Investigating Potential Regulators of the \textit{S. cerevisiae} Deacetylase, Sir2

By

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Chapter 1

Introduction

All the DNA in a cell is packaged into chromatin, a dynamic DNA-protein complex that enables fine-tuned genetic regulation (Kornberg, 1974). Through modulation of chromatin structure a cell is able to prevent some of its genome from producing RNA transcripts, a process referred to as transcriptional silencing. This repression is important to proper cellular function and regulates regions of the genome that are never expressed, such as the telomeres. Transcriptional silencing is also involved in processes such as cellular differentiation and environmental response (Margueron & Reinberg, 2010). Thus, understanding repression at the transcriptional level is essential to determining how cells produce stable, heritable phenotypes. The budding yeast, *S. cerevisiae*, provides a useful model for studying this process. Yeast has three major silenced regions: the telomeres, the mating type loci and the rDNA repeats (Nasmyth, 1982; Gottschling et al., 1990; Smith & Boeke, 1997). The NAD⁺-dependent histone deacetylase, Sir2, is responsible for establishing silencing at these locations (Imai et al., 2000). Prior to this study, the ribosomal proteins, Rpl22a and Rps7a, as well as the metabolic protein, Tdh3, were identified through a genetic screen as potential regulators of Sir2 (Matecic et al., 2002). Tdh3 was then characterized as a regulator of telomeric and rDNA silencing, as well as a Sir2 interactor (Ringel et al., 2013). In this study we investigate a potential role for these ribosomal proteins in Sir2-mediated silencing.
We use mutational analysis in order to investigate the mechanism by which Tdh3 regulates silencing. A potential role in silencing for the Tdh3 paralog, Tdh2 is also examined.

## 1.1 Chromatin Structure

In order to ensure proper regulation, replication, and organization within the nucleus, genetic material is packaged into chromatin (Kornberg, 1974). This is accomplished by wrapping 145-147 base pairs of DNA around a histone octomer called the nucleosome (Luger et al., 1997) forming an open “beads on a string” structure (Olins & Olins, 1974). The amino terminus of these histone proteins contains an unstructured region referred to as a tail which provides sites for post-translational modifications and is thought to interact with DNA as well as other nucleosomes (Luger et al., 1997). Nucleosome-nucleosome interactions then drive condensation to form higher order chromatin structure through mechanisms that are poorly understood (Margueron & Reinberg, 2010). A variety of different features characterize domains of chromatin within a given cell based on the regulatory needs of each particular region of DNA. These features include nucleosomal density, composition and modification state (Margueron & Reinberg, 2010). Different combinations of these features are associated with different regulatory outcomes. From the nucleosomal level up, a variety of specialized molecular machines work to create appropriate chromatin landscapes throughout the genome, thus establishing and maintaining the gene expression patterns necessary for proper cellular function.

The smallest unit of chromatin is the nucleosome, which is comprised of two copies of each histone: H2A, H2B, H3 and H4 (Margueron & Reinberg, 2010;
Luger et al., 1997). Dimers of histones H3 and H4 form a tetramer through interactions between each H3 protein. Dimers of H2A and H2B then attach to this tetramer to form the full nucleosome octomer through interactions between H2B and H4 (Luger et al., 1997). DNA is packaged into chromatin one 145-147 base pair loop at a time by wrapping around the nucleosome (Figure 1.1). Within this loop the alpha helical histone proteins are optimally positioned to interact positive side chains and helix dipoles with the negatively charged DNA phosphodiester backbone (Luger et al., 1997). The association between DNA and the nucleosome is strengthened by the amino terminal tails of histones H3 and H4 which have been observed to pass through the channels created by the DNA superhelix structure (Luger et al., 1997).

In addition to the crystal structure shown in Figure 1.1, which was produced using human histone proteins and DNA (Luger et al., 1997), the structure of the *S. cerevisiae* nucleosome has also been determined (White et al., 2001). The *S. cerevisiae* nucleosome crystal structure shares the same overall organizational features previously described in the human and *X. laevis* nucleosomes, suggesting that the mechanism by which nucleosomes package DNA is conserved throughout these organisms (White et al., 2001). However, closer inspection of the crystal structure indicates that several stabilizing histone-histone interactions observed in the human and *X. laevis* nucleosomes are not found in the *S. cerevisiae* nucleosome. This idea of a less stable *S. cerevisiae* nucleosome is consistent with previous studies suggesting that yeast chromatin is more open overall than other systems (White et al., 2001). Although simple organisms such as *S. cerevisiae* can be used as models from which we can begin to understand chromatin structure regulation and associated processes in a variety of organisms, it is also important to understand the particular characteristics of each organism.
Beyond the basic structure provided by the core nucleosome, chromatin architecture can very greatly. Different features associated with different regions of chromatin are classified into two main types: euchromatin and heterochromatin (Clapier & Cairns, 2009; Kouzarides, 2007; Margueron & Reinberg, 2010). While euchromatin is set up to be accessible to transcriptional machinery, heterochromatin is generally more compact and consequently more difficult to access (Kouzarides, 2007; Margueron & Reinberg, 2010). These different conditions are established through organization or modification of histones, as well as the addition of alternative histone proteins.
One of the main ways in which a cell can modulate chromatin structure is through directly repositioning nucleosomes. This work is done by proteins called chromatin remodelers, which all share ATP-dependent function (Clapier & Cairns, 2009). The activity of chromatin remodelers can be associated with both activation and repression. One key way in which chromatin remodelers promote transcription is through establishment of the nucleosome free region upstream of the transcriptional start site (Clapier & Cairns, 2009). Conversely, chromatin remodelers can also contribute to transcriptional repression through promoting increased compaction of chromatin structure. There are four main families of chromatin remodelers: SWI/SNF, ISWI, CHD and INO80 (Clapier & Cairns, 2009). Each family is characterized by conserved domains important to remodeling function or targeting (Clapier & Cairns, 2009).

There are several other structural proteins, in addition to the core nucleosome, that contribute to the formation of chromatin. A fifth histone, histone H1, often called the linker histone, has been found to bind DNA at the nucleosome entry and exit sites (Izzo et al., 2008). Histone H1 is often thought to have a stabilizing effect on the nucleosome and is usually associated with chromatin folding (Izzo et al., 2008). Variant forms of the core histones also exist. For example, the protein H2A.Z can replace canonical H2A in the nucleosome. H2A.Z is inserted into nucleosomes by the INO80 related chromatin remodeler, SWR1 (Clapier & Cairns, 2009). This replacement has been associated with an increase in gene expression; H2A.Z containing nucleosomes are often observed flanking the transcriptional start site (Kouzarides, 2007).

The third major control feature of chromatin is post-translational modifications. The unstructured, amino-terminal tails of histones provide multiple sites for the addition of different chemical groups or small modifying proteins. Histone
modifications have the ability to affect chromatin structure directly through promoting or obstructing histone-histone or histone-DNA interactions (Kouzarides, 2007). These marks can also have an indirect effect on chromatin structure by recruiting protein complexes such as chromatin remodelers or histone modifiers (Zentner & Henikoff, 2013). Histone modifications range from the addition of small chemical groups such as acetylation, methylation, phosphorylation, glycosylation, or ADP-ribosylation (Zentner & Henikoff, 2013) to the addition of much larger modifications. For example, in the processes of ubiquitination and sumoylation the modifier which is added is actually a small protein (Kouzarides, 2007). Post-translational modifications can also involve facilitation of a reaction within an existing group, as is the case during deimination and proline isomerization (Kouzarides, 2007). Some modifications are associated with fairly predictable outcomes. Acetylation, for example, has been shown to destabilize chromatin and lead to an increase in transcription (Kouzarides, 2007). Other modifications, such as methylation, can lead to variety of different outcomes depending on the context (Kouzarides, 2007). Additionally, many regions of chromatin contain several different modifications. The varied and combinatorial nature of post-translational modifications makes them a key method by which chromatin is able to perform its role as a dynamic structure.

Changes in chromatin structure lead to changes in expression state in order to best serve the current needs of the cell. These needs can vary depending upon the metabolic state of the cell and its surrounding environment. Thus, processes must exist that link chromatin structure to the metabolic state of the cell in order to appropriately change expression patterns. Histones have been proposed to be metabolic sensors due to the fact that they are modified by chemical groups that are derived from metabolites and are deposited by enzymes that use metabolites
1. Introduction

(Katada et al., 2012). For example, in mammals histone acetylation has been shown to be dependent upon the activity of ATP-citrate lyase, a metabolic enzyme responsible for converting citrate into acetyl-CoA (Wellen et al., 2009). This dependency on metabolites serves to connect processes that have been previously thought of as distinct: chromatin structure and metabolism.

Although the addition of histone modifications has a wide variety of important affects on chromatin structure, the removal of these marks has an equally important role to play in this process. The group of proteins responsible for removing acetylation marks from histone tails, called histone deacetylases (HDACs), have long been characterized as key proteins in the establishment of heterochromatin (Kouzarides, 2007; Zentner & Henikoff, 2013; Katada et al., 2012). Acetylation marks are found on the lysine residues of histone tails, where they serve to destabilize chromatin (Kouzarides, 2007). This destabilizing effect has been attributed to acetylation’s ability to neutralize the positively charged lysines, consequently decreasing a histone’s affinity for DNA (Zentner & Henikoff, 2013). It follows that the removal of acetyl groups would lead to increased stabilization of chromatin, and indeed hypoacetylation has been observed to be associated with regions of chromatin that are silenced (Braunstein et al., 1993) while hyperacetylation is enriched at actively transcribed regions (Zentner & Henikoff, 2013).

1.2 S. cerevisiae histone deacetylase, Sir2

The S. cerevisiae protein, Sir2, is an NAD$^+$-dependent histone deacetylase and founding member of the highly conserved sirtuin family, found in both prokaryotes and eukaryotes (Imai et al., 2000; Frye, 1999; Martinez-Redondo & Vaquero, 2013). Bacterial and archaeal organisms usually have one or two proteins in the
Sirtuins have been shown to modulate DNA regulatory proteins as well as metabolic proteins in these types of organisms (Yuan & Marmorstein, 2012). Eukaryotes typically possess several different and specialized sirtuins. In addition to the eponymous Sir2, budding yeast possesses four other sirtuins: Hst1-4 (Yuan & Marmorstein, 2012). Humans have even more sirtuins; these proteins are referred to as SIRT1-7 (Martinez-Redondo & Vaquero, 2013). Although yeast Sir2 is primarily characterized as a histone deacetylase involved in transcriptional silencing, sirtuins have been found to perform a variety of activities. A weak ADP-ribosyltransferase activity has been observed in yeast sirtuins and appears to be the main function of SIRT4 in humans (Martinez-Redondo & Vaquero, 2013). Sirtuins also use their deacetylase activity in processes other than transcriptional silencing: SIRT6, Hst3 and Hst4 promote DNA damage repair through deacetylation of H3K56 (Martinez-Redondo & Vaquero, 2013). Additionally, SIRT5 has been shown to be directly involved in metabolism and is found in the mitochondria (Martinez-Redondo & Vaquero, 2013). Both prokaryotic and eukaryotic sirtuins operate at the interface of genetic regulation and metabolism, suggesting they have an evolutionarily conserved role as metabolic sensors (Yuan & Marmorstein, 2012).

