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INVESTIGATING THE ROLE OF TORC1 AND THE TRANSCRIPTION FACTOR SFP1P IN THE REGULATION OF *HMO1* GENE IN *SACCHAROMYCES CEREVISIAE.*

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical Sciences
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Department of Biological Sciences

by
Mathew Smith
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ABSTRACT

HMGB proteins are eukaryotic, chromatin-associated proteins that play roles in both DNA dynamics and transcription regulation. Hmo1p is an HMGB protein in *Saccharomyces cerevisiae* that behaves somewhat like a hybrid between mammalian HMGB proteins and the metazoan linker histone H1. mTORC1, a protein complex containing the Tor1p kinase and a major regulator of cellular growth, is inhibited by both rapamycin and stress. It has also been shown to not only associate with Hmo1p at various gene promoters, but also regulate the *HMO1* gene itself through direct binding. In this study, the Hmo1p-mTORC1 relationship was further investigated through two questions: 1) Does the transcription factor Sfp1p play a role in relaying mTORC1's signal to the *HMO1* promoter, and 2) Is the reduction in *HMO1* transcripts during stress dependent on mTORC1? Gene expression analyses revealed that Sfp1p is not required for normal *HMO1* transcription; however, it does appear to play a role in transmitting the mTORC1 stress signal to the promoter, as transcripts are only significantly decreased during stress when Sfp1p is present. Survival tests revealed that Sfp1p might be hindering the cell's ability to repair DNA double-strand breaks, as there is a slight increase in cell survival during double-strand break-induction when Sfp1p is knocked-out; however, there is some uncertainty as to whether this is Hmo1p-related. Chromatin Immunoprecipitation techniques were then used to demonstrate that RNA polymerase II is evicted from the *HMO1* gene over the course of one hour during stress when Tor1p is knocked-out. This same phenomenon had previously been shown in wild-type cells; however, *HMO1* transcripts are only attenuated in wild-type cells, and not when Tor1p is knocked-out. This suggests that mTORC1 is responsible for the reduction of *HMO1* mRNA during stress.

We propose the possibility that mTORC1 is participating in active mRNA degradation at the *HMO1* gene, and that transcription-inhibition techniques can be utilized to confirm this.

CHAPTER 1. INTRODUCTION AND BACKGROUND

1.1. Chromatin

The genetic material of eukaryotic organisms is comprised of DNA compacted into higher-order structures by means of nucleoprotein complexes, thus forming chromatin (1,2,3). These complexes, the nucleosomes, consist of approximately 146 DNA base pairs wrapped 1.7 times around 2 copies each of H2A, H2B, H3, and H4, altogether forming a histone octamer (1,2). Each H2A and H2B histone are dimerized, forming two H2A/H2B heterodimers, while the H3 and H4 histones collectively form a $(H3/H4)_2$ heterotetramer (2). The negatively-charged phosphodiester backbone of the DNA is attracted to the positively charged amino acid residues (notably those of lysine and arginine) that are common in histone core particles, creating a first-order compaction of chromatin known as the 11-nanometer (nm) “beads on a string” (26). In this conformation, the N-terminal tails of each histone jut out from the nucleosome and contact neighboring chromatin proteins as a means of regulating higher-order structures (21).

Linker DNA connects adjacent nucleosomes and may be bound by linker histones. Metazoan cells contain the linker histone H1, which is localized to where linker DNA enters/exits the nucleosome (1). H1 allows the “beads on a string” to further compact by means of its lysine-rich C-terminus as well as its globular “winged-helix-turn-helix” domain (1). This causes the DNA to coil into a 30-nm fiber (secondary structure), which can further compact into still higher order (tertiary) structures depending on the stage of the cell cycle. Chromatin itself can be classified as being in one of two states: heterochromatin or euchromatin. Heterochromatin is heavily supercoiled and highly condensed, preventing the access of RNA polymerases and transcription factors, rendering it transcriptionally-

inactive (21). Its dense nature allows it to resist nuclease activity, providing some means of protection (25). On the other hand, euchromatin is accessible to gene expression proteins due to its relatively loose coiling and unpacked nature. Therefore, euchromatin is generally transcriptionally-active (21).

Histones can be modified through the attachment of several different possible functional groups, which can affect the heterochromatin-euchromatin dynamics. Among the more commonly detected modifications are the acetylation of lysine residues' ϵ-amino groups. The negative charge of the DNA phosphodiester backbone makes it highly attracted to positively charged side chains, such as lysine; therefore, the neutralization of these side chains would attenuate the interactions between the two. The addition of acetyl groups via histone acetyltransferases (HATs) does exactly that, and this is generally accompanied by an increase in gene transcription. This process is reversed via histone deacetylases (HDACs), and the coordination of these two enzymes helps regulate the transcriptional output of affected genes. In addition, the acetylated lysines of histones serve as binding sites for certain binding-domains, such as PHD fingers and bromodomains. For example, the SWI/SNF remodeling complex, which opens the DNA around the nucleosome core, uses the bromodomain of Swi2/Snf2 to recognize acetylated lysines (9).

In addition to acetylation, methylation of histones has been shown to affect transcription regulation, albeit by a different mechanism. Rather than altering chromatin dynamics, the addition of methyl groups to lysine and arginine residues has been shown to recruit specific proteins that perform specific functions (9). Histone methylation has also been shown to play a role in regulating the cell cycle during DNA repair as well as RNA

interference (10). Another type of histone modification, phosphorylation, has been shown to be important in various DNA-related processes, including the regulation of gene expression, particularly of proliferative genes. There is also a significant connection between histone phosphorylation and chromatin compaction associated with mitosis and meiosis (11).

Of particular note, however, is the role of histone phosphorylation in DNA repair processes. DNA double-strand breaks (DSBs) are cuts in the phosphodiester backbones of both DNA strands at a complementary location, which thereby sheer a single double-strand DNA molecule into two. In mammalian cells, if left unaided for too long, these DSBs can prove detrimental to cellular health by promoting cancer and other diseases. Therefore, when DSBs occur, the cell responds by generating a sequence of events, known as the DNA-damage response, in order to repair the damage. In order for DNA repair to occur, however, chromatin remodeling must take place to allow repair proteins to access the damage site. Histone phosphorylation is one of the earliest steps in this process, taking place on H2A at serine-129 in yeast and on H2AX (an H2A variant) at serine-139 in mammals, thereby generating γ-H2AX. This phosphorylation is the initial step in activating homologous recombination, non-homologous end joining, and other DNA-damage responses (2,3,8,11,12).

