STRUCTURE/FUNCTION ANALYSIS OF THE HIF1 HISTONE CHAPERONE IN *SACCHAROMYCES CEREVISIAE*

by

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Abstract

Structure/Function Analysis of the Hif1 Histone Chaperone in *Saccharomyces cerevisiae*

Nora Saud Dannah, Ryerson University, 2014, Master of Science in Molecular Science

Understanding the regulation of chromatin structure is a vital aspect of molecular biology regarding its influences on biological processes such as DNA replication, transcription (gene expression), DNA repair, chromosome segregation and recombination. In the budding yeast *Saccharomyces cerevisiae*, a histone chaperone called Hif1 has been found in the nuclei as having a functional role in chromatin assembly. Hif1 is a homolog of the human protein NASP that is involved in the maintenance of genome stability. Previously, Hif1 has been shown to physically interact with Hat1, Hat2 and H3/H4 to form the NuB4 complex directly involved in chromatin assembly. A molecular genetic approach was conducted to determine which domain of Hif1 is involved in the interaction with the HAT1 complex.

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List of Abbreviations

AP	Affinity purification
Acd	Acidic region
ACF	ATP-utilizing chromatin remodeling factors
ADH1	alcohol dehydrogenase 1
APS	Ammonium persulfate
Asf1	Anti-silencing function 1
BSA	Bovine serum albumin
bp	Base pair
B.P.	Basic patch
BLAST	Basic Local Alignment Search Tool
C-	Carboxy
CAF-1	Chromatin assembly factor 1
CBP	Calmodulin binding peptide
CCR	Cell cycle control region
CenH3	Centromeric H3
ChIP	Chromatin immunoprecipitation
Co-IP	Co-immunoprecipitation
Crn1	CoRoNin
ddH_2O	Double distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
GFP	Green fluorescent protein
HATs	Histone acetyltransferases
HAT-A	Histone acetyltransferase type A
HAT-B	Histone acetyltransferase type B
Hat1	Histone acetyltransferase 1
Hat2	Histone acetyltransferase 2

HC	Histone chaperones
Hif1	Hat1p-interacting factor 1
HIRA	Histone interaction protein A
HMG	High mobility group
HPC	Histone promoter control
HPR	Horseradish peroxidase
HU	Hydroxurea
H3	Histone H3
H4	Histone H4
IgG	Immunoglobin G
IP	Immunoprecipitation
K	Lysine
kb	Kilo base pair
kDa	Kilodalton
LiOAc	Lithium acetate
Lsm1	Like Sm 1
µ	Micro
MCS	multiple cloning site
MP	Mini-preparation (DNA)
mRNA	Messenger RNA
MW	Molecular weight
N-	Amino
NEG	Negatively regulated element
NLS	Nuclear localization sequence
NP-40	Nonidet P-40
ORF	Open reading frame
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PMSF	Phenylmethanesulfonylfluoride
PAGE	Polyacrylamide gel electrophoresis

PMTs	Post-translational modifications
RD	Replication dependent
RI	Replication independent
RNA	Ribonucleic acid
RT	Room temperature
rpm	Rotations per minute
Rtt106	Regulator of ty1 transposition 6
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate poly-acrylamide gel electrophoresis
SGD	*Saccharomyces* genome database
SL	Synthetic lethal
Spt2	Suppressor of ty's 2
Sro9	Suppressor of rho3
SS	Synthetic sick
TAP	Tandem affinity purification
TBE	Tris borate EDTA
TBS	Tris-buffered saline
TEMED	Tetramethylethylenediamine
TEV	Tobacco etch virus
TPR	Tetratricopeptide repeat
UAS	Upstream activating sequence
UTR	Untranslated region
UV	Ultraviolet
V/V	Volume/volume
WCE	Whole cell extract
WT	Wild-type
W/V	Weight/volume
YNB	Yeast nitrogen base
YPD	Yeast extracts peptone dextrose

Chapter 1: Introduction

1.1. Chromatin

Over the last 100 years, since the DNA was introduced to the world of science, scientists have been fascinated with its molecular structure and meaning. The DNA of eukaryotic cells is assembled into chromatin and ranges from 10 million to 100 billion base pairs (bps). The chromatin bound DNA is stored in the nucleus which is a small number of micrometers in diameter (Richmond, 2006). DNA packing into chromatin is critical for stability of the genome and for its ability to condense and be packaged into the nucleus. Many proteins participate in and facilitate chromatin assembly, including a set of small, basic proteins, named histones, and other nuclear proteins through not well-understood mechanisms (Luger and Hansen, 2005). The DNA material can be targeted by several cellular processes such as DNA replication, damage repair, recombination and gene transcription, must overcome the physical limitations posed by the condensed chromatin structure. Thus, it is evident that mechanisms capable of changing the levels of genome compaction regulate DNA accessibility (Luger and Hansen, 2005; Luger, 2006).

