SIW14 IS A NOVEL 5-DIPHOSPHOINOSITOL PENTAKISPHOSPHATE (5PP-IP₅) PHOSPHATASE THAT NEGATIVELY REGULATES THE STRESS RESPONSE

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ABSTRACT

Inositol pyrophosphates are high energy signaling molecules involved in cellular processes such as energetic metabolism, telomere maintenance, vesicle trafficking, and the stress response. While the inositol kinases underlying inositol pyrophosphate biosynthesis are well characterized, the phosphatases that selectively regulate their cellular pools are not fully described. The DIPP (diphosphoinositol phosphate phosphohydrolase) enzymes of the Nudix protein family have been demonstrated to dephosphorylate inositol pyrophosphates; however, the Saccharomyces cerevisiae homolog Ddp1 prefers inorganic polyphosphate over inositol pyrophosphates. Additionally, a proper functioning cellular stress response is important for cell survival. In yeast, the presence of inositol pyrophosphates is a requirement for the induction of a stress response. I identified a novel phosphatase of the recently discovered Atypical Dual-Specificity Phosphatase family as a physiologic inositol pyrophosphate phosphatase. Purified recombinant Siw14 hydrolyzes the β-phosphate from 5-diphosphoinositol pentakisphosphate (5PP-IP₅) in vitro. In vivo, siw14Δ yeast mutants possess increased IP₇ levels while heterologous SIW14 overexpression eliminates IP₇ from cells. IP₇ levels increased proportionately when siw14Δ was combined with ddp1Δ or vip1Δ, indicating independent activity by the enzymes encoded by these genes. Interestingly, we also found that the stress response is partially on in the unstressed siw14Δ mutant strain and it can still mount a stress response. I show this by using microarray analysis, reverse transcriptase-quantitative PCR (qRT-PCR), and cell growth assays under heat, osmotic, and oxidative treatments. Consistent with these data and role of Siw14 as an
IP7 phosphatase, the levels of inositol pyrophosphates change when wild-type cells are under stress. I conclude that Siw14 is a physiologic phosphatase that modulates inositol pyrophosphates metabolism by dephosphorylating the IP7 isoform 5PP-IP5 to IP6 which is the reason why the deletion mutant is resistant to stresses.
DEDICATION

I dedicate this work to the loving memory of my grandmother, Nanny (Martha Clark 1923-2014). She was always supportive of me and on her dying day wanted to make sure I planned on finishing my Ph.D. During the hard times I remembered that and it kept me going.

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Many people have supported me the last six years of my doctoral work and I want to thank each of you for your encouragement, great or small. I have family and friends in and out of science that have been there for me. I need to thank my family, especially, my mom who pushed me to apply to graduate school and my dad who pushed me to stay in the program. My grandparents, Dorothy Steidle and Walter Steidle. Grandpa has his Ph.D. and has always been so interested to hear how my classes or research were going.

Before coming to Georgetown I had two professors that believed in me so much I feel compelled to thank them here. Dr. Wallace Martin from Randolph-Macon College believed I could be a great professor. Dr. Jon Monroe at James Madison University made science so fun and interesting that I wanted to become a professor because I aspired to be like him.

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CHAPTER 1

Introduction

This chapter is a literature review that will provide the necessary background information to understand inositol pyrophosphates, the cellular stress response, the phosphatase Siw14, and how they connect to one another. The structure and metabolism of inositol pyrophosphates will be explained by first discussing the kinases and phosphatases that are involved in their synthesis and breakdown (Section 1.1). Inositol pyrophosphates are involved in many different cellular processes and are multi-functional: they can donate phosphates, bind proteins, and affect cell signaling. One process in which inositol pyrophosphates are known to play a role is the cellular stress response. In section 1.2 this response will be broken into several specific stress responses including the general, osmotic, heat, and oxidative stress responses. Within each stress the main transcription factors that are responsible for gene expression changes are discussed, as well as any other major cellular processes that help cells respond to the particular stress in question. Next the biochemical and genetic features of the phosphatase Siw14 will be discussed (Section 1.3). Finally, the last section will link all three major topics together (Section 1.4).

1.1 Inositol Pyrophosphates

Structure and Synthesis of Inositol Pyrophosphates

Myo-inositol is a six-membered carbon ring with hydroxyl groups at each carbon making it a sugar alcohol. There are many conformations of inositol but myo-inositol is most commonly found in the chair conformation where the 2-position hydroxyl group is in the axial position and the remaining hydroxyl groups are in the equatorial position (Figure 1A). When myo-inositol is phosphorylated, it is known as an inositol phosphate and it can be phosphorylated at one or more of the carbons. The most well-known inositol phosphate is inositol trisphosphate (IP₃) because of
its role as a second messenger in Ca\(^{2+}\) signaling (Streb et al. 1983; Furuichi et al. 1989; Berridge 1993). In yeast and mammals, IP\(_3\) is formed by the hydrolysis of membrane-associated phosphatidylinositol 4,5-bisphosphate [PI(4,5)P\(_2\)] by the enzyme phospholipase C. IP\(_3\) is a substrate for a number of kinases, resulting in phosphorylation at all six carbons making it inositol hexakisphosphate (IP\(_6\)). In order to synthesize IP\(_6\) in yeast, the inositol phosphate multikinase Arg82 phosphorylates IP\(_3\) to generate inositol tetrakisphosphate (IP\(_4\)) and inositol pentakisphosphate (IP\(_5\)) on carbons 3 and 6. Inositol phosphate kinase Ipk1 synthesizes IP\(_6\) by phosphorylating IP\(_5\) on carbon 2 (Figure 1B; reviewed in Hatch and York 2010).

This dissertation focuses on the molecules that are synthesized using IP\(_6\) as a substrate, known as inositol pyrophosphates (Figure 1). In addition to being fully phosphorylated at each carbon these molecules have β-phosphates at either the 5-position, 1-position or both. Diphosphoinositol pentakisphosphate (PP-IP\(_5\) or IP\(_7\)) is the most abundant isoform of inositol pyrophosphate, ranging from 0.5-5 µM in human cells and 300-500 µM in amoeba (Albert et al. 1997; Lin et al. 2009). The two physiologically relevant isomers of IP\(_7\) are 1PP-IP\(_5\) and 5PP-IP\(_5\) and sequential phosphorylation of IP\(_7\) results in bisdiphosphoinositol tetrakisphosphate (1,5PP-IP\(_4\) or IP\(_8\)) (reviewed in Wilson et al. 2013; Thomas and Potter 2014). Mennetti et al. (1993) and Stephens et al. (1993) were the first to discover inositol pyrophosphates in cellular extracts using strong anion exchange (SAX) HPLC analysis. The two research groups observed peaks that eluted from the HPLC column after the IP\(_6\) peak, suggesting the existence of inositol-containing molecules more negatively charged than IP\(_6\). In the past 23 years, researchers have uncovered many but not all mechanisms for catabolism and anabolism of these molecules, and the many processes they influence in eukaryotes.
The enzymes that regulate the pools of inositol pyrophosphates have not been fully described. The inositol hexakisphosphate kinases (IP6K) synthesize IP\(_7\) by catalyzing the addition of a β-phosphate to IP\(_6\) at the 5-position generating 5PP-IP\(_5\) (reviewed in Thomas and Potter 2014). Yeast possess a single IP6K gene, KCS1, while mammals possess three homologous genes (Figure 1B,C). The Kcs1 kinase of yeast has the ability to pyrophosphorylate the 5-carbon on other inositol polyphosphate molecules, such as IP\(_5\) to make bisdiphosphoinositol tetrakisphosphate (5PP-IP\(_4\)), a pyrophosphorylated IP\(_6\) (Draskovic et al. 2008; Wundenberg and Mayr 2012). This pyrophosphorylated IP\(_6\) is believed to occur \emph{in vivo} when the gene that codes for the kinase that makes IP\(_6\), \emph{IPK1}, is absent (Saiardi et al. 2002).

IP\(_8\) is synthesized by a PP-IP\(_5\) kinase (PPIP5K) that adds the β-phosphate to IP\(_7\) at the 1-position to make 1,5PP-IP\(_4\); this enzyme can also use IP\(_6\) as a substrate, generating 1PP-IP\(_5\) (Figure 1B). Mammalian genomes have two genes for PPIP5K and the yeast genome has a single gene, \emph{VIP1} (Figure 1B,C). The structure of PPIP5K enzymes is rigid and does not allow for flexibility in substrate like the previously mentioned IP6K enzymes (Wang et al. 2012). These enzymes have an insignificant amount of activity with IP\(_5\) or 5PP-IP\(_4\) which are among the inositol polyphosphates that are most like IP\(_6\) and 5PP-IP\(_5\) (Wang et al. 2012). Interestingly, the human version of PPIP5K1, designated human PPIP5K1 (hPPIP5K1) can bind to the insoluble phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P\(_3\)] which affects signaling and pools of inositol pyrophosphates at the plasma membrane (Gokhale et al. 2013).

To date, phosphatases described to target the β-phosphate of IP\(_7\) and IP\(_8\) include members of the Nudix family of hydrolases, (reviewed in Thomas and Potter 2014) named DIPP for diphosphoinositol phosphate phosphohydrolase (Figure 1C). In mammals, the DIPP enzymes display a preference for the β-phosphate at the 1-position and are encoded by four genes (Figure...
One orthologue is found in yeast, encoded by $DDP1$; this phosphatase was recently shown to prefer polyphosphate (poly-P) over inositol pyrophosphates although it is able to cleave the $\beta$-phosphate at the 1-position on both IP$_7$ and IP$_8$ in vitro (Lonetti et al. 2011). The preferred pathway from IP$_6$ to IP$_8$ is via the addition of the $\beta$-phosphate at the 5-position and then the $\beta$-phosphate at the 1-position (Kilari et al. 2013) (Figure 1B). However, it seems that there are distinct roles for the two isoforms of IP$_7$. For example, proteins with pleckstrin homology (PH) domains (discussed later) cannot bind to 1PP-IP$_5$ but can bind to 5PP-IP$_5$ and IP$_6$ (Gokhale et al. 2013). In addition, 1PP-IP$_5$ but not 5PP-IP$_5$ can directly bind to the Pho80-Pho85-Pho81 complex inhibiting the complex from sensing phosphate availability, (discussed later) (Lee et al. 2008). Therefore, cells may direct the inositol pyrophosphate pathway through the 1-position first in specific situations.
Mammalian Orthologs of the Inositol Phosphate Pathway.

<table>
<thead>
<tr>
<th>Enzyme Names</th>
<th>Yeast Proteins</th>
<th>Mammalian Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inositol phosphate multikinase</td>
<td>Arg82</td>
<td>IP3K</td>
</tr>
<tr>
<td>Inositol polyphosphate kinase</td>
<td>Ipk1</td>
<td>IP5K</td>
</tr>
<tr>
<td>Inositol hexakisphosphate kinases</td>
<td>Kcs1</td>
<td>IP6K1, IP6K2, IP6K3</td>
</tr>
<tr>
<td>Diphosphoinositol polyphosphate kinase</td>
<td>Vip1</td>
<td>PPIP5K1, PPIP5K2</td>
</tr>
<tr>
<td>Diphosphoinositol phosphate phosphohydrolase</td>
<td>Ddp1</td>
<td>hDIPP1, hDIPP2, hDIPP3, hDIPP4</td>
</tr>
<tr>
<td>5-diphosphoinositol pentakisphosphate phophatase</td>
<td>Siw14</td>
<td>No known ortholog</td>
</tr>
</tbody>
</table>

Figure 1. Inositol pyrophosphates synthesis and degradation in yeast. (A) Chair conformation of myo-inositol. Image from Figure 1B, Thota and Bhandari (2015). (B) In yeast, soluble inositol polyphosphates and inositol pyrophosphates are generated by phosphorylation of IP3 (myo-inositol-1, 4, 5-triphosphate) by the inositol phosphate multikinase Arg82 that produces IP5 (inositol-1,3,4,5,6-pentakisphosphate) after two phosphorylation steps. Inositol phosphate kinase Ipk1 phosphorylates the 2 position to produce IP6 (inositol hexakisphosphate). The inositol pyrophosphates are synthetized by adding a β-phosphate to the 5-position preferentially by the enzyme IP6 kinase Kcs1 (indicated by the heavier arrows; Kilarl et al. 2013), and then at the 1-position by the PP-IP5 kinase Vip1. Our data show that Siw14 opposes the action of Ksc1 by preferentially hydrolyzing the β-phosphate at the 5 position; Ddp1 preferentially hydrolyzes the β-phosphate at the 1-position (6). This schematic was modified from Hatch and York (2010). (C) List of the enzymes in the inositol phosphate pathway and their yeast and mammalian homologs (Hatch and York 2010).
Processes Affected by Inositol Pyrophosphates

Inositol pyrophosphates are a novel class of signaling molecules that carry energy-rich diphosphate bonds that have been implicated in an array of processes, including cellular energy metabolism, ribosome synthesis, DNA repair, chromatin remodeling, glycolysis, phosphate metabolism, and vesicle trafficking in animals and fungi (Figure 2; reviewed in Wilson et al. 2013; Thota and Bhandari 2015). Inositol pyrophosphates have also been implicated in human diseases, such as cancer and diabetes (Barker et al. 2004; Nagata et al. 2005; Illies et al. 2007; Morrison et al. 2009). Therefore, understanding the basic function of inositol pyrophosphates in the cell is critical to better understand and treat these diseases.

Cell cycle and Growth: Inositol pyrophosphate levels change during the cell cycle in both mammalian and yeast cells. In yeast, IP$_7$ levels are at their highest during S-phase and recently, it was observed that overexpression of KCS1 causes yeast cells to enter G2/M phase sooner than wild-type cells (Banfic et al. 2013; Banfic et al. 2016). Rat mammary tumor cells also have high levels of IP$_7$ during the G$_1$-phase (Barker et al. 2004) and when apoptosis is induced in ovarian cancer cells, both IP$_7$ and IP$_8$ levels increase (Nagata et al. 2005). Finally, mice with homozygous ip6k2 deletions are more prone to tumorigenesis through defects in cell cycle and reduced apoptosis (Morrison et al. 2009). These findings suggest that inositol pyrophosphates may act as cancer suppressors to regulate growth.

Cellular energy metabolism: Inositol pyrophosphates are required for functional mitochondria in both yeast and mouse fibroblast cells (Szijgyarto et al. 2011). In spite of a lack of mitochondrial function, kcs1A mutants have increased transcription of glycolytic enzymes
leading to an increase in ATP synthesis (Szijgyarto et al. 2011). It was proposed that inositol pyrophosphates reduce the activity of the transcription factor Gcr1 by pyrophosphorylation, which in turn reduces the transcription of glycolytic genes in wild-type cells (Szijgyarto et al. 2011). Thus, the $kcs1\Delta$ mutant, which has lower levels of inositol pyrophosphates and an absence of 5PP-IP$_5$ would lack the inhibition. These findings suggest that inositol pyrophosphates are metabolic regulators by influencing the levels of ATP by changing the glycolytic versus mitochondrial metabolic ratio (Szijgyarto et al. 2011).

**DNA repair:** Yeast cells that cannot pyrophosphorylate IP$_6$ at the 5-position (i.e. in a $kcs1\Delta$ mutant), are sensitive to the DNA damage reagent phleomycin (Onnebo and Saiardi 2009). Phleomycin is an antibiotic that binds and intercalates DNA which in turn damages the overall structure. $kcs1\Delta$ mutant yeast are also defective in DNA recombination (Huang and Symington 1995; Luo et al. 2002). In addition, mice carrying the homozygous deletion of $IP6K1$ are also defective in homologous recombination (Jadav et al. 2013). These findings suggest that either the IP6K enzymes or 5PP-IP$_5$ is important for DNA repair mechanisms.

**Phosphate Metabolism:** Polyphosphates (poly-P) are long chains of inorganic phosphate linked by anhydride bonds. Inositol pyrophosphates positively regulate poly-P metabolism because a depletion of 5PP-IP$_5$ leads to a complete loss of poly-P (Lonetti et al. 2011), whereas there is no relationship between 1PP-IP$_5$ and the levels of poly-P because poly-P does not change in a $vip1\Delta$ mutant (Auesukaree et al. 2005; Lonetti et al. 2011). In the amoeba *Dictyostelium discoideum*, energy is stored in the form of poly-P (Livermore et al. 2016). *D. discoideum* mutants that are unable to synthesize poly-P have increased levels of inositol pyrophosphates
(Livermore et al. 2016). This might suggest that in the absence of their preferred energy storage molecule, the cells are storing energy in the form of pyrophosphate bonds in inositol pyrophosphates (Livermore et al. 2016).

Inositol pyrophosphates also play a role in phosphate deprivation. Though controversial one research group reported that during low phosphate conditions 1PP-IP$_5$ inhibits the Pho80-Pho85-Pho81 complex in yeast (Lee et al. 2008; Saiardi 2012). This cyclin dependent kinase (CDK) complex activates Pho4-dependent transcription of phosphate metabolism genes including $PHO85$, which encodes the high affinity phosphate uptake transporter and $PHO5$ which encodes the acid phosphatase involved in inorganic phosphate mobilization. Likewise, in a $vip1\Delta$ mutant Pho4 does not get activated during phosphate starvation (Lee et al. 2007). On the contrary, the expression of Pho5 remains on in a $kcs1\Delta$ mutant and overexpression of $KCS1$ inhibits the expression of Pho5 during phosphate deprivation (Auesukaree et al. 2005). In addition, $kcs1\Delta$ mutants have reduced uptake of inorganic phosphate (Saiardi et al. 2004), and in vertebrates, overexpression of $IP6K$ results in higher uptake of inorganic phosphate (Norbis et al. 1997). These data suggest that phosphate levels are important for directing which IP$_7$ isoform is synthesized first (Figure 1B).

**Cellular Stress Response:** Recent work found that production of inositol pyrophosphates is essential for mounting environmental stress responses including the down-regulation of ribosomal biogenesis in yeast (Worley et al. 2013). Consistent with these data, the Kcs1 kinase is necessary for the human fungal pathogen *Cryptococcus neoformans* to adapt to the host environment and invade (Lev et al. 2015). This topic will be discussed further in section 1.4.
**Vesicular Trafficking:** Cells with mutations in *ARG82* and *KCS1* have unusually small vesicles, vacuoles that are fragmented, and defective endocytic membrane trafficking (Saiardi et al. 2000; Dubois et al. 2002; Saiardi et al. 2002). Since these mutants also do not produce inositol pyrophosphates and specifically 5PP-IP₅, these findings suggest a role for inositol pyrophosphates in these processes but mechanisms have not been determined. In mammals, IP₇ is necessary for the release of insulin in pancreatic β-cells (Illies et al. 2007). In addition, *ip6kΔ* knockout mice are unable to secrete insulin and the phenotype is rescued by exogenous IP₇ (Bhandari et al. 2008). Thus, IP6K and/or IP₇ may be an important aspect to study in the development of diabetes (Illies et al. 2007).

**Figure 2. Inositol pyrophosphates affect many different cellular processes.** Redlines with blunt ends represent negative regulation of processes and black arrows represent positive regulation of processes by inositol pyrophosphates. Adapted from Thota and Bhandari, (2015).
The Function of Inositol Pyrophosphates

It is obvious now that there are an extensive number of processes in which inositol pyrophosphates play a role. Also, numerous reports showed that the cellular levels change in response to various stimuli. However, the direct function of inositol pyrophosphates in these processes is not fully known.

Pleckstrin Homology (PH) Domains

Inositol pyrophosphates bind to pleckstrin homology (PH) domains and this may affect protein trafficking (Wilson et al. 2013; Thomas and Potter 2014). Proteins with PH-domains are important for cell signaling processes such as protein synthesis, actin polymerization, metabolic homeostasis, cell survival, and cell cycle entry (Cantley 2002; Shears 2015). PH-domains are made up of 120 amino acids that form two perpendicular anti-parallel beta sheets, with a C-terminal amphipathic helix (reviewed in Riddihough 1994). Proteins with PH-domains bind to specific phosphatidylinositol phosphates to aid in protein localization to the plasma membrane. The 5PP-IP$_5$ isoform of IP$_7$ and IP$_6$ are able to successfully compete with phosphatidylinositol phosphates to bind PH-domains (Luo et al. 2003; Chakraborty et al. 2010; Gokhale et al. 2013). These studies argue that proteins with PH-domains have a higher affinity for 5PP-IP$_5$ over IP$_6$, but the important point is that any of the soluble forms of inositol polyphosphates bind to PH-domain containing proteins (reviewed in Shears 2015). If these proteins are unable to bind to phosphatidylinositol phosphates and instead bind to the soluble inositol phosphates their function is inhibited. For example, the mammalian protein Akt, which is part of the TORC pathway is inhibited by 5PP-IP$_5$ (Chakraborty et al. 2010; Chakraborty et al. 2011).

Not only can soluble inositol polyphosphates bind PH-domain containing proteins but mammalian PPIP5K1 can also bind the insoluble PI(3,4,5)P$_3$ (Gokhale et al. 2011; Gokhale et al.
The protein binds using a catalytically inactive phosphatase-like domain when the levels of PI(3,4,5)P₃ are high (Gokhale et al. 2011; Gokhale et al. 2013). Erneux and Elong Edimo (2013) suggested that this function maybe be important for concentrating pools of soluble inositol pyrophosphates near the plasma membrane or allow translocation of PPIP5K1. Interestingly, inositol pyrophosphates have been shown to affect the processes that could be influenced by a competition between the soluble and insoluble inositol polyphosphates binding to proteins that are currently unrecognized as binding each molecule respectively.