1.2. *Saccharomyces cerevisiae*

The budding yeast *Saccharomyces cerevisiae*, commonly known as baker's yeast (hereafter simply referred to as "yeast"), is a unicellular fungus [1] that is regularly used as a laboratory model organism, due to its relatively fast life cycle and the evolutionary conservation of several of its genes and molecular pathways. The life cycle of yeast

consists of both haploid and diploid stages, depending on environmental factors. In the haploid stage, its DNA is compacted into 16 nuclear chromosomes (22) that altogether form a genome approximately 24 Mbp in length (23). In this stage, yeast reproduces through budding of a new daughter cell from the parent cell, ultimately leading to nuclear mitosis and the separation of the bud to form a new diploid cell. During environmental stress, diploid cells begin the process of sporulation by means of meiosis. The cell then becomes an ascus, forming four spores within itself before its wall breaks down (24).

The spores are the yeast haploid cells, each one containing a copy of the Mating-Type Locus (*MAT*). Cell mating-types are distinguished through two non-homologous alleles, designated as "**a**" and "*α*". Depending on its mating-type, each haploid cell produces one of two types of pheromones: the 13 amino acid **a**-factor or the 12 amino acid *α*-factor. Special receptors recognize the pheromone of the cell's opposite mating-type, and the cell cycle is arrested at G1. Proteins essential for the mating process are synthesized in each cell, and an extension of each cell's plasma membrane (and cell wall) grow toward the targeted mate (24). These "shmoos" meet at their projections, where they fuse their cytoplasms to form a single cell (27). Afterward, karyogamy, or nuclear fusion, occurs, generating a diploid zygote (*MAT***a**/*α*) and completing the life cycle (24).

In addition to *MAT*, yeast cells contain the transcriptionally-silent loci denoted as *HMR***a** and *HMLα* that contain the genetic information for expression of the "**a**" and "*α*" phenotypes, respectively. Silent information regulator (*SIR*) genes prevent these loci from being expressed. However, their information can be copied to *MAT* via duplicative transposition in order to switch the mating-type of the cell. This process is initiated by the *HO* (homothallic) gene, which encodes the *HO* endonuclease, a site-specific protein that

targets an 18 bp sequence in the *MAT* locus and generates a DSB (24) with sticky ends (28). DSB repair mechanisms delete the mating-type cassette in *MAT*, after which it is repaired through the replication of the opposite mating-type cassette within its locus (24). This mechanism can be exploited for experimental purposes to test the effects of DSBs in yeast (Figure 1).

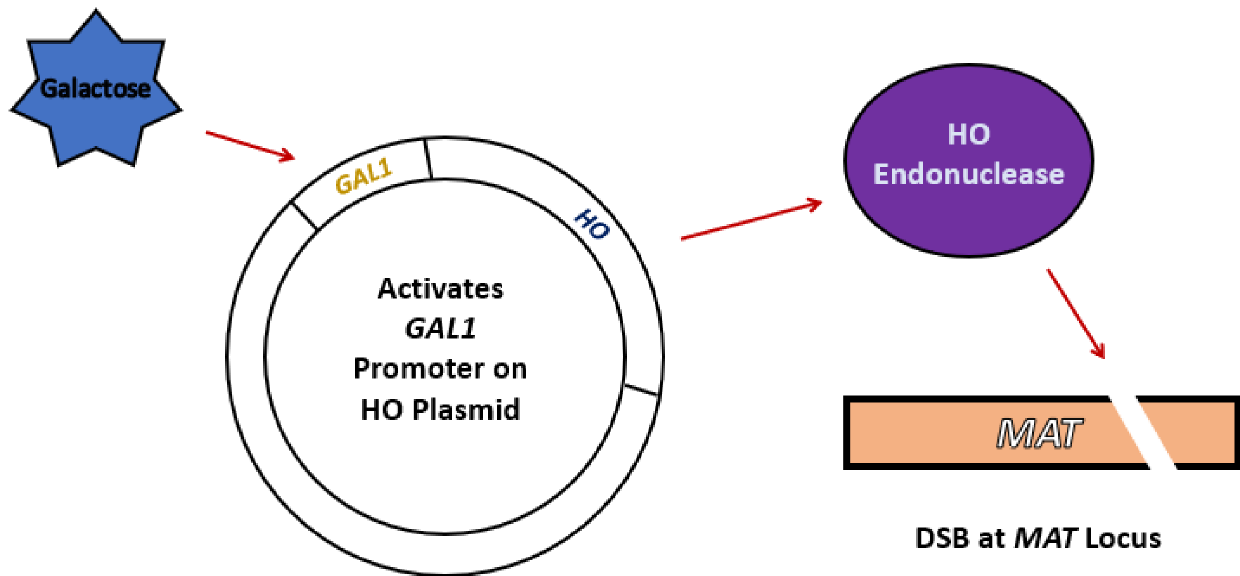

Figure 1. Mechanism of Controlled Induction of DSBs. A plasmid encoding *HO* under control of the *GAL1* promoter is transformed into yeast and galactose is supplied as a carbon source in order to activate the promoter and induce transcription of HO endonuclease. The endonuclease then targets the *MAT* locus and creates a DSB. As the target site of HO endonuclease is specific, this provides a means of experimental control, preventing the DSB from occurring at random sites, which would potentially damage essential genes.

Yeast also contains a special linker histone called Hho1p, which is distinguished from metazoan H1 by its possession of two globular domains. One of these domains is very similar to that of H1, suggesting homology between the two proteins. In yeast, this domain, which adopts a similar fold to that of H1, has been shown to protect against nuclease activity by means of nucleosome-binding (2,8). Hho1p's second globular domain, which is unstructured in neutral conditions and adopts a winged-helix motif in the

presence of concentrated tetrahedral anions, binds most efficiently to DNA four-way junctions (2). During stationary phase, Hho1p assists in genomic compaction by forming loops along the length of the nuclear DNA, further linking it to metazoan H1 functions. Unlike H1, however, Hho1p does not contain a basic C-terminus; rather, its lysine-rich domain is enclosed between its two globular domains, preventing it from participating in chromatin reorganization (2,8). In addition, the stoichiometry of Hho1p to nucleosomes in yeast is significantly lower than that of its metazoan counterpart (2).

