Characterization of Genetic and Physical Interactions Between the SWI/SNF Complex and U2 snRNP Protein Components as Facilitators of Co-transcriptional pre-mRNA Splicing

A thesis submitted in partial satisfaction of the requirements for the degree of Master of Science in Biology by Erik Randolph Soule

Committee in Charge:

Professor Tracy Johnson, Chair
Professor Lorraine Pillus
Professor Ella Tour

2013
The Thesis of Erik Randolph Soule is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2013
DEDICATION

This work is dedicated to family, friends, and four-dimensional spacetime.
EPIGRAPH

“Live as if you were to die tomorrow. Learn as if you were to live forever.”

- Mahatma Gandhi
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ABSTRACT OF THE THESIS

Characterization of Genetic and Physical Interactions Between the SWI/SNF Complex and U2 snRNP Protein Components as a Facilitator of Co-transcriptional pre-mRNA Splicing

by

Erik Randolph Soule

Master of Science in Molecular Biology

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Professor Tracy Johnson, Chair

Recent findings have demonstrated that the spliceosome assembles onto nascent pre-mRNA co-transcriptionally and that, as a consequence, modifications in
the chromatin can influence this co-transcriptional spliceosome assembly. The purpose of this study was to test the hypothesis that the SWI/SNF chromatin-remodeling complex, which has been shown to bind acetylated histones, is an adaptor protein that links Gcn5 catalyzed histone acetylation with co-transcriptional splicing through physical interactions with U2 snRNP components such as Msl1 and Lea1. Synthetic lethality dependent on the catalytic activity of Gcn5 was demonstrated between snf2Δ and gcn5Δ. Additionally, deletion of a SWI/SNF component, SNF5, produced a negative genetic interaction with both msl1Δ and lea1Δ, and a strong growth defect was observed between snf2Δ and msl1Δ. Co-IP experiments have further demonstrated physical interactions between Snf5 and Msl1, as well as Snf5 and Lea1. These data suggest that the SWI/SNF complex has both physical and functional interactions with the U2 snRNP, a hypothesis that was corroborated when an IP experiment was coupled with an RNA primer extension, revealing a physical interaction between Snf2 and the U2 snRNP and, unexpectedly, the U1 snRNP. Finally, chromatin immunoprecipitation using strains tagged for U2 proteins Msl1 and Lea1 demonstrated that, similar to GCN5 deletion, deletion of SNF2 decreases co-transcriptional recruitment of the U2 snRNP to several intron-containing genes. These data, analyzed together, support a model whereby the SWI/SNF complex interacts with U2, directly or indirectly, facilitating co-transcriptional spliceosome assembly and perhaps dynamic splicosomal rearrangements.
INTRODUCTION

Overview of Pre-mRNA Splicing:

Pre-mRNA splicing is the process by which the non-coding portions of an mRNA transcript, or introns, are removed, and the remaining coding regions or exons, are fused together.

Figure 1: Splicing: first and second step schematic

94% of human protein-coding genes contain introns, and mutations that affect splicing can cause disease directly or contribute to the susceptibility or severity of disease. For example, spinal muscular atrophy is the most common cause of infant mortality and is caused by an exonic mutation that disrupts splicing (Tazi et. al., 2009). Additionally, cancer tissues have been shown to contain different splice
variants compared to the surrounding normal tissue (Ward et al., 2010). These facts establish a need to understand the mechanisms of splicing at the molecular level.

In human cells, alternative use of potential splice sites is abundant, with the average gene containing 8.8 exons with a mean length of 145 nucleotides, while the average intronic length is a staggering 3365 nucleotides (Tazi et al., 2009). Depending on cell type and time in development, different exons are expressed, expanding the proteome drastically (Black, 2003). Since alternative splicing makes the study of splicing in multicellular eukaryotes more difficult, it is beneficial to study splicing in an organism with a streamlined genome such as Saccharomyces cerevisiae, or budding yeast. The streamlined genome of S. cerevisiae means that there are fewer introns (6% of genes contain introns), however, more than 30% of all messenger RNAs are derived from intron-containing genes (Ares, et al., 1999). Furthermore, the core components of the splicing machinery, or snRNPs, share a high degree of evolutionary conservation (Meyer et al., 2009).

The S. cerevisiae genome contains 6,275 genes, and 23% of its genome is shared with humans. Many of these genes can be deleted without killing the cell, and the ability of yeast to grow as a haploid organism expedites the process of transforming new genes or deleting a gene of interest. This ease of manipulation is another reason that yeast is such a valuable model organism, in addition to its short doubling time.
Nucleosomes and gene regulation:

DNA is wrapped around protein complexes known as nucleosomes, which play key roles in gene expression. Nucleosomes are composed of four histone proteins, which make up the histone core octamer with two subunits of each distinct protein, H2A, H2B, H3, and H4.