*Protein Pyrophosphorylation*

Inositol pyrophosphates are thought to be multifunctional and reports from several labs suggest that inositol pyrophosphates can donate the β-phosphate to protein without the action of a kinase (Saiardi et al. 2004; Bhandari et al. 2007; Szijgyarto et al. 2011). The P-O-P bond on the inositol pyrophosphate has a calculated energy bond of 6.6 kcal/mol, slightly higher than the same bond on an ADP molecule (6.4 kcal/mol) (Stephens et al. 1993; Wundenberg and Mayr 2012). After incubating radiolabeled (³²P)P-IP₅ with yeast cell extracts, the yeast nucleolar protein Nsr1 was pyrophosphorylated (Saiardi et al. 2004) and similar results were observed with respect to the yeast transcription factor Gcr1 (Szijgyarto et al. 2011). In order for this post-translational modification to occur, the protein requires an adjacent serine to be previously phosphorylated (Bhandari et al. 2007). Unfortunately, these findings have not been observed *in vivo*; therefore, further research needs to be conducted in order to discover whether this modification is biologically relevant (Shears 2015).
1.2 Cellular Stress Response

The ability of cells to cope with different types of stress is crucial for cell survival whether they are unicellular organisms or part of a multicellular organism. Extrinsic stresses include environmental conditions such as temperature extremes, nutrient limitation, acidic or basic conditions, osmotic stress and others. These extrinsic stresses can cause intrinsic stresses which occur from disrupted processes inside the cell, leading to oxidation and macromolecule damage, i.e. damage to DNA, proteins or lipid membranes (reviewed in Kültz 2005). For instance, the cell membrane can induce a stress response because its lipid composition, fatty acid saturation, and membrane fluidity can change under stressing conditions (Steels et al. 1994; Swan and Watson 1999; Chatterjee et al. 2000). Intrinsic cellular damage activates different stress signaling pathways depending on the level and type of stress (Kültz 2005; Morano et al. 2012).

There are ~300 proteins conserved over the three domains of life, and 15% of those conserved proteins are required to function during a stress response (Kültz 2005). This 15% however, represents a relatively small number of stress response genes, and in reality many more genes are up-regulated by individual species during stressful conditions. Within species there are many different types of stress responses and, depending on the organism, a different set of proteins might be categorized as part of the general stress response, the heat stress response, the osmotic stress response, etc. Overall, the genes that are up-regulated during a stress response are involved in carbohydrate metabolism, protein metabolism, intracellular signaling, defense against reactive oxygen species (ROS), DNA damage repair, and others. Each of these processes help cells defend against stress. For example, carbon metabolism is up-regulated because cells need to maintain a viable energy source during stressful conditions (Hohmann and Mager 2003).
Additionally, regular cell functions may be negatively affected by stress and need to be turned off. Protein synthesis is a down-regulated process during stress because macromolecules like RNA and ribosomes can be damaged, which could lead to further intracellular stresses if they continue unchecked. Growth-promoting processes are also down-regulated because DNA damage can lead to improper DNA replication, mutations, and debilitated daughter cells (Gasch 2007; Martínez-Montañés et al. 2010). Together, down regulating these processes help to protect the mass and energy of the cell while it responds to the stressful environment (Hohmann and Mager 2003).

**General Stress Response**

The general stress response defines a set of genes that are activated under most stress conditions including low nutrients, osmotic stress, heat stress, and oxidative stress among others (Morano et al., 2012). In yeast, the general stress response uses the transcription factor Msn2 and its partially redundant paralog Msn4 (referred collectively as Msn2/4) to bind to the stress response element (STRE) found in the promoters of the target genes (Martínez-Pastor et al. 1996; Schmitt and McEntee 1996; Boy-Marcotte et al. 1998; Smith 1998). The STRE has the sequence CCCCT (Wieser et al. 1991). Msn2/4 up regulates ~200 general stress response genes under multiple stress conditions (Marchler et al. 1993; Martínez-Pastor et al. 1996; Schmitt and McEntee 1996; Gasch et al. 2000; Causton et al. 2001).

The regulation of Msn2/4 is complicated involving many different kinases and phosphatases: Msn2/4 is inactivated by phosphorylation and held in the cytoplasm, and is activated by dephosphorylations and shuttled to the nucleus (Figure 3; Riddihough 1994; Beck and Hall 1999; Görner et al. 2002; Kaida et al. 2002; Santhanam et al. 2004; De Wever et al.)
During non-stress conditions, Msn2/4 is hyperphosphorylated by protein kinase A (PKA) (Görner et al. 2002), and Snf1 (De Wever et al. 2005). Phosphorylated Msn2/4 binds to Bmh2, which acts as an anchor to sequester it in the cytoplasm. Bmh2 itself is regulated by the TOR pathway (Beck and Hall 1999).

Under stress conditions the protein phosphatase Psr1 is activated by a regulating subunit Whi2 to dephosphorylate Msn2/4. Simultaneously, Tor1 becomes inhibited, allowing the protein phosphatase PP2A to become active and dephosphorylate Msn2/4 (van Zyl et al. 1992; Shu et al. 1997; Wang and Burke 1997; Gentry and Hallberg 2002). Finally, a third protein phosphatase PP1 (encoded by \textit{GLC7} and \textit{REG1}; Stark 1996) dephosphorylates Msn2, working as the direct antagonist of protein kinase A (PKA) (De Wever et al. 2005). These dephosphorylations allow Msn2/4 to move into the nucleus and activate transcription (Kaida et al. 2002; Santhanam et al. 2004).

Msn2/4 has C-terminal and N-terminal domains that are both important in allowing Msn2/4 to react to different types of stress (Boy-Marcotte et al. 1998). The C-terminal domain is phosphorylated by PKA and is important to the stress response under glucose limitation but not oxidative or heat shock; the latter stresses seem to affect dephosphorylation of the N-terminal domain (Boy-Marcotte et al. 1998; Santhanam et al. 2004).

Beyond phosphorylation and dephosphorylation of Msn2/4, the histone deacetylase (HDAC) complex Rpd3L is necessary for the repression of genes during a heat stress but also the activation of general stress response genes (Ruiz-Roig et al. 2010). This was a surprising result because the canonical role of HDACs are to turn off transcription. Nevertheless, in order for full induction of a stress response via Msn2/4, Rpd3L is recruited to the promoters of the general stress response genes (Alejandro-Osorio et al. 2009; Ruiz-Roig et al. 2010). In addition, while
Rpd3L is at the promoters it likely recruits more Msn2/4 proteins during stress conditions (Ruiz-Roig et al. 2010). Interestingly, Rpd3 has been found to be implicated in more than just the general stress response and will be discussed more later.

**Figure 3. The basic regulation of the stress response transcription factors Msn2 and Msn4.** The left side of the diagram shows regulation of Msn2/4 under non-stress conditions when Msn2/4 is being held in the cytoplasm. The right side of the diagram shows the regulation of Msn2/4 when a cell is under stress moving Msn2/4 into the nucleus. Faded objects represent when a protein or pathway is inactive or not present in that location during each condition. Green arrows represent positive regulation of the PKA pathway and blue arrows represent positive regulation of the TORC1 pathway. Blue lines with a blunt end represent negative regulation of the TORC1 pathway.

**Osmotic Stress Response**

The activation of the high-osmolarity glycerol (HOG) pathway is the first response to osmotic stress, leading to an increase in the synthesis of glycerol. Glycerol biogenesis and
transport are thought to contribute the most to the rapid changes in glycerol levels upon osmotic stress. Glycerol is a water soluble solute that restores the osmotic balance and turgor pressure in cells (reviewed in Chen and Thorner 2007). For example, cells that have defects in glycolysis are sensitive to osmotic stress (Hohmann et al. 2007). Glycerol export transporters like Fbs1 are inactivated during osmotic stress to keep glycerol inside the cell (Tamás et al. 1999); and Stl1, a sugar transporter-like protein is also induced by the HOG pathway to accumulate exogenous glycerol (reviewed in Saito and Posas 2012).

Transmembrane osmosensors receive stimuli to activate the HOG pathway. Sln1, a histidine kinase, is the osmosensor for one branch and Sho1, Opy2, and genetically redundant Msb2 and Hkr1 are the osmosensors for the second branch. If one branch is defective cells depend on the other for osmosensing (Saito and Posas 2012). Under osmotic stress these osmosensors activate signaling cascades to phosphorylate and activate the mitogen-activated kinase (MAPK) Hog1. Once activated, Hog1 is localized to the nucleus where it can affect the expression of hundreds of genes (de Nadal et al. 2007). The general stress response is also activated during osmotic stress. However, 80% of the genes that are induced during an osmotic stress response are due to the interaction of Hog1 with several transcription factors that include Hot1, Smp1, Msn1, Msn2, and Msn4 (Rep et al. 1999; de Nadal et al. 2003; Capaldi et al. 2008). Hog1 also converts the transcriptional repressor Sko1 to an activator (Proft et al. 2001; Proft and Struhl 2002). Hog1 directly phosphorylates these transcription factors or it binds to the promoters of specific genes that the transcription factors regulate (Saito and Posas 2012). Each of the transcription factors only regulate a portion of the genes needed for an osmotic stress response. The genes either encode proteins that directly respond to the stress or they encode proteins that work in conjunction with other osmotic stress response factors (Saito and Posas 2012). For
example, the transcription factor Hot1 is responsible for regulating genes that are important for glycerol biosynthesis like \( GPD1 \) and \( GPP2 \) (Rep et al. 1999; 2000).

It has been suggested that during osmotic stress Hog1 can bind to an Rpd3 complex (likely Rpd3L) and recruit it to some genes (Saito and Posas 2012). Usually HDACs repress genes, but when an Rpd3 complex binds to promoters it results in histone deacetylation, entry of RNA polymerase II, and induction of gene expression (de Nadal et al. 2004). However, the role of the Rpd3 complex during osmotic stress at promoters might not be limited to changing the structure of chromatin, it might also act as a unique binding surface or recognition motif for the recruitment of transcription factors such as Msn2 (Alejandro-Osorio et al. 2009; Saito and Posas 2012).

**Heat Stress Response**

The heat shock response (HSR) is primarily a transcriptional response regulated mainly by the transcription factors Hsf1 and Msn2/4. There are four paralogs of the heat shock factor (HSF) proteins in mammals and one in yeast (Akerfelt et al. 2010). Hsf1 regulates genes that contain the heat shock element (HSE) which contains five repeats of the sequence \( nGAAn \) (Sorger and Pelham 1987). Each gene has multiple HSE sites and Hsf1 constitutively binds to one of the HSE sites but under heat stress will bind to the additional sites (Gross et al. 1990; Erkine et al. 1999; Hahn et al. 2004). The constitutively bound Hsf1 rids the promoter of nucleosomes preparing for transcriptional initiation before a heat stress occurs (Gross et al. 1993).

It is hypothesized that an increase in misfolded proteins activates Hsf1 (Rowley et al. 1993; Lee and Goldberg 1998; Trotter et al. 2001; Trotter et al. 2002; Takemori et al. 2006). However, during a 37°C heat stress bulk misfolded protein accumulation does not occur (Nathan
et al. 1997). Trotter and colleagues (2001; 2002) observed that it is specifically newly synthesized misfolded proteins that activate Hsf1. When cells are treated with azetidine 2-carboxylic acid (AZC), a proline analog that causes newly synthesized proteins to misfold, it results in the induction of genes regulated by Hsf1 but not Msn2/4 genes (Trotter et al. 2001; Trotter et al. 2002). Additionally, treatment with ethanol or inhibition of proteasomal degradation also activates Hsf1 (Lee and Goldberg 1998; Takemori et al. 2006). Therefore, Morono and colleagues (2012) suggested that ribosome-proximal HSPs/chaperones have to sense an increase of newly-made misfolded polypeptides to activate the HSR.

**Oxidative Stress Response**

Oxidative stress is defined as a cell’s inability to cope with reactive oxygen species (ROS) and the damage caused by them. In order for *S. cerevisiae* to defend against oxidative stress, it has to detoxify and reduce the production of reactive oxygen species (ROS) as well as repair the damage (reviewed in Morano et al. 2012). As found in other stresses the general stress response transcription factors Msn2/4 are involved; however, Yap1 is the primary transcription factor for gene regulation under oxidative stress (Harshman et al. 1988). Many reports have shown that Yap1 is required for cells to defend against oxidants such as H$_2$O$_2$ and diamide as well as heavy metals like cadmium (Schnell and Entian 1991; Kuge and Jones 1994; Wu and Moye-Rowley 1994). Yap1 specifically activates genes based on the type of oxidative stress that a cell is experiencing and, because of this, regulation of Yap1 is very complex (reviewed in Morano et al. 2012). Under non-stress conditions, Yap1 interacts with the protein Crm1, which aids in exporting Yap1 from the nucleus (Isoyama et al. 2001) (Figure 4). Yap1 has two cysteine rich domains, one at each terminus (Wemmie et al. 1997; Coleman et al. 1999). The oxidant causing
the stress determines which terminus or if both are necessary for gene induction by Yap1 (Kuge and Jones 1994; Coleman et al. 1999; Delaunay et al. 2000) (Figure 4). When a cell is stressed with diamide for example, only the N-terminal cysteine-rich domain is modified which prevents Crm1 binding, resulting in retention of Yap1 in the nucleus (Figure 4; Isoyama et al. 2001). However, further modification of Yap1 occurs during H$_2$O$_2$ stress and Yap1 undergoes a conformational change because disulfide bonds form between the N- and C-termini (Delaunay et al. 2002; Veal et al. 2003; Gulshan et al. 2004). This inhibits Crm1 interaction, increases retention in the nucleus, and leads to altered patterns of gene expression (Figure 4; Wood et al. 2004).

An additional transcription factor, Skn7, is necessary for proper induction of many genes regulated by Yap1 during an H$_2$O$_2$ stress response. These include the genes that encode thioredoxin and superoxide dismutase (Morgan et al. 1997; Lee et al. 1999). Skn7 binds to some of the same promoters as Yap1 except that Skn7 binds at a different site (Morgan et al. 1997). Skn7 interacts with Mediator, a multi-subunit complex that is necessary for regulating the expression of most RNA polymerase II transcripts, which include protein-coding and most non-coding RNA genes (reviewed in Conaway and Conaway 2013). Yap1 does not interact with Mediator and so Skn7 is able to recruit components of Mediator to the promoters of crucial target genes (reviewed in Morano et al. 2012). Therefore, Skn7 facilitates Mediator interactions in order to activate full induction of stress response mechanisms through Yap1.

Transcriptional regulation is not the only response to oxidative stress. Another important process that is changed during this type of stress is the reprogramming of carbohydrate metabolism (reviewed in Morano et al. 2012). This evolutionarily conserved response to oxidative stress includes redirecting a cell’s metabolism from glycolysis to the pentose phosphate pathway,
generating NADPH as a result (Ralser et al. 2006). NADPH serves as the reducing potential for most antioxidant and redox regulatory enzymes (Morano et al. 2012). Glycolysis and the pentose phosphate pathway are directly linked by the oxidation of glucose-6-phosphate. The pentose phosphate pathway begins with glucose-6-phosphate dehydrogenase (G6PDH) catalyzing the conversion of glucose-6-phosphate to 6-phosphate-gluconolactone. Subsequently, 6-phosphate-gluconolactone is converted to ribose-5-phosphate by 6-phosphogluconate dehydrogenase (6PGDH). Both of these steps convert NADP\(^+\) to NADPH (Morano et al. 2012). In yeast, G6PDH and 6PGDH continue to be active during an oxidative stress response (Slekar et al. 1996; Izawa et al. 1998; Shenton and Grant 2003). Cells become resistant to oxidative stress when the glycolytic enzymes that are important for steps downstream of the oxidation of glucose-6-phosphate are inhibited (Ralser et al. 2006; Ralser et al. 2007). This occurs because the pentose phosphate pathway has been activated and there is a shift in the NADPH/NADP\(^+\) ratio to a more reduced state (Ralser et al. 2006; 2007). Furthermore, the production of many glycolytic enzymes are repressed during H\(_2\)O\(_2\) stress (Godon et al. 1998). Therefore, yeast cells defend against an oxidative stressor by preventing the activity of glycolytic enzymes and by down regulating gene expression of new glycolytic enzymes (reviewed in Morano et al. 2012). The combination of protein modification and gene expression allows for quick inhibition of glycolysis in a reversible manner.
Figure 4. The regulation of the transcription factor Yap1 during oxidative stress. The blue rectangles together represent the Yap1 transcription factor with two cysteine-rich domains (green). In unstressed conditions Yap1 moves into the nucleus at basal levels and is exported by Crm1. During oxidative stresses like diamide the N-terminus cysteine-rich domain is modified such that it cannot interact with Crm1 so it is not exported from the nucleus. However, during a stress like H$_2$O$_2$ it causes Yap1 to interact with two protein chaperones (orange boxes) that help Yap1 fold such that the two cysteine-rich domains form disulfide bonds.

1.3 SIW14

Biochemical Features

Siw14 of Saccharomyces is a member of the recently discovered plant and fungi atypical dual-specificity phosphatase (PFA-DSP) subfamily in the broader phosphotyrosine phosphatase super family. Members of this protein family are found in fungi, plants and protists but not in...
animals (Romá-Mateo et al. 2011). The PFA-DSP subfamily has the consensus sequence HCxxGxxR, which includes signature phosphatase catalytic residues in the yeast Siw14, C214 and R220 as well as two additional conserved residues, H213 and G217 (Romá-Mateo et al. 2011). The active site of Siw14 is basic and shallow, suggesting that the substrate for Siw14 is larger than a phosphorylated amino acid and is negatively charged (Figure 5; Romá-Mateo et al. 2011). Siw14 has no activity with phosphotyrosine (Romá-Mateo et al. 2011), which is consistent with the shape and charge of the active site.

Siw14 has 58% sequence similarity with an Arabidopsis protein, At1g5000, also found in the PFA-DSP subfamily. The Arabidopsis protein has catalytic activity with phosphatidylinositol 3-phosphate [PI(3)P], phosphatidylinositol 5-phosphate [PI(5)P], and small chain polyphosphates (poly-P) (Aceti et al. 2008; Romá-Mateo et al. 2011). Interestingly, the PFA-DSP subfamily has active site sequence homology with the mammalian protein PTEN (Romá-Mateo et al. 2011); the physiological substrate for PTEN is phosphatidylinositol 3, 4, 5-trisphosphate [PI(3,4,5)P3]. PIP3 is not found in S. cerevisiae (reviewed Odorizzi et al. 2000) but these data are consistent with Siw14 having a minimal amount of activity with phosphatidylinositol 3, 5-bisphosphate [PI (3,5)P2] (Romá-Mateo et al. 2011). However, soluble inositol polyphosphates could also be substrates for the enzymes in PFA-DSP subfamily because PTEN can also dephosphorylate the soluble inositol tetrakisphosphate (IP4) in vitro (Caffrey et al. 2001).

In S. cerevisiae, Siw14 has two paralogs, Oca1 and Oca2 that exhibit 51% and 58% amino acid sequence similarity throughout the catalytic core with Siw14 (Wishart and Dixon 1998). Thus, these proteins are also thought to be phosphatases. Purified recombinant GST-Oca1 showed no detectable activity with a generic phosphatase substrate para-nitrophenyl phosphate (pNPP) or with PI(3,4,5)P3 (Romá-Mateo et al. 2011). The Oca2 protein is predicted to be
catalytically inactive due to substitution of the active site cysteine to serine (Wishart and Dixon 1998). All three proteins (Siw14, Oca1 and Oca2) have genetic and physical interactions with each other as detected by two-hybrid assays and co-immunoprecipitation experiments (Ho et al. 2002; Gavin et al. 2006; Krogan et al. 2006; Breitkreutz et al. 2010).

![Model of the Saccharomyces cerevisiae Siw14 based on the PFA-DSP1 ortholog in Arabidopsis thaliana.](image)

**Figure 5. Model of the Saccharomyces cerevisiae Siw14 based on the PFA-DSP1 ortholog in Arabidopsis thaliana.** Yellow arrow points to the positively charged active site. Positive and negative charges are represented by blue and red shading, respectively. Figure taken from Figure 3C, Roma-Mateo et al. (2011).

**Genetics of SIW14**

The *siw14Δ* mutant is pleiotropic with a broad range of phenotypes suggesting Siw14 is important for many different cellular processes (Table 1). Siw14 also has genetic and physical interactions with genes and proteins involved in endocytosis, nutrient level detection, stress response, RNA binding, and aging (Fromont-Racine et al. 2000; Uetz et al. 2000; Ito et al. 2001;
This dissertation will focus on the phenotypes and interactions that suggest that Siw14 plays a role in the stress response. The phenotypes include resistance to heat stress at 37°C, high salt (1 mM NaCl), and H$_2$O$_2$ treatment (Care et al. 2004; Altıntaş et al. 2016). Siw14 also plays a role in the cellular response to low nitrogen by affecting the localization of Gln3, the transcriptional activator of nitrogen catabolite repression genes (Hirasaki et al. 2008). Under low nitrogen conditions, Gln3 is localized to the nucleus; but in an siw14Δ mutant, Gln3 stays in the cytoplasm (Courchesne and Magasanik 1988; Hirasaki et al. 2008). In addition, Oca1, the paralog and putative partner of Siw14, is also involved in the stress response; it is necessary for proper cellular response to oxidized lipid products (Alic et al. 2001). Interestingly, there is an additional genetic interaction between SIW14 and OCA1; mutants lacking each gene are sensitive to caffeine and this phenotype can be rescued in the oca1Δ mutant by the overexpression of SIW14 (Care et al. 2004; Romá-Mateo et al. 2011).

Altogether, these findings point to a role of Siw14 in the cellular stress response.
Table 1. Phenotypes associated with a mutation in *SIW14*.