1.3. The High-Mobility Group Box Protein Hmo1p

High-mobility group box (HMGB) proteins are eukaryotic nuclear proteins that are highly involved in DNA-dependent processes. They are typically defined by their “HMG-Box” domains (2,3,8), which consist of three α-helices that are arranged in an L-shaped motif that plays significant roles in DNA interactions (2). There are two categories of HMGB proteins: sequence-specific and non-sequence-specific. By definition, sequence-specific HMGB proteins are specialized transcription factors, only being expressed in specific cell types. In contrast, non-sequence-specific HMGB proteins are common to all eukaryotic cells and play a major role in chromatin dynamics. They are considered “architectural” by definition, as their HMG-Box domains act to bind/bend DNA targets. As a consequence of this, they tend to display a high affinity to pre-bent DNA. DNA-bending via HMGB proteins is accomplished through the energy derived from the electrostatic and van der Waals interactions made between the HMG-Box domain and the DNA minor groove. As the minor groove widens due to the insertion of the HMG-Box, the major groove narrows, generating a bend in the overall structure (2).

Non-sequence-specific HMGB proteins usually contain two HMG-Box domains (Box A and Box B) that have higher affinity for non-B-form DNA, including four-way junctions, somewhat linking their functionality to Hho1p. In mammals, HMGB proteins 1-4 contain acidic C-termini, which interact with Box A and Box B to block them from being able to bind to DNA. In the case of nucleosomes, however, it is believed that the N-terminus of histone H3, which contacts linker DNA by passing through the DNA entry-exit point, interacts with the acidic C-terminus to allow the binding of the box domains to the entry-exit point, distorting the nearby DNA. In turn, this creates a chain reaction of DNA-unwinding around the nucleosome core, generating a loosely-packed chromatin environment associated with transcriptional activation. As a result, mammalian HMGB proteins engage in competitive nucleosome-binding against the linker histone H1, leading to dynamic chromatin-regulation methods (2,8).

Of the many HMGB proteins found in yeast, Hmo1p and Hmo2p are notable as having two separate globular domains that heavily resemble HMG-Boxes (hereafter referred to as Box A and Box B) (2). Hmo1p, in particular, has been found to not only possess HMGB characteristics, but also those of linker histones, due in part to its lysine-rich C-terminus. Along with its terminal domain, Hmo1p's Box A and Box B domains further contribute to chromatin compaction (2,3,8,14). Box A domains dimerize in order to bend Hmo1p-bound DNA (2,14), and although it has low affinity for B-form DNA, it possesses structural specificity for other forms, particularly four-way junctions (similar to HMGB proteins and Hho1p) (2). Box B, on the other hand, is mostly responsible for the protein's overall DNA-binding affinity, and it does not directly play a role in DNA bending (2,14).

Two models of Hmo1p-mediated DNA stabilization have been proposed. The first, as mentioned above, involves the dimerization of Hmo1p's Box A domains, which happens particularly in nucleosome-free DNA. The second, which occurs at the nucleosome dyad when it is bound by Box A, involves the lysine-rich C-terminus preventing DNA unwinding around the nucleosome through its association with the linker DNA. This particular characteristic contrasts Hmo1p with typical mammalian HMGB proteins, which typically bind to nucleosome dyads to loosen the DNA (2). Hmo1p has been shown to localize to the *MAT* locus, and of particular interest is its tendency to become evicted following 2 hours of continuous DSBs (3,5,8). This provides a simple means of control while performing experiments relating to DSBs. In addition to DNA compaction, Hmo1p has been shown to be involved in transcriptional activity, particularly at the promoters of ribosomal biogenesis (Ribi) genes, various ribosomal protein (RP) genes, and even the *HMO1* gene itself (2,3,8,5,14).

Due to its ability to stabilize chromatin, Hmo1p has a significant effect on DNA repair efficiency. It has been demonstrated that in the absence of Hmo1p, cell survival is significantly increased during continuous DSBs. It is believed that when chromatin is heavily compacted by Hmo1p, DNA repair proteins have a more difficult time accessing the DSB sites. Thus, a less-condensed chromatin environment would allow for a greater chance of DNA-damage repair and, therefore, continued cell survival (2,3,8)

1.4. The Target of Rapamycin Complex 1 (TORC1)

In order for cells to carry out a response to a particular stimulus, specialized proteins must relay the signal within the cell. Protein kinases accomplish this by phosphorylating downstream targets, such as proteins targeted to gene promoters or

other cellular proteins. The yeast serine-threonine kinase TORC1 (target of rapamycin complex 1), a multi-protein complex containing the catalytic Tor1p (5,20) is a significant regulator of cellular development, playing roles in the expression of ribosome-related genes (2,5,14,16,17,18,20) as well as various RNA polymerase II (Pol II)-transcribed genes (5,17,18). rRNA genes are regulated by TORC1 through direct contact (2,5), while RP and Ribi genes are regulated indirectly via downstream phosphorylation targets, such as the AGC family protein kinase Sch9p (2,5,9) and the zinc-finger transcription factor split finger protein 1 (Sfp1p). Sfp1p itself has been shown to be required for maximal ribosome production in yeast, which makes sense in context with its tendency to bind to RP promoters (2,5,17,18,19). Furthermore, Sfp1p's overall localization within the cell appears to be dependent on TORC1: while TORC1 is inactive, Sfp1p remains in the cytoplasm, hindering it from participating in gene regulation. Its localization to the nucleus is reserved for instances of TORC1 activation (2,5,18,19).

Several different proteins make up the overall complex of TORC1. In yeast, these subunits are called Tor1p (or possibly Tor2p), Kog1p, Tco89p, and Lst8p (5,13,20). In mammalian cells, the complex, originally referred to as mammalian TORC1, contains a specific mammalian TOR (mTOR) protein along with the regulatory-associated protein of mTOR (RAPTOR), mLst8, the proline-rich Akt substrate of 40 kDa (PRAS40), and the disheveled, Egl-10, and Pleckstrin domain-containing mTOR-interacting protein (DEPTOR). RAPTOR is the ortholog of Kog1p, while PRAS40 and DEPTOR take the place of Tco89p (13,20). The catalytic subunits, Tor1p/Tor2p and mTOR, are phosphatidylinositol kinase-related kinases (PIKKs). In yeast, both Tor1p and Tor2p can act as the catalytic subunit due to their similar domains (5,13,20).

TORC1 has been heavily conserved throughout evolution from yeast to mammals, making it a commonality among eukaryotes under the collective name “mechanistic TORC1” (hereby referred to as “mTORC1”) (5,13,20). As its name implies, mTORC1 is inhibited by the *Streptomyces hygroscopicus*-produced macrolide rapamycin, which binds to the 12-kDa FK506-binding protein (FKBP12). This complex gains the ability to block the Tor1p/Tor2p active site by binding to its FKBP-rapamycin-binding (FRB) domain, thus inhibiting catalytic potential (13,20). It has further been demonstrated that cellular stress (DSBs in particular) mimics this inhibition response (5). In regards to *HMO1*, mTORC1 is responsible for relaying the stress signal to the promoter, as a decrease in *HMO1* transcript levels is only observed after induction of DSBs when Tor1p is present (5). This was surprising, considering how Tor2p has been shown to take the place of Tor1p as the catalytic subunit when necessary. This suggested that Tor1p had a more specific function in regulating the *HMO1* promoter; in particular, Tor1p may be localizing to the promoter and performing some direct activity. Chromatin immunoprecipitation (ChIP) techniques have confirmed that it binds directly to the *HMO1* promoter (5), and while a similar behavior has been demonstrated at other genes (including the aforementioned rRNA genes), its role in binding to this particular promoter is currently unknown.