Chromatin packaging prevents access of DNA-binding proteins to DNA. DNA-binding proteins help initiate gene transcription by binding to a promoter, therefore modulation of chromatin structure can dictate what genes are expressed in the cell. Chromatin remodeling is the term used to refer to changes in chromatin structure. One mechanism of chromatin remodeling is the direct covalent modification of specific amino acids residues within histone proteins. Post-translational modifications (PMTs) of histones include acetylation, methylation, and ubiquitination (Turner, 2000). The highly systematic structure of chromatin can be in a coiled or

relaxed state along different regions of the chromosome, depending on what genes need to be transcribed and expressed. As a result, this organization is tightly regulated to ensure accurate cellular functioning and preserve the genome integrity (Campos and Reinberg, 2009).

1.2. Heterochromatin and Euchromatin

Through the usage of specific dyes, giemsa, that bind DNA, chromatin can be observed at the microscopic level in the interphase stage of the cell cycle as existing in two different forms: heterochromatin and euchromatin (Fransz et al., 2000; Fransz et al., 2003). Originally, the term heterochromatin was assigned to areas on chromatin that remained heavily stained with dye and is extremely concentrated throughout the cell cycle. However, with the growing knowledge of chromatin structure and function, other features of heterochromatin have been elucidated such as being transcriptionally inactive (Hennig, 1999; Fransz et al., 2003). In contrast to heterochromatin, euchromatin does not stain with these dyes and remains loosely packed during the interphase stage and contain genes which are being actively transcribed (Fransz et al., 2003).

Heterochromatin exists in two varieties either constitutive or facultative. Constitutive heterochromatin is extremely condensed and includes great amounts of frequent DNA sequences. Whereas facultative heterochromatin includes activated loci in certain stage and inhibited in others (Brown, 2002). Recently, research on heterochromatin has resulted into dramatic developments in understanding epigenetic regulation of gene expression (Lund et al., 2004; Bernstein and Allis, 2005). Nonetheless, the exact structural modifications and molecular interfaces of higher-order chromatin folding are intensely unclear (Grigoryev et al., 2006).

1.3. Levels of DNA Packaging

The fundamental repeating subunit of chromatin is the nucleosome in all eukaryotes (Figure 1). The nucleosome is the primary level of DNA packaging, condensing the length of the DNA molecule by a factor of five (Luger and Harsen, 2005). Linker histones, which hold the loci in between nucleosomes, enable the DNA to condense further into a fibrous structure with a 30 nm of diameter in such a way that is not currently fully understood (Luger, 2006). Euchromatin containing actively transcribed loci that is compacted at this level and can be targeted by several nuclear processes in this state of condensation (Hayes and Harsen, 2001). Domains comprising either intergenic repeat sequences or transcriptionally inert loci are more compressed into higher-order hierarchical structure with the involvement of additional nuclear proteins thereby forming heterochromatin (Woodcock, 2006).