<table>
<thead>
<tr>
<th>Category</th>
<th>Strain Background</th>
<th>Phenotype</th>
<th>Citation</th>
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<tr>
<td><strong>Stress</strong></td>
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<tr>
<td></td>
<td>W303</td>
<td>Resistance to 37°C</td>
<td>Care et al. 2004</td>
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<tr>
<td></td>
<td>W303</td>
<td>Sensitive to 53°C heat shock</td>
<td>Care et al. 2004</td>
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<td></td>
<td>BY4741</td>
<td>Resistant to 50°C heat shock</td>
<td>Chapter 2, Figure 22</td>
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<tr>
<td></td>
<td>BY4741</td>
<td>Long lived</td>
<td>Chapter 2, Figure 24</td>
</tr>
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<td></td>
<td>W303</td>
<td>Resistance to MgCl</td>
<td>Care et al. 2004</td>
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<td></td>
<td>W303</td>
<td>Arrest in G1 phase in response to nutrient deprivation</td>
<td>Care et al. 2004</td>
</tr>
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<td></td>
<td>W303 &amp; BY4742</td>
<td>Sensitive to caffeine</td>
<td>Care et al. 2004; Hirasaki et al. 2008</td>
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<td></td>
<td>W303</td>
<td>Resistant to 0.7 M CaCl</td>
<td>Care et al. 2004</td>
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<td></td>
<td>BY4741</td>
<td>Resistant to 1.3 M KCl</td>
<td>Chapter 2, Figure 23</td>
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<td></td>
<td>Y7092 &amp; BY4741</td>
<td>Resistant to ( \text{H}_2\text{O}_2 )</td>
<td>Altıntaş et al. 2016; Chapter 2, Figure 18</td>
</tr>
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<td><strong>Cell Wall</strong></td>
<td>BY4742</td>
<td>Sensitive to Hygromycin B</td>
<td>Hirasaki et al. 2010</td>
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<tr>
<td></td>
<td>W303</td>
<td>Resistant to 50 ( \mu \text{M} ) Calcofluor White</td>
<td>Care et al. 2004</td>
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<td><strong>Cytoskeleton</strong></td>
<td>W303</td>
<td>Actin clumping; cortical actin defects</td>
<td>Care et al. 2004</td>
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<td></td>
<td>W303</td>
<td>Septal protein accumulation</td>
<td>Care et al. 2004</td>
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<td><strong>TOR Pathway</strong></td>
<td>BY4741</td>
<td>Sensitive to Rapamycin</td>
<td>Appendix A, Figure 28</td>
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<td><strong>Respiration</strong></td>
<td>BY4741</td>
<td>Normal ( \text{O}_2 ) consumption</td>
<td>This study (Figure 20)</td>
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<td></td>
<td>W303 &amp; BY4741</td>
<td>Sensitive to glycerol</td>
<td>Care et al. 2004; Appendix B, Figure 30A,B</td>
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<td></td>
<td>BY4741</td>
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<td>Appendix B, Figure 30A</td>
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<td></td>
<td>BY4741</td>
<td>Normal growth on potassium acetate</td>
<td>Appendix B, Figure 30A</td>
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<td>BY4741</td>
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<td>Defective in fluid phase endocytosis</td>
<td>Care et al. 2004</td>
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<td></td>
<td>BY4741</td>
<td>Resistant to Canavanine</td>
<td>Appendix C, Figure 35</td>
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<tr>
<td></td>
<td>BY4741</td>
<td>Secretes excess Inosine and Hypoxanthine</td>
<td>Appendix C, Figure 34</td>
</tr>
<tr>
<td></td>
<td>BY4741</td>
<td>Sensitive to 5-Fluorocytosine</td>
<td>Appendix C, Figure 33</td>
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<tr>
<td><strong>Autophagy</strong></td>
<td>W303</td>
<td>Low levels of glycogen</td>
<td>Care et al. 2004</td>
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1.4 Stress, Inositol Pyrophosphates, and SIW14

To induce the general stress response in yeast, three key players are required in the nucleus: the transcription factors Msn2/4, the HDAC Rpd3L, and inositol pyrophosphates. Msn2/4 are necessary to up-regulate ~200 genes in the presence of general stress (Marchler et al. 1993; Martínez-Pastor et al. 1996; Schmitt and McEntee 1996; Gasch et al. 2000; Causton et al. 2001). The HDAC Rpd3 is required in order for Msn2 to mount a proper general stress response including the down-regulation of ribosomal biogenesis genes as well as the up-regulation of stress response genes (Alejandro-Osorio et al. 2009; Ruiz-Roig et al. 2010). Interestingly, a four amino acid substitution in an rpd3 mutant mimics an rpd3Δ null mutant when comparing gene expression profiles (Worley et al. 2013). These amino acids are in a putative inositol polyphosphate binding pocket that was identified in the human orthologue of Rpd3, HDAC3. HDAC3 requires IP₄ to interact with co-repressors which interact with transcription factors (Watson et al. 2012). Intriguingly, Watson and colleagues have shown via modeling that inositol pyrophosphates would also fit in the binding pocket of the HDAC3 protein based on stereochemistry (personal communication). Inositol pyrophosphates are required in yeast because strains lacking both KCS1 and VIP1 are unable induce a cellular stress response (Worley et al. 2013). These data together indicate that yeast cells need Rpd3L with an intact inositol phosphate binding pocket, as well as inositol pyrophosphates to induce a stress response through Msn2/4.

Many of the mutant phenotypes of an siw14Δ mutant align with processes that inositol pyrophosphates affect. For example, the siw14Δ mutant strain does not respond to low nutrients by arresting the cell cycle in G1 in stationary phase (Care et al. 2004). Likewise, inositol pyrophosphates are known to play a role in the cell cycle (Barker et al. 2004; Nagata et al. 2005; Banfic et al. 2013; Banfic et al. 2016). The roles that Siw14 specifically plays in the stress
response and how that role is linked to inositol pyrophosphates, although previously investigated is not thoroughly understood (Care et al. 2004; Worley et al. 2013). In-depth analysis of this link is the focus of this dissertation. One genetic interaction with the gene *WHI2* encoding an activator of the phosphatase Prs1 (Care et al. 2004) is of particular interest because it suggests that Siw14 affects the general stress response specifically. Prs1 helps to regulate the localization of Msn2 to the nucleus by dephosphorylation (Figure 3; Sneddon et al. 1990; Santhanam et al. 2004). The whi2Δ mutant has a slow growth phenotype that is exacerbated by deleting the *SIW14* gene during stress conditions (Care et al. 2004). This finding suggests that Siw14 is in a parallel pathway to Whi2 affecting the stress response.

**Major Findings**

The work described in this dissertation links inositol pyrophosphates to the stress response through Siw14. In chapter 1, the function of Siw14 is described as a phosphatase whose role is to dephosphorylate soluble inositol pyrophosphates. Using *in vitro* assays and *in vivo* characterization, Siw14 is shown to hydrolyze the β-phosphate at the 5-position on IP$_7$ (5PP-IP$_5$). In chapter 2, the *siw14Δ* mutant is shown to be is resistant to several different environmental stresses, including heat, osmotic, and oxidative stresses and that it has a longer life span. Furthermore, the *siw14Δ* mutant is resistant to these stresses because the stress response is partially up-regulated even in the absence of stress as assessed by global gene expression patterns. Importantly, the levels of inositol pyrophosphates increase when cells are treated with hydrogen peroxide. This latter finding suggests that the levels of IP$_7$ and IP$_8$ are regulated during oxidative stress. These data support a model that inositol pyrophosphates are an intracellular
signal of oxidative stress. The regulatory mechanisms for altering IP$_7$ and IP$_8$ levels can occur through the Siw14 phosphatase.
CHAPTER 2

A Novel Inositol Pyrophosphate Phosphatase in *Saccharomyces cerevisiae*:

Siw14 Selectively Cleaves the β-phosphate From

5-diphosphoinositol Pentakisphosphate (5PP-IP$_3$)

Contributing Authors

Lucy Chong, Mingxuan Wu, Elliott Crooke, Dorothea Fiedler, Adam Resnick, and Ronda Rolfes

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2.1 Introduction

Inositol pyrophosphates are a novel class of signaling molecules that carry energy-rich diphosphate bonds. In wild-type yeast, the most abundant isoform, diphosphoinositol pentakisphosphate (PP-IP$_5$ or IP$_7$), consists of a fully phosphorylated six carbon *myo*-inositol ring further pyrophosphorylated at one of the carbons. Two physiologically relevant isomers of IP$_7$ include 1PP-IP$_5$ and 5PP-IP$_5$ in which the pyrophosphate group is found at the 1-position or the 5-position, respectively. Sequential phosphorylation of IP$_7$ results in bisdiphosphoinositol tetrakisphosphate (1,5PP-IP$_4$ or IP$_8$) that is pyrophosphorylated at both the 1- and 5-positions (reviewed in Wilson et al. 2013; Thomas and Potter 2014). Since their discovery more than 20 years ago, inositol pyrophosphates have been implicated in an array of processes including cellular energetic metabolism, telomere maintenance, oxidative stress response, and vesicle trafficking in animals and fungi (Wilson et al. 2013). Recent work in *Saccharomyces cerevisiae* found that production of inositol pyrophosphates is essential for mounting environmental stress
responses (Worley et al. 2013). Inositol pyrophosphates are thought to be metabolic regulators displaying expanded cellular roles beyond classic second messengers (Wilson et al. 2013).

The enzymes that regulate the pools of inositol pyrophosphates are not fully described. The inositol hexakisphosphate kinases (IP6K) synthesize IP7, the most abundant inositol pyrophosphate in the cell; they add the β-phosphate to IP6 at the 5-position generating 5PP-IP5 (reviewed in Thomas and Potter 2014). Yeast possess a single IP6K gene, KCS1, while mammals possess three homologous genes encoding IP6K (Figure 1C; adapted from Hatch and York 2010). IP8 is synthesized by a PP-IP5 kinase that adds the β-phosphate to IP7 at the 1-position to make 1,5PP-IP4; this enzyme can also use IP6 as a substrate, generating 1PP-IP5. Mammalian genomes have two genes for PPIP5K and the yeast genome has a single gene, VIP1, which encodes this enzyme. To date, phosphatases described to target the β-phosphate of IP7 and IP8 include members of the Nudix family of hydrolases, (reviewed in Thomas and Potter 2014) named DIPP for diphosphoinositol phosphate phosphohydrolase. In mammals, the DIPP enzymes display a preference for the β-phosphate at the 1-position and are encoded by four genes (Kilari et al. 2013). One orthologue is found in yeast, encoded by DDPI; this phosphatase was recently described to prefer polyphosphate (poly-P) over inositol pyrophosphates although it is able to cleave the β-phosphate at the 1-position on both IP7 and IP8 in vitro (Lonetti et al. 2011).

Siw14 of Saccharomyces is a member of the recently discovered plant and fungi atypical dual-specificity phosphatase subfamily, with members in fungi, plants and protists (Romá-Mateo et al. 2011). The SIW14 gene encodes a phosphatase with a basic, shallow active site that was previously shown to have low activity with PI(3,5)P2 and no activity with phosphotyrosine (Romá-Mateo et al. 2007). In this chapter, Siw14 is described to be a soluble inositol phosphatase dephosphorylating inositol pyrophosphates. Using in vitro assays and in vivo
characterization Siw14 is shown to remove the \( \beta \)-phosphate at the 5-position on IP\( _7 \) (5PP-IP\( _7 \)). These results define Siw14 as an important regulator of inositol pyrophosphates and establish its role as an IP\( _7 \) phosphatase.

### 2.2 Experimental Procedures

**Yeast Strains and Plasmids**

Yeast strains were purchased from Open Biosystems and Invitrogen, including parental strain BY4741 (MATa his3\( ^{\Delta} \) leu2\( ^{\Delta} \) met15\( ^{\Delta} \) ura3\( ^{\Delta} \)) and isogenic mutant strains: siw14\( ^{::KanMX} \), vip1\( ^{::KanMX} \), ddp1\( ^{::KanMX} \), oca1\( ^{::KanMX} \), and oca2\( ^{::KanMX} \). The double mutants vip1\( ^{::KanMX} \) siw14\( ^{::URA3} \) (siw14\( ^{\Delta} \) vip1\( ^{\Delta} \)), ddp1\( ^{::KanMX} \) siw14\( ^{::URA3} \) (siw14\( ^{\Delta} \) ddp1\( ^{\Delta} \)), oca1\( ^{::KanMX} \) siw14\( ^{::URA3} \) (siw14\( ^{\Delta} \) oca1\( ^{\Delta} \)), and oca2\( ^{::KanMX} \) siw14\( ^{::URA3} \) (siw14\( ^{\Delta} \) oca2\( ^{\Delta} \)) were constructed by replacing SIW14 with siw14\( ^{\Delta} ::URA3 \) using homologous recombination after amplification of the allele using the primers: 5’-AGTTCGGCTTTTTA TCTACTCTCTTCTGGATCAATTTTTTTTCTCTAAAGTTAAAAGGAGTCATATTC ATCATTTT-3’ and 5’-TATCAATAAACATCATTTTCGAAGAGACTAGTTACGTAAGGT AATCACTGTCATATATTACCATTAGTTTTGCTGGCC-3’. oca2\( ^{::KanMX} \) oca1\( ^{::LEU2} \) (oca1\( ^{\Delta} \) oca2\( ^{\Delta} \)) was constructed by replacing OCA1 with oca2\( ^{\Delta} ::LEU2 \) using homolog recombination after amplification of the allele using the primers: 5’TCCATTTCACACAAGAGGTGTCCACCTGGAAATAAACAAGCTTCTCTTTCCCTTACAAAGGACTGTGGGAAT ACT’3’ and 5’ 5’GTAAATGGTCATAAAGCTCAGTGGCTTTTTCTTTATCTTTTGCAATT TTCTTTCTGGCTTTAAGCAAGGATTCTTTT3’. Transformation of *Saccharomyces* used the protocol of Ito et al. (Ito et al. 2001).
Plasmid pR492 carries the *SIW14* gene with its native promoter in the centromeric pRS316 vector (Sikorski and Hieter 1989) for expression in *Saccharomyces*. It was cloned after amplification using primers *SIW14* Forward (5’-ACTCTTCAAGCTTGCTATAGATGGAGCT3’), *SIW14* Reverse (5’-AGCTCTCGAGCCTATGCATTTAGACTGG-3’), BY4741 genomic DNA as template, and PrimeStar HS Polymerase (Clontech). PCR fragments were purified (Qiagen Clean-up Kit), digested with *Hin*DIII and *Xho*I (New England Biolabs), and ligated into pRS316. The catalytically dead allele, *SIW14*-C214S (Care et al. 2004), was made by fusion PCR (Cha-Aim et al. 2012). Initial synthesis of fragments was completed using PrimeSTAR® HS DNA Polymerase (Clontech), following the manufacturer’s instructions, pR492 as template, and primer pairs *SIW14* Forward with C214S Reverse (5’-CAACCGATACTGATACATAGTAATAGAGGCAAACATAGAACG-3’) and C214R Forward (5’-CGTTCTATGTTTGCCTCTATTACTATGATCAGTATCGGTTG-3’) with *SIW14* Reverse. Fragments were purified and combined as templates for the fusion PCR using the *SIW14* Forward and Reverse primers. The fusion product was purified, digested and ligated as above, and its DNA sequence was determined, generating plasmid pR496.

For expression of proteins in *E. coli*, plasmid vectors pGEX-4T-1 and pGEX-4T-*SIW14* were obtained (Romá-Mateo et al. 2011). The mutant allele of *SIW14* (*SIW14*-C214S) was constructed using fusion PCR (Cha-Aim et al. 2012), as described above, using primers pGEX Forward (5’-GCAGGGCTGGCAAGCCAC-3’) and Reverse (5’-CGAAACGCGCGAGGCGCCAG-ATC-3’) in place of the *SIW14* primers. Fusion products were purified, digested with *Bam*HI and *Xho*I (New England Biolabs), ligated into pGEX-4T-1, and confirmed by DNA sequencing.

For expression in mammalian cells, the *SIW14* gene was cloned into the donor vector plasmid pDONR221 using BP Clonase II and sequentially transferred to a Gateway compatible
N-terminally tagged pCMV-Myc destination vector using LR Clonase II, following the manufacturer’s directions in the Gateway Cloning Technology Instruction Manual (Life Technologies). The \textit{SIW14}-C214S allele was constructed in the pCMV-Myc plasmid via site-directed mutagenesis using the following primer pairs Forward 5’CTATTACTATGTATCTATTGTATCAGTATCGGGTTGATTGCAGG3’ and Reverse 5’GATACATAGTAATAGGCAAGGCAACACATA-GAACGGGG3’. The sequence of all plasmid inserts was determined and confirmed to be correct.

\textbf{Expression and Purification of Recombinant Proteins}

Plasmids pGEX-4T-1, pGEX-4T-\textit{SIW14}, and pGEX-4T-\textit{SIW14-C214S} were transformed into the \textit{Escherichia coli} strain Rosetta\textsuperscript{TM}(DE3)pLysS (EMD Millipore). Overnight bacterial cultures were inoculated 1:20 into fresh LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) with 100 \(\mu\)g/\(\mu\)L ampicillin and 34 \(\mu\)g/\(\mu\)l chloramphenicol. Cells were grown at 37°C with shaking for 2 hr (~0.3 OD\textsubscript{600}) and protein expression was induced at room temperature for 3 hr with 0.1 mM IPTG. Transformed cells were lysed as described (Romá-Mateo et al. 2011), substituting Tris buffered saline [19.98 mM Tris-HCl, pH 8.0, 135 mM NaCl] containing 0.1% Triton X-100, 5 mM dithiothreitol (DTT), and protease inhibitors (Halt\textsuperscript{®}, ThermoScientific) as the lysis buffer. Proteins were purified using glutathione agarose (Pierce) resin following the manufacturer’s instructions with the following change: resin with bound protein was incubated in wash buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl) containing 28 mM reduced glutathione (Sigma) for 5 min at room temperature to elute. Three elutions were combined and proteins were concentrated using Amicon Ultra centrifugal 30 kDa filters for 15 min at 4,000 \(\times\) g at 4°C (Sorvall RC6+ centrifuge). Proteins were examined using SDS-PAGE (Any kD\textsuperscript{TM} Mini-PROTEAN\textsuperscript{®} TGX\textsuperscript{TM} Gel, BioRad) followed by Coomassie Blue staining or immunoblotting.
analysis with rabbit α-GST-HRP antibody (Abcam) at a 1:5000 dilution in phosphate buffered saline (10 mM sodium phosphate, pH 7.4, 137 mM NaCl, 2.7 mM KCl), 2% milk, and 0.01% Tween-20 and were compared with Kaleidoscope Prestained Standards (BioRad).

**Phosphatase Activity Assays**

*Substrates*

*p*-Nitrophenyl phosphate (pNPP) was purchased from Sigma-Aldrich. The phosphatidylinositol phosphates phosphatidylinositol 3-phosphate (PI(3)P), phosphatidylinositol 4-phosphate (PI(4)P), phosphatidylinositol 5-phosphate (PI(5)P), phosphatidylinositol 3,4-bisphosphate (PI(3,4)P₂), phosphatidylinositol 3,5-bisphosphate (PI(3,5)P₂), phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂), and phosphatidylinositol 3,4,5-triphosphate (PI(3,4,5)P₃), each with caprylic acid (8:0) as the two acyl chains, were purchased from Eschelon Biosciences. Inositol hexakisphosphate (IP₆) was purchased from Sigma-Aldrich. Four isoforms of IP₇ (5-diphosphoinositol pentakisphosphate [5PP-IP₅], non-hydrolyzable 5-methylene-bisphosphonate inositol pentakisphosphate [5PCP-IP₅], 1-diphosphoinositol pentakisphosphate [1PP-IP₅], and non-hydrolyzable 1-methylene-bisphosphonate inositol pentakisphosphate [1PCP-IP₅]) and IP₈ (3,5-bisdiphosphoinositol tetrakisphosphate [3,5PP-IP₄]) were synthesized following the methods of (Wu et al. 2013; Capolicchio et al. 2014; Wu et al. 2014).

[^32P]-polyphosphate (poly[^32P]) was prepared as described in (Akiyama et al. 1993) using purified recombinant *E. coli* polyphosphate kinase (gift of E. Crooke) and 5 μCi of [δ[^32P]] ATP (3000 Ci/mmol; Perkin-Elmer). Substrate preparations were analyzed by scintillation counting to calculate the concentration of poly-P synthesized.
Detection of orthophosphate

Orthophosphates cleaved from the phosphatidylinositol phosphates were detected with the EnzChek Phosphate Assay Kit (LifeTechnologies) following the manufacturer’s instructions, except reaction volumes were scaled down to 100 μL and the absorbance at 360 nm was measured using a NanoDrop ND-1000. The orthophosphates cleaved from the inositol pyrophosphates were measured using the Malachite Green Phosphate Assay Kit (Cayman Chemical Company) following the manufacturer’s guidelines.

Assay conditions with p-nitrophenyl phosphate and phosphatidylinositol phosphates

Purified recombinant Siw14 (10 μg) was incubated with 10 mM of pNPP in 25 mM HEPES, pH 6, 50 mM NaCl, 10 mM MgSO₄, and 1 mM DTT for 90 min at 37°C. pNPP assays were analyzed by the colorimetric change at 420 nm using the GloMax Multi Detection System (Promega).

Purified recombinant Siw14 (50 μg) was incubated with 100 μM of the mono-, di-, and triphosphorylated phosphatidylinositol phosphates PIPₙ in reaction buffer (0.1 M Tris-HCl, pH 6.0, 10 mM DTT, 40 mM NaCl) for 90 min at 37°C (Romá-Mateo et al. 2011). PTEN (0.08 μg; Eschelon Biosciences) was used with PI(3,4,5)P₃. The specific activity of both Siw14 and PTEN with PIPₙ were normalized to their activities with pNPP.

Assay conditions with soluble inositol pyrophosphates (IPₙ)

Purified recombinant Siw14 (10 μg) was incubated with each inositol polyphosphate substrate, as indicated in the figure, 90 min at 37°C in 25 mM HEPES, pH 6.8, 50 mM NaCl, 10 mM MgSO₄, and 1 mM DTT. Human DIPP1 (hDIPP1) enzyme (1 μg) (gift of A. Saiardi) was
incubated for 15 min under the same conditions. Reaction products were separated by electrophoresis in 35% acrylamide gels in TBE buffer (89 mM Tris, pH 8.0, 89 mM boric acid, 2 mM EDTA) and stained with Toluidine Blue (20% methanol, 0.1% Toluidine Blue, and 3% glycerol) as described (Losito et al. 2009; Lonetti et al. 2011).