Pol II eviction throughout the *HMO1* gene has been shown to occur within 1 hour of DSB induction in wild-type (WT) yeast cells (5), providing what was once a possible explanation for the accompanied decrease in transcript levels. However, preliminary data has suggested a similar phenomenon in *tor*Δ cells (Ashish Gupta, unpublished data), where DSBs have been shown to have no effect on *HMO1* transcript levels. The

observation that *HMO1* transcript levels do not decrease in response to DNA damage when Tor1p is absent, even though Pol II leaves the gene, indicates that mTORC1 is somehow responsible for the response. As such, this may be a key piece of evidence for explaining why mTORC1 binds to particular genes.

The relationship between Hmo1p and mTORC1 has been of particular interest in our lab; therefore, my aim was to further study this relationship in regards to DNA damage. With evidence suggesting that Sfp1p may also be involved in this interaction, I first looked into the possibility of Sfp1p's involvement in *HMO1* regulation via mTORC1 signaling by conducting both survival and gene expression analysis experiments. I then sought to determine the precise role of mTORC1-binding at the *HMO1* gene by demonstrating Pol II eviction from the locus during DSB.

CHAPTER 2. MATERIALS AND METHODS

2.1. Gene Expression Analysis

Cultures of two *Saccharomyces cerevisiae* strains, *DDY3* (our experimental WT) and *DDY3-sfp1*Δ, were grown in synthetic minimal uracil-dropout (-URA) media and tryptophan-dropout (-TRP) media, respectively, at 30°C with constant shaking to exponential phase (OD600~0.4-0.8). Both strains had previously been transformed with a plasmid encoding HO endonuclease under control of the *GAL1* promoter and marker genes for uracil (*DDY3*) and tryptophan (*DDY3-sfp1*Δ) (See 5). Both were supplemented with raffinose as a carbon source (2% final concentration). 15 mL of culture were pelleted and washed with diethyl pyrocarbonate (DEPC)-treated water (0-hour control). The remaining culture was then treated with galactose (2% final concentration) and incubated at room temperature for 1 hour with constant shaking, after which 15 mL were pelleted and washed with DEPC-treated water. Pellets were stored at -80°C overnight.

Pellets were thawed and total RNA was extracted via illustra RNAspin Mini RNA Isolation Kit (GE Healthcare), after which TURBO DNase (Ambion) was used to degrade contaminating genomic DNA. PCR was used to confirm complete removal of DNA. NanoDrop (Thermo Scientific) was used to determine total RNA concentrations and dilutions were made. 1 µL of 20 mM reverse primer (5'-TCTCCAACTCGACGTTGTAAGCCTGC-3') was added to ~100 ng of total RNA in a separate 1.5 mL Eppendorf tube and sterile ddH_2O was added to make the total volume 13 µL. Samples were incubated at 65°C for 5 minutes and then placed on ice for 5 minutes to allow primer annealing and fixation. AMV Reverse Transcriptase (New England Biolabs) was used to generate cDNA. 2 µL 25 mM magnesium chloride, 2 µL 25 mM

dNTPs, 2 μL 10x AMV Reverse Transcriptase Reaction Buffer, and 1 μL AMV Reverse Transcriptase (10,000 units/mL) were added to make a 20 μL reaction volume. The entire mixture was placed in a 42°C water bath for 1 hour, and resulting samples were stored at -20°C. cDNA was verified using PCR.

For quantitative-PCR (qPCR), samples were prepared in triplicate, 20 μL reactions in a 96-well plate using Luna Universal qPCR Master Mix (New England Biolabs). Primer sequences used for the open reading frame (ORF) were 5'-AAGATAGAGGCTTTCACCACTTTGAC-3' (forward) and the aforementioned cDNA-generating primer (reverse). A QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems) was used to carry out qPCR. The *IPP1* gene was used as a reference (primers: 5'-CCCAATCATCCAAGACACCAAGAAGG-3' [forward], 5'-AGCAATAGTTTCACCAATTTCCAACACATC-3' [reverse]). Relative expression levels were calculated via the $\Delta\Delta C_t$ method.

2.2. Survival Tests

The aforementioned strains were both grown to exponential phase (OD_{600}~0.5-0.8) and treated with galactose as described above. 1 mL of culture was collected prior to treatment and every hour thereafter for up to 4 hours and diluted to 1000x, after which 50 μL were plated on either synthetic minimal -URA or -TRP agar media with raffinose (2% final concentration) and incubated at 30°C for 2-5 days. Colonies were counted and used to determine the concentration of the treated, undiluted sample through the following equation: (# of Colonies * 1000μL * 1000x) / (50μL * OD_{600})

2.3. Chromatin Immunoprecipitation

ChIP was performed using the same protocol as described in (5) with the following alterations. Cultures of *DDY3-tor1*Δ containing the aforementioned HO/uracil marker plasmid were grown in synthetic minimal -URA media with raffinose (2% final concentration) in 30°C with constant shaking to exponential phase (OD_{600}~0.5-0.8). A 50 mL sample of culture (0-hour control) was collected and treated with formaldehyde (3% final concentration) and left to shake at room temperature for 20 minutes to allow DNA-protein crosslinking, after which the sample was pelleted and washed with 1x phosphate-buffered saline (PBS). The remaining culture was treated with galactose (2% final concentration) to induce DSBs and incubated at room temperature for 1 hour with constant shaking. 50 mL samples were collected at 15, 30, and 60 minutes, treated with formaldehyde as described above, pelleted, and washed with 1x PBS. Pellets were stored at -80°C overnight.