Nucleosome Components:

- H2A
- H2B
- H3
- H4
- DNA

Figure 2: Crystal structure of the nucleosome (Harp et al., 2000)

Figure 2 shows the 4 different protein components of the nucleosome, histone H2A, H2B, H3, and H4, as well as the DNA that is wrapped around the histone core octamer. The N-terminal tails of the histone proteins protrude from the nucleosome and can undergo modifications such as methylation, acetylation, etc. Histone modifications are known to have differential effects on the genes that house them. Some histone marks are correlated with transcriptional activation, such as trimethylation of histone H3, lysine 4 or lysine 36 (Vermeulen and Timmers, 2010).
Acetylation of histone lysine residues is also associated with activation (Rando and Winston, 2012). Histone acetyltransferases, or HATs, such as Gcn5 are responsible for marking histones with acetyl groups. Gcn5 acetylates lysines on histones H2B, H3, and H4, and carries out its HAT function as part of the SAGA complex. Gcn5 contains a bromodomain, a protein domain that has been implicated in binding to lysine residues (Dyson, 2001). It has been shown that the Gcn5 bromodomain is necessary for the cooperative acetylation of nucleosomes (Li et al., 2009).

One protein, Snf2, was shown to bind to acetylated nucleosomes via its bromodomain (Awad et al., 2008). Snf2 is part of the SWI/SNF complex, which functions to activate genes for transcription by remodeling the chromatin via either nucleosome disassembly and removal (Brown et al., 2011), or an intranucleosomal DNA loop-formation mechanism (Zhang et al., 2006). After a gene has been acetylated by GCN5, and SWI/SNF has performed its chromatin remodeling function, GCN5 acetylates SNF2, which causes the SWI/SNF complex to dissociate from the chromatin (Kim et al., 2010). Notably, Gcn5 acetylates histones in gene promoter regions as well as in the body of genes (DeSimone and Laney, 2010).

Once nucleosomes are rearranged and RNA polymerase II accesses the gene’s promoter, the DNA is transcribed to pre-messenger RNA in the 5’ to 3’ direction. Modifications are made to the messenger RNA during and after transcription. These modifications include pre-mRNA splicing, 5’ end capping, and polyadenylation of the 3’ end of the mRNA to form a mature message.
Mechanisms of pre-mRNA splicing including co-transcriptional spliceosome assembly:

Splicing is accomplished by the spliceosome, a large protein-RNA complex that exists in the nucleus of eukaryotic cells. The spliceosome is composed of five snRNPs, or small nuclear ribonucleoproteins, which interact with the pre-mRNA transcripts in order to excise their introns via the two transesterification reactions. The five snRNPs must bind the pre-mRNA and undergo specific coordinated rearrangements in order to catalyze splicing. First, U1 base pairs to the 5’ splice site. Protein interactions play a role, but RNA complementarity between the U1 snRNP and the 5’ splice site drive the interaction of the U1 snRNP with the pre-mRNA. BBP and Mud2 identify the branch-site and subsequent ATP-dependent rearrangements in the snRNA and associated proteins facilitate U2 snRNP interaction with the branchpoint. In the first ATP dependent step of splicing, U2 base pairs near the branchpoint of the intron, to form a complex referred to as the ‘A complex.’ A B1 complex is formed when the tri-snRNP, or U4/U6:U5 also associates with the pre-mRNA. A C1 complex is formed when U4 dissociates and U2/U6 forms the catalytic center of the spliceosome. In the first transesterification step, a lariat is formed by the linkage between the 5’ end of the intron and the adenosine at the branch-site (Johnson and Vilardell, 2012). In the second transesterification step, the 3’ splice site is cleaved, and the exons are ligated together. Finally, the mature message dissociates from the post catalytic spliceosome. This sequence of events illustrates a key feature of the splicing
reaction; namely that the spliceosome undergoes dramatic but ordered, energy-driven rearrangements that are central to its activity (McKay and Johnson, 2010).

Figure 3: Dynamic rearrangements of splicing

Each color-coded circle denoted U1, U2, U4, U5, or U6 represents a snRNP, a complex composed of RNA and protein.

Remarkably, all of this can occur co-transcriptionally, while the RNA polymerase is still associated with the template and the nascent transcript. Co-transcriptionality is thought to improve the efficiency of the overall gene expression process (Aitken et. al., 2011). In order for splicing to occur co-transcriptionally, the Cap Binding Complex binds to the 5’ 7-methylguanylate cap after it has been synthesized and recruits the U1 snRNP to the 5’ splice site. In the absence of the CBC, U2 snRNP accumulation at the branch-point is abrogated. (Görnemann et. al., 2006)
These and subsequent steps of splicing can occur co-transcriptionally, and our lab has previously shown that these events are driven by changes in the acetylation state of histones (Gunderson et. al., 2011); specifically, co-transcriptional recruitment of components of the U2 snRNP to the pre-mRNA of an intron-containing gene is affected by deletion of GCN5. However, although genetic interactions were observed between the heterodimeric U2 snRNP components MSL1 and LEA1 and either GCN5 or histone residues targeted by Gcn5, a direct physical interaction was not observed. Moreover, RNA polymerase II occupancy within the gene was relatively unaffected (Gunderson et. al., 2009), suggesting that Gcn5’s effect on spliceosome assembly is not simply due to a change in transcription. Ms11 and Lea1 do not contain bromodomains, so it is unlikely that they would be able to directly bind these acetylated histones.

One hypothesis that would be consistent with these results is that there might be an adaptor protein which binds to acetylated histones via a bromodomain and recruits the U2 protein complex co-transcriptionally. Previous reports demonstrate roles for such chromatin associated adaptor proteins in coordinating chromatin modifications and spliceosome assembly (Sims et. al., 2007).
Figure 4: Adaptor protein hypothesis (Gunderson et. al., 2011)

This is a hypothetical mechanism which illustrates an unknown adaptor protein binding to Gcn5-acetylated histones and interacting with the U2 snRNP to facilitate co-transcriptional spliceosome assembly.