Sirtuins are characterized as class III histone deacetylases, a class unique in its requirement for NAD\(^+\) (Borra et al., 2004). Deacetylation is not a redox reaction, therefore the use of a valuable redox cofactor by Sir2 is noteworthy. It has been hypothesized that the NAD\(^+\)-dependency of Sir2 functions to link its activity to the metabolic state of the cell (Denu, 2003). Sirtuins share a conserved catalytic core domain of approximately 250 amino acids, which is flanked in eukaryotes by variable N and C terminal regions (Martinez-Redondo & Vaquero, 2013). Analysis of several sirtuin crystal structures has revealed key structural similarities
within the catalytic core. Sirtuins bind NAD\(^+\) in a large Rossmann fold which is

Figure 1.2: Sir2 is shown here in complex with a portion of Sir4, one of its binding partners. The proteins were crystalized in the presence of NAD\(^+\), however in the crystal ADP ribose is bound in the NAD\(^+\)-binding pocket. It is likely that the nicotinamide group was hydrolyzed during crystallization (Hsu et al., 2013).

linked to a smaller domain containing helical and zinc-binding modules (Yuan & Marmorstein, 2012). The linking loops between these domains form a cleft where the substrate meets NAD\(^+\) and undergoes catalysis (Yuan & Marmorstein, 2012). Yeast Sir2 contains a unique N-terminal helical domain, which is responsible for interacting with binding partners (Hsu et al., 2013). A crystal structure of yeast Sir2 in complex with a portion of Sir4, one of its binding partners, is shown in Figure 1.2.
Sir2 has long been implicated in transcriptional silencing however the mechanism through which it affects chromatin structure proved difficult to elucidate. Some of the first studies of the enzymatic activity of Sir2 identified Sir2 as having weak ADP-ribosyltransferase activity (Tanny et al., 1999; Frye, 1999). However it was unclear how this ADP-ribosyltransferase activity could contribute to transcriptional repression. Additionally, Sir2 has been observed to promote hypoacetylation at silenced regions (Braunstein et al., 1993), indicating that there is more to Sir2’s role in transcriptional silencing than simply ADP-ribosylation.

The idea of Sir2 as an ADP-ribosyltransferase was disrupted when Imai et al. (2000) determined that Sir2 can act as a histone deacetylase. Sir2 was observed to remove acetyl groups from lysines on histones H3 and H4 in an NAD$^+$-dependent manner in vitro. These two different Sir2 activities were reconciled when Tanny & Moazed (2001) proposed a mechanism by which Sir2 would be able to perform both activities. Over the course of a deacetylation reaction performed by Sir2, the release of nicotinamide followed by acetyl-ADP-ribose was observed (Tanny & Moazed, 2001). These observations, along with additional biochemical analyses, lead to the promotion of a mechanism whereby Sir2 uses cleavage of the high energy ribose-nicotinamide bond to drive transfer of an acetyl group from the protein substrate to the ADP-ribose generated by NAD$^+$ breakdown (Tanny & Moazed, 2001). This model necessitates an intermediate step where ADP-ribose is bound to the acetyl group on the protein substrate. The authors propose that under certain circumstances the molecule could be allowed to stay in this conformation, thus accounting for Sir’s observed weak ADP-ribosyltransferase activity (Tanny & Moazed, 2001). This model of Sir2 enzymatic activity has been supported by further study and a proposed mechanism is shown in Figure 1.3.

Sir2 was originally identified as a deacetylase of histones H3 and H4 (Imai
Lysines 9 and 14 of histone H3 and 5, 8, 12 and 16 of histone H4 have been observed to be acetylated \textit{in vivo} (Csordas, 1990). Borra et al. (2004) tested the preference of Sir2 for these different acetylated residues \textit{in vitro}. They determined that Sir2 has the strongest affinity for acetylated H4K16 and the weakest affinity for H4K12 (Borra et al., 2004). These results are consistent with previous observations of hypoacetylation patterns in heterochromatin (Braunstein et al., 1993). However, sirtuins are not limited to deacetylating histones. The first evidence of a nonhistone sirtuin substrate was found by Vaziri et al. (2001), who showed that the human homolog of Sir2, SIRT1, plays a role in DNA-damage induced apoptosis through deacetylation of the protein p53. Like deacetylation of histones, this activity was found to be NAD\textsuperscript{+}-dependent (Vaziri et al., 2001). Since this discovery many more nonhistone substrates of mammalian sirtuins have

**Figure 1.3:** Proposed mechanism of Sir2 NAD\textsuperscript{+}-dependent deacetylation activity (Denu, 2003).
been found (Martinez-Redondo & Vaquero, 2013). Finding nonhistone substrates in *S. cerevisiae* proved to be more difficult, however, two recent studies suggest that yeast Sir2 also has the ability to deacetylate nonhistone proteins (Lin et al., 2009; Downey et al., 2013). Sir2 has been shown to deacetylate both the gluconeogenesis protein Pck1 (Lin et al., 2009) and a transcription factor that regulates ribosomal protein genes called Ifh1 (Downey et al., 2013). Not only do these findings expand the role of the yeast Sir2 protein but they also link the function of Sir2 to processes outside of heterochromatin formation, especially processes associated with metabolism.

### 1.3 Sir2-Mediated Silencing

In budding yeast the telomeres and mating type loci are transcriptionally silenced; meaning that despite the presence of functional genes at the mating type loci and proximal to the telomeres, production of RNA transcripts from these regions is attenuated (Rine & Herskowitz, 1987; Gottschling et al., 1990). Analysis of the chromatin at these regions has revealed that they are hypoacetylated when compared to transcriptionally active regions of chromatin (Braunstein et al., 1993; Suka et al., 2001). However when silencing was perturbed by knockout of *SIR2*, acetylation of these regions was found to be comparable to that found at transcriptionally active regions (Braunstein et al., 1993). Conversely, higher levels of Sir2p lead to an observed decrease in histone acetylation (Braunstein et al., 1993). This phenomenon of hypoacetylation was therefore linked both to transcriptional silencing and the activity of Sir2. Deacetylated lysines allow histone proteins to more strongly interact with the negatively-charged DNA backbone and have been observed to be associated with heterochromatin in a variety of
situations (Kouzarides, 2007; Zentner & Henikoff, 2013). In the case of the silenced telomeres and mating type loci in *S. cerevisiae*, heterochromatin is formed through histone deacetylation by Sir2 (Grunstein, 1998).

The *SIR2* gene was originally identified as a member of a group of genes found to be necessary for proper mating type silencing through several genetic screens (Haber & George, 1979; Klar et al., 1979; Rine et al., 1979). The other genes in this group are called *SIR1, SIR3* and *SIR4* and they give rise to protein products that work together with Sir2 to establish silencing at the mating type loci and telomeres (Nasmyth, 1982; Aparicio et al., 1991). The interaction between these proteins and the importance of these interactions to proper silencing has been extensively studied. Biochemical assays indicate that Sir2 and Sir4 form a complex that dynamically associates with Sir3 (Moazed et al., 1997; Hoppe et al., 2002). Sir3 and Sir4 have also been shown to interact both with each other and with the tails of histones H3 and H4 (Hecht et al., 1995, 1996). The final Sir protein, Sir1 is involved in establishment of the silenced state at the mating type loci and is thought to play a stabilizing role in the assembly of the Sir protein complex (Rusche et al., 2002). Sir1 has not been observed to participate in telomeric silencing (Aparicio et al., 1991).

The Sir proteins work together to change the chromatin state at target locations, leading to transcriptional silencing. In the case of mating type silencing, heterochromatin formation is initiated at regions of the DNA near the mating type loci called the E and I silencers (Abraham et al., 1984; Feldman et al., 1984). The silencers consist of binding sites for the DNA-binding proteins, ORC, Rap1 and Abf1 (Buchman et al., 1988; Diffley & Stillman, 1988). Binding of these proteins to the silencers allows subsequent recruitment of the Sir proteins. Sir4 binds to Rap1 independent of the other Sir proteins, which leads to the subsequent recruit-
ment of Sir2 and Sir3 (Hoppe et al., 2002; Rusche et al., 2002). ORC provides a binding site for Sir1 (Triolo & Sternglanz, 1996) which helps to stabilize the SIR complex assembly. Sir2 then establishes silencing at that initial region through deacetylation and then silences the rest of the region through sequential spreading with Sir3 and Sir4, while Sir1 stays confined to the silencer (Rusche et al., 2002). Sir3 prefers binding to deacetylated histone tails and thus drives spreading in a manner dependent upon the activity of Sir2 (Liou et al., 2005; Rusche et al., 2002). Under this model, after Sir2 deacetylates the histone tails of the nearest nucleosome Sir3 binds these deacetylated tails and the SIR complex assembles on this nucleosome (Rusche et al., 2002). This then allows Sir2 to deacetylate the neighboring nucleosome and the process continues, leading to silencing of the entire region (Rusche et al., 2002). This spreading process is depicted in Figure 1.4:

**Figure 1.4:** In this model the SIR complex is formed through association of Sir3 with a Sir2/Sir4 complex. This complex binds silenced chromatin, allowing Sir2 to deacetylate adjacent active chromatin. Deacetylation leads to changes in chromatin structure through Sir3 binding and subsequent SIR complex spreading (Liou et al., 2005).
Silencing at the telomere is also thought to spread through this type of sequential mechanism (Luo et al., 2002; Hoppe et al., 2002). In this case, silencing is initiated solely by Rap1 (Luo et al., 2002). Rap1 binds the telomeric repeats, which contain the same binding motif found in the silencers of the mating type loci. As at the mating type loci, Sir4 binds Rap1 leading to the recruitment of Sir2 and Sir3 (Luo et al., 2002). Once recruited to the telomere, the SIR complex spreads to silence the telomere as well as the region within approximately 5 kb of the telomere (Gottschling et al., 1990).

Sir2 also operates independently of the other Sir proteins to mediate silencing at an additional locus in the yeast genome: the rDNA repeats (Smith & Boeke, 1997). The ribosomal DNA is a highly repetitive region localized to the nucleolus (Smith & Boeke, 1997). Reporter genes inserted at the rDNA locus were found to be partially silenced in a Sir2-dependent manner (Smith & Boeke, 1997). In addition to promoting silencing, Sir2 has been found to be necessary for repression of homologous recombination at this highly repetitive locus (Gottlieb & Esposito, 1989). Despite being Sir2-dependent, silencing at the rDNA does not require Sir3 or Sir4 (Smith & Boeke, 1997). Instead of functioning as part of the SIR complex like it does at the telomeres and mating type loci, Sir2 operates at the rDNA with a different complex, termed RENT (Straight et al., 1999). The protein, Net1, is important to proper nucleolar formation and was identified as a potential member of this complex due to its specific interaction with Sir2 (Straight et al., 1999). Net1 localizes specifically to the rDNA and is necessary for rDNA silencing (Straight et al., 1999). The phosphatase, Cdc14, associates with Net1 and Sir2 in the RENT complex and is also found specifically at the rDNA locus (Shou et al., 1999; Straight et al., 1999). This complex, like the SIR complex at the mating type
loci and telomeres, serves to hypoacetylate the rDNA locus and modify chromatin structure (Hoppe et al., 2002).