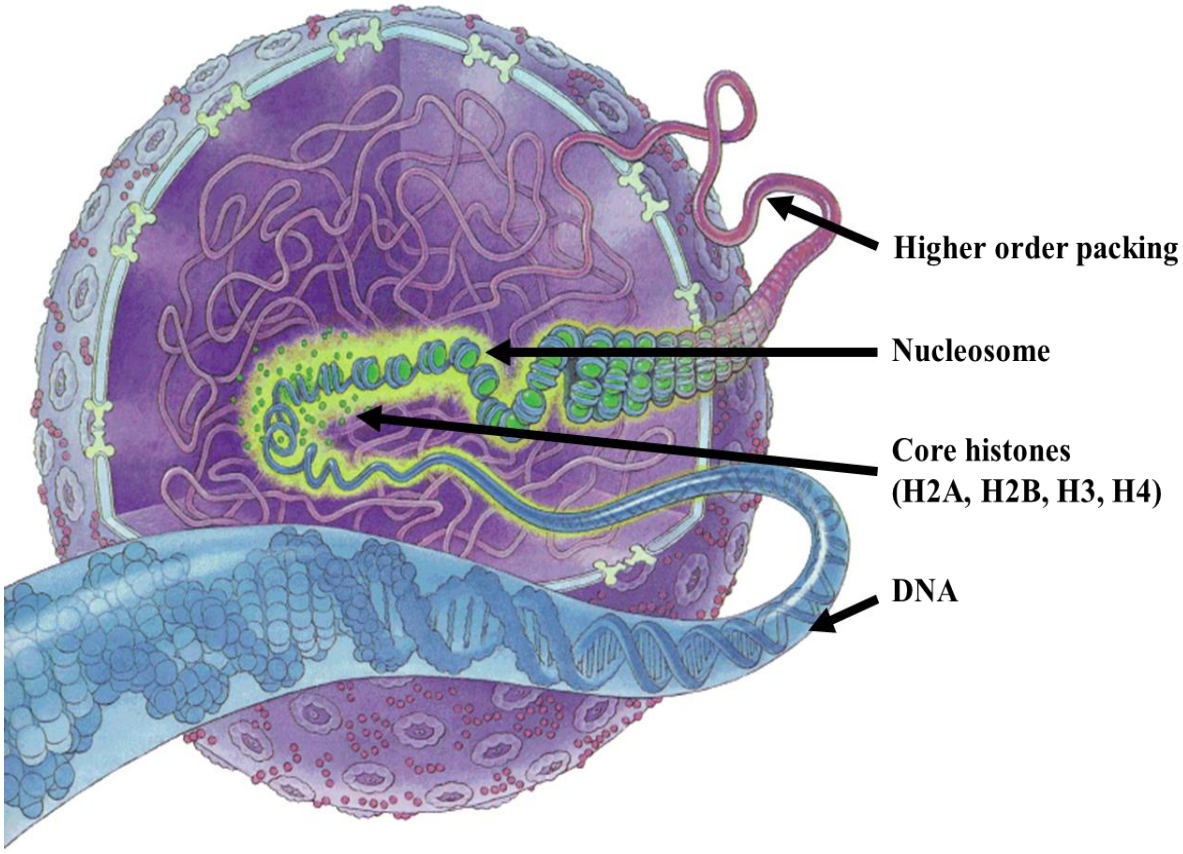


Figure 1: Chromatin is the Physiological Template of DNA.

The combination of DNA and histone proteins material is chromatin which is found in the nuclei of eukaryotic cells. Condensed chromatin makes up chromosomes which are composed of nucleosome units. The nucleosome consists of DNA wrapped around core histone proteins H2A, H2B, H3 and H4. Then the repeating units of nucleosomes give us the structure of chromatin that protects DNA (Adapted from Wolffe, 1995).

1.4. Histones

Histones are the most conserved proteins among eukaryotes which are involved in chromatin assembly. Histones H2A, H2B, H3, and H4 are the core histones, and H1 is linker histone that joins the nucleosome at the entry and exit spots of the DNA (Luger et al., 1997; Campos and Reinberg, 2009). The core histones are distinctive with a high percentage that basic amino acids (20% lysine/arginine residues) and are smaller than 20kDa in size. These histones have a structurally conserved motif near the C-terminus called the histone fold domain. This fold, which is about 70 amino acids in length, is required for non-specific DNA interactions to regulate histone-histone interactions (Luger et al., 1997; Arents et al., 1991). In addition to histone fold chromatin, each core histone possesses a highly charged N-terminal tail domain that protrudes out of the nucleosome surface, as well as a short C-terminal domain that differs in length between the different histone classes (Khorasanizadeh, 2004; Park and Luger, 2006).

1.5. The Nucleosome

The nucleosome is composed of approximately a 147 base pairs (bp) stretch of DNA coiled 1.67 times around the highly conserved and basic core histone proteins arranged as a hetero-octamer composed of a H3-H4 tetramer flanked by two H2A-H2B dimers (Figure 2) (Luger et al., 1997; Campos and Reinberg, 2009). Negatively charged DNA is able to bind non-specifically to the positively charged histone octamers, while H1, the extra-nucleosomal or linker histone, ties or condenses each “bead-on-a-string” nucleosomal unit further, ensuring tighter packaging arrangements (Campos and Reinberg, 2009). The length of linker DNA between nucleosome can vary from about 160 bp in yeast to further than 200 bp in higher organisms (Hayes and Harsen, 2001; Luger, 2006).