Central to such a model would be the identity of proteins that bind acetylated histones and interact with the U2 snRNP. The SWI/SNF complex appeared an ideal candidate. Genome-wide analysis of protein-protein interactions indicated that Msl1 had two-hybrid interactions with a component of SWI/SNF (Fromont-Racine et. al., 1997).
Furthermore, the catalytic component of SWI/SNF is the bromodomain containing protein SNF2, which is known to bind Gcn5-acetylated histones (Awad et. al., 2008).

Figure 5: The SWI/SNF complex

The different components of the SWI/SNF complex are illustrated here. The catalytic component, Snf2, is shown in blue. Other components necessary to maintain the integrity of the complex are shown in orange. Components involved in transcription are shown in green. Components that are though to maintain proper chromatin structure are shown in purple. In yellow are components whose exact functions have not yet been determined.

Hence we considered that Snf2 could directly or indirectly recruit spliceosomal components to Gcn5 acetylated chromatin, and that this coordinated regulation of chromatin modification and splicing could facilitate co-transcriptional splicing and the production of the gene products needed for proper cellular function. To test this hypothesis, we’ve taken a combined genetics and biochemical approach to examining the interactions between SWI/SNF and the spliceosome.
MATERIALS AND METHODS

Generation of yeast strains:

Tagged and knockout yeast strains were obtained from Open Biosystems or in the cases of \textit{SNF2} and \textit{SNF5}, the genes were knocked out using standard molecular techniques. The desired mutants with opposite mating types were streaked together with 5μl ddH2O and grown for one day. Subsequently, diploids were selected via replica plating onto media that neither haploid could grow on without forming a diploid. Sporulation media lacking uracil was inoculated with the selected diploids and incubated for 8 days on a sporulation wheel in order to ensure Uracil plasmid retention if applicable. Next, cells were incubated with zymolase for 6 minutes at 30°C and tetrads were dissected under a microscope outfitted with a dissecting needle. Finally, the resulting tetrads were analyzed via growth on selective media, and haploid strains containing the necessary markers were selected.

Dilution series growth assay:

Cells were streaked from frozen stock onto selective media to select for any plasmids present, including SC-Ura in the case of strains containing a \textit{SNF2} or \textit{SNF5} pRS316 plasmid, and SC-Leu in the case of \textit{GCN5} or \textit{CUP1} pRS315 plasmids. The cells were then grown in the corresponding liquid media at 30°C until an OD600 of 0.5 was reached. At that point, the liquid culture was diluted 5 times, each 1/10 the concentration of the previous dilution in liquid media. Finally, the cells were spotted
via micropipette onto the proper plate needed for experimental conditions and controls and grown for 4 or 5 days at the indicated temperatures. To select against the SNF2 or SNF5 pRS316 plasmids, 5FOA media was used.

RNA Preparation:

100mL of HA-tagged cells were grown to an OD600 of 0.5 along with any appropriate control strains. 2.7mL of 37% formaldehyde was added, and the culture was shaken slowly on a shaker at room temperature for 15 minutes. Next, 11mL of 2 M glycine was added to stop the crosslinking and the culture was incubated an additional 5 minutes on the shaker. The cells were spun down at 2500g for 5 minutes at 4°C and washed in ice-cold TBS twice then re-suspended in 0.5 mL of ice-cold FA lysis buffer. The cells were pelleted by centrifugation at 15,000g for 30 seconds at 4°C. This pellet was resuspended in 0.75 mL of ice-cold FA lysis buffer containing 40 U/mL RNasin. 500 µL of zirconia/silica beads were added. The cells were lysed in a FastPrep cell disruptor using a speed setting of 5.5 for 30 seconds followed by a one-minute incubation on ice, which was repeated 5 times. A syringe needle was used to make a hole in the bottom of the 1.5 mL tube, which was then placed into a 2 mL tube and centrifuged at 2000g for 2 minutes at 4°C. The liquid was transferred from the 2 mL tube to a 15 mL tube, and 0.75 mL of FA lysis buffer containing 40 U/mL RNasin was added. The tube was placed in an ice/salt mixture in a beaker and the tip of a sonicator probe was placed near the bottom of the tube. The samples were sonicated with two 15-second pulses of 50% amplitude with a one-minute break between them.
The sonicated extract was transferred to a 1.5 mL tube and centrifuged at maximum speed for 10 minutes at 4°C, then transferred to a new tube and the centrifugation was repeated. This extract was transferred to a new 1.5 mL tube and 50 µL of Sepharose CL-4B beads were added. The extract was pre-cleared by incubating with end over end rotation for 1 hour at 4°C after which it was centrifuged at 1000g for 2 minutes at 4°C and the supernatant was transferred to a new 1.5 mL tube. The extract was adjusted to 25 mM MgCl₂ (from 1 M stock) and 5 mM CaCl₂ (from 2 M stock). 3 µL of RNasin and 6 µL of RNase-free DNase I were added, and the sample was incubated for 20 minutes at 37°C. The reaction was stopped by adding 0.5 M EDTA to a final concentration of 20 mM and the sample was centrifuged at maximum speed for 10 minutes at 4°C after which it was transferred to a new 1.5 mL tube.