1.4 Sir2 and Metabolism

The activity of Sir2 at the rDNA has an impact that extends beyond simply ensuring proper regulation and stability of the region. This process has been implicated in control of the yeast replicative lifespan, which is measured as the number of times a yeast cell buds off a daughter before it senesces (Wierman & Smith, 2014). Mutations in Sir2 have been shown to decrease replicative lifespan while increasing rDNA recombination (Kaeberlein et al., 1999). Conversely, an increase in Sir2 expression stabilizes the rDNA and promotes longevity (Kaeberlein et al., 1999). Recombination at the rDNA leads to the release of circular DNA from the region, referred to as extrachromosomal rDNA circles or ERCs (Sinclair & Guarente, 1997). These rDNA fragments have been shown to preferentially segregate to the mother cell during budding and thus accumulate over the course of a yeast lifespan (Sinclair & Guarente, 1997). Researchers have also used a plasmid based system to induce ERC production and found that ERCs decrease lifespan (Sinclair & Guarente, 1997). However, a more recent study found that rDNA instability in general is more closely correlated with replicative lifespan reduction than the actual number of ERCs produced (Ganley et al., 2009). Regardless, it is clear that Sir2 is necessary to repress rDNA recombination and maintain a normal lifespan in yeast.

Reparative lifespan can also be extended through calorie restriction, accomplished in yeast by reducing the amount of glucose present in the growth media (Wierman & Smith, 2014). Sir2 has been found to be necessary for low levels of
calorie restriction to increase lifespan, however this requirement can be overcome by increasing calorie restriction (Wierman & Smith, 2014). Calorie restriction induces stress on the cell and affects many metabolic and signaling pathways as a result (Wierman & Smith, 2014). The NAD$^+$ salvage pathway, which reproduces previously broken down NAD$^+$, is one of the two pathways used to make NAD$^+$ in yeast cells and has been shown to be affected by calorie restriction (Kato & Lin, 2014; Medvedik et al., 2007). Specifically, calorie restriction leads to an increase in the NAD$^+$ salvage enzyme, Pnc1, through activation of transcription factors Msn2/4 (Medvedik et al., 2007). Sir2 produces nicotinamidase as a result of deacetylation which can be salvaged by Pnc1 (Tanny & Moazed, 2001; Kato & Lin, 2014). Pnc1 is responsible for converting nicotinamidase to nicotinic acid. Npt1 then converts nicotinic acid to nicotinic acid mononucleotide which feeds back into the de novo pathway to produce NAD$^+$ (Kato & Lin, 2014). High levels of these proteins have been shown to stimulate Sir2 activity and increase lifespan (Anderson et al., 2002) indicating that they link Sir2 to calorie restriction.

It seems as though Sir2 has the ability to act as a metabolic sensor; one popular model suggests that Sir2 is activated by an increase in the NAD$^+$/NADH ratio (Wierman & Smith, 2014). This idea is supported by observations that increasing the NAD$^+$/NADH ratio extends lifespan while decreasing the ratio does not (Wierman & Smith, 2014). However changes in the NAD$^+$/NADH ratio do not appear to affect Sir2 activity in vitro (Anderson et al., 2003). It has been suggested, therefore, that Sir2 activation through calorie restriction has more to do with flux through the NAD$^+$ salvage pathway than changes in overall NAD$^+$ or NADH levels (Anderson et al., 2002). Although further study is necessary in order to fully elucidate this mechanism, the activity of Sir2 certainly appears to be linked to NAD$^+$ production and availability.
1.5 Screen for Regulators of Sir2

Previous members of the Holmes laboratory were interested in learning more about the regulation of Sir2 and its associated silencing processes. To do this they took advantage of the fact that strong overexpression of $SIR2$ has a toxic effect on the cell (Holmes et al., 1997). This toxicity is likely the result of chromosome instability, as high levels of Sir2 have been shown to increase chromosomal loss (Holmes et al., 1997). This lethal phenotype provides a useful basis for a genetic screen. Matecic et al. (2002) screened for overexpression suppressors of this phenotype in the hopes of identifying potential negative regulators or substrates of Sir2. Overexpression of a negative regulator of Sir2 could be predicted to rescue lethality through restoration of wild type Sir2 regulation. Another possibility is that a substrate of Sir2 could titrate Sir2 away from its lethality causing activity, thus restoring cellular viability. Matecic et al. (2002) conducted this screen using plasmid born $SIR2$ and a random yeast gene on an additional plasmid. Both genes were driven to overexpress by galactose-inducible promoters. Figure 1.5 shows that overexpression of $SUR2$, $HHF2$, $RPS7A$, $RPL22A$ and $TDH3$ was able to rescue the lethal phenotype observed when $SIR2$ alone was overexpressed. $HHF2$ codes for histone H4, a known substrate of Sir2. $RPS7A$ and $RPL22A$ both code for ribosomal proteins, while $TDH3$ gives rise to a glycolytic protein. This observation connecting ribosomal and glycolytic proteins to Sir2 was novel and may be part of the larger story connecting Sir2 and metabolism.
1. Introduction

Figure 1.5: While the vector lane (overexpressed SIR2 only) does not grow well in the presence of galactose, additional overexpression of SUR2, HHF2, RPS7A, RPL22A or TDH3 restores wild type growth (Matecic et al., 2002).

1.6 S. cerevisiae Glyceraldehyde-3-Phosphate Dehydrogenase

The gene TDH3, identified as a suppressor of SIR2 overexpression induced lethality, codes for one of the three S. cerevisiae glyceraldehyde-3-phosphate dehydrogenase (GAPDH) enzymes (McAlister & Holland, 1985b). GAPDH catalyzes the conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate during glycolysis (Seidler, 2013). GAPDH uses NAD$^+$ as a redox cofactor in order to accomplish this reaction, producing NADH as a byproduct (Seidler, 2013). Studies have suggested that GAPDH functions as a homotetramer, with all subunits involved during catalysis (McAlister & Holland, 1985a; Seidler, 2013). A prediction of what such a tetramer of Tdh3 would look like is shown in Figure
1.6. Beyond its canonical function in glycolysis, GAPDH has been implicated in other processes, including gene expression regulation, oxidative stress response and apoptosis (Sirover, 2011).

![Figure 1.6: Crystals of Tdh3 were made from purified tetrameric protein. The structure was resolved for a single subunit. A tetrameric biological assembly was then predicted and shown above (Liu et al., 2012).](image)

*Figure 1.6:* Crystals of Tdh3 were made from purified tetrameric protein. The structure was resolved for a single subunit. A tetrameric biological assembly was then predicted and shown above (Liu et al., 2012).

*S. cerevisiae* contains three genes that code for GAPDH: *TDH1*, *TDH2* and *TDH3* (McAlister & Holland, 1985a,b). Mutational analysis of these genes has suggested that Tdh2 and Tdh3 perform the majority of glycolytic activity in the cell as evidenced by the fact that cells lacking functional *TDH1* have wild type growth, an indicator of normal glycolysis (McAlister & Holland, 1985b). However, cells lacking both functional *TDH2* and *TDH3* are inviable, suggesting that at
least one version of GAPDH is necessary for glycolysis to proceed (McAlister & Holland, 1985b). \(TDH3\) and \(TDH2\) are very similar with 96% sequence identity. Analysis of the expression of these different genes has suggested that Tdh1 is specifically expressed during stationary phase (Boucherie et al., 1995). Although under most circumstances \(TDH2\) and \(TDH3\) are similarly expressed, the presence of Tdh2p but not Tdh3p is decreased during heat shock (Boucherie et al., 1995). This differential stress response appears to be due to differential modification of these enzymes (Grant et al., 1999). When oxidative stress was induced through addition of \(H_2O_2\), Tdh3p was found to be S-thiolated and recovered enzymatic activity after the stress. However, Tdh2p was not modified and thus subject to irreversible oxidation, preventing recovery of activity (Grant et al., 1999). Despite their high sequence similarity and metabolic redundancy, Tdh3 and Tdh2 appear to play different roles under some circumstances.

1.7 The GAPDH-Sir2 Connection

After identification of Tdh3 as a potential Sir2 regulator, the role of Tdh3 in Sir2-mediated silencing was investigated. Knockout of \(TDH3\) was found to decrease silencing at the telomeres and rDNA, as well as increase rDNA recombination (Ringel et al., 2013). A less extreme decrease in telomeric silencing was observed in cells lacking \(TDH2\) (Ringel et al., 2013). A role for Tdh3 at the mating type loci was also suggested; overexpression of \(TDH3\) led to an increase in silencing at this region (Ringel et al., 2013). In addition to these results, Tdh3 was found to interact with Sir2 through two-hybrid and co-immunoprecipitation assays as well as associate with silenced chromatin in a Sir2-dependent manner (Ringel et al., 2013). Thus it seems clear that Tdh3 has the ability to promote silenc-
ing, likely through interaction with Sir2. Mutational analysis of TDH3 suggested that the catalytic activity of Tdh3 as a glycolytic enzyme is at least partially uncoupled from this observed silencing activity (Ringel et al., 2013). However, we believe Tdh3’s ability to bind NAD$^+$ is important; we have evidence to suggest that Tdh3 regulates nuclear NAD$^+$ levels (Ringel et al., 2013). Based on these findings we have proposed that Tdh3 promotes silencing through a separate moonlighting function that involves controlling the access of Sir2 to NAD$^+$. An interaction in yeast between Sir2 and Tdh3 has been suggested by other groups as well. Ralser et al. (2009) observed a genetic interaction between Sir2 and the three yeast GAPDH genes based on plasmid recombination. This interaction was shown to be dependent upon the catalytic activity of GAPDH (Ralser et al., 2009). Additionally, Sir2 and Tdh3 were observed through whole proteome mass spectrometry to be in the same complex (Gavin et al., 2002).

Homologs of these two proteins have also been linked in mammalian studies. Mammalian GAPDH and the Sir2 homolog, SIRT1, both appear to be involved in an oxidative stress signaling pathway (Tristan et al., 2011). Like yeast Tdh3, mammalian GAPDH is S-nitrosylated in response to oxidative stress (Tristan et al., 2011). This modification leads GAPDH to associate with the protein Siah which is responsible for translocating GAPDH into the nucleus (Tristan et al., 2011). Once there, GAPDH initiates a signal cascade that can lead to apoptosis through the nitrosylation of nuclear proteins, including SIRT1 (Tristan et al., 2011; Kornberg et al., 2010). GAPDH and SIRT1 were further linked through the finding that SIRT1 levels control GAPDH nuclear localization (Joo et al., 2012). This study demonstrated an interaction between the two proteins through coimmunoprecipitation (Joo et al., 2012). Another connection between GAPDH and the sirtuin family has been found in a recent mammalian study where nuclearly
localized GAPDH was found to activate PARP1 through a physical interaction both in vitro and in vivo (Nakajima et al., 2015). The PARP family of poly ADP-ribose polymerases has been suggested to be related to the sirtuin family due to their related mechanisms and catalytic domains (Martinez-Redondo & Vaquero, 2013). These results suggest that GAPDH serves to connect sirtuins, as well as sirtuin related proteins, to metabolism.

1.8 Questions Addressed in this Study

In this study we hope to further understand Sir2-mediated silencing through investigation of potential Sir2 regulators. The ribosomal proteins Rpl22a and Rps7a were identified through a genetic screen for overexpression suppressors of Sir2-induced lethality (Matecic et al., 2002). This finding suggests that Rpl22a and Rps7a could be involved in Sir2 related processes. Do these proteins play a role in Sir2-mediated silencing? Rpl22a and Rps7a could suppress Sir2-induced lethality through a physical interaction with Sir2. Is such an interaction observed between either of these proteins and Sir2? Through answering these questions we can begin to understand why Rpl22a and Rps7a were identified in the genetic screen as well as potentially expand our knowledge of Sir2 regulation.