Assay conditions with polyphosphate (poly-P)

Recombinant Siw14 (20 μg) was incubated with 600 μM $^{32}$P in the form of poly-$^{32}$P for 90 min at 37°C in 50 mM HEPES-KOH, pH 7, 5 mM MgCl$_2$, 0.4% β-mercaptoethanol and 2% glycerol. As a positive control, 2.5 μg of *E. coli* polyphosphate phosphatase (PPX; gift of E. Crooke) was incubated for 15 min under the same conditions. Reaction products were spotted onto thin layer chromatography plates and assayed as described (Akiyama et al. 1993). Plates were exposed to phosphorimaging screens, and the density of each spot on the TLC plate was measured using ImageQuant software on a STORM 840 Molecular Dynamics instrument.

Thrombin Protease Assay

In order to test whether the GST-tag affects the activity of Siw14 it was cleaved using 15 μg of thrombin protease. GST-SIW14 was incubated with thrombin or 1X TBS for 3 hrs at 4°C. Both cleaved protein and fusion protein were examined using SDS-PAGE (followed by Coomassie Blue staining or immunoblot analysis with rabbit α-GST-HRP antibody. Activity of the proteins was measured with pNPP and 5PP-IP$_5$.  

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**Extraction of $^3$H-Inositol Phosphates ($^3$H-IP$_n$) and HPLC Analysis**

Myo-inositol pools in yeast cells were uniformly radiolabeled by culturing strains overnight in YPD (1% yeast extract, 2% peptone, and 2% dextrose) and 0.005 OD$_{600}$ cells were inoculated into 10 mL of synthetic complete (SCD) medium lacking inositol (0.17% YNB-inositol (MP Biomedicals), 0.5% ammonium sulfate, 0.069% CSM-Leu amino acid mix (MP Biomedicals), 0.76 mM leucine, and 2% dextrose) and containing 75 μCi $^3$H-myoinositol (>60 Ci/mmol, PerkinElmer). Cultures were grown for 20-24 hr until cells were in mid-log phase (~0.6 OD$_{600}$). Tritiated soluble inositol phosphates ($^3$H-IP$_n$) were extracted as described (Azevedo and Saiardi 2006), except that cells were broken via vortex in the presence of glass beads for four cycles of 2 min with cooling between for 2 min on ice. The inositol phosphates in extracts were separated using HPLC using a Hichrom 110 x 4.6mm Partisphere SAX column and collecting 1 mL fractions per min for 90 min. The cpm of each fraction was measured by scintillation counting (Beckman Coulter) in Ultima-Flo AP (PerkinElmer) scintillation fluid. All inositol phosphate profiles were corrected for baseline cpm before analysis.

**Extraction of $^3$H-Inositol Phosphates ($^3$H-IP$_n$) in Mammalian Cells and HPLC Analysis**

One mL of HEK-293T cells were seeded in 6 cm well plates at 0.2 × 10$^6$ cells/mL confluency in medium [1X DMEM (Corning cellgro), 10% FBS (HyClone), 1% L-alanyl-L-glutamine (Life Technologies)] containing 1% penicillin-streptomycin (Lonza) and 0.1 mg/mL Normocin (InvivoGen). The following day the medium was exchanged with 1 mL inositol-free medium supplemented with $^3$H-myoinositol [1X DMEM without L-glutamine and without inositol (MP Biomedicals), 10% FBS (HyClone), 1% L-alanyl-L-glutamine (Life Technologies), 1% penicillin-streptomycin (Lonza), 0.1 mg/mL Normocin (InvivoGen), and 20 μCi/mL $^3$H-myoinositol].
inositol (PerkinElmer)] and labeled for 72 hours. Cells were transfected in duplicate with mammalian expression plasmids, empty vector (pCMV-Myc) or expressing myc-tagged Siw14 (Myc-Siw14) or myc-tagged Siw14-C214S for 48 hr in 2 mL of the $^3$H-myoinositol labeling medium with Lipofectamine 2000 (Invitrogen) as per manufacturer’s instructions. Soluble $[^3$H]-inositol phosphates were extracted as described (Azevedo and Saiardi 2006) with the modifications that 250 μL of extraction buffer was added to each well and duplicate transfected wells were combined; and, neutralization occurred overnight at 4°C.

**Extraction of Poly-P and Quantification**

Poly-P was extracted as described (Lonetti et al. 2011) and ~150,000 cpm of poly-$^{32}$P was spiked into the lysis buffer per sample in order to measure extraction efficiency; recovery was measured by scintillation counting. Cells were lysed for 8 min in 2 min cycles. To measure the total poly-P pools, poly-P was converted to ATP using 3.4 μg PPK, 10 mM ADP in 50 mM Tris-HCl, pH 7.0, 4 mM MgCl$_2$, 40 mM (NH$_4$)$_2$SO$_4$ for 2 hours. Reactions were stopped with 4 mM EDTA and 80 mM Tris-HCl, pH 8.0. ATP was measured using an ATP Luciferase Assay Kit (Invitrogen) on a Victor$^3$V 1420 Multi Label counter (PerkinElmer) plate reader. Twelve biological replicates were analyzed over four experiments; averages were calculated after samples had been normalized to extraction efficiency.

**Extraction of Phosphatidyl($^3$H)inositol Phosphates (P[$^3$H]IP$_n$) and HPLC Analysis**

Cells were grown overnight in YPD and 0.005 OD$_{600}$ cells were inoculated into SCD-inositol medium containing 75 μCi $^3$H-inositol. Cultures were grown for 20-24 hr until cells were in log phase (~0.6 OD$_{600}$). Tritiated phosphatidylinositides (P[$^3$H]IP$_n$) were extracted and
guidelines. Briefly, cells were killed by mixing them with 2 volumes of methanol:HCl (100:1)
for 5 min on ice, and harvested by centrifugation at 6,000 × g for 10 min at 4°C. Lysis was
accomplished by 16 rounds of vortexing in 300 μL of H₂O:methanol:HCl (66:100:1) with glass
beads for 30 sec and resting on ice for 1 min between rounds. Phosphatidylinositol phosphates
were isolated by extracting the samples with 1.33 mL of ice cold chloroform and 470 μL of
methanol:HCl (100:1) on ice for 10 min. Samples were mixed with 20 μg nonradioactive bovine
brain phosphatidylinositol phosphate as a carrier (Sigma-Aldrich) and extracted with 0.8 M HCl, 5
mM tetrabutylammonium hydrogen sulfate and 1 mM EDTA. The lower layer was dried in a
vacuum dryer and resuspended in 750 μL methanol. To deacylate the PIP₅, samples were
incubated with methylamine reagent (Sigma-Aldrich) at 53°C for 50 min, cooled to room
temperature and dried under vacuum. Samples were washed with water and then the lower
aqueous phase was removed using n-butanol:petroleum ether:ethyl formate (20:4:1) two times
and dried again. Once deacylated, the IP₅ were separated using a Waters 1525 Binary HPLC
pump with a Hichrom 125 × 4.6mm Partisphere SAX column as described above except that 120
fractions of 1 ml each were collected. The ³H-inositol was quantified for each elution as
described above.

Statistical Analysis of Data

Enzyme assays were performed in triplicate except for the PIP₅, which were performed in
duplicate; this is noted in the figure legends. Experiments with radiolabeled cells and extraction
of soluble or lipid inositol phosphates were performed between four and nine times, as indicated
in the figure legends, using an overnight culture that had been inoculated with a freshly grown
colony of \textit{S. cerevisiae} with the indicated genotype. For the mammalian transfection experiments, three to five samples containing HEK293T cells were transfected with the indicated constructs and were analyzed for inositol polyphosphates. The mean and standard error of the mean are reported graphically. The paired Student T-test was used to determine significance, and \emph{p}-values are reported using the following scheme: *\emph{p}-value \leq 0.05, **\emph{p}-value \leq 0.01, ***\emph{p}-value \leq 0.001, and ****\emph{p}-value \leq 0.0001.

2.3 Results

\textbf{Biochemical Characterization of Siw14}

The \textit{SIW14} gene of \textit{S. cerevisiae} encodes a phosphatase with a basic, shallow active site (Aceti et al. 2008; Romá-Mateo et al. 2011). In order to characterize substrates of the yeast \textit{SIW14} encoded phosphatase, GST-tagged Siw14 was expressed in \textit{E. coli} and purified by affinity chromatography. The predicted mass of the recombinant fusion protein was 52 kDa and it migrated at \~{}50 kDa by SDS-PAGE (Figure 6A). The purified recombinant protein displayed activity using the generic phosphatase substrate \textit{p}-nitrophenyl phosphate (\textit{pNPP}, Figure 6B). Site directed mutagenesis was performed to generate a phosphatase inactive mutant of Siw14 by mutating the conserved cysteine within the putative active site (position 214) to serine (Care et al. 2004). The activity of the purified GST-Siw14-C214S mutant protein was undetectable (Figure 6B), confirming that the phosphatase activity detected in the purification of GST-Siw14 was due to Siw14 itself. The optimal conditions for the Siw14 phosphatase reaction are 37°C in a buffer of pH 6.0 (Figure 6C). Treatment with thrombin to remove the GST moiety had no effect on activity towards \textit{pNPP} (Figure 11). The activity of Siw14 for \textit{pNPP} was low, with a Km of 3 mM, \textit{k}_{\text{cat}} of \textbf{4.4 \times 10^{-7}} \text{ s}^{-1}, and specificity constant of \textbf{1.5 \times 10^{-4}} \text{ M}^{-1}\text{s}^{-1} (Figure 7A & B; Table 2).
Figure 6. Enzymatic activity of purified GST-Siw14 with pNPP. (A) Purification of GST-Siw14 from E. coli. A representative Coomassie stained gel (top panel) and anti-GST immunoblot (bottom panel) of crude extracts (crude) and purified empty vector (vector), GST-Siw14, and GST-Siw14 carrying the substitution C214S (GST-C214S). Ten µg of each protein sample was used for both panels; marker, Kaleidoscope Prestained Standards. (B) Activity of the crude extract before purification and after purification, GST-Siw14 and a catalytically dead version of GST-Siw14 (GST-C214S). (C) GST-Siw14 activity with pNPP while varying the pH. All enzymes assays were completed in triplicate; error bars represent the standard error of the mean.
Figure 7. Kinetic characterization of Siw14 with pNPP. (A) Michaelis-Menten and (B) Hanes-Woolf plots showing the rate of GST-Siw14 activity over a range of substrate concentrations. Each bar or point represents an average of triplicate reactions using 10 µg of protein in buffer (25 mM HEPES, pH 6, 1 mM DTT, 50 mM NaCl, and 10 mM MgSO₄).

Table 2. Kinetic measurements for Siw14.

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<th>pNPP</th>
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<tr>
<td>Kₘ</td>
<td>3 mM</td>
<td>35 µM</td>
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<tr>
<td>kₗcat</td>
<td>4.4 x 10⁻⁷ s⁻¹</td>
<td>2.5 x 10⁻³ s⁻¹</td>
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<tr>
<td>Specificity Constant</td>
<td>1.5 x 10⁻⁴ M⁻¹ s⁻¹</td>
<td>74 M⁻¹ s⁻¹</td>
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</table>
**Siw14 Has a Low Activity Towards poly-P**

To identify the substrate(s) of Siw14, the activity with poly phosphate (poly-P) were tested first (Aceti et al. 2008; Romá-Mateo et al. 2011). Siw14 is homologous to the *Arabidopsis thaliana* protein AtPFA-DSP1 that is able to cleave short chain poly-P molecules (Aceti et al. 2008). Siw14 cleaved orthophosphate from radiolabeled poly-P but only with the use of more protein and longer reaction times as compared with pNPP and with a control phosphatase, *E. coli* polyphosphate phosphatase (PPX) (Figure 8). Thus, these data lead to the conclusion that poly-P is a poor substrate for Siw14 and is not its specific substrate.

![Figure 8. Siw14 has low activity with poly-P. (Top panel)](image)

GST-Siw14 (20 µg) was incubated with 0.614 mM $^{32}$P in the form of poly-$^{32}$P for up to 90 min in buffer at 37°C; PPX (2.5 µg) alone or with Siw14 (20 µg) was incubated for 15 min under the same conditions. Orthophosphate (in 4 µL of the reaction volume) was separated from poly-P by thin layer chromatography and (Bottom panel) quantified using ImageQuant software.
Siw14 Has Low Activity with Phosphatidylinositol Phosphates (PIPₙ)

The ability of Siw14 to dephosphorylate phosphatidylinositol phosphates (PIPₙ) was tested because the purified enzyme was previously described to act upon PI(3,5)P₂ (Romá-Mateo et al. 2011). Each form of the mono-, di-, and triphosphorylated phosphatidylinositol phosphates with two caprylic acid (8:0) acyl chains were used as potential substrates and measured the release of orthophosphate. As shown in Figure 9A, recombinant Siw14 protein had only minimal activity with each of the PIPₙ, showing no clear preference for any of these substrates; the greatest activity occurred with PI(3,5)P₂ as substrate. The activity of Siw14 was compared to PTEN, a well-characterized PI(3,4,5)P₃ phosphatase, after normalizing their activities to pNPP as a common substrate. The specific activity of Siw14 for PI(3,5)P₂ was nearly 20-fold lower than that of PTEN for PI(3,4,5)P₃ (Figure 9A). While these data replicated the results of Romá-Mateo and colleagues (Romá-Mateo et al. 2011), they suggested that none of the PIPₙ are the authentic physiologic substrates for Siw14.

To investigate this further, we radiolabeled cells with ³H-myo-inositol, extracted phosphatidylinositol phosphates from wild-type and siw14Δ mutant cells, and examined the deacylated products by HPLC; using this method, we can detect the inositol head groups from PI(3,5)P₂ and PI(4,5)P₂. We found no difference in the levels of tritiated PI(3,5)P₂ or PI(4,5)P₂ in the siw14Δ mutant as compared to the isogenic wild-type strain, consistent with the in vitro experiments (Figure 9B, C).
Figure 9. Siw14 has low activity with phosphatidylinositol phosphates. (A) Specific activity of duplicate samples of Siw14 (50 μg) with each of the seven isoforms of PIP$_n$, and PTEN (0.08 μg) with PI(3,4,5)P$_2$, normalized to their specific activity with pNPP. Error bars represent the standard error of the mean. (B) Representative PIP$_n$ profiles for WT and siw14$\Delta$ mutant; this experiment was repeated four times. (C) Average PI(3,5)P$_2$ and PI(4,5)P$_2$ expressed as a percentage of the total of the total $^3$H-inositol counted in four biological replicates, error bars represent the standard error of the mean. The differences were not significantly different using the Student T-test; $p$-value = 0.22 for PI(3,5)P$_2$ and $p$-value = 0.63 for PI(4,5)P$_2$. 
**Soluble Inositol Pyrophosphates are the Physiologic Substrates for Siw14**

We considered whether soluble inositol polyphosphates might be the natural substrates for Siw14 because of their structural similarity to phosphatidylinositol phosphates for which Siw14 had low preference and the shallow and basic active site of Siw14 that might accommodate them (Romá-Mateo et al. 2011). Purified recombinant GST-Siw14 was tested with commercially prepared inositol hexakisphosphate (IP\(_6\)) and two different isoforms of chemically synthesized IP\(_7\) (5-diphosphoinositol pentakisphosphate [5PP-IP\(_5\)] and 1-diphospho-inositol pentakisphosphate [1PP-IP\(_5\)]). The human DIPP1 (hDIPP1) enzyme was used as a positive control during quantitation because it is a known inositol pyrophosphate phosphatase (Lonetti et al. 2011; Kilari et al. 2013).

As shown in Figure 10A and B, Siw14 exhibited no detectable activity with IP\(_6\) as a substrate and very low activity with 1PP-IP\(_5\) as a substrate. However, Siw14 dephosphorylates 5PP-IP\(_5\) to IP\(_6\) as shown by the change in mobility of bands on a 35% acrylamide gel (Figure 10A, B). These reactions were quantified by using a malachite green assay (Figure 10C), and found that Siw14 exhibited greater activity with 5PP-IP\(_5\) than with 1PP-IP\(_5\) or with IP\(_6\). The activity was assessed from purified recombinant GST-Siw14 and GST-Siw14-C214S proteins with 5PP-IP\(_5\) as substrate; and no orthophosphate product formed from the C214S mutant protein (Figure 10E). These data showed that 5PP-IP\(_5\) is a preferred substrate for Siw14.

To confirm that Siw14 was cleaving the pyrophosphate from 5PP-IP\(_5\), the ability of Siw14 to utilize analogs of IP\(_7\) that contain non-hydrolyzable methylene-bisphosphonate moieties was measured (Wu et al. 2013; Wu et al. 2014). In these analogs, carbon replaces the oxygen in the anhydride linkage to the β-phosphate. In semi-quantitative gel assays, GST-Siw14 hydrolyzed 5PP-IP\(_5\) to IP\(_6\) but was unable to use 5PCP-IP\(_5\) as a substrate (Figure 10B). This
finding is consistent with Siw14 hydrolyzing the β-phosphate at the 5-position. To quantify this, malachite green assays were performed with GST-Siw14 using 5PP- and 1PP- isomers of IP₇ as well as the bisphosphonate analogs; hDIPP1 was used as a positive control. As can be seen in Figure 10C, no product formed when 5PCP-IP₅ and 1PCP-IP₅ were used as substrates for GST-Siw14, indicating that Siw14 is unable to cleave any of the phosphates from these molecules. Together with the above data presented in Figure 8 and Figure 9, our results provide support that Siw14 is capable of dephosphorylating the β-phosphate of inositol pyrophosphates as the physiologic substrate, preferring 5PP-IP₅ over 1PP-IP₅, to produce IP₆.

The biochemical properties of GST-Siw14 using 5PP-IP₅ were characterized. Siw14 has highest activity at a pH of 6.0 (Figure 10D), similar to pNPP; and the affinity of Siw14 for 5PP-IP₅ and its kinetic parameters were assayed under these conditions (Figure 10E, F). Siw14 has a Kₓ of 34 μM, a kₓ of 2.5 × 10⁻³ s⁻¹, and a specificity constant of 74 M⁻¹ s⁻¹ with 5PP-IP₅ using Hanes-Woolf plot analysis. It is noted that the Kₓ for 5PP-IP₅ is ~100x lower than for pNPP and the kₓ for 5PP-IP₅ is ~6000× faster, supporting an enhanced kinetic efficiency of 5 orders of magnitude with 5PP-IP₅ (compare the specificity constants of 74 M⁻¹ s⁻¹ for 5PP-IP₅ vs. 1.5 × 10⁻⁴ M⁻¹ s⁻¹ for pNPP) (Table 2). This affinity value of 74 M⁻¹ s⁻¹ was lower than expected. We wondered if the GST-tag was inhibiting the activity of Siw14; therefore, the GST-tag was cleaved from the GST-Siw14 fusion protein using thrombin. Greater than 95% of the fusion protein was separated from the tag (none visible by Coomassie stain and ~5% was visible as fusion protein by immunoblot analysis, Figure 11A,B). No difference was observed in activity towards either pNPP or 5PP-IP₅ as compared with the tagged protein (Figure 11C,D).
Figure 10. Siw14 dephosphorylates IP7. (A and B) Reactions containing 10 µg GST-Siw14 (+) or water (-) were incubated with 400 µM IP6, 1PP-IP5, 5PP-IP5 or the non-hydrolyzable analog, 5PCP-IP5 (as indicated) and then separated on a 35% acrylamide gel. The mobility of IP6, IP7, and IP8 are indicated. Representative gels are shown; experiments with 1PP-IP5 and 5PP-IP5 were repeated three times; experiments with 5PCP-IP5 were repeated twice. (C) Reactions containing 10 µg of GST-Siw14 or 1 µg of hDIPP1 was incubated with 50 µM of IP6, 5PP-IP5, 1PP-IP5, or the non-hydrolyzable analogs 5PCP-IP5 or 1PCP-IP5. The cleaved orthophosphate was quantified using a malachite green assay. Reactions were performed in triplicate, and were performed with three separate enzyme preparations (data shown are from one of the preparations). (D) GST-Siw14 activity with 5PP-IP5 in buffers with the pH varying from pH 4 to pH 10. The liberated orthophosphate was quantified using malachite green. (E) Michaelis-Menten and (F) Hanes-Woolf plots assessing the affinity and kinetics of Siw14 with 5PP-IP5. Bars or points represent the average of triplicate reactions normalized to the no enzyme control for each substrate. Error bars for graphs in panels C-E represent the standard error of the mean.
Figure 11. The GST-tag does not affect the activity of Siw14. GST-Siw14 protein was incubated with thrombin protease or 1XTBS for 3 hrs. (A) A coomassie stained gel (B) and anti-GST immunoblot of fusion GST-Siw14 protein compared to cleaved Siw14. Ten μg of each protein sample was used for both panels; marker, Kaleidoscope Prestained Standards (C-D) Activity of 10 μg fusion GST-Siw14 compared to 10 μg of cleaved Siw14 with (C) pNPP and (D) 5PP-IP₅.
*Siw14 Modulates IP₇ Levels in vivo*

Our *in vitro* studies demonstrated Siw14 to be a 5PP-IP₅ phosphatase. In order to evaluate the *in vivo* relevance of these results, the intracellular inositol polyphosphate levels in *siw14Δ* mutant strains were examined. The relative inositol phosphate levels were quantified by radiolabeling cells with $^3$H-*myo*-inositol, extracting soluble inositol phosphates, separating fractions by HPLC, and quantifying tritium by scintillation counting. The inositol pyrophosphate levels were determined relative to IP₆ and then expressed as normalized to the WT strain. In the *siw14Δ* mutant, levels of IP₇ increased by an average of 6.5 ± 1.0 fold (Figure 12A) and the levels of IP₈ also increased by about 1.6 ± 0.2 fold (Figure 12B); both were statistically significant increases. Alternatively, when these data are expressed as a ratio of IP₆: IP₇: IP₈ in each cell type, the WT cells have a ratio of 96.4: 2.1: 1.5 whereas the ratio in the *siw14Δ* mutant changed to 85.5: 12.8: 1.7. Thus, we saw substantial changes in the pools of both IP₆ and IP₇.