ChIP lysis buffer (50 mM HEPES [pH 7.5], 140 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate) with protease inhibitor was prepared using one tablet of Roche cOmplete protease inhibitor cocktail (mini, EDTA-free) dissolved in 1.5 mL sterile ddH_2O, which was then added at 10 µL/mL lysis buffer (mixture hereby referred to as PI). 400 µL PI was added to each thawed pellet along with 500 µL of acid-washed glass beads (~500 µm) and left to vortex in 1.5 mL Eppendorf tubes at 4°C for 40 minutes. 10 µL 100 mM phenylmethylsulfonyl fluoride (PMSF) dissolved in isopropanol was added to each sample, and flame-heated 26G syringe needles were used to poke through the bottoms of the tubes, after which each pierced tube was placed within a second tube and briefly centrifuged to allow the total cell extract to filter through. Samples were sonicated for 10 seconds (0.9 ON, 0.1 OFF) at 25% amplitude a total of 6 times while being left on ice for

1 minute between sonications. Remaining cell debris was removed through centrifugation, and 10 µL PMSF was added. 20 µL of the resulting whole-cell extract (WCE) was used to check for chromatin fragment size, while the rest was stored in -80°C.

The 20 µL WCE was added to 200 µL of immunoprecipitation elution buffer (IPEB) (100 µL 1 M $NaHCO_3$, 50 µL 20% SDS, 850 µL sterile ddH_2O) and 10 µL 5M NaCl, and the total mixture was incubated at 65°C for 4 hours to reverse crosslinking. Phenol/chloroform was used to extract DNA, and RNase A (final volume 60 µg/mL) was used to remove total RNA in 25 µL 1x TE at 37°C for 30 minutes. Samples were run on 1% agarose gels to determine fragment sizes (target size: 300-1000 bp).

Protein G-Sepharose 4 Fast Flow (GE Healthcare) beads were prepared by taking 35 µL of beads per sample (including antibody immunoprecipitation [AIP] and no antibody control [NA]) and washing them with PI (105 µL per sample) 3 times via centrifugation (8000 RPM, 3 minutes). Bead resuspension buffer (2.4 mL PI, 88 µL salmon sperm DNA [200 µg/mL], and 120 µL bovine serum albumin [500 µg/mL]) was used to dilute beads back up to their original volume (35 µL per sample). For each sample, 100 µL WCE was mixed with 300 µL PI and 30 µL beads and left to rock on a nutator for 1 hour at 4 °C for preclearing. Beads were then removed via centrifugation and 2 µL anti-RNA polymerase II CTD was added to each AIP sample. AIP and NA samples were then left to rock on a nutator at 4°C for 10 hours, and after the addition of 30 µL prepared beads, samples were left to rock for another 12 hours.

Beads were separated from solution via centrifugation and washed for 15 minutes using lysis buffer via rocking on nutator at 4°C. Centrifugation was used to separate beads, and process was repeated using IP wash buffer 1 (lysis buffer, 500 mM NaCl, filter

sterilized), IP wash buffer 2 (10 mM Tris [pH 8], 250 mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, filter sterilized), and 1x TE (twice). After removing second TE, each sample was treated with 250 µL IPEB and shaken at 150 RPM at room temperature to elute crosslinked protein-DNA from beads. Samples were centrifuged and 225 µL of the resulting supernatant of crosslinked protein-DNA was transferred to a separate sterilized Eppendorf tube. The process was repeated with the left-over beads and the supernatant added to the first collection. 100 µL of remaining WCE per sample was thawed and mixed with 475 µL IPEB and 20 µL 5 M NaCl, acting as the input control (IC). Each bead-eluted sample was also treated with 20 µL 5 M NaCl, and all samples were subjected to 65°C incubation for 4 hours to undo formaldehyde crosslinking. All samples were then treated with 20 µL 1 M Tris (pH 8), 10 µL 0.5 EDTA, and 1 µL Proteinase K and left to incubate at 55°C for 30 minutes. Phenol/chloroform extraction was used to remove remaining protein, and DNA was collected using ethanol precipitation. DNA was resuspended in 200 µL 1x TE and treated with RNase A (final volume 60 µg/mL) at 37°C for 30 minutes. PCR was performed using 17.8 µL sterile ddH_2O, 1 µL Taq DNA Polymerase with 3 µL Standard Taq Buffer (New England Biolabs), 1 µL each of the aforementioned ORF 20 mM forward and reverse primers, 0.6 µL 25 mM dNTPs, and 3 µL 25 mM magnesium chloride (27.4 µL reaction). Samples were run on a 1% agarose gel, and ImageJ imaging software was used to quantify band intensities. IC and NA bands were used to standardize AIP bands.

CHAPTER 3. RESULTS AND DISCUSSION

3.1. Sfp1p is Involved in Regulating the *HMO1* Promoter

Based on the idea that DNA damage inhibits mTORC1 signaling, our lab has previously shown that DNA damage reduces *HMO1* transcript levels, but only when Tor1p is present, indicating that mTORC1 is involved in transmitting the stress signal to the promoter (5). As mentioned above, we had reason to believe that Sfp1p may be involved in *HMO1* promoter regulation, and that inducing DSBs may have a different effect on transcript levels depending on whether Sfp1p was present or not. We used WT and *sfp1*Δ strains containing plasmids encoding the HO endonuclease under control of the *GAL1* promoter to induce DSBs by adding galactose as a carbon source. Total RNA was extracted from cells and subjected to qPCR to measure C_t values. to Using the $\Delta\Delta C_t$ method, it was determined that Sfp1p does not have a significant effect on *HMO1* expression in the absence of DSBs, indicating that Sfp1p is not involved in normal *HMO1* expression during exponential growth (Figure 2A). However, after inducing DSBs, it was clear that Sfp1p is somehow involved in relaying the stress signal to the promoter, as expression levels fail to go down in a *sfp1*Δ strain (Figures 2B and 2C). Thus, there is a similar response to DSBs between *tor1*Δ and *sfp1*Δ cells, indicating that the two proteins may be interacting at the promoter. This makes sense in context with Sfp1p's role as a downstream phosphorylation target for mTORC1 at RP gene promoters, and this further emphasizes a similarity in gene regulation between RP genes and other Pol II-transcribed genes (in this case, *HMO1*).