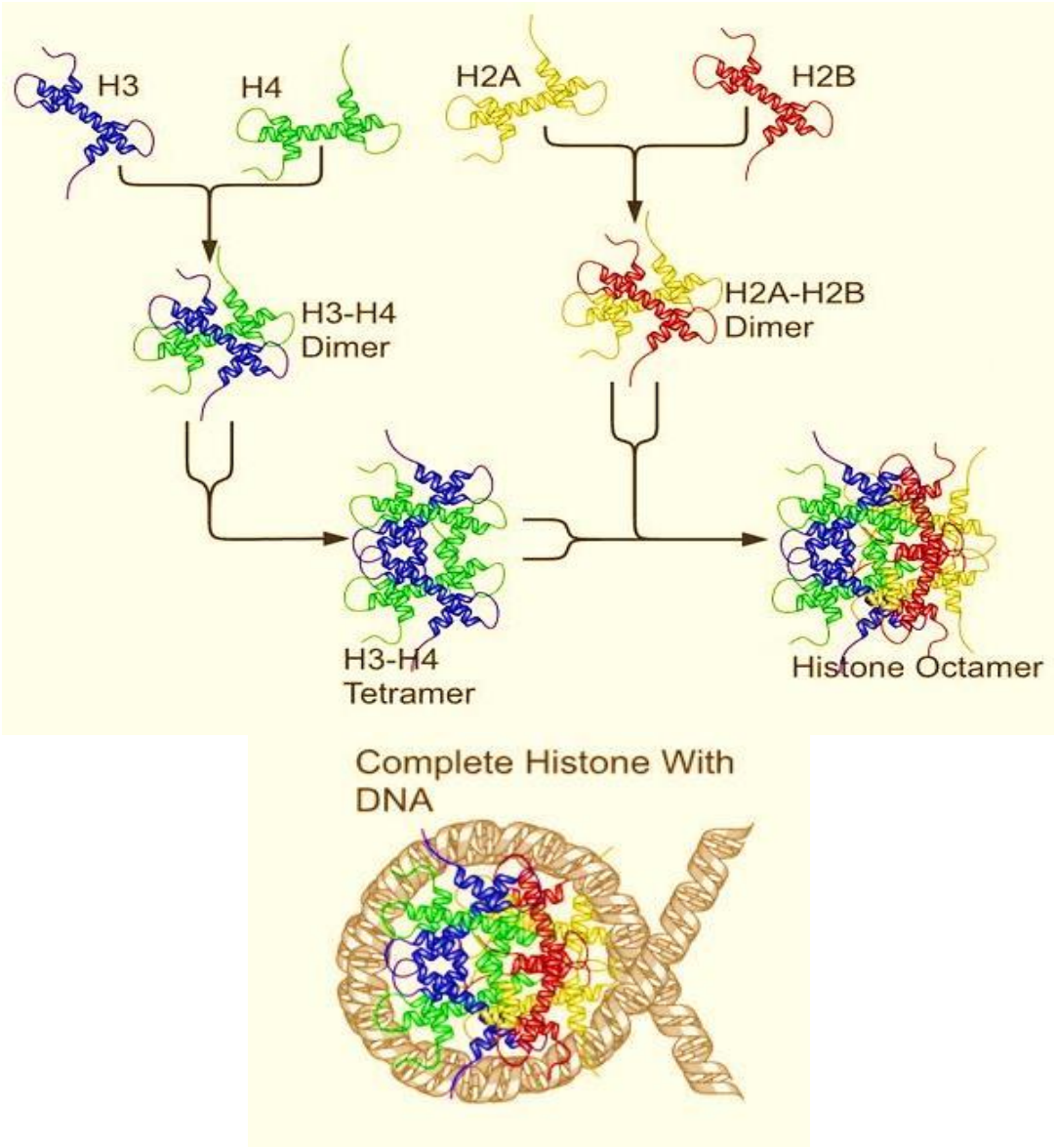


Figure 2: Nucleosome assembly.

Histones H2A-H2B form a dimer, whereas histones H3-H4 form a tetramer. The H2A/H2B dimer associates with the H3/H4 tetramer as a result of the interactions between histone H4 and histone H2B. The histone octamer is made up by a central H3/H4 tetramer flanked by two H2A/H2B dimers.