The *siw14Δ* mutant was complemented with the wild-type and C214S mutant alleles of *SIW14*, radiolabeled and levels of soluble inositol polyphosphates were measured. As shown in Figure 13, the wild-type allele of *SIW14* restored native levels of IP₇ to the strain, but the catalytically dead allele of *SIW14* (C214S) was unable to complement and the IP₇ levels were not significantly different from the null mutant (Figure 13 A, B).

We extended this analysis by testing whether Siw14 is sufficient for *in vivo* regulation of IP₇ by overexpression in mammalian cells. Vector (pCMV-Myc) or *myc*-tagged *SIW14* expression constructs were transfected into HEK-293T cells radiolabeled with $^3$H-*myo*-inositol. In the presence of Siw14, there was a reduction in endogenous IP₇ levels compared to the cells transfected with the empty vector control pCMV-Myc. Furthermore, expression of the catalytically dead protein (*myc*-tagged *SIW14*-C214S did not lead to a change in the endogenous
IP\(_7\) pools (Figure 13C, E). Together, these *in vivo* findings are consistent with our *in vitro* data supporting a role for Siw14 as an IP\(_7\) phosphatase.

Figure 12. *In vivo* IP\(_7\) and IP\(_8\) levels increase in the *SIW14* yeast deletion mutant. (A) Representative inositol polyphosphate profiles of WT and *siw14Δ*. (B) Ratios of IP\(_7\) or IP\(_8\) to IP\(_6\) in yeast *siw14Δ*, normalized to WT. Average values are from nine replicates; error bars represent the standard error of the mean. Significance was determined by Student T-test; **p*-value ≤ 0.01, ****p*-values ≤ 0.0001.
Figure 13. The catalytic activity of Siw14 is necessary to modulate IP$_7$ levels. (A) Representative inositol polyphosphate profiles of WT and the siw14Δ mutant complemented with empty vector, wild-type gene *SIW14*, and an allele expressing the catalytically dead Siw14-C214S. (B) Ratio of IP$_7$ to IP$_6$ normalized to WT, performed in triplicate. (C) Representative inositol phosphate profiles from empty vector, Myc-SIW14 and Myc-SIW14-C214S transfected HEK-293T cells. (D) Western blot showing successful expression of the two alleles of Myc-Siw14 relative to α-GAPDH. (E) Ratios showing a significant fold decrease of IP$_7$ in Myc-Siw14 transfected cells compared to empty vector. The control and Myc-SIW14 experiment was performed on five samples of transfected cells, the Myc-SIW14-C214S experiment was performed in triplicate. For graphs in B and E, the bars represent the average of the replicates with standard error. Significance was determined using the Student T-test, **$p$-values ≤ 0.01, **** $p$-values ≤0.0001.
Higher Levels of Inositol Pyrophosphates Do Not Affect poly-P Pools

Yeast cells that are unable to synthesize inositol pyrophosphates have undetectable levels of poly-P (Lonetti et al. 2011). The opposite might be true; that is, whether increased levels of inositol pyrophosphates might result in higher levels of poly-P. To examine this, the relative poly-P levels were measured by extracting poly-P, enzymatically converting it to ATP, and quantifying ATP via the release of light using a luciferase assay (Ault-Riché et al. 1998; Lonetti et al. 2011). There was no difference in poly-P pools in the siw14Δ mutant as compared to the parental strain (insignificant decrease, \( p\)-value, 0.09), over four separate experiments (Figure 14). Higher levels of inositol pyrophosphates do not lead to higher levels of poly-P.

![Figure 14. Poly phosphate levels do not change in the siw14Δ mutant.](image)

The levels of poly-P were determined in WT and siw14Δ mutant strains after extraction, conversion into ATP, quantification with a luciferase assay. Bars represent the average of 12 biological replicates over 4 experiments. Error bars represent the standard error of the mean. \( p\)-value = 0.095.
*Siw14 and Other Enzymes That Metabolize IP₇ Act Independently*

A number of mutant yeast strains have been shown to accumulate IP₇ (Mulugu et al. 2007; Onnebo and Saiardi 2009; Lonetti et al. 2011). Vip1 is the kinase that adds the β-phosphate at the 1-position to convert IP₇ (5PP-IP₅) to IP₈ (1,5PP-IP₄) (Mulugu et al. 2007). *DDP1* encodes the *Saccharomyces* homolog of hDIPP1. *In vitro*, the Ddp1 enzyme is able to dephosphorylate the β-phosphate from IP₇ only at prolonged time points or at non-physiological concentrations; however, *in vivo* IP₇ levels increase in the *ddp1* mutant demonstrating a potential biological role for this enzyme (Lonetti et al. 2011). We measured the levels of IP₇ and IP₈ in the *ddp1Δ* and the *vip1Δ* mutants, and compared them with the *siw14Δ* mutant. The increase in IP₇ levels for the *siw14Δ* mutant (6.5 ± 1.0 fold) was of the same magnitude as the accumulation in the *ddp1Δ* (7 ± 1.1 fold) and *vip1Δ* (4.0 ± 1.7 fold) mutants (Figure 15A-C); as had been seen previously (Lonetti et al. 2011). The levels of IP₈ increased in the *ddp1Δ* mutant 2 ± 0.4-fold, similar to the *siw14Δ* mutant (1.6 ± 0.2-fold). As was to be expected, the *vip1Δ* mutant strain had a decrease in IP₈ by more than half of the WT strain.

We wondered if these enzymes acted independently. We assessed accumulation of IP₇ in the double *siw14Δ ddp1Δ* and *siw14Δ vip1Δ* mutants. Both double mutants accumulated higher levels of IP₇ than either single mutant alone (Figure 15A-C); we found a 21.3 ± 3.2-fold increase for the *siw14Δ ddp1Δ* mutant that was slightly more than additive, and an 11.8 ± 2.6-fold increase for the *siw14Δ vip1Δ* mutant that was additive. We also measured the levels of IP₈ in these mutants. The *siw14Δ ddp1Δ* double mutant strain had a 2.8 ± 0.6-fold increase in IP₈ compared to the WT strain (significant at *p*-value = 0.001) and the difference from the *siw14Δ* single mutant (*p*-value = 0.054). The *vip1Δ siw14Δ* double mutant had the same low levels of IP₈ as the *vip1Δ* single mutant as anticipated.
Figure 15. The levels of IP$_7$ increase in single and double kinase and phosphatase mutants. (A) Representative inositol polyphosphate profiles of WT, and single and double mutants of $siw14\Delta$ and $ddp1\Delta$. (B) Representative inositol polyphosphate profiles of WT, and single and double mutants of $siw14\Delta$ and $vip1\Delta$. (C) Levels of IP$_7$ in each mutant, normalized to WT and IP$_6$, over four separate experiments. Error bars represent standard error of the mean and significance was determined by Student T-test; $p$-values $\leq 0.001$ for each mutant with WT, *$p$-values $\leq 0.05$, **$p$-values $\leq 0.01$. 
Mutations in the SIW14 Paralogues OCA1 and OCA2 Only Marginally Alter IP7 Levels

The OCA1 and OCA2 genes of S. cerevisiae encode paralogs of SIW14, although their ability to function as active enzymes is still in question (see above; Wishart and Dixon 1998; Romá-Mateo et al. 2011). All three proteins (Siw14, Oca1 and Oca2) have genetic and physical interactions and share some phenotypes (Ho et al. 2002; Gavin et al. 2006; Krogan et al. 2006; Breitkreutz et al. 2010; Romá-Mateo et al. 2011; Altıntaş et al. 2016). We wondered if the Oca1 and Oca2 proteins also affected inositol pyrophosphate pools. Yeast cells were radiolabeled with 3H-myoinositol and soluble inositol polyphosphates were isolated. We found a 1.7 ± 0.3-fold increase in IP7 for the oca1Δ mutant and 2.1 ± 0.4-fold increase in IP7 for the oca2Δ mutant in the IP7 levels as compared with the wild-type (Figure 16A, B). There was a small increase that was much lower than that seen in the siw14Δ mutant (6.5-fold increase). Overexpression of OCA1 and OCA2 in mammalian HEK-293T cells yielded no change in the levels of IP7 (Figure 16C, E). Together these results suggest that Oca1 and Oca2 individually, under normal growth conditions, do not regulate inositol pyrophosphates.

Siw14, Oca1, and Oca2 are likely to form a complex (Ho et al. 2002; Gavin et al. 2006; Krogan et al. 2006; Breitkreutz et al. 2010), therefore we wondered if the levels of IP7 changed in double mutants compared to the single mutants. We examined inositol pyrophosphate levels in all three double knockout combinations: siw14Δ oca1Δ, siw14Δ oca2Δ, and oca1Δ oca2Δ. Consistent with the single oca1Δ and oca2Δ mutants the double oca1Δ oca2Δ mutant has a slight increase in IP7 levels of 2.1 ± 0.1-fold, indicating again that Oca1 and Oca2 do not regulate inositol pyrophosphates (Figure 17A,B). Interestingly, when OCA1 or OCA2 are deleted in the siw14Δ mutant the level of IP7 decreases compared to the siw14Δ single mutant (~6.5-fold) (Figure 12). The oca1Δ siw14Δ double mutant has a 4.5 ± 0.1-fold increase in IP7 levels and the
*oca2Δ siw14Δ* double mutant has a 3 ± 0.4-fold increase in IP7 levels compared to the wild-type (Figure 17A,B). These data suggest that *OCA1* and *OCA2* are epistatic to *SIW14* and that they modify the activity of Siw14.

We observed an increase in IP8 levels in the *siw14Δ* single mutant and even though deletions in *OCA1* and *OCA2* only slightly affect the levels of IP7 they could be playing a more significant role in regulating IP8 levels. Therefore, we measured the level of IP8 for all three single and double mutants. Unfortunately, there were no significant changes in the single *oca1Δ* and *oca2Δ* single mutants concerning IP8 (Figure 17C). However, in all of the double mutant strains there was a significant increase in IP8; the *siw14Δ oca1Δ* strain had a 1.6 ± 0.1-fold increase, the *siw14Δ oca2Δ* strain had a 1.7 ± 0.1-fold increase, and the *oca1Δ oca2Δ* strain had a 1.4 ± 0.1-fold increase. Consistent with the IP7 data, the IP8 data indicate that Siw14 contributes the most activity to regulating inositol pyrophosphates.
Figure 16. The levels of IP$_7$ do not change in either the oca1Δ or oca2Δ yeast mutants compared to the WT or when they are overexpressed in mammalian cells. (A) Representative inositol phosphate profiles of WT, siw14Δ, oca1Δ, and oca2Δ. (B) Ratios showing the fold increase of IP$_7$ in each mutant compared to WT over 5 separate experiments. *$p$-values $\leq$ 0.05 and *** $p$-values $\leq$ 0.001. (C) Representative inositol phosphate profiles of empty Myc vector, Myc-Siw14, Myc-Oca1, and Myc-Oca2 transfected HEK-293T cells (D) with a corresponding Western blot showing successful transfection of Myc-Siw14, Myc-Oca1, Myc-Oca2, and α-GAPDH as a loading control. (E) Ratios showing no change in IP$_7$ levels in Myc-Oca1 and Myc-Oca2 transfected cells compared to empty Myc vector. ** $p$-values $\leq$ 0.01.
Figure 17. **OCA1** and **OCA2** are epistatic to **SIW14**. (A) Representative inositol polyphosphate profiles of WT, *siw14Δ*, *oca1Δ*, *oca2Δ* single mutant strains and *oca1Δ siw14Δ*, *oca2Δ siw14Δ*, and *oca1Δ oca2Δ* double mutant strains. (B) Levels of IP₇ and (C) levels of IP₈ in each mutant, normalized to WT and IP₆, of 6 biological replicates. *p-values ≤ 0.05, **p-values ≤ 0.01, ***p-values ≤ 0.001, ****p-values ≤ 0.0001.
2.4 Summary of Results

This chapter showed Siw14 to be a regulator of the soluble inositol pyrophosphate 5PP-IP$_3$ (Figure 1B). Siw14 displays pyrophosphate-specific phosphatase activity \textit{in vitro} with 5PP-IP$_3$ over any other substrate tested (Figure 10). The affinity of Siw14 for 5PP-IP$_3$ was surprisingly low at a K$_m$ of 34 µM (Table 2). However, 34 µM is not out of the biologically relevant range. Even though intracellular concentrations of IP$_7$ have not been measured in yeast to date, IP$_7$ levels have been measured to range from 0.5-5 µM in humans and from 300-500 µM in amoeba (Albert et al. 1997; Lin et al. 2009).

In the absence of \textit{SIW14}, the \textit{in vivo} IP$_7$ levels increase $\sim$6.5-fold (Figure 12). This phenotype cannot be rescued by complementing the mutant with an allele lacking the catalytic cysteine, indicating that the phenotype is due to the loss of the catalytic activity of Siw14 (Figure 13A-B). Consistent with the result, the levels of IP$_7$ decrease in a mammalian cell line when \textit{SIW14} is overexpressed; this does not occur if Siw14 is catalytically dead (Figure 13C-D).

The deletion of two other genes, \textit{VIP1} and \textit{DDP1} also cause an increase in IP$_7$ levels in yeast (Mulugu et al. 2007; Onnebo and Saiardi 2009; Lonetti et al. 2011). Vip1 phosphorylates the phosphate on the 1-carbon of 5PP-IP$_3$ or IP$_6$ to make 1,5PP-IP$_5$ (IP$_8$) or 1PP-IP$_5$ (an isoform of IP$_7$), respectively (reviewed in Hatch and York 2010). Ddp1 can dephosphorylate all inositol pyrophosphates and has the highest activity with 1PP-IP$_5$ of the IP$_7$ and IP$_8$ isoforms, but Ddp1 has the most activity with poly-P \textit{in vitro} (Lonetti et al. 2011). When the \textit{siw14Δ} mutant is combined with the \textit{ddp1Δ} mutant strain or the \textit{vip1Δ} mutant strain levels of IP$_7$ increase further in an additive manner suggesting that the enzymes are working on IP$_7$ independently (Figure 15).

Finally, \textit{SIW14} has two paralogs in \textit{Saccharomyces cerevisiae}, \textit{OCA1} and \textit{OCA2}. The proteins encoded by these genes are thought to interact with each other based on two-hybrid and
high throughput affinity purification studies (Ho et al. 2002; Gavin et al. 2006; Krogan et al. 2006; Breitkreutz et al. 2010). We wondered if OCA1 or OCA2 affect the pools of IP₇. We found that in the absence of OCA1, OCA2 or both, the pools of IP₇ have a slight increase (Figure 16 & Figure 17). Moreover, overexpressing OCA1 or OCA2 in mammalian cells did not decrease IP₇ levels (Figure 16). These data suggest that Siw14 is the primary phosphatase and that neither Oca1 nor Oca2 have substantial phosphatase activity. Interestingly, in siw14Δ oca1Δ and siw14Δ oca2Δ double mutant strains the level of IP₇ decreased when compared to the siw14Δ single mutant; these results suggests that Oca1 and Oca2 modify the activity of Siw14 (Figure 16 & Figure 17).

In conclusion, Siw14 is a novel inositol pyrophosphate phosphatase. It acts as the antagonist to the Kcs1 kinase by dephosphorylating 5PP-IP₅ back to IP₆. Further research will determine if Siw14 works with its putative partner proteins to maintain inositol pyrophosphate levels in Saccharomyces cerevisiae.
CHAPTER 3
Siw14 Negatively Regulates the Environmental Stress Response Through Inositol Pyrophosphates

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3.1 Introduction

The ability of cells to respond to different types of intrinsic and extrinsic stresses is crucial for cell survival. Inositol pyrophosphates are high energy signaling molecules involved in many cellular processes including the cellular stress response (Fleischer et al. 1994; Ali et al. 1995; Alic et al. 2001; Holt et al. 2009; Worley et al. 2013). Inositol pyrophosphates are fully phosphorylated myo-inositol rings with one or more additional β-phosphates. In yeast, the kinase Kcs1 synthesizes IP7 by adding the β-phosphate to IP6 at the 5-position, generating 5PP-IP5 and Vip1 adds the β-phosphate at the 1-position resulting in 1,5PP-IP4 (IP8) (reviewed in Wilson et al. 2013; Thomas and Potter 2014). Vip1 can also use IP6 as a substrate, generating 1PP-IP5 an isomer of IP7. The kcs1Δ vip1Δ double mutant is unable to make inositol pyrophosphates and is unable to induce a cellular stress response (Worley et al. 2013).

This chapter connects inositol pyrophosphates to the stress response through the phosphatase Siw14. The previous chapter showed that SIW14 encodes a newly described inositol pyrophosphate phosphatase that specifically cleaves the β-phosphate from the 5-position of IP7; and IP7 levels increase in a siw14Δ mutant strain (Steidle et al. 2016). This chapter shows that the siw14Δ mutant is resistant to a range of environmental stresses including high temperature,
oxidative stress, and osmotic stress. The mutant also exhibits an increase in chronological aging. Using gene expression analysis, we see that the environmental stress response (ESR) is partially up-regulated in unstressed \textit{siw4}\textsubscript{Δ} mutant cells and that application of additional stress further increases expression of the ESR genes. In addition, when wild-type cells were stressed with hydrogen peroxide and radiolabeled with tritiated \textit{myo}-inositol it resulted in an increase in both IP\textsubscript{7} and IP\textsubscript{8} levels. These findings demonstrate that inositol pyrophosphates are necessary for generating the stress response.

3.2 Materials and Methods

\textit{Strains and Plasmids}

BY4741 and the \textit{siw14::KANMX} mutant strains were purchased from OpenBioSystems. The plasmids carrying the \textit{SIW14} gene or the phosphatase-dead allele \textit{siw14-C214S} were constructed in the pRS316 vector (Steidle et al. 2016).

\textit{Growth Conditions}

Cells were grown in YPD (1% yeast extract, 2% peptone, 2% dextrose) and plasmid-bearing cells were grown in SC-URA (0.17% YNB (Sunrise Science Products), 0.5% ammonium sulfate, 0.069% CSM-Ura amino acid mix (MP Biomedicals), 0.75 \textmu M adenine and 2% dextrose). Overnight cultures were inoculated into fresh medium at an OD\textsubscript{600} of 0.1-0.15, and were allowed to grow to log phase (OD\textsubscript{600} of ~0.6) or for 24-hr for post-diauxic shift. Cultures were normalized to the same OD\textsubscript{600} and serially diluted 1:10 four times in buffered saline with glucose (10 mM Tris-HCl, pH7.5, 85 mM NaCl, 10 mM glucose) unless otherwise noted. Post-diauxic shift cultures were serially diluted 1:10 in 10 mM Tris-HCl, pH 7.5, 85 mM NaCl.
without glucose unless otherwise noted. For semi-quantitative measurement of yeast growth, 2.5 
µL of each dilution was spotted onto solid medium. For quantitative measurement of yeast 
growth 25 µL of the 10⁻⁴ dilution were spread onto solid medium. All plates were allowed to 
grow for 2 days at 30°C unless otherwise noted.

For osmotic stress, cells were plated on YPD medium containing 1.3 M KCl (Marešová 
et al. 2012). For oxidative stress, cultures were grown to early log phase (OD₆₀₀ of 0.3) or post-
diauxic shift, and 1 mM H₂O₂ was added to the medium for 3 hr (modified from Vernis et al. 
2009). Cells were serially diluted 1:10 in sterile deionized H₂O, and were plated onto solid YPD 
medium. For heat shock, cells were subjected to 50°C for 10 min, serially diluted, and plated on 
SC-URA solid medium (modified from Care et al. 2004).

**Chronological Aging Assays**

Using Pereira and Saraiva (2013) as a guide, cells were grown overnight in YPD and then 
normalized in SC media at an OD₆₀₀ of ~0.1. Cultures were allowed to grow for 14 days at 30°C 
with shaking. Samples were removed every 24-hr and normalized to an OD₆₀₀ of 0.3 which 
equaled about 10⁶ cells. They were stained with ~6 µg/mL propidium iodide to test for dead cells. 
Using flow cytometry (Becton Dickinson FACSort), stained cells were counted and the percent 
alive were calculated for each strain at each time point in triplicate.

**Endogenous Levels of Reactive Oxygen Species**

We measured endogenous levels of superoxide using dihydroethidium (DHE, Santa Cruz 
Biotechnology) and H₂O₂ using dihydrorhodamine (DHR, Santa Cruz Biotechnology) in yeast by 
modifying the protocols from Drakulic et al. (2005), Mesquita et al. (2010), Lam et al. (2011),
Heeren et al. (2004), and Katragkou et al. (2010). Overnight cultures were grown in YPD, and inoculated into fresh YPD media at an OD\textsubscript{600} of 0.1 and grown to log phase (~0.6 OD\textsubscript{600}) or post-diauxic shift (24 hours). About 10\textsuperscript{7} cells were incubated with either 29.3 µM DHE or 30 µM DHR for 10 min in the dark at 30°C. Cells were pelleted, washed once with an equal volume of 1X PBS, and were counted using flow cytometry (Becton Dickinson FACSort). Data are shown as the average of the median cell fluorescence of triplicate samples.

**Oxygen Consumption**

Overnight cultures were inoculated into fresh YPD, and were grown to log phase (~0.6 OD\textsubscript{600} or post-diauxic shift (24 hrs). For log phase growing cells, 1 mL of culture was placed in an airtight chamber at 30°C and O\textsubscript{2} was measured for 6 min using an oxygraph system (Hansatech Instruments, Ltd.). For post-diauxic shift cells, cultures were diluted 10-fold in spent media that was obtained by centrifuging a portion of the culture for 2 min at 13,000 rpm. One mL of the diluted cells was placed in the airtight chamber to measure O\textsubscript{2} consumption. The rate of O\textsubscript{2} consumption was calculated by the following calculation: O\textsubscript{2} (nmoles) / Volume of culture (mL)/ Time in chamber (min)/OD\textsubscript{600} of cells. All samples were normalized to the O\textsubscript{2} consumption of growth medium. For each experiment biological triplicates were measured.