We now know that Tdh3 is a Sir2 interacting factor with a positive effect on Sir2-mediated silencing (Ringel et al., 2013). In order to learn more about the silencing role of Tdh3 we will strive to determine the nature of the Sir2-Tdh3 interaction. We have suggested that Tdh3 promotes Sir2 activity by controlling nuclear NAD$^+$ levels in a manner separate from its role in glycolysis. However, the mechanism by which Tdh3 is able to regulate Sir2 activity is not clearly understood. Past mutational analysis has suggested that the role of Tdh3 in
silencing is at least partially uncoupled from its glycolytic role (Ringel et al., 2013). In order to further understand the GAPDH-Sir2 connection in yeast it is necessary to distinguish the glycolytic and silencing functions of Tdh3 through mutational analysis. Although Tdh3 and Tdh2 have mostly redundant glycolytic function (McAlister & Holland, 1985a) they do not appear to have the same silencing function (Ringel et al., 2013). Investigation into the different contributions to silencing of these two proteins will also help us to understand the unique silencing function of Tdh3, as well as add to our knowledge of Tdh2’s role in this process.
Chapter 2

Materials and Methods

2.1 Strains and Plasmids

Strains used in this study are listed in Table 2.1. Gene knockouts were created through PCR-mediated gene deletion as described in Wach et al. (1994) using either MX series plasmids (Goldstein & McCusker, 1999) or DNA derived from strains in the Yeast Knockout Collection (Open Biosystems YSC1053). C-terminal epitope tags were added through PCR-mediated fragment addition at the 3’ end of the gene (Knop et al., 1999). Sir2 was FLAG tagged through gap repair into pKAM1, using a fragment derived from pJR2659 (Table 2.2).

Mutations were introduced into the \textit{TDH3} gene through hybrid PCR (Horton et al., 1989). We used CRISPR-Cas for allele replacement at the endogenous \textit{TDH3} locus (DiCarlo et al., 2013). A guide RNA targeted Cas9 to the KanMX sequence and the hybrid PCR product was used as the rescue fragment.

Two-hybrid experiments were performed using strains expressing Sir2, Rpl22a or Rps7a fused to either the activating or binding domain and an additional vector either containing Sir2, Rpl22a or Rps7a fused to the opposite domain or an empty vector control (Table 2.2). An additional control strain containing both empty vectors was also used. These strains were constructed via gap repair in the YSH625 strain background lacking all endogenous Sir genes. Gap repair was performed
as described in Uetz et al. (2000), however Epindorf tubes were used in place of 96-well plates. A strain containing \textit{SIR2-BD} as well as several empty vector controls were previously created by Hannah Stubbs.

**Table 2.1:** Strains used in this study

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## 2. Materials and Methods

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Table 2.2: Plasmids used in this study

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Table 2.3: Primers used in this study

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<td>To create <em>TDH3</em> alleles.</td>
</tr>
<tr>
<td>SP591</td>
<td>ACGGGCACAACCTCAATG</td>
<td>To check for the presence of <em>TDH3</em> and create a fragment for sequencing.</td>
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<tr>
<td>SP599</td>
<td>CTACCGCTACTGAGTAAATG</td>
<td>For knockout of <em>RPL22A</em> using deletion strain as template.</td>
</tr>
<tr>
<td>SP600</td>
<td>TGAACGGGCAATTGAGCCC</td>
<td>For knockout of <em>RPL22A</em> using deletion strain as template.</td>
</tr>
<tr>
<td>SP601</td>
<td>TCGACGTTCCATTGCCTG</td>
<td>To check knockout of <em>RPL22a</em>.</td>
</tr>
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<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Usage</th>
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| SP602  | ACCCCACCAAACCCAAAAAA  
         | AGAGATCGAATTCCAGCTGAC  
         | CACCATGGCCCCCAAACGTAT  
         | GATAC | To create \textit{RPL22A-AD} gap repair fragment. |
| SP603  | CTACGATTCATAGATCTCTG  
         | CAGGTCGACGGATCCCCGGG  
         | AATTGCCATTTATTCTTCGT  
         | CTTCTTCTTC | To create \textit{RPL22A-AD} gap repair fragment. |
| SP604  | CCAATCACTTTGTTCGCG | For knockout of \textit{RPS7A} using deletion strain as template. |
| SP605  | TCACCTAGAAAGCCACCC | For knockout of \textit{RPS7A} using deletion strain as template. |
| SP606  | GAGAGAACGCCGAATGTAC | To check knockout of \textit{RPS7A}. |
| SP607  | ACCCCACCAAACCCAAAAAA  
         | AGAGATCGAATTCCAGCTGA  
         | CCACCATGTCTGCTCCACAA  
         | GCCAAG | To create \textit{RPS7A-AD} gap repair fragment. |
| SP608  | CTACGATTTCATAGATCTCTG  
         | CAGGTCGACGGATCCCCGGG  
         | AATTGCCATCTAATGAGTTT  
         | CACTTGGAA | To create \textit{RPS7A-AD} gap repair fragment. |
### Materials and Methods

<table>
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<tr>
<th>Primer</th>
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<td>SP617</td>
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<td>To check for the presence of <em>TDH3</em>.</td>
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<td>SP618</td>
<td>GGTCTCAACTCAGCTTTCA</td>
<td>To check for the presence of <em>RPS7A</em>.</td>
</tr>
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<td>SP620</td>
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<td>To check for the presence of <em>RPL22A</em>.</td>
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<td>SP638</td>
<td>ATCGTTATGTCCCGTGTACC</td>
<td>For ChIP qPCR, <em>ACT1</em>.</td>
</tr>
<tr>
<td>SP639</td>
<td>TGGAAGATGGAGGCAAGGC</td>
<td>For ChIP qPCR, <em>ACT1</em>.</td>
</tr>
<tr>
<td>SP877</td>
<td>ATTTGTCTCCAAGTCTTCGAC</td>
<td>To check for the presence of <em>TDH3-myc</em>.</td>
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<td>SP886</td>
<td>TTGTAAATGACGAGCATATCGGTG</td>
<td>For ChIP qPCR, telomere of chromosome V.</td>
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<tr>
<td>SP887</td>
<td>CGTCAACAGTTCTAAATTTCGGGT</td>
<td>For ChIP qPCR, telomere of chromosome V.</td>
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| SP895  | ATAAAAAGAAAAATTATTTAA  
AATGCAAGATTTAAGTAAA  
TTCACATCGATGAATTCGAGCTCG | To myc tag *TDH3*. |
| SP1032 | CAGGACATGCGCTTTGATATG | For ChIP qPCR, *NTS1*. |
| SP1033 | CGCCGCGTCGCCAAAAATT | For ChIP qPCR, *NTS1*. |
| SP1087 | GCCTGGAGTAAATGATG | To sequence *TDH3* alleles. |
| SP1089 | GCTTTTCAATCAATGAATCG | To sequence *TDH3* alleles. |
| SP1113 | GCGAAAAAGACAAGAAC | To create a *TDH3* fragment for sequencing. |
### 2. Materials and Methods

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<tr>
<td>SP1123</td>
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<td>To myc tag <em>TDH3</em>.</td>
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<td>SP1171</td>
<td>GAATTGCAAGGTAAGTTGGC CGGTATGGGCTTTTC</td>
<td>To create <em>tdh3-T227A</em>.</td>
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<tr>
<td>SP1172</td>
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<td>To sequence <em>TDH3</em> alleles.</td>
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<td>SP1331</td>
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<td>To create <em>tdh3-T227K</em>.</td>
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<td>SP1332</td>
<td>GAAAGCCATACCTTTCAACT TACCTTGGAATTC</td>
<td>To create <em>tdh3-T227K</em>.</td>
</tr>
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<td>SP1371</td>
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</tr>
<tr>
<td>SP1372</td>
<td>TGTAAATTGATATTAATTTG GCACTTTTAAAATTATTAAT TGCCTTCTACCTTAGAAAAC TCATCGAGCA</td>
<td>To create SIR2-FLAG gap repair fragment.</td>
</tr>
<tr>
<td>SP1564</td>
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<td>To create <em>tdh3-S22Q</em>.</td>
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<td>SP1565</td>
<td>CAACCTCGACGTTTGGTCTTT TGCAAAGCAATTCTCATGAC</td>
<td>To create <em>tdh3-S22Q</em>.</td>
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2. Materials and Methods

<table>
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<th>Sequence</th>
<th>Usage</th>
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| SP1566 | GAGAATTGCTTTTGCTAGAA
          AAAACGTCGAAGTTGTTGCT | To create tdh3-P24K. |
| SP1567 | AGCAACAACTTCGACGTTTT
          TTCTAGACAAAGCAATTCTC | To create tdh3-P24K. |
| SP1568 | TCGAAGTTGTTGCTTTGAAC
          GGTCATTCATCACCACGA | To create tdh3-D33G. |
| SP1569 | TCGTTGGTGATGATTGGACC
          GTTCAAAGGAACAACGCTTTGCA | To create tdh3-D33G. |
| SP1570 | CTCAAAAGACTGTTGAGGGCT
          TCTTCCACCAAGGACTGAG | To create tdh3-P189S. |
| SP1571 | CTCCAGTCTCTTGTGGAAGA
          ACCGTCACAGCTTTTGGAG | To create tdh3-P189S. |

2.2 GAPDH Activity Assay

Cells to be lysed were grown in a 2mL overnight culture. This culture was then diluted 1:20 in appropriate media and allowed to grow for 4 more hours. A 10mL aliquot of this culture was washed twice in 5mL of filter sterilized and chilled PBS. The cells were lysed in 200µL PBS by bead beating at maximum speed 3 times, for 2 minutes each. Lysate was separated from the beads and the soluble fraction was subsequently cleared through centrifugation at 14,000 RPM for 1 minute. Bradford assay was used to determine the protein concentration using the following equation: 

\[
\text{[Concentration protein mg/mL]} = \frac{\text{OD}_{595}}{0.066\times\mu L \text{ sample added to cuvette}}
\]  
(Ringel, 2009).
GAPDH activity was measured as the rate of NADH production. NADH has an absorbance peak at 340nm that NAD$^+$ does not, which allowed the increase in NADH concentration produced as a result of GAPDH activity to be tracked by determining the absorbance at 340nm. The assay was conducted using 10µg of protein lysate in a 30mM sodium pyrophosphate buffer containing 12mM sodium arsenate and 1mM NAD$^+$ (Ralser et al., 2007). The GAPDH reaction was started by adding 10µL of 40mM glyceraldehyde-3-phosphate (Ralser et al., 2007). The OD$_{340}$ was recorded every 15 seconds for 4 minutes. The recorded absorbances were used to calculate NADH concentration at each time point using Beer’s Law (Absorbance = extinction coefficient × path length × concentration); $6.22 \times 10^3 M^{-1} cm^{-1}$ was used as the extinction coefficient for NADH. These NADH concentrations were plotted versus time and the slope of the linear portion was calculated. The slopes calculated for the experimental strains were normalized to that of wild type in order to produce the final values reported.