Immunoprecipitation:

An amount of extract equivalent to 1x10⁶ cells from each sample was diluted with 150 µL of RIP elution buffer to prepare input RNA and snap-frozen in liquid nitrogen for use as input RNA. An amount of extract equivalent to 1x10⁸ cells was diluted to 500 µL with FA lysis buffer containing 40 U/mL RNasin. This was incubated overnight with 1-5 µL of the primary antibody against the protein or epitope of interest with end-over-end rotation at 4°C. The RIPs were then centrifuged at maximum speed for 10 minutes at 4°C and the supernatant was transferred to a new 1.5 mL tube. A 20 µL bead volume of Protein A-Sepharose beads was added to the sample and incubated with end-over-end rotation for 90 minutes at 4°C. The beads
were then washed with centrifugation at 1000g for 2 minutes at 4°C, removal of the supernatant, resuspension in 1 mL of ice-cold FA lysis buffer, incubation with end-over-end rotation for 5 minutes at 4°C, and another centrifugation at 1000g for 2 minutes at 4°C. The beads were resuspended in 1 mL of ice-cold FA500 buffer and incubated for with end-over-end rotation for 5 minutes at 4°C, and another centrifugation at 1000g for 2 minutes at 4°C was performed. The beads were then resuspended in 1 mL of ice-cold LiCl wash and incubated with end-over-end rotation for 5 minutes at 4°C, followed by centrifugation at 1000g for 2 minutes at 4°C. Finally the beads were resuspended in 1 mL of ice-cold TE buffer and incubated with end-over-end rotation for 5 minutes at 4°C, and centrifuged at 1000g for 2 minutes at 4°C. Most of the supernatant was removed and the remaining supernatant was aspired with a GELoader tip to prevent loss of beads. 75 µL of RIP elution buffer containing 40 U/mL RNasin was added and incubated in the Thermomixer at 1400 rpm for 10 minutes at 37°C. The beads were pelleted by centrifugation at 1000g for 2 minutes at room temperature. The supernatant was transferred to a new 1.5 mL tube and the elution was repeated with 75 µL of RIP elution buffer. The eluates were pooled.

Co-Immunoprecipitation:

For the Co-IP experiment, the immunoprecipitation eluate was then loaded into an SDS-PAGE gel and run for 15 minutes at 80 V then 90 minutes at 120 V. The gel was transferred onto a methanol-activated PVDF membrane for 80 minutes at 100 V. This membrane was then dried, re-activated in methanol, washed with TBS and
blocked for 30 minutes in 5% milk. The membrane was then incubated overnight in 5% milk with end-over-end rotation at 4°C with a 1:500 dilution of mouse anti-myc antibody clone 9E10 from Upstate. The membrane was washed 3x for 10 minutes each in TBS + 0.01% Tween and incubated with a 1:10000 dilution of goat anti-mouse HRP-conjugated secondary antibody in 5% milk for 60 minutes. The membrane was washed again 3x for 10 minutes each in TBS + 0.01% Tween and exposed with Thermo Scientific SuperSignal West Pico Chemiluminescent Substrate.

RNA purification:

6 µL of 5 M NaCL and 20 µg of proteinase K were added to the immunoprecipitation eluate and incubated for 60 minutes at 42°C, and the input RNAs were processed in parallel. 100 µL of nuclease-free water was added to the RIP sample along with 250 µL acid-phenol:chloroform. The phases were separated by centrifuging in MaXtract tubes at 10,000g for 3 minutes at room temperature. The aqueous layer was transferred to a new 1.5 mL tube and 25 µL of 3 M sodium acetate, 20 µg of glycogen, and 625 µL of ice-cold 100% ethanol were added. The RNA was precipitated for 90 minutes at -80°C, and the RIPv were centrifuged at maximum speed for 30 minutes at 4°C. The pellet was washed with 1 mL of ice-cold 70% ethanol and centrifuged at maximum speed for 5 minutes at 4°C, then the supernatant was discarded. The pellet was air-dried for 7 minutes then re-dissolved in 90 µL of RNase-free water. 10 µL of TURBO DNase buffer and 1 µL of TURBO DNase were added and the samples were incubated for 30 minutes at 37°C. 10 µL of DNase
Inactivation Reagent was added and was incubated with the sample with occasional agitation for 2 minutes at room temperature followed by centrifugation at 10,000g for 1.5 minutes at room temperature and transfer of the supernatant to a new 1.5 mL tube.

Primer Extension:

10 µmol of each primer oligo (5 µmol for U2) were incubated at 37°C for 30 minutes with 2 µL γ-labeled ATP, 1 µL 10x T4 buffer, 1 µL T4 Polynucleotide Kinase, and 2 µL of H2O. Then 15 µL H2O was added to each tube of primer oligo mix, and the tubes were combined to make the snRNA ladder primer set. The labeled primer set (125 µL) was then loaded into a prepped Tris P-30 column, and spun at 3,500 rpm for 4 minutes. Next, 5 µL of purified RNA was combined with 2.5 µL 100mM Tris pH 8.0, 1 µL 1M KCl, 0.2 µL RNasin, 5 µL snRNA ladder primers, and 6.3 µL of MilliQ H2O to make the annealing mix. This 20 µL sample was heated at 65°C for 10 minutes then put on ice. For the extension mix, the 20 µL samples was mixed with 10 µL 5x AMV RT buffer, 0.5 µL 1M DTT, 5 µL 10 mM dNTPs, 13.8 µL MilliQ H2O, 0.2 µL RNasin, and 0.5 µL AMV Reverse Transcriptase. This 50 µL reaction mixture was incubated for 60 minutes at 37°C, then 5 µL 1M NaOH was added, and the sample was incubated another 60 minutes at 65°C. 100 µL Primer extension buffer, 15 µL of 3M NaOAc, pH 5.2, and ice-cold 100% EtOH were added, and the mixture was allowed to precipitate at -20°C overnight. The sample was spun, washed, and dissolved in 20 µL UREA/TBE loading buffer, heated at 65°C for 5 minutes, vortexed, spun, resuspended, and run on a 6% urea gel.
ChIP:

Cells were grown in 150 mL of yeast extract-peptone-dextrose (YPD) to an OD600 of 0.6 to 0.8. One aliquot of 400 µL lysates was incubate with Roche anti-HA antibodies diluted 1:250 for 2 h followed by 1 h of incubation with 80 µL of Gammabind G Sepharose beads (GE Healthcare). RT-PCR was performed using the ABI Prism 7000 (Applied Biosystems) or Mastercycler ep realplex (Eppendorf) real-time PCR system, and amplicons were detected using SYBR green. The nontranscribed region (NTR) in chromosome V was used as a “no ORF” control for the ECM33 ChIP experiment. The other primers used in this experiment were; (1) specific for the promoter, (2) exon 1-intron junction, (3) intron-exon 2 junction, (4) toward the 5’ end of exon 2, and (5) the middle of exon 2. The occupancy of protein in a specific region of the ORF was determined using the following equation: occupancy of tagged protein = (IP value for ORF/IP value for NTR)/(input value for ORF/input value for NTR). The DBP2 primers used were specific to the promoter, as well as 2 sets of primers within exon 1, denoted 1.1 and 1.2, and one primer set within exon 2. For the DBP2 ChIP experiments, a no antibody control was used to calculate occupancy.
Chapter 1: Genetic analysis of *SWI/SNF* with U2 snRNP proteins Msl1 and Lea1

Genetic interactions are interpreted by observing the deviation of a double-mutant organism’s phenotype from the expected neutral phenotype of either single mutant alone. A double mutant with a more extreme phenotype than expected indicates a synergistic interaction between the corresponding mutations. In other words, the two mutated proteins may contribute to the same mechanism. Following this reasoning, if both of these proteins are deleted, the effect on the cell is very deleterious because the specific mechanism that both proteins carry out functions in is doubly affected. Conversely, if the double-mutant phenotype is less severe than expected based on the phenotypes of the single mutants, a phenomenon known as a genetic suppression, it is often the result of gene products operating within the same pathway. An example of this is when a mutation in one gene impairs the function of an entire pathway, masking the consequence of mutations in additional downstream members of that pathway since the cell has found a way to subvert its resources to maintain a similar function while avoiding the impaired pathway (Mani *et. al.*, 2008). Genes encoding proteins operating within a complex may also show synthetic interactions. Previous work showed a synergistic interaction between *gcn5Δ* and a deletion of either component of the *MSL1/LEA1* heterodimer (Gunderson *et. al.*, 2009). We next wanted to determine if *SWI/SNF* was responsible for mediating this effect, since no direct physical interaction between Gcn5 and Msl1/Lea1 was detected. To test this hypothesis, we performed several dilution series, the first of which was
designed to test whether the histone acetylation activity of Gcn5 acts synergistically with Snf2. A similar set of analyses were performed by Roberts and Winston (Roberts and Winston, 1997).

Figure 6: *gcn5Δ* exhibits a synthetic lethal genetic interaction with *snf2Δ*, which cannot be rescued by the presence of non-catalytic Gcn5 protein

Strains were streaked from frozen stock onto SC − Ura − Leu plates to select for the *WT SNF2* plasmids as well as the empty vector, *GCN5*, or non-catalytic *gcn5* KQL plasmids then grown in YPD liquid media at 30°C to an OD600 of 0.5 and tenfold serial dilutions were performed. The cells were plated on 5FOA − Leu to remove the *WT SNF2* plasmids but retain the *GCN5* Leu plasmids and grown for 4 days at three different temperatures.

Consistent with previous studies, the data shows a strong negative genetic interaction between *snf2Δ* and *gcn5Δ*. This effect could not be suppressed by a plasmid containing a non-catalytic copy of *gcn5* (*gcn5* KQL).

After demonstrating the interaction between *snf2Δ* and *gcn5Δ*, we wanted to test for a genetic interaction between *snf2Δ* and U2 components via viability analysis.
Figure 7A: *MSL1* deletion combined with deletion of *SNF2* or *SNF5* results in a severe negative genetic interaction. Figure 7B: Control experiment, WT versions of *SNF2* and *SNF5* restore growth of *snf2Δ* and *snf5Δ* mutants.