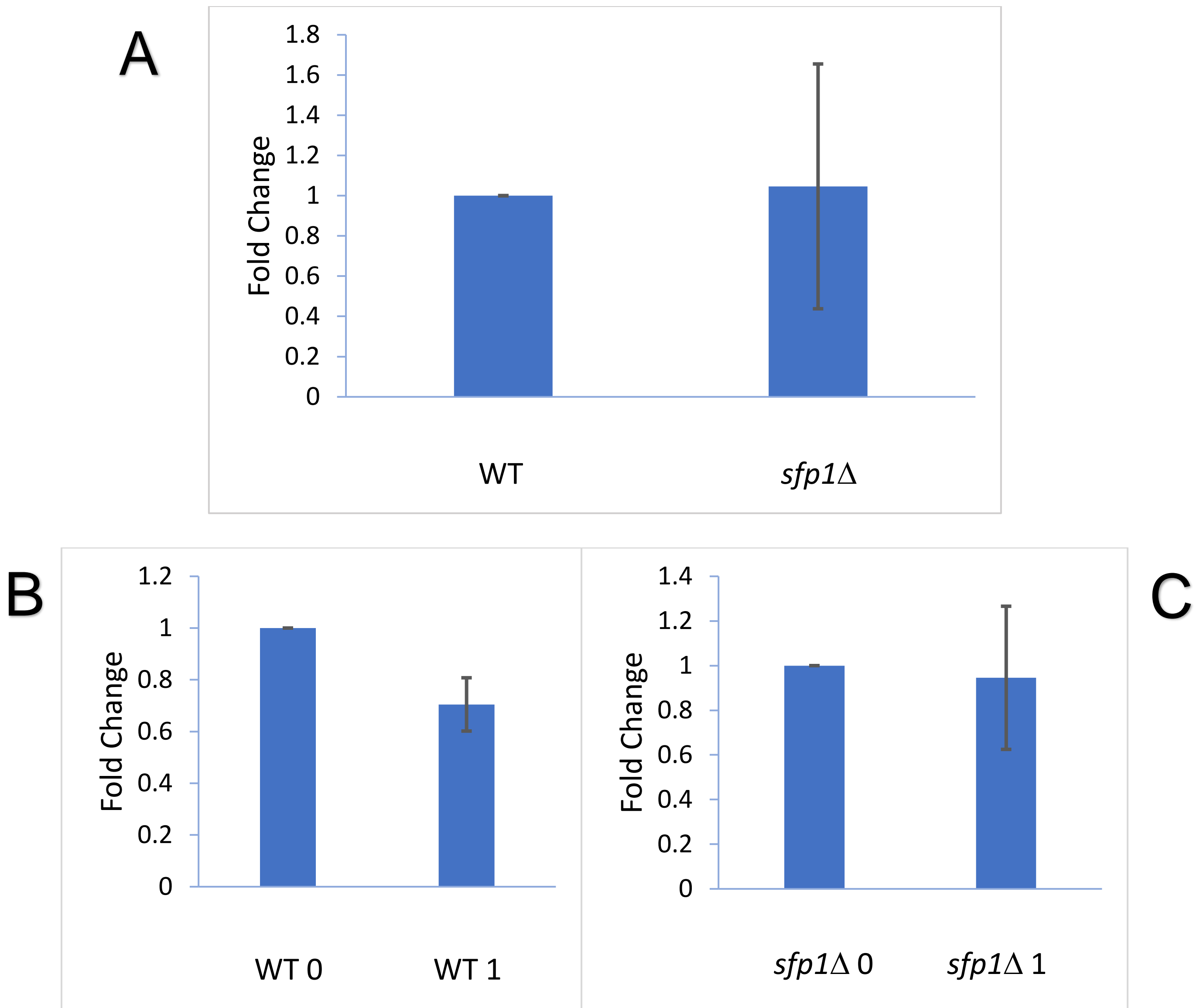

Figure 2. Sfp1p's Effects on *HMO1* Transcription. (A) Bar graph representing relative *HMO1* transcript levels between WT and *sfp1*Δ during exponential growth. (B) Bar graph representing relative WT *HMO1* transcript levels between untreated samples (WT 0) and samples that have undergone 1 hour of DSB induction via galactose-mediated expression of HO endonuclease (WT 1). (C) Bar graph representing relative *sfp1*Δ *HMO1* transcript levels between untreated samples (*sfp1*Δ 0) and samples that have undergone 1 hour of DSB induction via galactose (*sfp1*Δ 1). *IPP1* was used as a reference gene. Expression values normalized to untreated (0 Hr) cells. Means and standard deviations are representative of 3 replicates.

It is worth noting that cells were pelleted at a wide range of concentrations. Theoretically speaking, gene expression should remain relatively constant throughout all

of the exponential growth phase, as was the reasoning during this experiment. While this appears to hold true in WT, the *sfp1*Δ fold changes have exceptionally wide error bars around their means, indicating the likelihood of more concentration-sensitive transcription regulation. What we can conclude from this data is that Sfp1p is having some sort of effect on *HMO1* transcription during DSBs, likely acting as a signaling mediator between mTORC1 and the promoter.

Based on the idea that Hmo1p stabilizes chromatin and its absence results in more efficient DNA repair, we wanted to test the possibility that Sfp1p made a difference in cell survival during consistent DSBs. Based on the aforementioned gene expression data, it could be predicted that in a *sfp1*Δ strain, cell survival would be similar to that of WT, as the mTORC1 stress signal would fail to reach the *HMO1* promoter, thus permitting standard cellular levels of Hmo1p. Considering how Hmo1p remains at the *MAT* locus for 2 hours of DSBs, we first wanted to test if the absence of Sfp1p changed this behavior. WT and *sfp1*Δ cultures containing the *GAL1*-HO plasmid were grown to exponential phase and treated with galactose to induce expression of the HO endonuclease in order to generate DSBs, after which cells were diluted, plated, and incubated at 30°C for several days. WT and *sfp1*Δ cells that were subjected to 2 hours of DSBs before being plated showed no significant difference in survival (Data not shown), prompting us to extend DSB treatment to 4 hours. Conducting 4-hour survival tests in WT revealed expected results, as judging simply by the generated error bars, cell survival clearly decreases after 4 hours of consistent DSBs (Figure 3). The results of *sfp1*Δ survival, on the other hand, generated more complicated results. While the average number of colony-forming units per milliliter (CFU/mL) had decreased, there is a significant overlap among standard

deviations between untreated and DSB-induced cells (Figure 3). Based on this observation alone, we could conclude that the absence of Sfp1p slightly enhances the DNA repair process, which contradicts the logic of our hypothesis. Considering the previous data that suggests that Sfp1p is not required for standard *HMO1* transcription, cellular levels of Hmo1p should remain the same in *sfp1Δ* as in WT. Taken altogether, the data suggests that Sfp1p may possibly be hindering DNA repair through other biochemical pathways; in particular, its role in ribosome biogenesis may be producing some effect that makes DSB repair more difficult.