2.3 *S. cerevisiae* Fractionation

Whole cell, cytoplasmic and nuclear protein fractions were separated as described by Keogh et al. (2006) with the exception that 50mL cultures with an optical density at 600nm of approximately 0.8 were used. Care was taken to ensure that all cultures used were at a similar optical density when lysed. Before western blot analysis samples were boiled for 5 minutes. Insoluble material was then removed from the whole cell and nuclear fractions through a 10 second spin at 14,000 RPM. A Bradford assay was performed and samples were diluted to an equal optical density in water. Diluted samples were loaded onto 10% acrylamide gels and run at 45 Volts constant for two hours and then increased to 90 Volts
constant for 30 minutes. They were then transferred to nitrocellulose membranes for 1 hour at 0.03 Amps, blocked in 5% nonfat dry milk and probed overnight in 1:1,000 anti-myc (Cell Signaling 2276) or 1:500 anti-histone H3 (Abcam 1791). Goat anti-mouse IgG-HRP (SC2005) or goat anti-rabbit IgG-HRP (SC2004) secondary was used at a concentration of 1:1,000 and applied for one hour. Blots were treated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific 34087) immediately prior to imaging in a SynGene G:Box.

2.4 Fluorescence Size Exclusion Chromatography

Proteins lysates were prepared as described by Rudner et al. (2005). Lysates were run on a Superdex 200 10/300 GL column (GE 17-5175-01). An RF-10AXL fluorescence detector from Shimadzu was used for detection and data was collected using UNICORN 5.31 software (GE).

2.5 Native Gel Analysis

Protein lysates were prepared as described by Rudner et al. (2005) with the exception that 50mL cultures with an optical density at 600nm of approximately 0.8 were used. Care was taken to ensure that all cultures used were at a similar optical density when lysed. An equal volume of 2X sample buffer (0.625 mM Tris pH 6.8, 4% glycerol, 0.02% bromophenol blue) was added to all samples before a Bradford assay was used to normalize protein concentrations (Institute of Molecular Development LLC, 2001). Non-denaturing gels (7.5% acrylamide) were purchase from Bio-Rad (456-1026S). Gels were pre-run at 4°C at 75 Volts
constant for 30 minutes. The samples were loaded and run for 1 hour at 75 Volts constant and then increased to 90 volts for 30 minutes. Transfer and anti-myc blotting was performed as previously described.

2.6 Chromatin Immunoprecipitation

Chromatin immunoprecipitation was performed as described by Martins-Taylor et al. (2011). Myc-tagged proteins were precipitated using 1µL anti-myc (Cell Signaling 2276). Serial dilutions of input, precipitated and mock precipitated DNA were used as templates for quantitative PCR reactions, primers used are listed in Table 2.3. PCR products were run on 8% acrylamide gels, and stained with SYBR Gold dye. The gels were scanned with TYPHOON 9400 (GE Healthcare) using the 520nm filter. Each band was quantified using ImageQuant TL (GE Healthcare) and averages of values in the dynamic range were used for subsequent calculations.

2.7 Co-immunoprecipitation

Protein lysates were prepared as described in Rudner et al. (2005). Myc-tagged proteins were purified from 50µL of lysate by incubation overnight in 100µL anti-myc gel (Sigma A7470). GFP-tagged proteins were purified from 50µL of lysate by incubation overnight in 1 µL anti-GFP (20µL/mL Abcam ab290) and subsequent 1 hour incubation in Protein G magnetic beads (NEB S1430S). Samples were loaded onto 10% acrylamide gels. Transfer and blotting were performed as previously described. In addition to anti-myc blotting, anti-FLAG (F1804), anti-GFP (Roche 11814460001) and anti-acetyl lysine (Cell Signaling 9681) antibodies were used.
with goat anti-mouse IgG-HRP secondary (SC2005).
Chapter 3

Results

3.1 Does Rpl22a or Rps7a affect silencing?

The discovery of Rpl22a and Rps7a as overexpression suppressors of Sir2-induced lethality indicated that these proteins could be involved in Sir2-mediated silencing (Matecic et al., 2002). To investigate this possibility further, the *RPL22A* and *RPS7A* genes were removed in a strain containing reporter genes at the *HMR* mating type locus and adjacent the telomere of chromosome V. If the *TRP1* gene, placed at the *HMR* locus, is expressed the cells will be able to grow in the absence of tryptophan. The second panel in Figure 3.1 shows that the ∆*sir2* cells grow more than wild type on media lacking tryptophan (SDC-trp). This is because loss of Sir2 has eliminated silencing at the mating type locus and thus increased expression of *TRP1*. In contrast, the ∆*rpl22a* cells grow less than wild type on SDC-trp. This indicates that *TRP1* is less expressed in these cells and therefore loss of Rpl22a leads to an increase in silencing at the mating type loci. The ∆*rps7a* cells grow similarly to wild type on SDC-trp, suggesting that Rps7a has no effect on silencing at this locus.

Telomeric silencing was also assayed in these strain backgrounds by tracking expression of the *URA3* gene. Expression of *URA3* can be counter-selected for by the use of media containing 5-fluoroorotic acid (FOA). If cells are expressing *URA3*
3. Results

Figure 3.1: Loss of Rpl22a increases silencing. Serial dilutions (1:10) of strains containing a TRP1 reporter at the HMR mating type locus and a URA3 reporter adjacent to a telomere are plated on several different types of media. The first panel shows equal growth on SDC, a complete media containing all necessary nutrients. The second panel shows growth on plates lacking tryptophan, and the third shows growth on media containing 5-fluoroorotic acid (FOA). Growth assays were repeated a minimum of three times.

and performing uracil biosynthesis, the presence of FOA will have a toxic effect. This can be observed in the third panel of Figure 3.1 where cells lacking Sir2 grow much less than wild type due to the loss of telomeric silencing. Again the ∆rpl22a cells show the opposite result, growing more than wild type. This result suggests that at the telomeres as well as the mating type loci, loss of Rpl22a leads to an increase in silencing. The ∆rps7a cells grow similar to wild type on FOA media, indicating that Rps7a does not affect silencing at the telomeres. Taken together these results indicate a mechanism where Rpl22a functions to negatively regulate Sir2-mediated silencing. This idea is consistent with our previous hypothesis that overexpression suppressors of Sir2-induced lethality could function as negative regulators of Sir2 activity. Rpl22a could function to modulate Sir2 through direct interaction or indirectly by affecting other Sir2 regulators.
3.2 Does Rpl22a or Rps7a interact with Sir2?

Rpl22a and Rps7a have both been previously observed to genetically interact with Sir2 (Matecic et al., 2002) and we have now shown that Rpl22a inhibits silencing. We were interested in the possibility that either of these proteins physically interacts with Sir2. We chose to assess this possibility using a two-hybrid assay. In a two-hybrid assay, one protein of interest is fused to a DNA binding domain that binds upstream of a reporter gene, in this case HIS3. The other protein of interest is fused to an activating domain, which if brought near to the gene promoter through an interaction between the two proteins will activate expression of the reporter gene. This process is illustrated in Figure 3.2. We performed these experiments in the YSH625 strain background, which lacks all of the SIR genes. This choice was made because if present, the Sir proteins will repress the reporter, preventing the observation of an interaction (Hickman et al., 2007; Ringel et al., 2013).

The two-hybrid experiments were performed both with Sir2 fused to the activating domain and to the binding domain. In both cases no interaction was observed between either Rpl22a or Rps7a and Sir2. The experimental strains, containing both fusion proteins, did not grow significantly more on media lacking histidine than the strains containing empty vector controls. This indicates HIS3 expression is not significantly activated by the presence of these fusion proteins, and an interaction is not occurring. Interestingly, the strain containing Rps7a-BD and empty pOAD grew significantly more on media lacking histidine than the other strained assayed. This suggests that when Rps7a is tethered to the HIS3 promoter it has the ability to promote transcription in some way. Our results do not suggest that either protein physically interacts with Sir2. However, two-
hybrid assays have a high rate of false negative results therefore it is possible that Rpl22a or Rps7a could interact with Sir2 but we are not detecting this interaction through this assay. In particular Rpl22a and Rps7a could be substrates of Sir2, because enzyme-substrate interactions have proved difficult to detect via two-hybrid. Alternatively, Rpl22a and Rps7a could affect Sir2 through an indirect mechanism either related to or separate from their function in the ribosome.

![Diagram showing protein interactions](image)

**Figure 3.2:** No interaction between Rpl22a or Rps7a and Sir2 has been observed via two hybrid. A schematic shows activation of *HIS3* through an interaction between proteins of interest. Serial dilutions of pOBD and pOAD containing strains are plated on media lacking tryptophan and leucine to select for the presence of both plasmids (left panels). In the right panels activation of the *HIS3* gene was assessed through plating on media that additionally lacks histidine. Growth assays were repeated a minimum of three times.
3.3 Is Tdh2 involved in silencing?

After identification as an overexpression suppressor of Sir2-induced lethality, Tdh3 was determined to promote Sir2-mediated silencing (Matecic et al., 2002; Ringel et al., 2013). Tdh3 has been characterized as a Sir2-interacting factor that localizes to the rDNA repeats and telomeres in a Sir2-dependent manner (Ringel et al., 2013). Mutational analysis has suggested that the silencing function of Tdh3 is at least partially uncoupled from its catalytic activity (Ringel et al., 2013). In order to build upon these results, we were interested in determining the silencing role of the closely related GAPDH isozyme, Tdh2. A strain background containing a *URA3* reporter gene integrated at the rDNA locus was used to assay silencing.

![Figure 3.3: Tdh3 and Tdh2 promote rDNA silencing.](image)

Serial dilutions of cells containing *URA3* at the rDNA are plated on complete (SDC) and 5-fluoroorotic acid (FOA) containing media. Growth assays were repeated a minimum of three times.
at this region (Figure 3.3). The \( \Delta tdh3 \) cells grew less than wild type on FOA, indicating a loss of silencing consistent with previous observations (Ringel et al., 2013). The \( \Delta tdh2 \) cells, grew only slightly less than wild type, suggesting that \( Tdh2 \) also promotes silencing at this locus. The differential growth patterns of \( \Delta tdh3 \) and \( \Delta tdh2 \) observed on FOA suggest that \( Tdh3 \) promotes rDNA silencing more strongly than \( Tdh2 \).

![Figure 3.4](image)

**Figure 3.4: Tdh3 promotes telomeric silencing.** Serial dilutions of cells containing \( URA3 \) adjacent the telomere of chromosome V are plated on complete (SDC) and 5-fluoroorotic acid (FOA) containing media. Growth assays were repeated a minimum of three times.

Using a strain background with \( URA3 \) integrated adjacent a telomere, cells lacking \( Tdh3 \) were again observed to grow less than wild type on FOA (Figure 3.4). This result is consistent with previous observations that \( \Delta tdh3 \) cells lose telomeric silencing (Ringel et al., 2013). In Figure 3.4, the \( \Delta tdh2 \) cells are shown to have comparable growth to wild type on FOA, suggesting that \( Tdh2 \) does not affect silencing at the telomeres. However, RT-PCR analysis of an endogenous telomere proximal gene by a previous lab member showed that removal of \( Tdh2 \) leads to a small increase in the expression of this gene (Ryznar, 2014). It therefore seems
likely that Tdh2 contributes to telomeric silencing but this contribution is small enough that a knockout phenotype is not observed in this nutritional reporter gene assay. Taken together these results suggest that although both Tdh3 and Tdh2 contribute to Sir2-mediated silencing, the contribution of Tdh3 is larger.

### 3.4 Investigating the difference between Tdh3 and Tdh2

![Figure 3.5: Alignment of Tdh3 and Tdh2.](image)

The two amino acid positions of note are boxed in red.