Strains were streaked from frozen stock onto SC – Ura plates to select for the *WT SNF2/SNF5* plasmids, then grown in YPD liquid media at 30°C to an OD600 of 0.5 and tenfold serial dilutions were performed. The cells were plated on 5FOA to select against the *WT SNF2/SNF5* plasmids and grown for 4 days. In the control experiment, the cells were plated on SC – Ura to select for the *WT SNF2/SNF5* plasmids and grown for 4 days.
This experiment demonstrates a negative genetic interaction between deletions of
SWI/SNF components SNF2 and SNF5, and deletion of MSL1, a component of the U2
snRNP. This suggests a functional relationship between SWI/SNF and MSL1 and hints
that the SWI/SNF complex may contribute to Msl1 function. Interestingly, deletion of
the ATPase component of the SWI/SNF, Snf2, has a strong interaction at all
temperatures tested, while at 37°C, the snf5Δ growth defect when combined with
msl1Δ was not as severe. The controls show that in the presence of the SWI/SNF
plasmid, all strains grow similar to wild type at all temperatures, with the exception of
snf2Δmsl1Δ. This is not surprising since msl1Δ has a slight slow growth phenotype, as
has been previously shown (Gunderson and Johnson, 2009).

These experiments were also performed with strains deleted for LEA1, the U2
component that forms a heterodimer with Msl1.
Figure 8: (A) LEA1 deletion combined with deletion of SNF2 suppresses the growth defect of snf2Δ. (B) snf2Δ lea1Δ or snf5Δ lea1Δ grow comparably to WT when SNF2 or SNF5 are provided on plasmids.

Strains were streaked from frozen stock onto SC – Ura plates to select for the WT SNF2/SNF5 plasmids, then grown in YPD liquid media at 30°C to an OD600 of 0.5 and tenfold serial dilutions were performed. The cells were plated on 5FOA to select against the WT SNF2/SNF5 plasmids and grown for 4 days. In the control experiment, the cells were plated on SC – Ura to select for the WT SNF2/SNF5 plasmids and grown for 4 days.
This experiment was performed exactly the same way as the experiment shown in Figure 7, the results, however, differ. It is evident that there is a suppression of the growth defect when both \textit{LEA1} and \textit{SNF2} are deleted. The results are surprising since Msl1/Lea1 is a heterodimer, and the proteins work together to bind the U2 snRNA (Caspary and Séraphin, 1998). When \textit{LEA1} deletion is combined with \textit{SNF5} deletion a synthetic growth defect is observed at 25°C, but at higher temperatures, genetic suppression is again observed. These data suggest that \textit{SWI/SNF} may have slightly different interactions with Msl1 and Lea1. Interestingly, our previous data showed that co-transcriptional association of Msl1 and Lea1 were slightly different in that a small amount of Msl1 associated in the 5’ region of the gene as measured by ChIP, (Gunderson \textit{et. al.}, 2009) perhaps via Lea1 independent interactions with the transcription machinery. Experiments to understand these differences between Msl1 and Lea1 are ongoing.
Chapter 2: Analysis of SWI/SNF interactions with the U2 snRNP

The functional interactions demonstrated in Chapter 1 led us to explore the possibility that the U2 snRNP interacted physically with SWI/SNF. Moreover, if SWI/SNF is an “adaptor,” we should detect interactions with the U2 snRNP.

Figure 9: Physical interactions between Snf5 and U2 components Msl1 and Lea1

Formaldehyde crosslinking was performed on cultures in which Snf5 was tagged with myc, and Msl1 (left) or Lea1 (right) were tagged with HA. The Immunoprecipitation was performed with Roche 12CA5 anti-HA antibodies diluted 1:250, and the western blot was probed with a 1:500 dilution of Upstate anti-myc clone 9E10 antibodies.

Cellular levels of Snf5-myc (Data not shown) as well as Lea1-HA and Msl1-HA (Gunderson and Johnson, 2009) were confirmed to remain consistent in the presence and absence of Gcn5 via western blotting. To evaluate whether the SWI/SNF complex interacts physically with the U2 snRNP, we performed a co-immunoprecipitation experiment. Formaldehyde crosslinking served to freeze all interactions within the cell. Next, the tagged Msl1/Lea1 proteins were immunoprecipitated with HA antibodies. Finally, the IP was run side by side with the input on a western blot which was probed with myc antibodies in order to detect any Snf5 protein that was physically interacting with our HA tagged proteins at the time of formaldehyde
crosslinking. A band was observed in the input lanes for the Msl1-HA experiment at higher exposures. Nonetheless, consistent with the genetic interaction demonstrated in figure 7A, there were also physical interactions between Snf5 and Msl1, as well as Snf5 and Lea1, as demonstrated by immunoprecipitation using strains containing tagged proteins. The physical interactions were at least partially independent of Gcn5, as when GCN5 was deleted, signal was still observed for the Myc-tagged Snf5 protein. This suggests that the SWI/SNF complex interacts with U2 but that this interaction does not depend on Gcn5’s acetylation of Snf2 (Jeong-Hoon et. al., 2010).

Furthermore, since Gcn5 is required for Snf2 to associate with chromatin, the interactions captured probably do not represent Snf2 on chromatin. These data are consistent with yeast two-hybrid results indicating a physical interaction between the U2 snRNP (Msl1) and the SWI/SNF. However we cannot rule out that the interactions are indirect and mediated through another protein/proteins.