Because of the highly basic charge of the four core histones, the histone octamer is stable only in the presence of the negatively charged DNA (Adapted from Wikipedia).

The association between histone proteins and the DNA backbone involves more than 120 non-covalent chemical interactions. Despite the fact that the actual DNA sequence wrapped around a given histone octamer can vary substantially, no clear changes in the complete nucleosome structure as a result of these variations have been found. Thus the actual sequence of DNA does not interfere within nucleosomal packaging. It is thought that water molecules participate in the uniform structure of the nucleosome via adjusting histone surfaces to the structural variations in the DNA (Luger, 2006; Davey et al., 2002). Nonetheless, histones, as the basic building blocks of the nucleosome, have a significant role in the overall nucleosome structure.

Post-translational modifications on histone residues modify nucleosome structure potentially through generation of small structural variations or through the recruitment of different chromatin-modifying factors, thereby altering DNA accessibility and higher-order structure. In response to dynamic cellular signaling, chromatin undergoes dramatic organizational changes where regions of the chromosome become relaxed or condensed, depending on working requirements of the cells, in response to chemical signals. Since histone modifications can regulate chromatin dynamics, they are essential to genomic integrity (Kouzarides, 2007).

1.6. Histone Variants

Most eukaryotic organisms have evolved histone variants whose gene sequences are non-allelic to genes encoding the canonical core histones. In fact, canonical core histone genes show high sequence similarity, have multiple copies of the genes present in the genome, are most expressed during the S-phase, a phase of the cell cycle when DNA is replicated, and are

incorporated into chromatin throughout the genome. On the other hand, histone variants generally exist only as single-copy genes, and are expressed at low levels throughout the entire cell cycle (Kamakaka and Biggins, 2005).

Following being transferred into the nucleus, histone variants are incorporated into specific functional domains of chromatin. For instance, the two main variants of histone H3 are centromeric H3 (CenH3) and H3.3 (Ahmad and Henikoff, 2002 and Malik and Henikoff, 2003). Besides H3, H2A has the largest number of variants, including the evolutionary conserved histone variant H2A.Z (Jackson et al., 1996). In general, most histone variants are involved in regulating chromatin dynamics and structure (Kamakaka and Biggins, 2005). Importantly, variants of histones H2A and H3 appear to be deeply involved in the arrangement of alternative chromatin structure and genome stability (Kamakaka and Biggins, 2005, and Henikoff et al., 2004).

1.7. Histone Chaperones (HC)

Histone chaperones (HC) are present during assembly and disassembly of nucleosomes when histones need to be replaced or changed or *de novo* as in DNA replication. These mechanisms are possible in part due to the acidity of the chaperone proteins. The chaperone binds to the basic histone proteins and as a result, prevents the histone from participating in other interactions with DNA or proteins (Das et al., 2010; De Koning et al., 2007; Park and Luger, 2008). Chromatin replication occurs during the S-phase of the cell cycle. Previously existing nucleosomes can be transferred randomly to daughter strands following the passing of replication fork machinery, whereas the remaining 50% of nucleosomes must be synthesized in a reaction known as *de novo* nucleosome assembly from the newly synthesized histones combined with

nuclear-imported histones (Verreault, 2000). To prevent this, a variety of histone chaperones that are negatively charged have evolved to assist in DNA packaging. These chaperones interact with histones and mask the basic charges to promote their deposition onto DNA during nucleosome assembly. Significantly, they are not present in the final product (e.g. chromatin) (Polo and Almouzni, 2006).

During the S-phase, nucleosome assembly involves importing a bulk of newly synthesized core histones into the nucleus and then depositing them onto newly replicated DNA, replication dependent. Core histones can also be assembled into chromatin through transcription and DNA damage repair, replication independent (Verreault, 2000). The assembly of histones into mature nucleosomes is a stepwise process. With the assist of histone chaperones a sub-nucleosomal particle is formed first by formation of a $(H3\text{-}H4)_2$ tetramer which then is deposited onto naked DNA. Subsequently, two dimers of H2A-H2B are deposited onto the $(H3\text{-}H4)_2$ tetramer via histone chaperones as well. This new nucleosome is immature and not regularly organized alongside with the template. As a result, chromatin remodeling is required in order to form a regular nucleosome assortment and functional regions. This process happens in the presence of ATP-utilizing chromatin remodeling factors (ACF) (Tyler, 2002; Nakagawa et al., 2001).