**Microarray**

BY4741 and the \textit{siw14Δ} mutant were grown to 0.6 OD\textsubscript{600}, were osmotically stressed by the addition of KCl (0.4 M) for 20 min, and were harvested (Worley et al. 2013). RNA was prepared and converted into cDNA before being labeled with Cy3 or Cy5 for transcript level measurement on Agilent G4813A DNA microarrays and an Axon 4000B scanner, as described.
previously (Worley et al. 2013). A list of up- and down-regulated genes was determined and a GO TERM analysis was performed using the FunSpec online tool (Robinson et al. 2002).

**Extraction of $^3$H-Inositol Phosphates and HPLC Analysis**

Overnight cultures were grown in YPD medium and normalized to an OD$_{600}$ of 0.005 in SC-inositol medium. Cells were radiolabeled with 75 µCi of $^3$H-myo-inositol for ~20 hr, stressed with either 0.4 M KCl or 1 mM H$_2$O$_2$ for 20 min, then harvested. Extracts were prepared and inositol pyrophosphates separated and assessed as described in chapter 2 (Steidle et al. 2016).

**qRT-PCR**

Overnight cultures were inoculated into fresh YPD medium and allowed to grow to log phase. Cultures were split and each set of cultures were treated with 1 mM H$_2$O$_2$, 0.4 M KCl or were untreated for 20 min at 30°C (Worley et al. 2013). As described in Ghosh et al. (2015) cells were immediately placed on ice and centrifuged at 2500 rpm for 2 min. Pellets were frozen at -80°C. RNA was extracted using the RiboPure Yeast Kit, AM1926 (Ambion) following the manufacturer’s instructions with slight modifications. Namely, 3 µg of RNA were treated with 1 µl of DNase I (RiboPure™ Yeast Kit) in a 50 µl reaction and treatment was incubated up to 1 hr at 37°C. cDNA was synthesized by following the manufacturers’ instructions (SensiFAST™ cDNA synthesis kit, Bioline). Two hundred ng of DNase I treated RNA was added into each reaction. The synthesized cDNA was then diluted 5 fold into the DEPC water. The RT-qPCR was performed following the manufacturers’ instructions (SensiFAST™ SYBR No-ROX Kit, BIO-98005). Each cDNA sample was performed with two technical replicates. Three µL of 5-fold diluted cDNA was added into the reaction with 15 µl final volume. The following primers
were used: ADH1- 5’ CAAGTCGTCAAGTCCATCTC3’ and 5’CAAGCCGACAACCT
TGAT3’, CTT1- 5’AGAGAGTTACGCAATACTCTGG3’ and 5’CCTTCAAGGTCAACAG
- GTTC3’, HSP12- 5’GCAGAC CAAGCTAGAGATTAC3’ and 5’TCTTGGTTGGGTCT
-TCTTC3’, GAR1- 5’GCTGACAACAATTCGCTATTG3’ and 5’GGCACCACCTTCTCT
-TCTTC3’, RPL16A- 5’GCCAAATTGGAAGCAAGAG3’ and 5’TTCAGCAGCAGTAGCAT
-TAG3’, and UBC6- 5’GATACTTGGAAATCTTGGCTGGGTCTGCTTC3’ and 5’AAAGGG
-TCTT CTGTTCATCACCCTATTTGC3’. The relative gene expression was calculated by
the ΔC\textsubscript{T} Method using UBC6 as the reference gene as described (Bio-Rad Laboratories. 2006;
Teste et al. 2009) and were normalized to the wild-type.

3.3 Results

The siw14Δ Mutant is Resistant to Oxidative Stress

The siw14Δ mutant was previously shown to be resistant to a heat stress at 37°C (Care et
al. 2004). Because heat stress can cause intrinsic oxidative stress (reviewed in Morano et al.
2012), I wondered if the siw14Δ mutant was also resistant to 1 mM hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}).
Therefore, cells were grown to either mid-log phase or post-diauxic shift, and were treated with
H\textsubscript{2}O\textsubscript{2} for 3 hr before plating onto solid YPD medium to determine survival. Approximately, 40%
of the siw14Δ mutant cells in log-phase survived H\textsubscript{2}O\textsubscript{2} treatment as compared to only 2% of the
treated wild-type cells, consistent with recent findings (Martins and English 2014; Altıntaş et al.
2016) (Figure 18). I found a similar cellular response in post-diauxic shift cells that had not been
previously reported; 60% of the siw14Δ mutant survived H\textsubscript{2}O\textsubscript{2}-treatment compared to 20% of
wild-type cells (Figure 18). These results indicated that the siw14Δ mutant is resistant to
oxidative stress.
Figure 18 The \textit{siw14Δ} mutant is resistant to oxidative stress. Wild-type and \textit{siw14Δ} mutant strains were grown to log phase or for 24 hr to the post-diauxic shift phase and were treated with 1 mM H$_2$O$_2$ for 3 hrs. Cells were plated on YPD medium and incubated at 30°C for 2 days. Colony forming units (CFUs) were determined for treated and untreated strains, and the % survival was calculated (CFU treated/CFU untreated *100). Bars represent the average of 12 biological replicates over 4 separate assays. Error bars are standard error of the mean. ***\textit{p-values} \leq 0.001

\textbf{Endogenous Levels of H$_2$O$_2$ Decrease in Post-Diauxic Shift \textit{siw14Δ} Mutant Cells}

The \textit{siw14Δ} mutant is resistant to external oxidative stress, therefore, I was interested in determining if the endogenous levels of reactive oxygen species (ROS) were also lower. I used flow cytometry to measure the levels of ROS from log-phase and post-diauxic shift cells after staining them with dihydroethidium (DHE) or dihydrorhodamine (DHR). DHE and DHR are fluorescent stains that bind superoxide and H$_2$O$_2$, respectively; the specific ROS oxidizes the molecule and it fluoresces. ROS naturally increase after transition from fermentation to respiration in post-diauxic shift cells. I found a this increase in both superoxide and H$_2$O$_2$ in the stained wild-type cells (Figure 19A and B). Interestingly, there was no difference in endogenous levels of superoxide in the \textit{siw14Δ} mutant as compared to the wild-type strain. However, a significantly lower increase in H$_2$O$_2$ was observed in the \textit{siw14Δ} mutant in the post-diauxic shift
cells. This result is consistent with the resistance of the siw14Δ mutant to exogenous H₂O₂ treatment (Figure 18).

One explanation for the decrease in H₂O₂ is a defect in mitochondrial function. To test mitochondrial function, I directly measured respiration by oxygen consumption in the siw14Δ mutant. I observed an increase in oxygen use in the post-diauxic shift cells as compared with mid-log phase cells (this increase is known as the Crabbetree effect), indicating a metabolic change from fermentation to respiration (Figure 20). There was no difference in oxygen consumption between the siw14Δ mutant and the wild-type strain (Figure 20). These results show that the resistance to H₂O₂ and the low level of endogenous H₂O₂ are not due to an intrinsic mitochondrial defect.

Figure 19. H₂O₂ decreases in post-diauxic shift siw14Δ mutant cells. (A) DHR staining and (B) DHE staining of wild-type and siw14Δ mutant cells. Cells were grown to either log phase or for 24 hr to the post-diauxic shift phase. About 10⁷ cells were stained for 10 min at 30°C and fluorescence was measured using flow cytometry. The bars represent the median fluorescence of biological triplicates. Error bars are standard error of the mean. ***p-value ≤ 0.001
Figure 20. The *siw14Δ* mutant does not have a mitochondrial defect. Oxygen consumption was measured using an oxygraph. \( \text{O}_2 \) was measured in an airtight chamber over 6 min for cells in log phase or post-diauxic shift. Bars represent the average \( \text{O}_2 \) (nmole) divided by the volume (mL) of culture divided by the time (min) in the chamber divided by the OD\(_{600}\) for biological triplicates. Error bars are standard error of the mean for triplicate samples.

*The siw14Δ Mutant Expresses More Catalase Than Wild-type*

The *CTT1* gene encodes the enzyme catalase that converts \( \text{H}_2\text{O}_2 \) to \( \text{H}_2\text{O} \) (Grant et al. 1998), and its transcription is regulated by Msn2 (Martinez-Pastor et al. 1996). Perhaps the \( \text{H}_2\text{O}_2 \) levels were down because of elevated *CTT1* expression in the *siw14Δ* mutant. Therefore, I measured the levels of *CTT1* in unstressed and oxidatively stressed *siw14Δ* mutant and wild-type cells. The expression of CTT1 was increased 5-fold in the unstressed *siw14Δ* mutant compared to unstressed wild-type (Figure 21). The addition of \( \text{H}_2\text{O}_2 \) led to an induction of 81-fold in *siw14Δ* mutant cells and a 13-fold increase in stressed wild-type (Figure 21). Thus, our data support the idea that the *siw14Δ* mutant is resistant to oxidative stress and has lower endogenous \( \text{H}_2\text{O}_2 \) levels because of increased catalase expression under both non-stressed and stressed conditions.
Figure 21. Expression of catalase is up-regulated in unstressed siw14Δ mutants. RT-qPCR fold changes represented by log2 values and normalized to the reference gene UBC6 and wild-type. Cultures were grown to log phase and half of the cultures were treated with 1 mM H2O2 for 20 min before RNA extraction. Bars represent the average fold change in log2 for biological triplicates for each condition. Error bars are standard error of the mean. *p-value ≤ 0.5, ***p-value ≤ 0.001.

The siw14Δ Mutant is Resistant to Environmental Stress

To determine the effect of additional environmental stresses, mutant cells were grown and subjected to heat shock and osmotic stress, and their chronological lifespan was determined as a measure of nutrient stress responses. Mutant and wild-type cells were grown to mid-log phase, subjected to heat stress at 50°C for 10 min and plated onto solid YPD medium. The siw14Δ mutant strain survived this acute heat stress whereas none of the wild-type cells survived (Figure 22). I complemented the siw14Δ mutant with a plasmid-borne allele, pRS316-SIW14 (SIW14+), which restored the wild-type phenotype (Figure 22). The siw14Δ mutant was also transformed with a plasmid expressing the catalytically dead allele of SIW14 in which the active-site cysteine at residue 214 was mutated to serine (C214S); this allele failed to complement the null allele (siw14Δ) and the transformed strain retained the mutant phenotype (Figure 22).
The *siw14Δ* mutant was also tested for resistance to osmotic stress. Wild-type and mutant strains were grown to mid-log phase or post-diauxic shift in YPD medium, and were plated on YPD containing 1.3 M KCl for high osmotic conditions. The *siw14Δ* mutant showed an increased survival when grown on medium containing 1.3 M KCl as compared to the wild-type strain (Figure 23). This was a statistically significant difference in growth in both log phase and post-diauxic shift cells.

Lack of nutrients stresses cells and they transition to stationary phase (Pereira and Saraiva 2013). One way to measure response to stationary phase is to measure life span using a chronological aging assay (Pereira and Saraiva 2013) which measures the survival of cells after being in stationary phase for a prolonged period of time. It was hypothesized that the *siw14Δ* mutant would survive stationary phase better than the wild-type strain because of its resistance to other environmental stresses. Wild-type and mutant strains were inoculated into minimal medium and were cultured at 30°C with shaking for 14 days; aliquots were removed daily. Cells were stained with propidium iodide (which is excluded by living cells) and the percent of living cells were counted relative to the total. I found that the *siw14Δ* mutant strain had fewer propidium iodide stained cells compared to the wild-type strain after the 14-day period, demonstrating that more cells of the *siw14Δ* mutant were still alive (Figure 24). Together these data suggest that the stress response in the *siw14Δ* mutant strain is enhanced compared to the wild-type strain.
Figure 22. The catalytic activity of Siw14 is necessary to revert the heat resistance of the siw14Δ mutant back to wild-type. Representative growth of the wild-type + pRS316, siw14Δ + pRS316, siw14Δ complemented with SIW14+ and SIW14-C214S (C214S) strains treated with 50°C heat stress for 10 min. Cells were normalized to the same OD600 and serially diluted in buffered saline with glucose before spotting 2.5 µL on to SC-URA medium.

Figure 23. The siw14Δ is resistant to osmotic stress. Cells were grown to log phase or post-diauxic shift phases and spread onto YPD agar plates without 1.3 M KCl. CFUs were quantified and % survival for each assay was calculated (treated CFU/untreated CFU*100). Error bars are standard error of the mean of 6 replicates over two separate assays. *p-values ≤ 0.05, **p-values ≤ 0.01
Figure 24. The *siw14Δ* mutant survives stationary phase better than the wild-type strain. Cultures were grown for 14 days to measure chronological aging. Aliquots were removed every 24 hr and were stained with propidium iodide and assayed by flow cytometry. Points represent the average of three biological replicates and error bars are standard error of the mean.

**The Stress Response is Partially Induced in the Unstressed *siw14Δ* Mutant and is Further Induced During Stress**

The *siw14Δ* mutant is resistant to imposed environmental stresses suggesting that the stress response pathway is partially induced in an unstressed *siw14Δ* mutant. This hypothesis is based on the facts that (1) Siw14 is an inositol pyrophosphate phosphatase and the mutant accumulates IP$_7$ and IP$_8$ (Steidle et al. 2016), and (2) IP$_7$ and IP$_8$ are necessary to induce the stress response because the *kcs1Δ vip1Δ* double knockout, which cannot make these molecules, cannot induce the stress response when treated with 0.4 M KCl (Worley et al. 2013). In collaboration with Andrew Capaldi and colleagues, DNA microarray analysis was performed. RNA was isolated from osmotically stressed and unstressed wild-type and *siw14Δ* mutant strains, and expression was measured using DNA microarray analysis. We observed that the osmotically stressed wild-type BY4741 strain behaved the same as the wild-type W303 strain in terms of up- and down-regulated genes (Worley et al. 2013).
About 50% of the up-regulated genes in an _siw14Δ_ mutant are induced by osmotic stress in the wild-type (Figure 25) however, these genes represent only about 10% of the total genes up-regulated in a wild-type stress response. These genes are involved in processes like oxidation-reduction processes, glycolysis, and gluconeogenesis among others (Table 3). The only genes that were commonly down-regulated in the unstressed _siw14Δ_ and in a stressed wild-type have unknown functions. However, there were a few genes that had opposite regulation in the unstressed _siw14Δ_ mutant compared to the stressed wild-type strain (Table 4). For example, _AGA2_, a protein involved in pH regulation, is up-regulated in the unstressed _siw14Δ_ and down-regulated in the stressed wild-type (Table 4).

I compared gene expression in stressed mutant to the stressed wild-type strain. About 60% of the up-regulated genes in the stressed _siw14Δ_ mutant have an even higher expression compared to the stressed wild-type (Figure 25). These genes affect many processes and among them are the same processes discussed above for the unstressed _siw14Δ_ mutant (Table 3 andTable 5). In addition, one process that the stressed _siw14Δ_ mutant up-regulates more than stressed wild-type is the pentose phosphate shunt (Table 5). This finding is consistent with cells shifting their carbon metabolism from glycolysis to the pentose phosphate pathway which changes the NADPH/NADP⁺ ratio to the more reduced state (Ralser et al. 2006; Ralser et al. 2007). This metabolic change is likely to be one of the reasons that the _siw14Δ_ mutant is resistant to oxidative stress. Similar to the up-regulated genes, about 40% of the down-regulated genes in a stressed _siw14Δ_ mutant have even lower expression when compared to the stressed wild-type (Figure 25). These genes are involved in translation and ribosomal biogenesis, among others (Table 6). This finding is consistent with the canonical cellular stress response that down regulates certain processes to help protect cellular mass and energy while it acclimates to the
stressful environment (Hohmann and Mager 2003). Finally, there are a few genes that are differentially regulated by the stressed \textit{siw14}\textDelta as compared to the stressed wild-type strain (Table 7); \textit{PLC1}, involved in septin organization, is down-regulated in the stressed \textit{siw14}\textDelta and up-regulated in stressed wild-type (Table 7). Interestingly, stationary phase \textit{siw14}\textDelta mutant cells were shown to have septin clumping (Care et al. 2004; Table 1).

The microarray results were confirmed using qRT-PCR, measuring the expression of \textit{HSP12}, \textit{CTT1}, \textit{GAR1} and \textit{RPL16A} (Figure 25B-E). I chose to analyze the expression of \textit{CTT1} under osmotic stress because of change in phenotype under oxidative stress that might be due to catalase (Figure 21). \textit{HSP12} is a downstream target of the general stress response transcription factor Msn2/4, which is known to activate genes during osmotic stress. \textit{GAR1} is classified as a gene involved in ribosomal biogenesis and \textit{RPL16A} encodes a protein found on the large ribosomal subunit. Based on the microarray data, I expected that the stress response genes to show increased expression and the protein synthesis and ribosomal biogenesis genes to have decreased expression under non-stress conditions in the \textit{siw14}\textDelta mutant (Alejandro-Osorio et al. 2009). Additionally, I expected that the imposition of stress would further up- or down-regulate the expression of these genes. Indeed, under osmotic stress both \textit{HSP12} and \textit{CTT1} expression increase in \textit{siw14}\textDelta cells, 81-fold and 152-fold, respectively. This is compared to the 31-fold and 91-fold increase of the same genes during osmotic stress in wild-type cells (Figure 25B &C). Similarly, both \textit{GAR1} and \textit{RPL16A} gene expression were further down-regulated in the stressed \textit{siw14}\textDelta mutant compared to the stressed wild-type strain (Figure 25D&E). Together these data indicate that the \textit{siw14}\textDelta mutant has a partially induced environmental stress response and is still able to mount an environmental stress response during stressful conditions.
Figure 25. The stress response is partially on in the unstressed *siw14Δ* mutant, but it is still able to mount a stress response. (A) Heat map of a preliminary microarray analysis. Genes in green are down-regulated and genes in read are up-regulated. Color scale represents, green ≤ -2.5-fold change and red ≥ 2.5-fold change. (B-E) qRT-PCR fold changes represented as Log₂ values normalized to normalized to both the reference gene *UBC6* and unstressed wild-type cells. Bars represent the average expression of triplicate samples and error bars are the standard error of the mean. *p-value ≤ 0.05, **p-value ≤ 0.01, *** p-value ≤ 0.001.
Table 3. GO TERM analysis of up-regulated genes in stressed wild-type and unstressed *siw14Δ* mutant strains.

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$k$ is the number of genes in the data set in the listed category and $f$ is the total number of genes in the listed category.
### Table 4. GO TERM analysis of oppositely regulated genes in stressed wild-type and unstressed \textit{siw14Δ} mutant strains.

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<th>Biological Process</th>
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<th>f</th>
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</tr>
<tr>
<td>helps regulate the cell integrity pathway</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

\(k\) is the number of genes in the data set in the listed category and \(f\) is the total number of genes in the listed category.
Table 5. GO TERM analysis of the genes that are further up-regulated in the stressed siw14Δ mutant compared to the stressed wild-type.

<table>
<thead>
<tr>
<th>Biological Process</th>
<th>GO:TERM</th>
<th>k</th>
<th>f</th>
<th>p-value</th>
<th>Gene</th>
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<tr>
<td>glycolysis</td>
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<td>28</td>
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<td>gluconeogenesis</td>
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<td>18</td>
<td>1.0E-07</td>
<td>PGK1 TDH3 ENO1 ENO2 TDH1 GPM1</td>
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<td>response to stress</td>
<td>GO:0006950</td>
<td>12</td>
<td>152</td>
<td>1.1E-06</td>
<td>HSP26 TPS1 FMP45 TPS2 HSP12 CTT1 XBP1 DDR48 HOR7 DDR2 TIR2 GRE1</td>
</tr>
<tr>
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<td>GO:0005992</td>
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<td>7</td>
<td>1.2E-06</td>
<td>TPS1 TPS2 UGP1 PGM2</td>
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<tr>
<td>cellular response to oxidative stress</td>
<td>GO:0034599</td>
<td>8</td>
<td>67</td>
<td>3.5E-06</td>
<td>GRX1 HSP12 TRX2 YJR096W SXR1 MCR1 AHP1 GCY1</td>
</tr>
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<td>mannose metabolic process</td>
<td>GO:0006013</td>
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<td>4</td>
<td>1.0E-05</td>
<td>GLK1 HXK1 AMS1</td>
</tr>
<tr>
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<td>GO:0010466</td>
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<td>4</td>
<td>1.0E-05</td>
<td>TFS1 PAI3 PBI2</td>
</tr>
<tr>
<td>cellular response to water deprivation</td>
<td>GO:0042631</td>
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<td>4</td>
<td>1.0E-05</td>
<td>CTT1 YJL144W SIP18</td>
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<td>glucose metabolic process</td>
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<td>272</td>
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<td>plasma membrane organization</td>
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<td>1.9E-04</td>
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<td>regulation of proteolysis</td>
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<td>2</td>
<td>1.9E-04</td>
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<td>12</td>
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<td>5.7E-04</td>
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<td>glucose import</td>
<td>GO:0046323]</td>
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<td>3</td>
<td>5.7E-04</td>
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<td>pentose-phosphate shunt</td>
<td>GO:0006098</td>
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<td>14</td>
<td>8.5E-04</td>
<td>TKL2 SOL4 GND2</td>
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<tr>
<td>D-xylose catabolic process</td>
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<td>4</td>
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<td>YJR096W GCY1</td>
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<td>1.1E-03</td>
<td>YJR096W GCY1</td>
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<td>negative regulation of protein kinase activity</td>
<td>GO:0056469</td>
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<td>4</td>
<td>1.1E-03</td>
<td>PIL1 LSP1</td>
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<td>glucose 6-phosphate metabolic process</td>
<td>GO:0051156</td>
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<tr>
<td>response to heat</td>
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<td>17</td>
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<td>PIL1 GAC1 LSP1</td>
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<tr>
<td>pentose-phosphate shunt, oxidative branch</td>
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<td>1.9E-03</td>
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<td>metabolic process</td>
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<td>425</td>
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<td>eisosome assembly</td>
<td>GO:0070941</td>
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<td>7</td>
<td>3.9E-03</td>
<td>PIL1 NCE102</td>
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<tr>
<td>cell redox homeostasis</td>
<td>GO:0045454</td>
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<td>31</td>
<td>8.9E-03</td>
<td>GRX1 TRX2 AHP1</td>
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<td>carbohydrate metabolic process</td>
<td>GO:0005975</td>
<td>5</td>
<td>94</td>
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<td>GLK1 HXK1 AMS1 SOL4 PGM2</td>
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</table>

k is the number of genes in the data set in the listed category and f is the total number of genes in the listed category
Table 6. GO TERM analysis of the genes that are further down-regulated in the stressed *siw14Δ* mutant compared to the stressed wild-type.