Tdh3 and Tdh2 only differ by twelve amino acids and appear to be redundant in terms of their metabolic function. However these very similar proteins have been shown to contribute differently to silencing in Figures 3.3 and 3.4, as well as in previous work by Ryznar (2014). Of these twelve differing amino acids most are conservative substitutions. However, the 22nd and 24th amino acids reveal two interesting differences between Tdh3 and Tdh2. The amino acid sequences of Tdh3 and Tdh2 were compared in a CLUSTALW alignment (Figure 3.5). At the 24th position (shown in the right red box in Figure 3.5) Tdh3 has a nonpolar and neutral proline while Tdh2 has a polar and basic lysine. Another notable difference between these two proteins is the fact that Tdh3 has been observed to be phosphorylated significantly more than Tdh2 (Tyers & Sadowski, 2015). One of the phosphorylated residues in Tdh3 is serine 22 (shown in the left red box of Figure 3.5). Tdh2 has a glutamine at this position which cannot undergo...
phosphorylation. These two amino acid differences are potential sources of Tdh3 and Tdh2’s differential silencing function.

![URA3](G_{1-3} \text{T Telomere Repeats})

**Figure 3.6:** S22Q and P24K mutants have the opposite effect on telomeric silencing. Serial dilutions of cells containing *URA3* adjacent the telomere of chromosome V are plated on complete (SDC) and 5-fluoroorotic acid (FOA) containing media. Growth assays were repeated a minimum of three times.

In order to determine the significance of these two amino acid differences to silencing function, we created point mutations in *TDH3* to change these residues to those found in Tdh2. The effect on silencing of these mutations was then assayed using the previously described telomeric *URA3* reporter strain. In Figure 3.6, growth of the *TDH3-S22Q* mutant is shown to be less than that of wild type when grown on FOA, indicating that this mutation induces a decrease in silencing at the telomere. Contrastingly, the *TDH3-P24K* mutant grows more on FOA than wild type, indicating this form of Tdh3 promotes an increase in telomeric silencing. Our results show that the 22nd amino acid of Tdh3 is necessary for wild type silencing at the telomere, while substitution of a lysine at the 24th residue
has the ability to enhance silencing.

![Graph showing GAPDH activity comparison](image)

**Figure 3.7: P24K mutant reduces GAPDH activity.** The rate of NADH production of each sample was normalized to wild type (shown in blue). The values shown here are the average of four independent experiments, error bars show one standard deviation above and below the average. $p<0.02$

Next, the GAPDH activity of strains containing the S22Q and P24K alleles was tested. Figure 3.7 shows the GAPDH activity of cells lacking Tdh3 is significantly lower than that of wild type cells. The GAPDH activity of cells containing the S22Q mutation is not significantly different than that of wild type cells. This result indicates that the S22Q mutation does not affect the ability of Tdh3 to perform its catalytic function. Thus the previously observed decrease in telomeric silencing is not a result of disruption of catalytic activity. $TDH3-P24K$ cells were observed to have GAPDH activity significantly less than wild type, though not as reduced as cells lacking Tdh3. Interestingly, GAPDH activity and silencing
activity appear to be inversely related in $TDH3-P24K$ cells.

### 3.5 What is the contribution of Tdh3’s NAD$^+$ usage to silencing?

Both Sir2 and Tdh3 use NAD$^+$ in order to perform their enzymatic function. Previous lab members observed that $\Delta tdh3$ cells have lower than wild type levels of nuclear NAD$^+$ (Ringel et al., 2013). Therefore we have hypothesized that the mechanism by which Tdh3 contributes to Sir2-mediated silencing is through controlling Sir2’s access to NAD$^+$. A version of Tdh3 that utilizes NADP$^+$ instead of NAD$^+$ would allow us to test this hypothesis. Bommareddy et al. (2014) have created several distinct mutations in *C. glutamicum* GAPDH in order to bias the protein towards using NADP$^+$ over NAD$^+$. They replaced the 35$^{th}$ aspartic acid with glycine in order to create more room in the NAD$^+$ binding pocket for the larger NADP$^+$ (Bommareddy et al., 2014). This mutation was found to create a version of GAPDH which uses both NAD$^+$ and NADP$^+$ sub-optimally (Bommareddy et al., 2014). The authors then optimized this NADP$^+$-dependent GAPDH activity through additional substitutions: L36T, T37K and P192S. The serine substitution at position 192 was chosen because this mutation is commonly observed in naturally occurring NADP$^+$-dependent GAPDH enzymes (Bommareddy et al., 2014). D35 and P192 are highly conserved residues and correspond to the 33$^{rd}$ and 189$^{th}$ residues in *S. cerevisiae*. We were interested in creating an NADP$^+$-dependent version of Tdh3 and decided to start with these two single mutations.

The telomeric silencing effect of these mutations was observed using the pre-
Figure 3.8: **D33G and P189S mutants reduce telomeric silencing.** Serial dilutions of cells containing *URA3* adjacent the telomere of chromosome V are plated on complete (SDC) and 5-fluoroorotic acid (FOA) containing media. Growth assays were repeated a minimum of three times.

Previously described *URA3* reporter strain. The D33G mutant grew significantly less than wild type on FOA, indicating a decrease in silencing (Figure 3.8). Cells containing this allele of Tdh3 grew even less than cells lacking Tdh3 altogether. This suggests that having the D33G form of Tdh3 is worse for silencing than no Tdh3 at all. The P189S mutant also grew less than wild type on FOA but this phenotype was not as that observed for the D33G mutant. The results of this silencing assay suggest that presence of both of these Tdh3 alleles leads to a decrease in silencing at the telomeres.

Next, we inquired whether these mutants were able to perform canonical NAD$^+$-dependent GAPDH activity. In Figure 3.9 the D33G mutant is shown to reduce NAD$^+$-dependent GAPDH activity to a level comparable to that found in cells lacking Tdh3. This result suggests that the D33G mutant does not con-
tribute to overall NAD\(^+\)-dependent GAPDH activity at all. In contrast, the P189S mutant does not reduce NAD\(^+\)-dependent GAPDH activity; the rate of NADH production observed in these cells is not significantly different from wild type. These results suggest that Tdh3-P189S retains the ability to reduce NAD\(^+\) while Tdh3-D33G does not. Additional experiments will need to be performed in order to determine whether these forms of Tdh3 have gained the ability to reduce NADP\(^+\). However, at this point we can conclude that the decrease in silencing observed in \textit{TDH3-P189S} cells is not a result of compromised catalytic activity.

![Figure 3.9: D33G mutant reduces GAPDH activity.](image)

The rate of NADH production of each sample was normalized to wild type (shown in blue). The values shown here are the average of four independent experiments, error bars show one standard deviation above and below the average. \(p<0.01\)
3.6 What is the oligomeric state of the T227K and T227A mutants?

Past mutational analysis by Ringel et al. (2013) revealed an interesting pair of Tdh3 alleles. TDH3-T227K cells were observed to have decreased GAPDH activity and wild type silencing while TDH3-T227A cells had wild type GAPDH activity and decreased silencing. These mutations were made based on a study of rat GAPDH which suggested the T227K mutation produces a monomeric form of GAPDH that accumulates in the nucleus (Park et al., 2009). Conversely, GAPDH-T227A was found to be tetrameric and predominantly localized to the cytoplasm (Park et al., 2009). We were interested in determining whether these mutations have the same effect in yeast. We decided to use fluorescence size exclusion chromatography (FSEC) to determine the oligomeric state of wild type Tdh3-GFP in order to start answering this question. Size exclusion chromatography allowed us to separate proteins in their native conformation by size, giving us insight into their oligomeric state. FSEC is a specific type of size exclusion chromatography that does not require purified protein. Instead we were able to track the elution of our protein of interesting using a fluorescent tag.

Figure 3.10 shows the elution profile of protein from TDH3-GFP cells. The green curve represents detection of GFP fluorescence and shows a peak at 14.3mL. This indicates that Tdh3-GFP elutes when proteins around 60kD are expected to elute, suggesting that Tdh3-GFP is monomeric. This is a surprising result because the catalytically active form of Tdh3 has been well characterized as tetrameric (Seidler, 2013). Thus we would expect monomeric Tdh3-GFP to be catalytically inactive but TDH3-GFP cells were shown to have wild type GAPDH activity by Ringel et al. (2013). It is possible that this result is an artifact to the FSEC
**Figure 3.10: Tdh3-GFP is monomeric.** Measurement of GFP fluorescence is shown in green. UV detection, which tracks the elution of all the molecules in the sample, is shown in blue.

Experiment, perhaps Tdh3-GFP travels through the column differently than expected. As an alternative to FSEC, we used native gel analysis to compare the oligomeric state of Tdh3-myc, Tdh3-T227K-myc and Tdh3-T227A-myc. Western blotting allowed us to observe the banding pattern of these tagged proteins that occurs when samples are separated under non-denaturing conditions. However, the banding patterns observed were inconsistent from experiment to experiment, allowing no conclusion to be made thus far.
3.7 Is Tdh3 acetylated?

Previous work has identified a physical interaction between Tdh3 and Sir2 (Ringel et al., 2013). We were interested in determining the nature of this interaction. It is possible that the Sir2-Tdh3 interaction is an enzyme-substrate interaction. In order to be deacetylated by Sir2, Tdh3 would have to first be acetylated. We decided to test for Tdh3 acetylation using a pan-acetyl lysine antibody. Whole cell extracts and immunopurified Tdh3-GFP was used for these experiments. Smc3-GFP was used as positive control because it is known to be acetylated. In Figure 3.11, bands the size of Tdh3-GFP can be observed in lanes 3 and 6 of the anti-GFP blot, indicating that Tdh3-GFP can be detected in both the whole cell extract (lane 3) and immunoprecipitated sample (lane 6). Tdh3-GFP was not observed in the anti-acetyl lysine blot. Smc3-GFP was not observed in either blot. Despite the lack of a good positive control, our results suggest

![Figure 3.11: Acetylated Tdh3 cannot be detected via western blot. Lane 1: untagged whole cell extract, 2: Smc3-GFP whole cell extract, 3: Tdh3-GFP whole cell extract, 4: untagged mock IP, 5: Smc3-GFP IP, 6: Tdh3-GFP IP](image)

that acetylation of Tdh3 cannot be detected through this method. Several other
known acetylated proteins were used as potential positive controls in subsequent experiments but their presence was not detected by the anti-acetyl lysine antibody either. As an alternative approach, we also used this antibody to look at the acetylation pattern of crude whole cell lysates from $TDH3$-$GFP$ and untagged cells. Bands were detected, indicating this antibody is functional but no band shift consistent with the addition of a GFP tag to Tdh3 was observed. Our inability to detect a band shift supports the idea that Tdh3 is not acetylated.