1.8. Histones and their classes

There are two general classes of histones: replication dependent (RD) histones and replication independent (RI) histones. The expression of each of these histone types depends on the cell cycle stage. Genes encoding RD histones, which include core histones H2A, H2B, H3, and H4 and linker histone (H1), are expressed precisely prior to and throughout the replication of

DNA (S-Phase). In contrast, RI histones are expressed at fairly low levels during all stages of the cell cycle. RI histones include specialized histone variants of H2A and H3: H2A.X and H2A.Z, and H3.3, respectively. H2A.Z and H3.3 have a role within transcription while H2A.X is involved in genome stability (Billon and Côté, 2012; Fillingham et al., 2006).

RD core histone genes are expressed during the S-phase of the cell cycle. During this time period, the eukaryotic genome doubles in size and thus requires a subsequent doubling of histones in order to package the new DNA into chromatin. Experimentally this has been observed as a sudden elevated expression of core histones. To ensure sufficient quantities of histones are present in the cell, the expression of core histones begins during late G1 phase, just prior to S-phase in yeast (Hereford et al., 1981). Indeed, an insufficient amount of histones during replication is lethal to the *Saccharomyces cerevisiae* cell (Kim et al., 1988). On the other hand, an excessive abundance of free and soluble RD histones can lead to decreased fitness of the cell resulting in DNA that is more vulnerable to DNA damaging agents (Gunjan and Verreault, 2003). As a result, the synthesis of the RD core histone is required massively in S-phase, and then must be suppressed when the cell is not replicating its DNA, such as during G1 and G2 phases or during DNA damage repair stages. RD core histone expression must therefore be tightly regulated at the transcriptional and post-transcriptional levels (Gunjan and Verreault, 2003).

1.9. Modifications of Histones

The N-terminal tails of histones, which extend beyond the nucleosomal core, contain sites for several covalent post-translational modifications. These modifications can include

methylation on lysines and arginines, acetylation on lysines, as well as phosphorylation on serines, tyrosines and threonines (Peterson and Laniel, 2004).

Transcriptional regulation is tightly controlled by chromosome structure. ATP-dependent chromosome remodeling and post-transcriptional modification of histones are two distinct mechanisms by which structural modifications of nucleosome structure occur (Kornberg and Lorch, 1999; Strahl and Allis, 2000). The histone modification mechanism plays a critical role in the regulation of chromatin structure and function. Therefore, such studies have been of importance in understanding the regulation of gene expression.

Histone PMTs modifications occur at the N-terminal tail residues of the core histone proteins, and can control the chromatin accessibility and gene activity. Histones can be modified in several conditions based on the histone modifying enzymes involved and the specific residues being modified. These enzymatic regulators are coordinated by transcription factors and chromosomal proteins. Functional groups such as acetyl groups can be added or removed to control chromatin structure and subsequently the transcription of certain genes (Kornberg and Lorch, 1999; Strahl and Allis, 2000).

The addition of acetyl groups to histones is called histone acetylation, which is the most studied form of histone modification. Histone acetylation occurs primarily on lysine residues of the histone tails. The addition of the negatively charged acetyl group to positively charged lysine leads to a decreased affinity of the histone for DNA (Hongs et al., 1993). Consequently, the conformation of the nucleosome changes (Norton et al., 1989) and the accessibility of transcriptional regulatory proteins is increased (Lee et al., 1993; Vettese-dadey et al., 1996). Therefore, acetylated histones mostly correlate with the active chromatin during transcription

(Allfrey et al., 1964; Hebbes and Thorne, 1988; Pogo et al., 1966; Sealy and Chalkley, 1978; Vidali, 2000).

1.10. Histone Acetyltransferases (HATs)

Some chromatin remodeling enzymes, including histone modifying enzymes, are also transcriptional co-factors (Kuo and Allis, 1998), altering histone residues by adding or removing functional groups and thereby modulating chromatin structure and play a significant role during gene transcription (Campos and Reinberg, 2009). More specifically, the addition or removal of a functional group can modulate chromatin structure in such a way to facilitate or hinder the ability of the transcriptional machinery to bind to DNA. Recruitment of these enzymes is sometimes coordinated by transcription activators and co-activators (Kornberg and Lorch, 1999; Strahl and Allis, 2000).