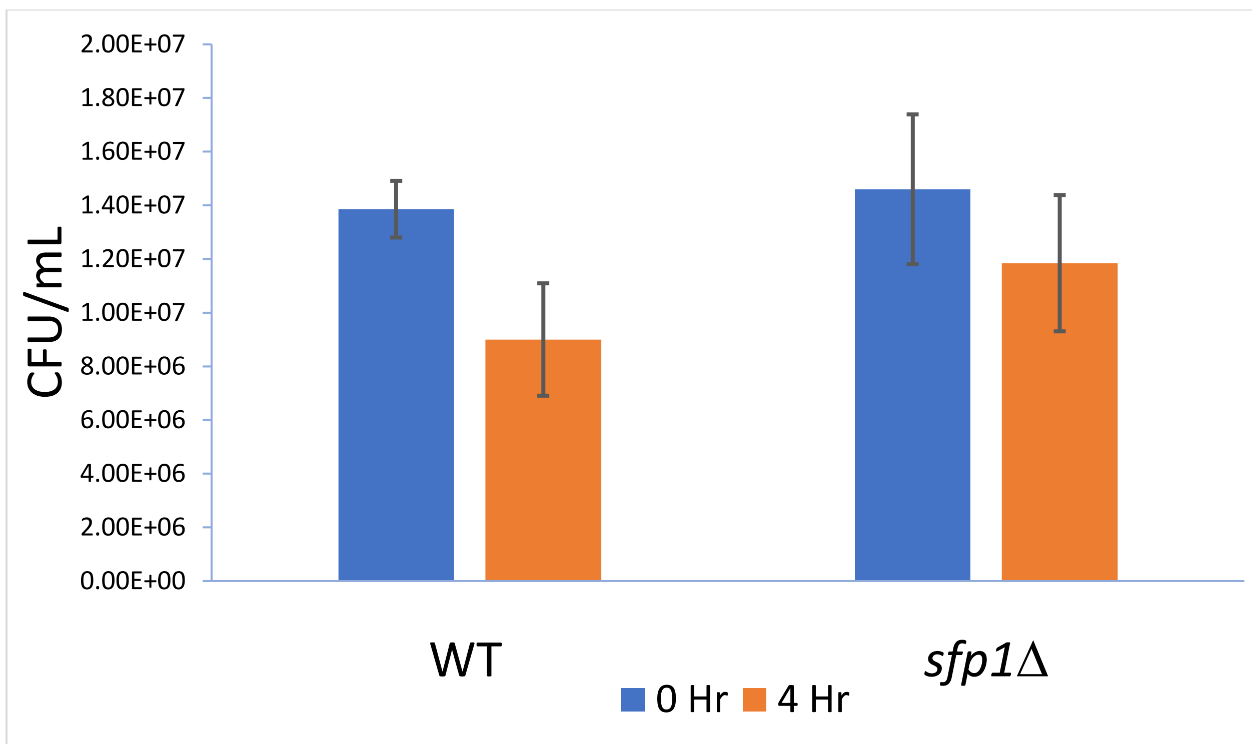

Figure 3. Sfp1p's Effects on DSB Survival. Bar graph representing the quantification of calculated CFU/mL of culture between WT and *sfp1Δ* cells before galactose treatment (0 Hr) and after 4 hours of DSB induction via galactose (4 Hr). Means and standard deviations are representative of 2 (WT) and 4 (*sfp1Δ*) replicates.

3.2. mTORC1 is Likely Inducing mRNA Degradation During Stress

As mentioned above, mTORC1 inhibition, whether through rapamycin-treatment or stress, leads to a reduction in *HMO1* transcript levels. Our lab has previously demonstrated an eviction of Pol II throughout the *HMO1* gene shortly after inducing mTORC1 inhibition, initially providing a possible explanation for the transcript decrease. However, preliminary data later suggested that the same Pol II eviction occurs in *tor1*Δ cells, where *HMO1* transcripts remain high, suggesting that the decrease in transcript levels is not simply the result of the removal of Pol II (Figure 4A). In order to verify this finding, multiple replicates of ChIP were performed using an anti-Pol II antibody to measure occupancy along the *HMO1* ORF in a *tor1*Δ strain. As expected, Pol II-binding was attenuated over time, with high significance ($p<0.05$) at the 60-minute mark (Figure 4B and 4C).

These observations highly suggest that the relative amounts of *HMO1* transcripts during DSBs is dependent on the presence or absence of mTORC1 rather than Pol II. One possible explanation is that mTORC1 is actively participating in transcript degradation. This is likely the case, as previous studies have demonstrated a similar function at other genes: Talarek, et al. demonstrated that mTORC1 uses the Rim15-Igo1/2-PP2A^{Cdc55} effector branch to manage the stability of transcripts that come from genes that use Msn2p/Msn4p and Gis1p as transcription factors (6), while Albig and Decker had shown that mTORC1-mediated degradation of certain mRNAs through deadenylation/reduced adenylation and decapping (7). Interestingly, mTORC1 has also been found to be a negative regulator of exosome release in mouse embryo fibroblasts, thus suggesting a possibility of exosome-enclosed mRNA regulation (29). The role of

mRNA degradation by mTORC1 may be justified by two possible theories. First, the cell may prefer to allocate the energy used during the gene translation process to that of DNA repair. Second, the cell realizes that high levels of Hmo1p would hinder the DNA repair process, and so it degrades its own *HMO1* transcripts to prevent an accumulation of Hmo1p within the cell (2,3,8; Figure 3).