<table>
<thead>
<tr>
<th>Biological Process</th>
<th>GO:Term</th>
<th>k</th>
<th>f</th>
<th>p-value</th>
<th>Gene</th>
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<td>translation</td>
<td>GO:0006412</td>
<td>12</td>
<td>318</td>
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<td>MAK16 RPL13A RPL27B RPL12A RPS26B RPL22B RPL8A YHR020W RPL16A RPS14B RPL31B RPL36A</td>
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<td>ribosome biogenesis</td>
<td>GO:0042254</td>
<td>7</td>
<td>170</td>
<td>2.4E-05</td>
<td>NOP1 UTP6 SNU13 RPL8A RPS14B NOP58 NOG1</td>
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<tr>
<td>rRNA processing</td>
<td>GO:0006364</td>
<td>7</td>
<td>170</td>
<td>2.4E-05</td>
<td>NOP1 UTP6 SNU13 NSR1 RPS14B NOP58 NOG1</td>
</tr>
<tr>
<td>transcription of nuclear rRNA large RNA polymerase I transcript</td>
<td>GO:0042790</td>
<td>3</td>
<td>17</td>
<td>8.8E-05</td>
<td>RPA49 RPA190 RPA135</td>
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<tr>
<td>rRNA processing</td>
<td>GO:0000028</td>
<td>2</td>
<td>14</td>
<td>2.4E-03</td>
<td>NSR1 RPS14B</td>
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<tr>
<td>maturation of SSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)</td>
<td>GO:0000462</td>
<td>3</td>
<td>60</td>
<td>3.8E-03</td>
<td>UTP6 SNU13 RPS14B</td>
</tr>
<tr>
<td>endonucleolytic cleavage in 5'-ETS of tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)</td>
<td>GO:0000480</td>
<td>2</td>
<td>27</td>
<td>8.8E-03</td>
<td>UTP6 NOP58</td>
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</table>

k is the number of genes in the data set in the listed category and f is the total number of genes in the listed category.

Table 7. GO TERM analysis of the genes that are oppositely regulated in the stressed *siw14Δ* mutant and stressed wild-type strains.

**Stressed Wild-type Up-regulated and Stressed *siw14Δ* Down-regulated Genes**

<table>
<thead>
<tr>
<th>Biological Process</th>
<th>GO:TERM</th>
<th>k</th>
<th>f</th>
<th>p-value</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>septin ring organization</td>
<td>GO:0031106</td>
<td>1</td>
<td>2</td>
<td>1.2E-03</td>
<td>PCL1</td>
</tr>
<tr>
<td>G1 phase of mitotic cell cycle</td>
<td>GO:0000080</td>
<td>1</td>
<td>7</td>
<td>4.2E-03</td>
<td>PCL1</td>
</tr>
<tr>
<td>glycogen biosynthetic process</td>
<td>GO:0005978</td>
<td>1</td>
<td>12</td>
<td>7.3E-03</td>
<td>GSY1</td>
</tr>
</tbody>
</table>

**Stressed Wild-type Down-regulated and Stressed *siw14Δ* Up-regulated Genes**

<table>
<thead>
<tr>
<th>Biological Process</th>
<th>GO:TERM</th>
<th>k</th>
<th>f</th>
<th>p-value</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>respiratory electron transport chain</td>
<td>GO:0022904</td>
<td>2</td>
<td>7</td>
<td>5.8E-06</td>
<td>B13 B14</td>
</tr>
<tr>
<td>mRNA processing</td>
<td>GO:0006397</td>
<td>2</td>
<td>170</td>
<td>3.8E-03</td>
<td>B13 B14</td>
</tr>
<tr>
<td>manganese ion transport</td>
<td>GO:0006828</td>
<td>1</td>
<td>8</td>
<td>4.8E-03</td>
<td>PHO84</td>
</tr>
<tr>
<td>polyphosphate metabolic process</td>
<td>GO:0006797</td>
<td>1</td>
<td>8</td>
<td>4.8E-03</td>
<td>PHO84</td>
</tr>
<tr>
<td>phosphate transport</td>
<td>GO:0006817</td>
<td>1</td>
<td>10</td>
<td>6.0E-03</td>
<td>PHO84</td>
</tr>
</tbody>
</table>

k is the number of genes in the data set in the listed category and f is the total number of genes in the listed category.
Inositol pyrophosphates are necessary to mount an environmental stress response (Worley et al. 2013). Therefore, I wondered whether inositol pyrophosphates levels increase when yeast strains are exposed to environmental stresses. Strains were radiolabeled with $^3$H-myo-inositol, grown to mid-log phase and treated with hydrogen peroxide or KCl for 20 min to induce oxidative or osmotic stress responses, respectively. After extracts were prepared, labeled inositol pyrophosphates were separated by HPLC and scintillation counting was performed. IP$_7$ and IP$_8$ levels were determined relative to IP$_6$, and then were normalized to the unstressed wild-type strain (Figure 26). There was a significant increase in both IP$_7$ and IP$_8$ in wild-type cells treated with H$_2$O$_2$; however, there was no increase in inositol pyrophosphates with KCl treatment (Figure 26). Interestingly, IP$_7$ or IP$_8$ were high in the siw14$\Delta$ mutant, as previously reported (Steidle et al. 2016), but their levels did not change under both oxidative and osmotic stresses (Figure 26). These data indicate that the levels of inositol pyrophosphates increase in response to oxidative stress, suggesting that they may be a signal to induce the environmental stress response.
Figure 26. Inositol pyrophosphate levels increase in wild-type cells during oxidative stress. Representative inositol phosphate profiles for wild-type and siw14Δ mutant stressed with (A) 1 mM H₂O₂ or (B) 0.4 M KCl for 20 min. Quantified levels of (C) IP₇ or (D) IP₈ relative to IP₆ in unstressed cells or cells stressed with 0.4 M KCl or 1 mM H₂O₂ in both wild-type or siw14Δ mutant strains. All fold changes were normalized to unstressed wild-type levels. Bars represent the average of 12 biological replicates in 4 separate experiments. *p-values ≤ 0.05
3.4 Summary of Findings

This chapter demonstrated that *SIW14* is a negative regulator of the stress response. These data include phenotypic (cell stress assays), gene expressions (global and gene specific transcription profiles) and metabolite assays (inositol pyrophosphate). When *SIW14* is deleted from yeast, the mutant is resistant to heat, oxidative, and osmotic stresses and it survives stationary phase better which is consistent with previous reports (Care et al. 2004; Altıntaş et al. 2016; Figure 18, Figure 22, and Figure 23). The deletion mutant induces stress response genes during non-stressful conditions, and over responds to stresses by amplifying the stress response (Figure 25). In addition, I previously demonstrated that *SIW14* codes for an IP$_7$ phosphatase (Steidle et al. 2016). Here the changes in the gene expression and the stress response are linked to the change in the levels of the inositol pyrophosphates. Finally, my data show that IP$_7$ and IP$_8$ levels increase in wild-type cells when they are placed under oxidative stress (Figure 22; Steidle et al. 2016).
CHAPTER 4

Discussion

This dissertation has shown that Siw14 is a regulator of the soluble inositol pyrophosphate 5PP-IP₅ (Figure 1B). Siw14 displays pyrophosphate-specific phosphatase activity both in vitro and in vivo. The Siw14 enzyme has a strong preference for inositol pyrophosphates over other previously described putative substrates (such as poly-P and PI(3,5)P₂) (Aceti et al. 2008; Romá-Mateo et al. 2011). When SIW14 is deleted from yeast, the level of IP₇ increases 6.5-fold and when SIW14 is overexpressed in mammalian cells, IP₇ levels almost completely disappear (Figure 12 Figure 13). This chapter discusses the limits of our biochemical assays as well as where they can be expanded.

In chapter 3, this dissertation demonstrated that SIW14 is a negative regulator of the stress response using gene expression and phenotypic assays. When SIW14 is deleted from yeast, the stress response is partially induced under non-stress conditions and the mutant can still mount a stress response during stressful conditions (Figure 25). Furthermore, chapter 2 showed that in wild-type yeast, the levels of both IP₇ and IP₈ levels increase when cells are subjected to oxidative stress (Figure 26). Therefore, this chapter also discusses that the change in the levels of the inositol pyrophosphates is likely the reason why the siw14Δ mutant strain has an improper response to stress.

4.1 Low in vitro Affinity for 5PP-IP₅

The Plant and Fungi Atypical-Dual Specificity Subfamily and the Nudix Family

Despite apparently similar roles in vivo, I found that recombinant Siw14 had a lower specific activity towards 5PP-IP₅ when compared with hDIPP1, the previously characterized inositol pyrophosphate phosphatase from humans. hDIPP1 is a member of the Nudix family. The
specificity constant for 5PP-IP₅ from our Siw14 data (74 M⁻¹ s⁻¹) is lower than the previously reported data for hDipp1, which ranges from 2.5 × 10⁶ to 4.76 × 10⁷ M⁻¹ s⁻¹ (Albert et al. 1997; Kilari et al. 2013). There are several possible reasons to explain this difference, the first is that the enzymes belong to different classes: Siw14 is a member of the Atypical Dual Specificity Phosphotyrosine Phosphatase Superfamily (EC 3.1.3, phosphoric monoester hydrolase) whereas hDipp1 is a member of the Nudix family (EC 3.6.1, acting on phosphorus-containing anhydrides).

The Nudix family was previously thought to be the only family of enzymes that could dephosphorylate inositol pyrophosphates. This family has orthologues in all domains of life and is characterized by having the Nudix motif in the active site, which is Gx₅Ex₅[UA]xREₓ₂EExGU (U represents an aliphatic, hydrophobic residue and the brackets denote that either the U or the alanine could be present) (McLennan 2005). The catalytic residue varies depending on the protein and many site-directed mutagenesis studies have shown the significance of different residues during catalysis (Mildvan et al. 2005; Ooga et al. 2005). The enzymes in this family can hydrolyze many different substrates including nucleoside di- and triphosphates, dinucleoside pyrophosphates, nucleotide sugars, RNA caps, and inositol pyrophosphates (reviewed in McLennan 2005). Within the Nudix family, the diphosphoinositol polyphosphate phosphohydrolase (DIPP) subfamily of enzymes hydrolyzes on the β-phosphate of inositol pyrophosphates. There are five DIPP isoforms found in mammals and their active site is a modified Nudix motif in which the typical loop–helix–loop fold is organized as a strand–loop–helix (Thorsell et al. 2009). This change causes the motif to have six residues instead of five between the N-terminal Gly and the first Glu meaning Gx₆E instead of Gx₅E (Thorsell et al. 2009). The human DIPPs have differing affinities for the inositol pyrophosphates; for example, hDIPPP1 has the highest affinity for 1PP-IP₅ whereas hDIPPP2 prefers 5PP-IP₅ (Kilari et al. 2013).
The yeast homolog *DDP1* encodes an enzyme that can dephosphorylate all inositol pyrophosphates tested (1PP-IP₅, 5PP-IP₅, 1,5PP-IP₄) but was recently shown to prefer poly-P *in vitro* (Lonetti et al. 2011). However, the *in vivo* accumulation of IP₇ suggests otherwise (Figure 15; Lonetti et al. 2011).

The PFA-DSP enzymes are distinct from the other characterized inositol pyrophosphate phosphatases of the Nudix hydrolase family based on amino acid sequence similarity and structure. The PFA-DSP subfamily has members in plants, fungi, kinetoplastids, and slime molds (Romá-Mateo et al. 2011). The PFA-DSP family has no members in animals because there is only limited homology with animal phosphatases outside of the signature phosphatase motif C(X)₅R in the catalytic center (Aceti et al. 2008). The proteins within the PFA-DSP subfamily have not been well studied and the evolutionary conservation of their inositol pyrophosphate phosphatase activity remains unknown.

Phosphatases in general are known to be promiscuous and the Nudix family is a good example of this because it has such a broad range of substrates. Therefore, the members in the PFA-DSP subfamily may also have these characteristics. For example, a potentially important role for these PFA-DSP enzymes is implied in plants because the *Arabidopsis thaliana* genome encodes five members in the PFA-DSP subfamily (Romá-Mateo et al. 2007; Romá-Mateo et al. 2011). Furthermore, the AtPFA-DSP1 protein was shown to dephosphorylate phosphotyrosine, phosphatidylinositol mono-, di-, and triphosphates, and short chain inorganic polyphosphate (poly-P) (Aceti et al. 2008; Romá-Mateo et al. 2011). However, it was not tested with soluble inositol pyrophosphates and thus it is unclear what substrates it would prefer. Siw14 and AtPFA-DSP1 are highly conserved throughout the catalytic core, and they share 61% identity and 76% similarity (Romá-Mateo et al. 2011; our unpublished data). The active sites are predicted to be
basic and shallow, consistent with our finding that the substrate is large and negatively charged (Aceti et al. 2008; Romá-Mateo et al. 2011). Unlike AtPFA-DSP1, the Siw14 only has minimal activity with phosphatidylinositol phosphates and poly-P (Romá-Mateo et al. 2011; Figure 8). Figure 9).

**Efficiency of Purification**

The difference between the activity of hDIPP and Siw14 with 5PP-IP₅ may be specific to the tagged recombinant form of the Siw14 enzyme. One potential handicap for recombinant proteins is the tag used to purify the protein. Siw14 is fused to GST that nearly doubles the size of the protein from 32 kD to 55 kD. To eliminate the GST-tag, I used the protease thrombin to cleave it from Siw14. The activity of the GST-fused Siw14 was compared with Siw14 lacking the tag; I found no difference in enzyme activity using both pNPP and 5PP-IP₅ as substrates (Figure 11). Unfortunately, that is not the only problem that can affect activity of proteins produced in an unnatural host. It is possible that the activity of Siw14 is regulated by post-translational modification; Siw14 has two putative phosphorylation sites (Holt et al. 2009) and *E. coli* is unable to make these post-translational modifications; potentially yielding substantial inactive protein. The protein could also be misfolding in *E. coli* causing aggregates to accumulate during expression. An alternative expression system in eukaryotes might solve this problem, such as in insect cells or yeast. Future work should include characterization of Siw14 to determine if it is post-translationally phosphorylated because it might increase enzymatic activity.
Siw14 Activity May Be Regulated by Oca1 and/or Oca2

An alternate hypothesis as to why Siw14 has low activity is that it may require additional partner proteins for full activity. In *S. cerevisiae*, Siw14 is the only member of the PFA-DSP family. Romá-Mateo and colleagues (2011) classify its two paralogs OCA1 and OCA2 as PFA-DSP-like proteins because they do not contain all four motifs used to define the PFA-DSP subfamily (Romá-Mateo et al. 2007). Siw14, Oca1 and Oca2 share slightly more than 50% identity (Wishart and Dixon 1998; Romá-Mateo et al. 2011); however this sequence identity extends throughout the entire protein. Using two-hybrid and affinity purification approaches, several high throughput studies found that Siw14 is can interact with Oca1 and Oca2 (Ho et al. 2002; Gavin et al. 2006; Krogan et al. 2006; Breitkreutz et al. 2010). It is possible that full activity from Siw14 may require these (or other) partner proteins.

Both Oca1 and Siw14 are necessary for proper cellular responses to environmental stresses: Oca1 is necessary to respond to oxidized lipid products (Alic et al. 2001) and Siw14 is necessary to respond to nutrient depletion, heat, oxidative and osmotic stresses (Figure 18, Figure 22, Figure 23, Figure 24; Care et al. 2004). In addition, both mutants are sensitive to caffeine, and the overexpression of *SIW14* in an oca1Δ mutant suppresses its caffeine sensitivity (Romá-Mateo et al. 2011). Although, deletion of *SIW14* in yeast or its overexpression in mammalian cells led to changes in inositol pyrophosphates levels, there were no effects on levels when *OCA1* was deleted from yeast or overexpressed in mammalian cells (Figure 16). Consistent with our results, Romá-Mateo et al. (2011) did not detect activity with pNPP or PI(3,4,5)P3. This may be due to a substitution in the active site at a potentially important histidine that is conserved within the PFA-DSP family (Aceti et al. 2008; Romá-Mateo et al. 2011). Oca2 is also thought to be catalytically inactive because the active-site cysteine has been replaced by serine (Wishart and
Thus far, it appears that Siw14 is the only protein of the three paralogs that is a catalytically active phosphatase on its own.

Interestingly, many phosphatases have partner proteins that are catalytically dead (reviewed in Wishart and Dixon 1998). The catalytically dead partner proteins can increase or decrease the activity of the catalytically active protein by acting as a substrate-presenting protein or they can function as an inhibitor (Wishart and Dixon 1998). For example, the MTMR5 protein is a catalytically dead member of the human myotubularin family, and it enhances the activity of MTMR2 to dephosphorylate PI(3)P (Kim et al. 2003). I hypothesize that Oca1 and/or Oca2 interact with Siw14 to modulate its activity (Wishart and Dixon 1998), functioning as redundant pseudophosphatases. When OCA1 or OCA2 are deleted in the siw14Δ mutant, to make double mutant strains, the levels of IP7 slightly decrease compared to the single siw14Δ mutant strain. This result suggests that Oca1 and Oca2 might increase the activity of Siw14. An alternative interpretation is that they might affect an additional enzyme of the pathway.

Because many phosphatases are known to be promiscuous, Oca1 and/or Oca2 could help Siw14 dephosphorylate a completely different substrate, such as insoluble phosphatidylinositol polyphosphates. This idea is consistent with the fact that Sw14 can dephosphorylate IP7 without the help of protein partners in mammalian cells (because OCA1 and OCA2 do not have mammalian orthologues) (Figure 13C, E). A third way that Siw14 might be modulated by Oca1 or Oca2 is that the Siw14 might localized differently depending on interaction with these subunits. This mechanism is based on the hypothesis that inositol pyrophosphates may be found localized into separate concentrated pools in the cell. This is consistent with the role of Oca1 in linoleic acid stress which causes lipids to become oxidized at the plasma membrane (Alic et al. 2001). Finally, the siw14Δ null mutant strain has different phenotypes than the catalytically dead
*siw14-C214S* mutant strain (Care et al. 2004); this results suggests that the presence of the protein is necessary for a particular role beyond catalysis, perhaps through Oca1 and/or Oca2. Future work will test whether the activity, substrate selection or localization of Siw14 changes in the presence of Oca1 and/or Oca2.

### 4.2 The Role of Siw14 in the Inositol Pyrophosphate Pathway

Consistent with the *in vitro* biochemical assays, IP\(_7\) levels rise in the *siw14\(\Delta\)* mutant. The increase in IP\(_7\) in the *siw14\(\Delta\)* mutant (Figure 15) was similar to that observed in the *vip1\(\Delta\)* and *ddp1\(\Delta\)* deletion mutants (Mulugu et al. 2007; Onnebo and Saiardi 2009; Lonetti et al. 2011). The IP\(_7\) detected is likely to be the 5PP-IP\(_5\) isoform because Kcs1 (the kinase specific for the pyrophosphorylation at the 5-position) is present. Small but significant accumulation of IP\(_8\) in the *siw14\(\Delta\)* mutant occurs, likely due to the increased amount of substrate for the Vip1 kinase (Mulugu et al. 2007). There is also a 2.8-fold increase in IP\(_8\) in the *siw14\(\Delta\) ddp1\(\Delta\)* double mutant (Figure 15). Our data are consistent with a model that indicates that the primary pathway from IP\(_6\) to IP\(_8\) is through the addition of a β-phosphate to the 5-position of IP\(_6\) by Kcs1 prior to the addition at the 1-position by Vip1 (Figure 1B). Because the *siw14\(\Delta\) vip1\(\Delta\)* deletion mutant has twice the IP\(_7\) levels as either single mutant, this finding indicates an additive effect on the IP\(_7\) pools due to independent activity by these enzymes. It is currently not possible to rule out additional pathway regulation in the *siw14\(\Delta\) ddp1\(\Delta\)* mutant because the IP\(_7\) increase was slightly more than additive in these cells (Figure 15). Future work should include systematically measuring the levels of inositol phosphates in all of the single and double kinase and/or phosphatase deletion mutants. For example, I expect that the *KCS1* deletion to be epistatic to the *SIW14* deletion mutant such that inositol pyrophosphate levels would become undetectable like
the \textit{kcs1Δ} single mutant strain. This is because Siw14 is the antagonist to Kcs1 and Ddp1 is the antagonist to Vip1 \textit{in vivo}. The supporting data are that inositol pyrophosphate levels can be seen in a \textit{kcs1Δ ddp1Δ} double mutant suggesting that Ddp1 has the same reaction rate as Vip1 leading to undetectable levels of inositol pyrophosphates in a \textit{kcs1Δ} single mutant (York et al. 2005). If there is no 5PP-IP\textsubscript{5} for Siw14 to dephosphorylate there should be no accumulation of IP\textsubscript{7} in the \textit{siw14Δ kcs1Δ} double mutant strain.