Histone acetyl-transferases (HATs) are histone modifying enzyme that covalently attach an acetyl group to the positively charged epsilon-amino group of certain lysines on core histones (Campos and Reinberg, 2009). The epsilon-amino group is so-called because it is attached to the fifth carbon in the carbon chain starting from the carbon attached to the carboxyl group (C=OOH) of the amino acid.

The mechanistic link between HATs and transcriptionally activated chromatin was first discovered in the ciliate *Tetrahymena thermophila* through the identification of the gene encoding p55, a nuclear HAT from the ciliate *Tetrahymena thermophila* (Brownell et al., 1996). Experiments examining the activity of the activity of p55's yeast homolog (Gcn5) showed that HATs are necessary due to their ability to act as a co-activator, a protein that increases the gene expression, during transcription (Candau et al., 1997; Kuo, Brownell, and Sobel, 1996).

Additional research using biochemical analysis of proteins implicated in transcriptional activation identified a significant number of HAT enzymes (Roth et al., 2001).

HAT enzymes are generally grouped into two classes based on their cellular location and exist in two distinct environments, cytoplasmic and nuclear, where they acetylate histones at different stages of the cell cycle. Cytoplasmic HAT-Bs acetylate histones before they are localized to the nucleus and assembled onto chromatin, whereas the nuclear HAT-As acetylate nucleosomal histones, in conjunction with transcription and other DNA-dependent processes (Allfrey et al., 1964).

1.11. Histone Assembly onto DNA

Previous work showed that the assembly of the nucleosomal complex occurs in an ordered fashion. Initially, either old or new histone H3-H4 tetramers are deposited onto the DNA, following by the incorporation of two H2A-H2B dimers (Smith and Stillman, 1991). Further research has shown non-nucleosomal intermediates with tetrasomes, a combination of H3-H4 tetramers and DNA, are attached to the DNA under incubation with histone chaperones (Torigoe et al., 2011).

Histone chaperones are the key proteins that aid in nucleosome formation by arranging chromatin assembly and disassembly through the regulation of binding to histones (Eitoku et al., 2008). Specifically, Chromatin Assembly Factor-1 (CAF-1), a histone chaperone, will mediate the deposition of the core histone H3 and H4 onto DNA during replication-coupled nucleosome assembly via physical interaction with the PCNA (Proliferating Cell Nuclear Antigen) sliding clamp that is found at the replication fork (Eitoku et al., 2008). The histone chaperones Histone Interacting Protein A (HIRA) and Daxx, an H3.3 specific histone chaperone, will incorporate the

histone variant H3.3 accompanied by H4 onto DNA during replication-independent nucleosome assembly (Goldberg et al., 2011; Tagami et al., 2004).

The most well studied histone chaperones are Anti-Silencing Function-1 (Asf1), CAF1, and HIR complex in *S. cerevisiae*. Asf1 is a H3/H4 chaperone involved in Replication Dependent and Replication Independent chromatin assembly in conjunction with CAF1 and HIRA (Figure 3) (Mousson et al., 2007). Importantly, Asf1 is not exclusive to this function as chaperones can have multiple and varying functions. Asf1 has also been observed in (RD) and (RI) nucleosome assembly, histone acetylation, histone exchange, control of transcription and chromatin silencing (Adkins et al., 2004; Daganzo et al., 2003; Green et al., 2005, 2010; Recht et al., 2006; Sharp et al., 2001).