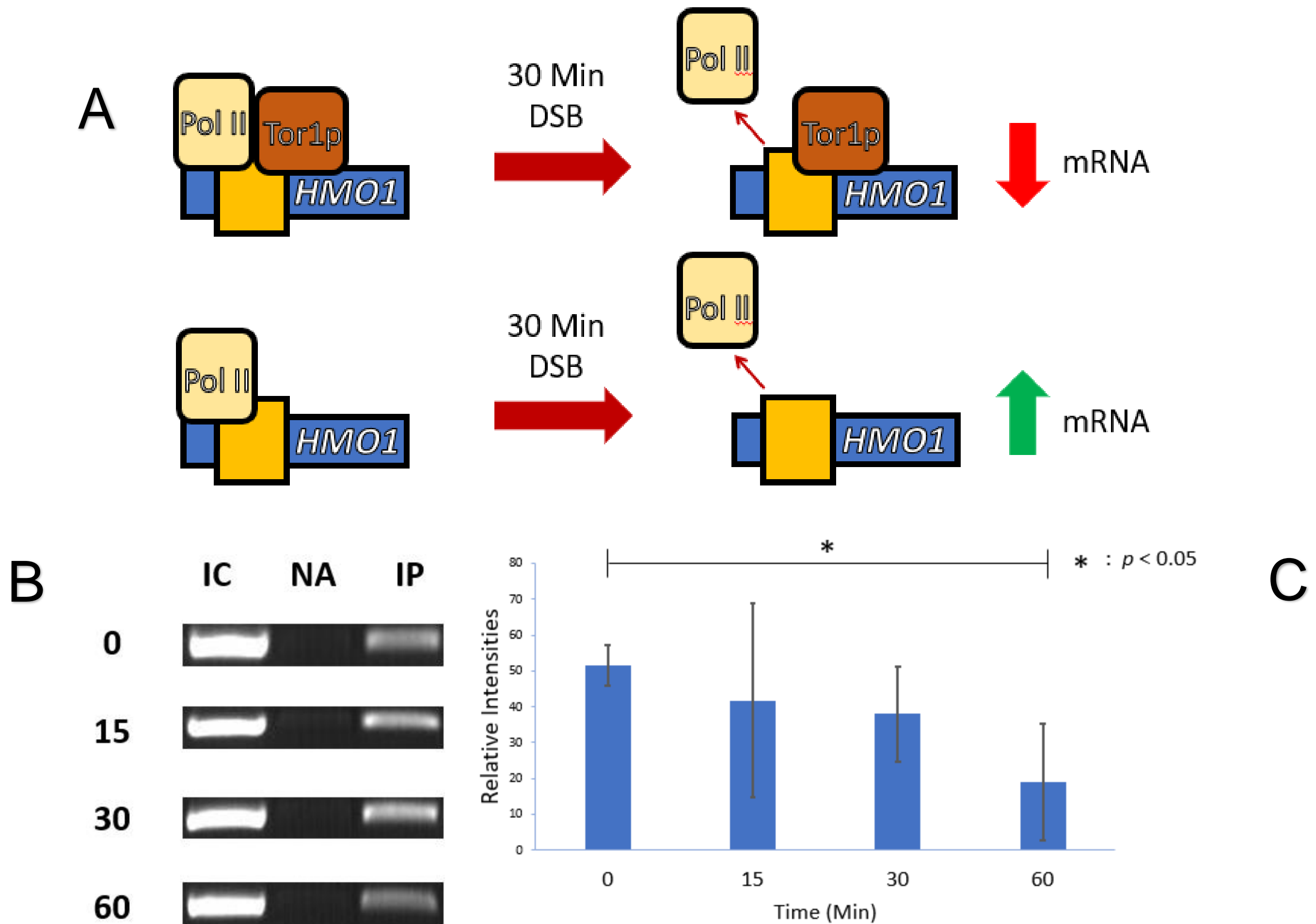

Figure 4. Pol II is Evicted from *HMO1* During DSBs. (A) Simplified visual model of the effects of DSBs on *HMO1* transcription in the presence (top) and absence (bottom) of mTORC1 signaling. (B) Gel images representing one replicate of ChIP performed at the *HMO1* promoter in *tor1*Δ using the Pol II antibody (IC = Input Control; NA = No Antibody Sample; IP = Antibody-Immunoprecipitated Sample). (C) Graphical data representing the relative ImageJ-quantified band intensities of standardized IP bands over the course of 60 minutes (Min). Means and standard deviations are representative of 3 (0, 15, and 30 Min) and 2 (60 Min) replicates. $p < 0.05$ determined by Student's t-test.

CHAPTER 4. CONCLUSION AND FUTURE DIRECTIONS

Hmo1p is an important component of both gene transcription and chromatin stability, and our experiments here further emphasize this. First, we show how the transcription factor Sfp1p is somehow involved in the biochemical pathway leading to *HMO1* regulation. More specifically, it is likely a factor in relaying the mTORC1 stress signal to the promoter. As our wide range of OD_{600} readings seems to have been the cause of the wide error bars around the *sfp1*Δ means (Figures 2A and 2C), redoing the experiment with narrower cell concentration limits is advised in order to determine Sfp1p's precise role in *HMO1* regulation. In addition, its presence may be a hinderance to DSB repair, although the means by which it is are unclear. Considering its role in binding to RP promoters as a means of activation, it is possible that Sfp1p could be doing the same thing at the *HMO1* promoter; therefore, we propose generating a FLAG-tagged Sfp1p strain in order to determine this phenomenon via ChIP. Furthermore, Western Blotting can be used to test the previously-stated hypothesis that there should be no difference in cellular Hmo1p concentrations between WT and *sfp1*Δ strains. In addition, predicted binding sites within the *HMO1* promoter (5) suggest the possibility of Reb1p being involved in transcription regulation. In particular, Reb1p is evicted from the *HMO1* gene during an overaccumulations of Hmo1p (5), which generates a negative-feedback response on its own promoter (5,14), pointing to the possibility of Reb1p being a transcriptional activator.

Second, we demonstrated that Pol II is evicted from the *HMO1* gene during stress regardless of whether Tor1p is present. Since we used only ORF-spanning primers, it is suggested that this experiment be redone using primers spanning the *HMO1* promoter

region in order to validate that Pol II is either being evicted at the transcription preinitiation complex or failing to reach the promoter altogether. Considering that *HMO1* transcript levels are significantly reduced during DSB only when Tor1p is present, we propose that mTORC1 may be playing a role in *HMO1* transcript degradation during stress. In order to test this hypothesis, we suggest using actinomycin D to halt the transcription process in both WT and *tor1*Δ cells. Actinomycin D is a commonly used transcriptional inhibitor that stabilizes the covalent bonds formed between topoisomerase I and DNA by intercalating into GC-rich sequences. As a result, Pol II will be stopped during the mRNA synthesis process, ultimately leading to transcriptional failure (15). After collecting a 0-hour control and adding actinomycin D, transcripts can be extracted and quantified in order to determine the difference in mRNA half-lives between the two strains. Previous studies have established how mTORC1's roles may go beyond signal transduction, and that all of these activities interplay to keep cellular metabolism in check. These findings may therefore have some medical significance, as faulty mTORC1 activity in mammals have been shown to be involved in diseases, particularly cancer.

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VITA

Mathew Damien Smith grew up in the small town of Hilmar, California and attended elementary, junior high, high school, and college in the neighboring city of Turlock. During his career at California State University, Stanislaus, he took part in faculty research projects, one being at Keck Graduate Institute in Claremont, California, as well as extracurricular activities that eventually granted him leadership positions. Eventually, he took an internship at Sensient Dehydrated Flavors, LLC. He was also a part of the founding of "Warriors Giving Back", a philanthropic program at CSU Stanislaus, and partook in various volunteer opportunities. In 2015, he received his Bachelor of Science in Biological Sciences with a concentration in Molecular/Microbial Biology and a minor in Chemistry. In 2017, he joined Dr. Anne Grove's lab at Louisiana State University to study HMGB proteins and the effects of stress in yeast gene regulation. He anticipates graduating from LSU with a Master of Science in Biological Sciences in 2020.