3.8 What part of Sir2 is responsible for Tdh3 interaction?

We were also interested in determining what part of the Sir2 protein is necessary for interaction with Tdh3. To do this we decided to utilize previously created N-terminally truncated forms of Sir2 (Hickman et al., 2007). However, the antibody to native Sir2 used by Ringel et al. (2013) to detect co-precipitation of Sir2 with Tdh3 only has the ability to detect Sir2 containing an intact amino terminus. We, therefore, created a FLAG tagged version of wild type Sir2. However, Sir2-FLAG was not able to co-precipitate with Tdh3. Figure 3.12 shows that Tdh3-myc can be detected in the immunoprecipitated sample but Sir2-FLAG cannot. Sir2-FLAG was detected in the whole cell extract, therefore the failure to detect this protein in the immunoprecipitated sample suggests that the presence of the FLAG tag disrupts the previously observed interaction between these two proteins.
Figure 3.12: Sir2-FLAG does not co-precipitate with Tdh3. Lane 1: untagged whole cell extract, 2: Tdh3-myc, Sir2-FLAG whole cell extract, 3: untagged mock IP, 4: Tdh3-myc, Sir2-FLAG IP.
Chapter 4

Discussion

4.1 Sir2 and Ribosomal Proteins

We have identified the ribosomal protein, Rpl22a, as a potential negative regulator of Sir2-mediated silencing. After previous lab members identified Rpl22a and Rps7a as overexpression suppressors of Sir2-induced lethality, we found that knockout of $RPL22A$ leads to an increase in silencing at the telomeres and mating type loci. However, silencing at these regions was not observed to be affected by loss of Rps7a. No physical interaction was observed between Rpl22a or Rps7a and Sir2 using a two-hybrid assay. These results suggest that the genetic interaction observed between the ribosomal proteins, Rpl22a and Rps7a, and Sir2 was not the result of a physical interaction. However, the possibility that these proteins are substrates of Sir2 should be investigated because two-hybrid often does not detect enzyme-substrate interactions. The results of our silencing assay suggest a role for Rpl22a in Sir2-mediated silencing. It is possible that Rpl22a could affect silencing through a novel function outside of its role in the ribosome. However, we must also consider the possibility that knockout of $RPL22A$ increases silencing through changes in translation of various chromatin factors. Future work on this project should include a comparison of Sir2 levels in wild type and $\Delta rpl22a$ cells, in addition to analysis of expression levels of other chromatin modifying proteins.
Both Rpl22a and Rps7a have paralogs, Rpl22b and Rps7b, that arose from the yeast whole genome duplication. It is interesting that neither pair gene was identified in our genetic screen for overexpression suppressors of Sir2-induced lethality. A previous lab member found that knockout of \textit{RPS7B} lead to a decrease in silencing at the mating type loci and telomeres, while knockout of \textit{RPL22B} does not affect silencing (Jacob Musinsky, unpublished). These results, as well as the fact that these genes did not appear in our previous genetic screen, suggest that these closely related pair genes have differential silencing function. Differential function of ribosomal pair genes has also been observed in studies of bud site selection (Ni & Snyder, 2001). Cells lacking Rpl22a or Rps7a were found to have a random budding pattern but those lacking the paralogs Rpl22b or Rps7b did not display a significant budding pattern phenotype (Ni & Snyder, 2001). These results suggest that the duplicated ribosomal proteins may play different roles in a variety of processes.

In their recent study, Downey et al. (2013) found that Sir2 deacetylates the ribosomal protein transcription factor, Ifh1, further connecting Sir2 and ribosomal proteins. Interestingly, Ifh1 has previously been linked to Sir2-mediated silencing. Singer et al. (1998) found that overexpression of Ifh1 decreases silencing at the telomeres, mating type loci, and rDNA repeats. These findings can help to explain our results because overexpression of Ifh1 also leads to overexpression of ribosomal proteins, including Rpl22a and Rps7a (Schawalder et al., 2004). We have evidence to suggest that overexpression of Rpl22a or Rps7a can inhibit Sir2 activity. It is possible that Ifh1 overexpression decreases silencing by increasing the expression of ribosomal proteins that negatively regulate Sir2 activity. This effect could either be specific to Rpl22a and Rps7a or a general effect of increasing ribosomal protein expression with Rpl22a and Rps7a the only ribosomal proteins capable
of producing a noticeable phenotype on their own. This theory suggests that absence of this negative regulation would increase Sir2 activity, consistent with our observation of increased silencing in ∆rpl22a cells. The idea of this negative regulation as a general effect by ribosomal proteins would suggest that we do not see a silencing phenotype in ∆rps7a cells because removal of Rps7a alone is not sufficient to decrease this negative regulation. Additionally, reduction of Ifh1 function has been shown to extend replicative lifespan (Cai et al., 2013), which is also associated with increased Sir2 activity.

Taken together, these data suggest that Sir2 is involved in and affected by ribosomal protein production. Ribosomal protein production is one of two major pathways that contribute to the formation of ribosomes. Sir2 is also linked to the second pathway, rRNA transcription, through its role at the rDNA locus. Ribosomal protein production and rRNA transcription are highly regulated and closely related processes that are sensitive to the metabolic state of the cell (Lempiäinen & Shore, 2009). It therefore makes sense that the activity of Sir2, a metabolic sensor, would be related to these processes. Although much more needs to be learned about the relationship between Sir2 and ribosomal protein production, this connection is certainly part of the larger story of Sir2 and metabolism that we and others are studying.

4.2 Sir2 and GAPDH

We have added to previous evidence to identify Tdh2 as a positive silencing factor. We observed a decrease in silencing at the rDNA locus when TDH2 was knocked out. Ryznar (2014) has also observed an increase in rDNA recombination in cells lacking Tdh2. These results indicate that Tdh2 positively contributes to
4. Discussion

Sir2-mediated activity at this locus. We were unable to observe a telomeric silencing phenotype for cells lacking Tdh2 using a nutritional reporter assay. However, Ryznar (2014) has used RT-PCR to observe an increase in the transcription of an endogenous telomere proximal gene when $TDH2$ is knocked out. This suggests that Tdh2 also positively contributes to silencing at the telomeres but this effect cannot be observed using our telomeric $URA3$ reporter strain.

Tdh2, like the closely related GAPDH isozyme Tdh3, is involved in Sir2-mediated silencing at the rDNA repeats and telomeres. However, our results suggest that Tdh2 does not contribute to this process as strongly as Tdh3. We have used mutational analysis to begin determining the source of this difference. Study of the Tdh3 and Tdh2 amino acid sequences revealed two significant substitutions. At the 22$^{nd}$ position Tdh3 was found to have a phosphorylation site not present in Tdh2. Mutation of this residue in Tdh3 to that found in Tdh2 created the $TDH3-S22Q$ allele, which was found to cause a decrease in silencing at the telomeres. Cells containing this allele were observed to perform wild type levels of GAPDH activity, demonstrating that this silencing defect is not the result of compromised catalytic activity. Our results indicate that the presence of a serine at the 22$^{nd}$ position of Tdh3 is necessary for proper silencing function. This residue has been found to be subject to phosphorylation (Tyers & Sadowski, 2015), suggesting that post-translational modifications may be important to the silencing difference between Tdh3 and Tdh2.

Tdh3 and Tdh2 have also been found to be differentially modified during oxidative stress. Cells treated with $H_2O_2$ protect Tdh3 from irreversible oxidation through S-thiolation but allow Tdh2 to become oxidized (Grant et al., 1999). S-thiolation allows Tdh3 to recover catalytic activity after an oxidative stress challenge, ensuring survival of the cell (Grant et al., 1999). The failure to modify...
Tdh2 is also essential to cell survival. When a cell is continuously treated with low levels of an oxidant, Tdh3 will be inactivated by S-thiolation but some unmodified Tdh2 will avoid oxidation, continuing glycolysis and allowing the cell to survive (Grant et al., 1999). If H$_2$O$_2$ is used to induce apoptosis the cells undergo nitric oxide stress which leads to the S-nitrosylation of Tdh3 but not Tdh2 (Almeida et al., 2007). S-nitrosylated Tdh3 is also involved in the apoptotic signaling pathway that occurs in chronologically aging yeast cells (Almeida et al., 2007). These findings suggest that differential modification of Tdh3 and Tdh2 is a key way in which the cell differentiates these two closely related proteins and expands their function.

In addition to the TDH3-S22Q allele we created TDH3-P24K; this version of Tdh3 was also made by substituting a residue of interest with that found at the same position in Tdh2. We were interested in the 24$^{th}$ amino acid substitution because of the eleven amino acid differences between Tdh3 and Tdh2 this is the only one that involves two amino acids with vastly different electrochemical properties. We postulated that the replacement of nonpolar, neutral proline with a polar, basic lysine was likely to affect protein function. We found that presence of Tdh3-P24K causes an increase in silencing but a decrease in overall GAPDH activity. This indicates that Tdh3-P24K represents a form of Tdh3 with enhanced silencing but compromised catalytic activity. Prior mutational analysis of this protein has only identified forms of Tdh3 that cause both activities to be compromised or one but not the other (Ringel et al., 2013). Although consistent with previous observations that catalytic activity and silencing function are not correlated, the inverse relationship observed in cells containing the Tdh3-P24K mutant is a novel finding that warrants further study.

Tdh3 has not only been identified as a positive silencing factor, but also a Sir2
interactor with the ability to control nuclear NAD$^+$ levels (Ringel et al., 2013). These findings suggest that Tdh3’s usage of NAD$^+$ is integral to its silencing function. We were therefore interested in two mutants predicted to bias Tdh3 towards NADP$^+$ usage. We created Tdh3-D33G, which was predicted to have more room in the NAD$^+$ binding pocket to accommodate the larger cofactor, and Tdh3-P189S, a substitution observed in GAPDH enzymes that naturally utilize NADP$^+$ (Bommareddy et al., 2014). Cells containing these forms of Tdh3 were found to have decreased silencing at the telomeres. Interestingly, the presence of Tdh3-D33G appeared to be worse for silencing than lacking Tdh3 altogether. We found that the NAD$^+$-dependent GAPDH activity of the D33G mutant was comparable to that of cells lacking Tdh3. Contrastingly, the P189S mutant was found to have NAD$^+$-dependent GAPDH activity that was not significantly different from that of wild type cells. These results indicate that while Tdh3-D33G has lost the ability to utilize NAD$^+$, Tdh3-P189S has retained this ability. We conclude that the decrease in silencing caused by Tdh3-P189S is not the result of defective catalytic activity.

The silencing defect observed in *TDH3-D33G* cells could be due, in part, to the loss of catalytic activity in general or simply the loss of NAD$^+$ dependency. However, the fact that this mutant causes a decrease in silencing stronger than that caused by lack of Tdh3 suggests that, in addition to being unable to promote silencing, Tdh3-D33G actively inhibits silencing. Perhaps in lieu of providing Sir2 with NAD$^+$, Tdh3-D33G brings Sir2 into contact with a metabolite that has an inhibitory effect on its activity. Alternatively, if Tdh3-D33G uses NADP$^+$ the consequential increase in NADPH levels would likely affect the NAD$^+$ and NADH levels in the cell as regulation of the two redox cofactors is related. Sir2 has been shown to be sensitive to changes in NAD$^+$ levels and could thus be indirectly
affected by Tdh3-D33G in this way. An NADP$^+$-dependent GAPDH activity assay will need to be performed in order to learn more about the effects of these mutations.

If these mutant forms of Tdh3 have the ability to utilize NADP$^+$ the findings of Bommareddy et al. (2014) would suggest that these mutants are not using NADP$^+$ optimally. The creation of a D33G, P189S double mutant would aid in our quest for an NADP$^+$-dependent version of Tdh3. We are also interested in examining NADP$^+$-dependent GAPDH enzymes found in other organisms. For example, the *K. lactis* GAPDH enzyme, Gdp1 has been found to use NADP$^+$ as a cofactor (Verho et al., 2003). We have obtained a plasmid containing the GDP1 gene which can be transformed into *S. cerevisiae* and used, in addition to protein extracts from *K. lactis*, as a positive control for NADP$^+$-dependent GAPDH activity in future assays. It would also be interesting to determine the effect of this gene in a Tdh3 null background. Given our current hypothesis of Tdh3’s role in silencing, one might predict that GDP1 would be able to rescue glycolytic but not silencing activity. Future examination of these versions of GAPDH will allow us to determine the role of NAD$^+$ in Tdh3’s regulation of Sir2-mediated silencing.

