPase at its core, serve as a nexus point through which the complexity of life is translated into cell fate decisions. With this dissertation, I have presented my contribution towards solving the puzzle of *SNF2* regulation, and in doing so, have also identified several intriguing areas of future study, which I hope will help us adjust how we interact with the world around us to bring health and true prosperity to humanity. Just like the complex web of gene regulation, the factors influencing our lives that might seem disconnected at a first glance, are actually intricately intertwined. By filling in the gaps in our understanding and using this information to educate the public, we can influence policies and empower people to protect their health.

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