### 4.3 Stress Response, Inositol Pyrophosphates, and SIW14

Inositol pyrophosphates have been linked to vesicle trafficking, ribosome biogenesis, telomere length, energy charge, cellular responses to nutrient limitation, and cellular stress response (Ali et al. 1995; Ye et al. 1995; Szijgyarto et al. 2011; Worley et al. 2013). When yeast cells are unable to synthesize inositol pyrophosphates, for example by deleting the kinases Kcs1 and Vip1 that phosphorylate IP\textsubscript{6} to IP\textsubscript{7} and IP\textsubscript{8}, they are unable to induce the environmental stress response (Worley et al. 2013). The \textit{kcs1Δ vip1Δ} double mutant had the most severe gene expression phenotype during H\textsubscript{2}O\textsubscript{2} treatment and it completely failed to mount a stress response, wherein a minor stress response was mounted during osmotic stress (Worley et al. 2013). Our observations here show that the opposite is true: higher levels of inositol pyrophosphates lead to a stronger induction of the stress response. I found that during H\textsubscript{2}O\textsubscript{2} treatment, the levels of inositol pyrophosphates increased in wild-type cells and the IP\textsubscript{8} increase was particularly high (5.3 \pm 0.8-Fold) (Figure 26A, C, D). Unexpectedly, neither IP\textsubscript{7} nor IP\textsubscript{8} levels changed during osmotic stress (Figure 26B-D). This might suggest that the transcriptional response to oxidative stress is more dependent on inositol pyrophosphate levels than other stress responses. Alternatively, oxidative stress affects multiple pools of inositol pyrophosphates (e.g. cytosolic,
membrane-associated, and nuclear) such that it is detectable, whereas osmotic stress affects only a smaller nuclear pool.

I found that the *siw14*Δ mutant is resistant to heat, osmotic, and H$_2$O$_2$ stresses. The *siw14*Δ mutant has additional phenotypes that link it to both both stress response and inositol pyrophosphates. For example, a phenotype that connects Siw14 to inositol pyrophosphates is that the *siw14*Δ mutant of *Saccharomyces* has actin clumps and cytoskeletal defects that are particularly evident during stationary phase (Care et al. 2004). Mutations in the *S. pombe* Asp1 gene, a member of the Vip1 family, also exhibits cytoskeleton structure defects (Pöhlmann et al. 2014). In *S. cerevisiae* *kcs1*Δ mutants, which have undetectable levels of inositol pyrophosphates, the opposite phenotype was observed and had diffuse actin cytoskeletons (Onnebo and Saiardi 2009). These data together suggest that inositol pyrophosphates might be playing a role in the polarization of actin filaments.

Yeast strains with mutations in *SIW14*, *VIP1* and *KCS1* genes share similar phenotypes such as resistance to H$_2$O$_2$ and other stresses (Onnebo and Saiardi 2009; Worley et al. 2013; Altıntaş et al. 2016; Steidle et al. 2016). However, the *kcs1*Δ single mutant results in undetectable levels of inositol pyrophosphates which is the opposite of what I observe in the *vip1*Δ and *siw14*Δ mutant strains (Onnebo and Saiardi 2009). The intrinsic levels of or the cycling through inositol pyrophosphates might be important for the regulation of the stress response in yeast, although it is yet to be determined if the accumulation of the different isoforms of IP$_7$ has the same or overlapping phenotypes. Onnebo and Saiardi (2009) found that the level of IP$_7$ and IP$_8$ decreases in wild-type cells stressed with H$_2$O$_2$; I repeated this experiment four times with 12 replicates and was unable to repeat their result. Our results are more consistent with the lack of inositol pyrophosphates inhibiting a stress response (Worley et al. 2013) and the
increase of inositol pyrophosphates in the \textit{siw14}Δ mutant and its resistance to stress. Likewise, I suspect that the biochemical mechanism that causes inositol pyrophosphates to increase during H$_2$O$_2$ treatment is due to the inhibition of the phosphatase and not the kinase because I previously showed that the levels of inositol pyrophosphates can be increased further in the \textit{vip1}Δ \textit{siw14}Δ and \textit{ddp1}Δ \textit{siw14}Δ double mutants (Figure 15). However, this is also contradictory to what Onnebo and Saiardi (Onnebo and Saiardi 2009) observed, such that the activity of Kcs1 becomes reduced in the presence of H$_2$O$_2$ which is consistent with their result but not ours. Therefore, the actual biochemical mechanism remains to be elucidated.

One reason a higher level of inositol pyrophosphates may lead to a higher induction of the oxidative stress response could be due to the HDAC Rpd3L. Three main nuclear components are required in yeast for the general stress response: the transcription factors Msn2/4, inositol pyrophosphates, and Rpd3L (Alejandro-Osorio et al. 2009; Ruiz-Roig et al. 2010; Watson et al. 2012; Worley et al. 2013). The HDAC Rpd3L has a putative inositol phosphate binding pocket; based on amino acid conservation seen in the crystal structure of the human ortholog HDAC3 that is unable to bind to a co-repressor, that inhibits transcriptional induction, without the presence of IP$_4$ (Watson et al. 2012; Worley et al. 2013). When Rpd3 is mutated at the putative inositol binding pocket, cells are unable to induce the environmental stress response (Worley et al. 2013). Therefore, increased level of inositol pyrophosphates in the \textit{siw14}Δ mutant could allow Rpd3 to more readily allow an increase in gene expression of stress response genes (Figure 27).
Figure 27. Model of the interaction between Rpd3L, inositol pyrophosphates and Siw14 during stress. The left panel shows a wild-type cell during stress inducing the environmental stress response (ESR) and down-regulating ribosomal biogenesis (RiBi) through the activation of Msn2 through a co-repressor binding to Rpd3L through inositol pyrophosphate (IP$_7$ in this case but could be IP$_8$) instead of Msn2/4 (depicted by the transparent co-repressor protein). The right panel depicts a siw14Δ mutant cell during stress further up-regulating the ESR and further down-regulating RiBi gens because there are more inositol pyrophosphates available for Rpd3L to bind the co-repressor allowing Msn2/4 to further induce the environmental stress response.
Inositol pyrophosphates may serve as negative regulators during oxidative stress because they can bind to proteins with plextrin homology (PH) domains (Luo et al. 2003; Chakraborty et al. 2010; Gokhale et al. 2013). PH-domains allow proteins to bind phosphatidylinositol polyphosphates at the plasma membrane, and promote signaling to an array of downstream targets to affect protein synthesis, actin polymerization, metabolic homeostasis, and cell cycle entry (reviewed in Shears 2015). H$_2$O$_2$ stress can damage macromolecules like DNA and membranes; therefore, it is important for cells to stop the cell cycle during an oxidative attack. The H$_2$O$_2$ stress response may be dependent on inositol pyrophosphates because they aid in down-regulating signals from the plasma membrane for cell cycle entry among others. For example, the mammalian protein AKT is a kinase with a PH-domain that is a part of the Target of Rapamycin Complex 1 (TORC1) pathway and is inhibited when bound to 5PP-IP$_5$ (Chakraborty et al. 2010). Interestingly, the yeast orthologue Sch9 is also in the TORC1 pathway, and the siw14Δ mutant shares phenotypes with the tor1Δ mutant: both mutants are long lived (Gomes et al. 2007; Figure 24), sensitive to rapamycin (Appendix A, Figure 28) and resistant to H$_2$O$_2$ (Wei et al. 2008; Appendix A, Figure 29). These preliminary data suggest that the siw14Δ mimics an H$_2$O$_2$ stressed wild-type cells by having increased levels of inositol pyrophosphates that inhibit AKT/Sch9 signaling from the plasma membrane.

4.4 Broader Implications

This dissertation project has strengthened the connection between inositol pyrophosphates and the stress response, an important process since inability to induce stress responses results in cell death. Therefore, understanding the regulation and metabolism of inositol pyrophosphates is also very important. Stress response genes are conserved throughout
all domains of life and inositol pyrophosphates have been found in all eukaryotes studied thus far. Consequently, the connection between inositol pyrophosphates and the stress response in yeast can have broader applications in other species (Illies et al. 2007). For example, Kcs1 was found to be necessary in order for the fungal pathogen *Cryptococcus neoformans* to adapt to host cell environments suggesting a defective stress response (Lev et al. 2015). In plants, the PPIP5K orthologue Vih2 plays a role in regulating plant defenses against herbivorous insects and necrotrophic fungi, and the *vih2Δ* mutants have reduced defenses against these stressors (Laha et al. 2015). Furthermore, cells in multicellular organisms that do not respond to stress can become cancerous, by not inducing the apoptosis pathway. The human ortholog of Kcs1, IP6K, is necessary in order to initiate the apoptosis pathway in human cells and the kinase is absent in some squamous cell carcinomas, further suggesting the biological link (Morrison et al. 2001; Nagata et al. 2005; Morrison et al. 2009). Finally, the presence of IP7 is necessary for the release of insulin from pancreatic β-cells (Illies et al. 2007). The *ip6kΔ* knockout mouse is unable to secrete insulin and the phenotype is rescued by addition of exogenous IP7 (Bhandari et al. 2008). Thus, IP6K and/or IP7 may be an important aspect to study in the development of diabetes. Therefore, manipulating the inositol pyrophosphate pathway may lead to better understanding in order to treat these diseases and infections.
The abbreviations used are:

PP-IP$_{5}$, diphosphoinositol pentakisphosphate (also known as IP$_{7}$); IP$_{6}$, inositol hexakisphosphate; 1,5PP-IP$_{4}$, bis-diphosphoinositol tetrakisphosphate (also known as IP$_{8}$); PCP-IP$_{5}$, methylene-bisphosphonate inositol pentakisphosphate; poly-P, polyphosphate; PI(3)P, phosphatidylinositol 3-phosphate; PI(4)P, phosphatidylinositol 4-phosphate; PI(5)P, phosphatidylinositol 5-phosphate; PI(3,4)P$_{2}$, phosphatidylinositol 3,4-bisphosphate; PI(3,5)P$_{2}$, phosphatidylinositol 3,5-bisphosphate; PI(4,5)P$_{2}$, phosphatidylinositol 4,5-bisphosphate; PI(3,4,5)P$_{3}$, phosphatidylinositol 3,4,5-triphosphate;

Siw14, phosphatase from SIW14 gene (also called Oca3); Kcs1, IP$_{6}$ kinase (IP6K); Vip1, PP-IP$_{5}$ kinase (PPIP5K); Ddp1, diadenosine and diphosphoinositol polyphosphate phosphohydrolase; DIPP, diphosphoinositol phosphate phosphohydrolase; Oca1 (oxidant-induced cell-cycle arrest-1), putative phosphatase; Oca2 (oxidant-induced cell-cycle arrest-2), putative phosphatase; Fig4 (factor-induced gene 4), phosphatidylinositol 3,5-bisphosphate phosphatase; PTEN, phosphatase and tensin homolog; PPX, polyphosphate phosphatase; PPK, polyphosphate kinase; Nudix (Nucleoside Diphosphate linked to X), phosphohydrolase; PFA-DSP, plant-fungal atypical-dual specific phosphatase;

YPD, yeast extract, peptone, dextrose; YNB, yeast nitrogen base; SCD, synthetic complete dextrose; CSM, complete synthetic medium (a mixture of amino acids); IPTG, isopropyl β-D-1-thiogalactopyranoside; p-NPP, para-nitrophenyl phosphate; SAX, strong anion exchange.
APPENDIX A

Preliminary Data Supporting the Connection Between the TORC1 Pathway, Sch9/PH-domains, and Siw14.

Figure 28. The siw14Δ mutant is sensitive to rapamycin like the tor1Δ mutant. A representative rapamycin growth assay of BY4741, siw14Δ and tor1Δ mutant strains. Cells were grown to log phase, normalized to the same OD_{600} before being serially diluted 10-fold in dH_{2}O, and spotted (2.5 µL) onto YPD or YPD containing 100 µM rapamycin. Strains were allowed to grow for 2 days at 30°C.

Figure 29. The siw14Δ and tor1Δ mutant share the H_{2}O_{2} resistance phenotype. Wild-type, siw14Δ, and tor1Δ mutant strains were grown to either log phase or post-diauxic shift and treated with 1 mM H_{2}O_{2} for 3 hrs. Colony forming units (CFUs) were determined for treated and untreated strains, and the % survival was calculated (CFU treated/CFU untreated *100). Bars represent the average of biological triplicates. Error bars are standard error of the mean.*p-values ≤ 0.05
APPENDIX B

The Deletion of *SIW14* Results in Cells Being Sensitive to Some But Not All Non-fermentable Carbon Sources.

The growth rate of the *siw14Δ* mutant in glycerol.

<table>
<thead>
<tr>
<th></th>
<th>YPD (hr)</th>
<th>YPG (hr)</th>
<th>YPG/YPD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1.87</td>
<td>4.61</td>
<td>2.46</td>
<td>-</td>
</tr>
<tr>
<td><em>siw14Δ</em></td>
<td>1.86</td>
<td>4.94</td>
<td>2.66</td>
<td>0.25</td>
</tr>
</tbody>
</table>

**Figure 30.** The *siw14Δ* mutant is sensitive to some non-fermentable carbons. (A) Spot assays on media containing a non-fermentable carbon source (as indicated) or glucose. (B) Quantification of the CFU found on glycerol containing media (YPG) versus glucose containing media (YPD). Error bars represent the standard error of the mean of biological triplicates. **p-value ≤ 0.01, ***p-value ≤ 0.001** (A&B) Cells were grown to either log phase or post-diauxic shift in YPD media and normalized all samples to 0.3 OD₆₀₀ and serially diluted them to 10⁻⁴ in buffered saline with glucose or without glucose for post-diauxic shift cells. Two and a half µL of each 10-fold dilution was spotted and/or 25 µL of the 10⁻⁴ dilution was spread onto solid medium containing 2% glucose or a non-fermentable carbon source (100 mM pyruvate, 2% acetate, 3% glycerol, and 2% ethanol). The glucose plates were allowed to grow for 2 days at 30°C and the non-fermentable carbon plates were incubated at 30°C for 4 days. (C) The growth rate represented is as doubling time of the *siw14Δ* mutant in YPD and YPG medium compared to the wild-type strain. Cells were grown in YPD over night before normalizing to the same OD₆₀₀ 0.1 and then making a further 1-1000 dilution into either YPD or YPG media in a 96-well plate such that ~1000 cells were in each well. Over 48 hours OD₆₀₀ readings were measured every 30 min at 30°C with shaking 15 sec before the read in a plate reader. OD₆₀₀ readings were converted to a doubling time by taking the log₂ of the readings and calculating the slope of the linear portion of the graph. Log₂ values represent the average of biological triplicates.
APPENDIX C

Siw14 Promotes Endocytosis

The *siw14Δ* mutant was originally discovered in the Rolfes lab screening for mutants that were sensitive to 4-aminopyrazolo[3,4-*d*] pyrimidine (4-APP), an adenine analog with the five-membered ring in the pyrazole configuration (Figure 31). 4-APP can disrupt cellular processes that utilize the adenine base such as nucleotides and ATP. The sensitivity of the *siw14Δ* mutant to 4-APP is consistent with the sensitivity of the *siw14Δ* mutant to caffeine treatment (Care et al. 2004; Hirasaki et al. 2008). In order to understand this sensitivity phenotype, I first measured the level of *ADE-5,7* expression, a gene that encodes two of the enzymes important for purine biosynthesis. I measured expression using a β-galactosidase reporter assay. In the pR116 plasmid the LacZ gene is under the control of the *ADE-5,7* promoter; therefore, the amount of β-galatosidase protein activity correlates to the expression level of *ADE-5,7*. I grew wild-type and *siw14Δ* mutant cells with the pR116 to log-phase in SC-URA media containing or lacking adenine, and extracted protein. When I assayed the crude extracts with o-nitrophenyl-β-D-galactopyranoside (ONPG), I saw an over-all decrease in β-galactosidase activity in the *siw14Δ* mutant, suggesting lower levels of *ADE-5,7* (Figure 32).

The *siw14Δ* mutant was previously shown to be defective in fluid phase endocytosis (Care et al. 2004; Table 1). The Fcy2 permease imports purine bases, cytosine and their chemical analogs like 4-APP and 5-fluorocytosine (5-FC) into yeast. I wondered if the *siw14Δ* mutant was sensitive to 4-APP because Fcy2 remains on the plasma membrane longer than in wild-types cells due to an endocytosis defect. This would allow for increased transport of 4-APP into the cell. Consistent with the 4-APP sensitivity, the *siw14Δ* mutant is also sensitive to 5-FC (Figure
I then measured the level at which the siw14Δ mutant could secrete the purine precursors, hypoxanthine and inosine, by performing a cross-feeding assay. A cross-feeding assay utilizes the ade2Δ mutant color change phenotype. When ade2Δ mutants are in a culture medium rich with purines, they are white but the cells become red in media lacking purines. This is due to exogenous purines inhibiting de novo purine biosynthesis via a negative feedback mechanism through the first enzyme of in the purine biosynthesis pathway, Ade4. To ensure that I would be able to observe hypoxanthine and inosine excretion, I blocked the negative feedback inhibition by expressing a dominant-acting mutant ADE4 allele (Rébora et al. 2001). Because the siw14Δ mutant is sensitive to 4-APP, I expected the mutant to secrete less inosine and hypoxanthine than wild-type. However, the siw14Δ mutant had 4.4 ± 0.7-fold more ade2Δ growth than the wild-type strain, indicating that it excretes more hypoxanthine and inosine under our test conditions (Figure 33). I wondered if other transporters, such as the arginine transporter Can1, were being miss-localized in the siw14Δ mutant. Canavanine is an arginine analog that can be imported into cells via the Can1 permease. I assayed growth of the siw14Δ mutant on canavanine and observed that the siw14Δ mutant had a smaller zone of inhibition of 6.2 ± 0.2 cm, when grown on solid medium containing 2 mg/mL canavanine compared to wild-type with a 7.9 ± 0.4 cm zone of inhibition (p-value = 0.004; Figure 35). These data together suggest that the siw14Δ mutant has an endocytosis defect that leads to transporters being retained on the plasma membrane.
Figure 31. The *siw14Δ* mutant is sensitive to 4-APP. Cells were grown to log phase and normalized to the same OD$_{600}$ before serially diluting 10-fold in sterile dH$_2$O. Two and a half µL of each dilution was spotted onto SC-ADE or SC-ADE containing 75 µM 4-APP. This is a representative result.

Figure 32. The *siw14Δ* mutant has overall lower levels of *ADE-5,7* expression. A β-galactosidase assay of BY4741+ pR116 and *siw14Δ* + pR116 strains grown to log phase in either SC-URA with adenine (+ADE) or lacking adenine (-ADE). Cells were lysed in 100 mM Tris-HCl, pH 8, 4% glycerol, 89.6 µM β-mercaptoethanol (β-ME), and 3.2 µM PMSF an equal volume of glass beads and 3 rounds of vortexing for 1 min. Crude extract was collected by centrifuging the lysate at 13,000 rpm for 15 min at 4°C. The amount of protein was measured using a Bradford assay and normalized before measuring the amount of β-galactosidase activity with 800 µg/mL ONPG in Z-buffer (60 mM NaSO$_4$ dibasic, 40 mM NaSO$_4$ monobasic, 6.2 mM MgCl, and 0.7 M β-ME) at 28°C. Samples were stopped with 500 µL of saturated NA$_2$CO$_3$ and read at OD$_{420}$. Bars represent the average β-galactosidase activity from yeast cell extracts expressing a *lacZ* gene under control of the *ADE-5,7* promoter (pR116). Error bars represent the standard error of the mean of biological triplicates.
Figure 33. The \textit{siw14Δ} mutant is sensitive to 5-FC. Cells were grown to log phase and normalized to the same OD$_{600}$ before serially diluting 10-fold in sterile DIH$_2$O. Two and a half µL of each dilution was spotted onto SC-ADE solid medium or SC-ADE containing 100 µM 5-FC. This is a representative result of biological triplicates.

The fold change of the total \textit{ade2Δ} growth for \textit{siw14Δ} normalized to wild-type.

<table>
<thead>
<tr>
<th>Inner white growth</th>
<th>Outer red growth</th>
</tr>
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<tbody>
<tr>
<td>2.6 ± 0.5</td>
<td>1.8 ± 0.2</td>
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</table>

Figure 34. The \textit{siw14Δ} mutant is able to secrete more inosine and hypoxanthine than the wild-type strain. A 2 mL \textit{ade2Δ} mutant culture was grown overnight in YPD, centrifuged at 13,000 rpm and resuspended in 500 µL sterile dH$_2$O. Top-agar was prepared with \textit{ade2Δ} mutants by adding 10 µL of the concentrated \textit{ade2Δ} mutant cells to 4 mL of liquefied SC-URA-ADE media with 1 % agar. Top-agar was poured onto SC-URA-ADE medium with 2% agar and allowed to solidify for 1 hr. Overnight cultures of BY4741+ pR433 and \textit{siw14Δ} + pR433 were concentrated in the same manner as the \textit{ade2Δ} mutant, counted with a hemocytometer, and ~10$^6$ cells in 30 µL were spotted onto the SC-URA-ADE with \textit{ade2Δ} mutant top-agar. They were allowed to grow for 3 days at 30°C. The blue arrows represent the diameter of the inner ring of the white \textit{ade2Δ} mutant cells and the black arrows represent the outer ring of the red growing \textit{ade2Δ} mutants that can grow but do not have enough adenine. The diameter of each ring was measured from three different points and then averaged. The fold change of the total \textit{ade2Δ} growth was calculated for the \textit{siw14Δ} and normalized to the wild-type strain. This is a representative assay of biological triplicates.
Figure 35. The *siw14Δ* mutant is resistant to canavanine. BY4741 and *siw14Δ* mutant cells were grown overnight in YPD and normalized to the same OD$_{600}$ using sterile dH$_2$O. Cells were centrifuged at 13,000 rpm for 5 min and resuspended in 500 µL of sterile dH$_2$O. Ten µL of each concentrated sample was mixed with 4 mL of liquified SC-ARG, 1% agar (top-agar) and poured onto SC-ARG 2% agar medium. Top-agar was allowed to solidify for 1 hr before placing a sterile piece of filter paper in the center of the plate and either pipetting 10 µL of dH$_2$O or 2000 µg/mL of canavanine on it. Cells were allowed to grow for 2 days at 30°C. This is a representative result in which the yellow line shows the diameter of the zone inhibition of the wild-type cells and the blue line depicts the zone of inhibition of the *siw14Δ* mutant. Three different diameters were measured for each biological replicate and averaged.
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