In light of SWI/SNF co-immunoprecipitation with Msl1 and Lea1, two U2 snRNP proteins, we wanted to determine if these interactions represent functional snRNPs, so we decided to examine Snf2’s interaction with the U2 snRNA via IP coupled with primer extension. All in vivo interactions were frozen with formaldehyde crosslinking, then HA-tagged Snf2, a representative of the SWI/SNF complex, was immunoprecipitated along with all its associated proteins, RNA, and DNA. The associated RNA was purified and amplified using primers specific to each snRNA, which was run on a 6% urea gel along with input RNA, untagged WT cells, and each condition in the absence of formaldehyde crosslinking to serve as controls.
Figure 10: Snf2 physical interactions with the U1 and U2 snRNAs with and without formaldehyde crosslinking

Snf2-HA and WT (C) cells were grown to an OD600 of 0.5, and either treated with formaldehyde (+HCHO) or not treated (-HCHO). RNA was prepared and treated with DNase in the presence of RNase inhibitors. Snf2-HA was immunoprecipitated using Roche anti-HA antibodies and the associated RNA was purified. Finally, a primer extension was performed with an snRNA primer ladder with primers specific to each snRNA. The products were run on a 6% urea gel loaded from left to right, input RNA +HCHO, IP +HCHO, WT +HCHO, Input -HCHO, IP -HCHO, and WT -HCHO.
As anticipated, Snf2 immunoprecipitated with the U2 snRNA with and to a lesser extent, without crosslinking. Surprisingly, Snf2 was also shown to interact with the U1 snRNA. Both interactions are enhanced by crosslinking, however, even without crosslinking, both interactions are still detected, especially U1. These data suggest that Snf2 interacts with snRNPs involved in early steps of splicing. Perhaps Snf2 is involved in two independent steps of splicing, U1 mediated interaction with the 5’ splice site and U2 interaction with the branch-point. Alternatively, SWI/SNF may be the link bridging U1 binding and recruitment of U2.
Chapter 3: *SWI/SNF* affects co-transcriptional recruitment of U2 snRNP components Msl1 and Lea1

In order to determine if *SWI/SNF* affects Msl1/Lea1 function, we analyzed co-transcriptional recruitment of Msl1 and Lea1 to an intron-containing gene. To accomplish this, Msl1 and Lea1 were tagged with HA, and immunoprecipitated after formaldehyde crosslinking. The associated DNA was purified and amplified with primers specific to different regions of an intron-containing gene. Previous work has demonstrated that U2 recruitment is Gcn5 dependent, and occurs after the branchpoint is formed. Most of the U2 enrichment is found near the 5’ end of exon 2 (Gunderson and Johnson, 2009). If *SWI/SNF* is acting as an “adaptor,” it could be predicted that this U2 enrichment will also be Snf2 dependent.

To first determine if Snf2 was present within the body of the gene, chromatin IP was performed to analyze Snf2 occupancy in several intron-containing genes. Importantly, *SWI/SNF* was found to be enriched not only at the promoter, but was also observed in the body of genes (Pradhan and Johnson, data not shown).

In order to determine if *SWI/SNF* affects Msl1/Lea1 function, we analyzed co-transcriptional recruitment of Msl1 and Lea1 to an intron-containing gene. ChIP analysis was performed in the presence and absence of Snf2.
Figure 11: SNF2 deletion affects cotranscriptional recruitment of U2 snRNP components Msl1 and Lea1 on ECM33

Msl1-HA (left), and Lea1-HA (right) cells were grown to an OD600 of 0.6-0.8, then crosslinked with formaldehyde. Next, immunoprecipitation was performed with Roche anti-HA (12CA5) diluted 1:250. After the DNA crosslinked to the HA-tagged protein was isolated, it was amplified via real-time PCR using 6 sets of primers; specific for the promoter (1), exon 1 intron junction (2), intron exon 2 junction (3), 5’ region of exon 2 (4), middle of exon 2 (5), and non-transcribed region (not shown) of ECM33, a representative for intron-containing genes.

Differences in recruitment of the HA-tagged U2 proteins when the snf2Δ strain was compared to strains with a wild type copy of SNF2 are illustrated in figure 11. The ChIP signal for each primer along the gene was compared to the signal at a non-transcribed region of the gene. The results of this experiment show that when SNF2 is deleted, Msl1 and Lea1 enrichment is decreased on ECM33. While intriguing, this work is still preliminary, and additional trials are underway for proper error analysis.
In order to confirm that this paucity of Msl1, and especially Lea1, along the intron-containing gene ECM33 was not a transcriptional effect of deleting SNF2, ChIP was performed on DBP2 using Rbp3 as a proxy for RNA Polymerase II.

![Diagram of DBP2 gene structure](image)

**Figure 12:** Rpb3 distribution does not change upon SNF2 or SNF5 deletion on DBP2, an intron-containing gene

WT, snf2Δ, and snf5Δ strains of yeast were grown to an OD600 of 0.6-0.8, then treated with formaldehyde. Immunoprecipitation was performed by incubating 400 µL of precleared lysates with 2.5 µl of anti-Rpb3 antibody (Neoclon) for 2 h at 4°C on a rotator followed by 1 h of incubation with 80 µL of Gammabind G Sepharose beads. After the DNA crosslinked to Rbp3 was isolated, it was probed via real-time PCR using four different primer sets. Primers specific to the promoter were used, as well as 2 sets of primers within exon 1, denoted 1.1 and 1.2, and one primer set within exon 2.

This experiment shows that when components of SWI/SNF are deleted, RNA polymerase II occupancy does not significantly change on an intron-containing gene.

Experiments to examine Rbp3 occupancy on ECM33 are ongoing, however, the data
suggest the effects of Snf2 on spliceosome assembly are not strictly due to a change in transcription caused by deleting *SNF2*.