In addition to making new mutations in Tdh3, we were also interested in further characterizing mutant forms of Tdh3 created in past studies. The T227K and T227A mutants were of particular interest due to their opposite effects (Ringel et al., 2013). Tdh3-T227K was observed to cause a decrease in GAPDH activity while Tdh3-T227A caused a decrease in silencing activity. We have begun the process of determining the oligomeric state and subcellular localization of these different forms of Tdh3. In the process we made the interesting observation that wild type Tdh3-GFP appears monomeric. We have previously found *TDH3-GFP* cells to have wild type GAPDH activity (Ringel et al., 2013). This information
suggests that Tdh3-GFP would be tetrameric because the catalytically active form of GAPDH has been well characterized as tetrameric (Seidler, 2013). Further study is necessary in order to explain this striking observation.

We have not yet been able to determine the oligomeric state and subcellular localization of the T227K and T227A mutants. However, based on a study conducted in mammalian cells we have predicted that $TDH3$-$T227K$ would encode a monomeric protein found primarily found in the nucleus and $TDH3$-$T227A$ a tetrameric and cytoplasmic protein (Park et al., 2009). These predictions, in combination with our previous observations of silencing and GAPDH activity, suggest the presence of a silencing-specific form of Tdh3. Canonical GAPDH operates in the cytoplasm and functions as a tetramer to catalyze a key step in glycolysis. The T227A mutant has been predicted to fulfill this description but is unable to perform wild type Tdh3’s silencing function. Tdh3-$T227K$, on the other hand, can promote silencing but has compromised GAPDH activity, likely because it is unable to form a tetramer and is not primarily localized to the cytoplasm. We postulate that wild type Tdh3 has the ability to exist in both a catalytically active, tetrameric form as well as a silencing-specific, monomeric form. This novel form of Tdh3 could promote silencing by acting as a carrier of nuclear NAD$^+$. Further characterization of these mutants will help us to determine whether such a form of Tdh3 truly exists.

Also essential to understanding the role of Tdh3 in Sir2-mediated silencing is characterization of the nature of the Sir2-Tdh3 interaction. We have previously shown a physical interaction between these two proteins through two-hybrid and co-immunoprecipitation assays (Ringel et al., 2013). We interrogated the possibility of an enzyme-substrate interaction between these two proteins by looking for evidence of Tdh3 acetylation. We were unable to detect acetylation of Tdh3,
suggesting that Tdh3 is not a Sir2 substrate. However, in a recent proteome-wide study, researchers immunopurified acetylated proteins and analyzed them via mass spectrometry. In contrast with our results, Tdh3 was identified as an acetyl protein using this method (Henriksen et al., 2012). It is possible that we were not able to detect acetylation do to low sensitivity of the anti-acetyl lysine antibody we used. Alternatively, Tdh3 could have been purified non-specifically in the whole-proteome experiment. We are therefore still considering the possibility that Tdh3 is a substrate of Sir2. In vitro deacetylation assays should be conducted in order to further test this idea.

We have also attempted to learn more about the Sir2-Tdh3 interaction by determining what part of Sir2 is necessary for this interaction. In order to test for the co-immunoprecipitation of Sir2 truncated alleles with Tdh3 we needed to add an epitope tag to Sir2. We choose the FLAG tag because it is a very small tag, but unfortunately found that this tag disrupts the interaction of Sir2 with Tdh3. Future lab members could pursue this experiment further by trying other epitope tags. Alternatively, two-hybrid assays could be performed using truncated forms of Sir2 in order to determine which mutations lead to a loss in Tdh3 interaction. Discovering the portion of Sir2 that is necessary for interaction with Tdh3 will allow us to learn more about the nature of this interaction. Whether the required portion of Sir2 is found in a highly conserved region or one specific to yeast will also give us insight into the evolutionary conservation of Sir2-GAPDH interactions. The Sir2 homolog, SIRT1, has been observed to interact with GAPDH in mammalian cells suggesting that this interaction is conserved (Joo et al., 2012). SIRT1 and GAPDH are part of the nitrous oxide apoptosis signaling pathway in mammalian cells (Tristan et al., 2011). Our study thus far has not characterized a Sir2-Tdh3 interaction in the context of this process. However, Tdh3 has been
implicated in the nitrous oxide apoptosis signaling pathway in yeast (Almeida et al., 2007). Determining the nature of the Sir2-Tdh3 interaction in yeast will allow us to decide whether this interaction is related to that found in mammalian cells.

Our study of the Sir2-GAPDH interaction is part of a larger body of studies on Sir2 regulation that present Sir2 as a fine-tuned sensor of changes in NAD$^+$ concentration and metabolism. Studies of calorie restriction induced replicative lifespan extension have linked Sir2 activity to the cellular NAD$^+$/NADH ratio as well as the activity of NAD$^+$ synthesis enzymes in the salvage pathway (Anderson et al., 2003; Wierman & Smith, 2014). However, the exact nature of this interaction has proved difficult to elucidate. Thus far most studies have primarily examined the concentrations of NAD$^+$, NADH and associated precursor molecules at the cellular level. A greater understanding of metabolite availability at the subcellular level could be necessary to determining the exact role these molecules play in regulating Sir2 activity. Our previous finding that Tdh3 regulates nuclear NAD$^+$ levels posits Tdh3 as a key player in the processes controlling subcellular localization of NAD$^+$. The silencing defects observed to be caused by our potential NADP$^+$-dependent forms of Tdh3 support this idea. Additionally, the differential silencing ability of Tdh3-T227K and Tdh3-T227A suggest the presence of a silencing-specific form of Tdh3 that is localized to the nucleus. Together our findings indicate that continuing to examine the Sir2-Tdh3 interaction is necessary in order to determine the role of NAD$^+$ control in Sir2 regulation and further our understanding of the connection between metabolism and chromatin structure.
Appendix A

Additional Questions

We have asked and attempted to investigate several other questions in addition to those discussed in the Results chapter of this thesis. These experiments could be continued by future lab members through additional troubleshooting. Despite the fact that these experiments did not yield additional information to this study, these questions are relevant to the story of how we are seeking to further understand the role of GAPDH proteins in Sir2-mediated silencing.

A.1 Strains for future use

Table A.1: I have created these strains for use in future experiments. These strains were designed for use in chromatin immunoprecipitation experiments but could be used to answer additional questions as well.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
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| YSH1215  | $\text{his3}\Delta1 \text{leu2}\Delta0 \text{met15}\Delta0 \text{ura3}\Delta0$  
TDH3-(3x)-myc-HIS3 $\Delta\text{hst2}$ |
| YSH1216  | $\text{his3}\Delta1 \text{leu2}\Delta0 \text{met15}\Delta0 \text{ura3}\Delta0$  
TDH2-(3x)-myc-HIS3 $\Delta\text{hst2}$ |
| YSH1217  | $\text{his3}\Delta1 \text{leu2}\Delta0 \text{met15}\Delta0 \text{ura3}\Delta0$  
TDH2-(3x)-myc-HIS3 $\Delta\text{sir2}$ |
| YSH1218  | $\text{his3}\Delta1 \text{leu2}\Delta0 \text{met15}\Delta0 \text{ura3}\Delta0$  
TDH2-(3x)-myc-HIS3 $\Delta\text{tdh3}$ |
A.2 Where are the T227K and T227A mutants localized?

In Section 3.6 we examined the oligomeric state of wild type Tdh3 as well as the T227K and T227A mutants. In addition to having different levels of self-assembly, these mutants were found to be differentially localized in rat cells (Park et al., 2009). We were interested in determining whether yeast Tdh3-T227K is predominantly found in the nucleus and Tdh3-T227A in the cytoplasm, as predicted by the mammalian study (Park et al., 2009). In order to do this we prepared protein from cells containing these mutations via cellular fractionation. Figure A.1 shows western blot analysis of these protein fractions. In the right panel we detect the presence of histone H3 in whole cell (lanes 1-4) and nuclear (lanes 9-12) fractions but not cytoplasmic fractions (lanes 5-8). This result indicates success-

![Western blot](image-url)
ful fractionation because histone H3 is known to be found in the nucleus but not the cytoplasm. The left panel suggests that the wild type, T227K, and T227A forms of Tdh3 are all found in both the cytoplasm and nucleus. We attempted to determine the nuclear to cytoplasmic ratios of each type of Tdh3 through band quantitation but found the results inconsistent from experiment to experiment. This inconsistency suggested that an internal loading control was necessary for reliable quantitative results. We were only able to detect tubulin in the whole cell fractions, indicating that tubulin cannot be used as an internal loading control for the cytoplasmic and nuclear fractions. We also tried using the proteasome component Rpn8 as an internal loading control for the cytoplasmic fraction but were unable to observe any signal using an anti-Rpn8 antibody (Abcam ab43730). Another internal loading control must be identified before we can reliably determine the ratio of nuclear to cytoplasmic Tdh3 and thus the localization pattern of these mutants.

A.3 Does Tdh2 associate with silenced chromatin?

Silencing assays in this study and others (Ryznar, 2014) have suggested a role for Tdh2 in Sir2-mediated silencing. Tdh3, a closely related positive silencing factor, has been found to associate with the rDNA locus and telomeres (Ringel et al., 2013). We were interested in determining whether Tdh2 can also be detected at these regions of silenced chromatin. In order to answer this question we performed chromatin immunoprecipitations of Tdh3-myc and Tdh2-myc and assessed the amount of DNA recovered from NTS1 (part of the rDNA locus) as well as the region 1kb upstream of the telomere of chromosome V. The amount of DNA recovered from these regions through immunoprecipitation (IP) and mock
IP is expressed as a percentage of the amount input DNA at these regions in Figure A.2. This figure suggests that more NTS1 and telomeric DNA is recovered from IPs of Tdh3 and Tdh2 than from mock IPs. This result indicates that Tdh3 and Tdh2 are found at the rDNA locus and telomeres.

![Graph showing percentage input of NTS1 and TELV1.0 for Tdh3 and Tdh2](graph.png)

**Figure A.2:** Tdh3 and Tdh2 are found at the rDNA and telomeres. Values shown are the average of two independent experiments.

In Figure A.3 the same results shown in Figure A.2 for NTS1 and telomeric DNA are expressed relative to the amount of DNA precipitated from the *ACT1* locus. *ACT1* is a transcriptionally active gene and thus found in euchromatin unlike the silenced regions of chromatin being tested. The results shown in Figure A.3 suggest that Tdh3 and Tdh2 are not enriched at the rDNA and telomeres relative to *ACT1*. This indicates that Tdh3 and Tdh2 do not specifically interact with silenced chromatin. However, this finding is inconsistent with previous results.
showing Tdh3 to be enriched at the rDNA and telomeres relative to ACT1 (Ringel et al., 2013). This inconsistency suggests that the lack of enrichment indicated by our results is due to some type of signal to noise issue, rather than a true observation. Further troubleshooting must be done to determine the nature of this issue.

Figure A.3: Tdh3 and Tdh2 nonspecifically interact with chromatin. Values shown are the average of two independent experiments.
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