A putative model involves the *SWI/SNF* complex responding to Gcn5’s histone acetylation activity by binding the acetylated histones and performing a role in facilitating co-transcriptional splicing. The unexpectedly strong physical interaction with the U1 snRNA combined with the physical interactions with the U2 snRNA and U2 components Msl1 and Lea1 suggest that *SWI/SNF* may be bridging the relationship between U1 base pairing and U2 snRNP recruitment.

Figure 13: Adaptor protein model
This figure depicts one interpretation of the data suggested from this study.

Once H3 is acetylated and bound by SWI/SNF, it may interact with U1 in order to facilitate its recruitment and base pairing with the 5’ splice site. Alternatively, once U1 has bound the 5’ splice site, it could interact with SWI/SNF in order to bridge the gap between U1 and U2 base pairing. Perhaps both the presence of U1 on the nascent transcript and SWI/SNF interacting with the U2 snRNP are required for optimal U2 recruitment.
DISCUSSION

Here we present a model in which *SWI/SNF* interacts with U1 and U2, facilitating co-transcriptional spliceosome assembly, possibly through binding to histones acetylated by Gcn5. It remains unanswered whether *SWI/SNF* is acting directly to recruit the U2 snRNP to the transcript or if it is acting indirectly through another protein. Moreover, a possibility that cannot be excluded is that *SWI/SNF* interacts with the U1 snRNP to cause a conformational change in U1 in concert with or independent of base pairing of the U1 snRNA with the 5’ splice site. This putative conformational change in the U1 snRNP could facilitate U2 binding to the nascent transcript.

It is also possible that another protein or protein complex other than the U1 snRNP assists *SWI/SNF* in stabilizing U2 snRNP interaction with the nascent transcript sometime after U1 base pairs with the 5’ splice site. The *PRP19* complex is currently being investigated as possibly being involved in this process, due to the genetic interaction that was recently observed between *snt309Δ* and *snf5Δ* (Düring *et al.*, 2012). Mutants have been generated which are deleted for both *SNT309*, a *PRP19* complex component, and *SNF2*.

Of course, it is possible that *SWI/SNF* has a direct role in recruiting the U2 snRNP to nascent intron-containing pre-messenger RNAs, which is supported by the genetic and physical interactions *SWI/SNF* has with U2 components Msl1 and Lea1 and the ChIP experiment in figure 11 which demonstrates that co-transcriptional Msl1
and Lea1 recruitment to *ECM33* are reduced, drastically in the case of Lea1, when *SNF2* is deleted.

Any of these non mutually-exclusive models may be occurring independently of, or dependent on *SNF2* binding acetylated H3 via its bromodomain. A very important future experiment for this project will be to mutate *SNF2*, either by deleting its bromodomain or abrogating its binding activity. This will allow us to demonstrate whether the effects of deleting *SNF2* on co-transcriptional Msl1 and Lea1 recruitment are occurring due to *SWI/SNF* binding to acetylated H3. Another informative experiment will be to mutate the ATPase domain of *SNF2*. This will allow us to determine whether the hydrolysis of ATP is important for co-transcriptional recruitment of Msl1 and Lea1.

Overall, we present a novel discovery that *SWI/SNF* affects co-transcriptional spliceosome recruitment and dynamic rearrangements. Future directions for this project involve determining the mechanism by which *SWI/SNF* is facilitating co-transcriptional splicing. The interaction between Snf2 and the U1 snRNP demonstrated in the IP/primer extension experiment raises the important question of whether *SWI/SNF* recruits the U1 snRNP to the nascent transcript. This could be tested by co-IP experiments between *SWI/SNF* components and protein components of the U1 snRNP, such as Nam8 or Luc7. Another approach would be to ChIP the U1 components in the presence and absence of Snf2 on an intron-containing gene.

An additional outstanding question is what effect the *SWI/SNF* complex has on splicing genome-wide. RNA-seq will be an important first step toward understanding
how SWI/SNF affects intron retention and exon ligation genome wide. Additionally, ChIP-seq will be a valuable tool to visualize the genome wide occupancy of Snf2 and understand whether there are specific patterns on intron-containing genes. Furthermore, an important analysis will be to perform ChIP-seq to assess Msl1 and Lea1 co-transcriptional association in the presence and absence of Snf2 throughout the genome. This will reveal whether the effect seen in figure 11 is specific to ECM33, or whether deletion of SNF2 affects recruitment of Msl1/Lea1 across all intron-containing genes. Since a possible mechanism by which SWI/SNF could affect spliceosome assembly is through changes in the chromatin and alterations in transcription, it is important to evaluate Rpb3 occupancy across the genome in the presence and absence of Snf2 and compare this to genes in which Msl1/Lea1 occupancy changes. By comparing this profile to Rbp3 ChIP-seq in the snf2Δ, snf2 bromodomain deletion, and WT, we can correlate the transcriptional effects of Snf2 with its effects on co-transcriptional recruitment of the U2 snRNP.

The novel findings in this master’s thesis have illustrated that the SWI/SNF complex interacts with the spliceosomal machinery, facilitating co-transcriptional spliceosome assembly. This brings us one step closer to understanding splicing, a conserved process, defects in which cause many human diseases. Knowledge of the intricacies of splicing lays the foundation that will give direction to the research that could one day develop treatments for these debilitating diseases.
WORKS CITED