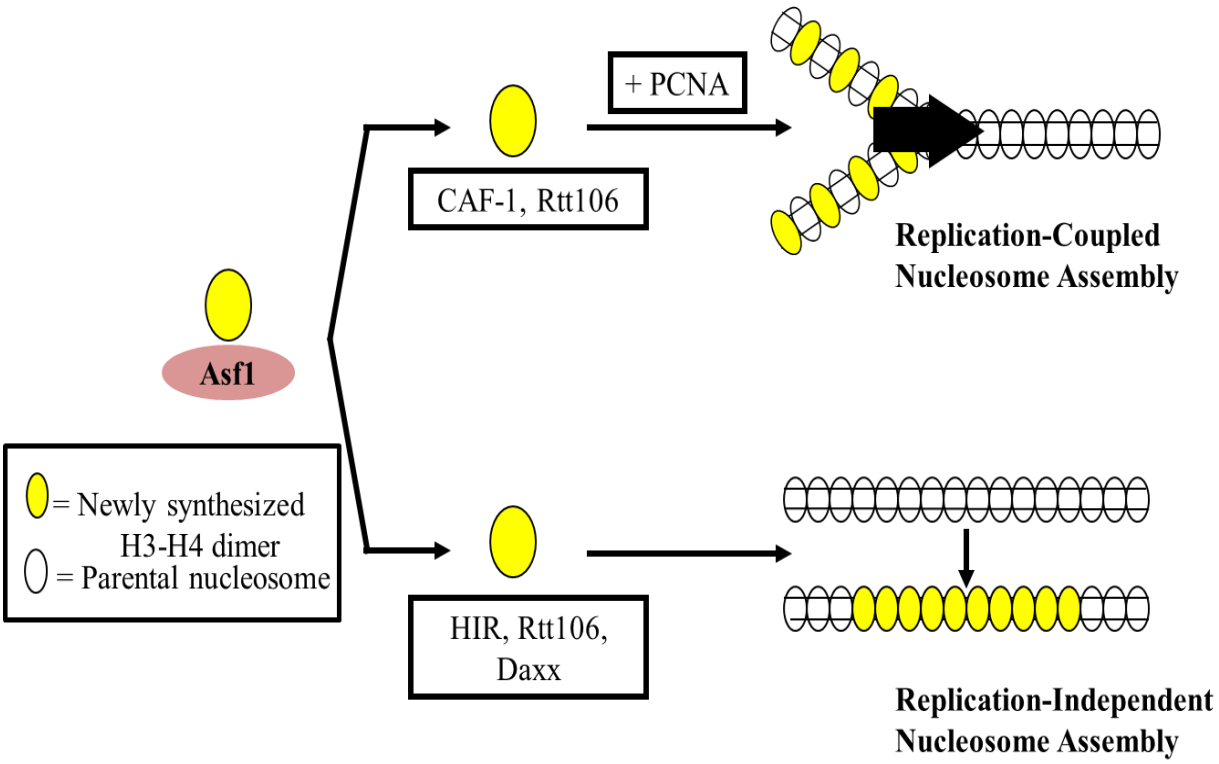


Figure 3: Nucleosome assembly of the newly synthesized H3–H4.

Replication dependent (RD) nucleosome assembly and replication independent (RI) nucleosome assembly pathways are shown. Histone chaperone Asf1 binds a H3–H4 dimer, which will be transferred to histone chaperones that are involved in RD or RI nucleosome assembly. Different sets of proteins are involved within the two different pathways (Adapted from Fillingham, unpublished).

1.12. Histone Acetyltransferase type B (HAT-B)

H3 and H4 histone binding factors are synthesized in the cytoplasm of yeast cells. The HAT-B histone acetyltransferase complex includes a chaperone called Histone Acetyl-Transferase 2 (Hat2), and a Histone Acetyl-Transferase 1 (Hat1). Hat1 and Hat2 together form Hat1/Hat2 complex (HAT-B complex) that functions to acetylate soluble histone H4 on lysine 5 and lysine 12 (Benson et al., 2007; Chang et al., 1997; Parthun, 2007; Poveda and Sendra, 2008). Following the acylation of H4, newly acetylated H3 interact with H4 and the Hat1/Hat2 complex to form Hat1-complex in the cytoplasm (Parthun, 2007).

Hat1 only acts on soluble histone H4 and does not interact with chromatin-bound nucleosomal histones. This is because Hat1 belongs to a family of type B HATs that exist in the cytoplasm and only bind free histones. Studies show that the HAT-B complex carries histones from the cytoplasm to the nucleus mediated by the Kap123 protein (Blackwell et al., 2007).

In the nucleus, the histone chaperone and chromatin assembly factor Hif1 binds to the Hat1-complex to form the NuB4 complex (Figure 4). The NuB4 complex also binds other chaperones such as Asf1 although the function of this interation remains unclear (Campos et al., 2010; Fillingham et al., 2008). Whether the NuB4 complex deposits histones itself or transfers them to another histone chaperone is not known. Rtt109 is also a HAT that acetylates soluble histones, such as lysine 56 of H3 with the help of Asf1, before they are deposited (Das et al., 2010; De Koning et al., 2007). Asf1 then successfully deposits these histones at the replication fork with the aid of CAF1 and histone chaperone Rtt106 (Fillingham et al., 2009; Lambert et al., 2010; Sutton et al., 2001).

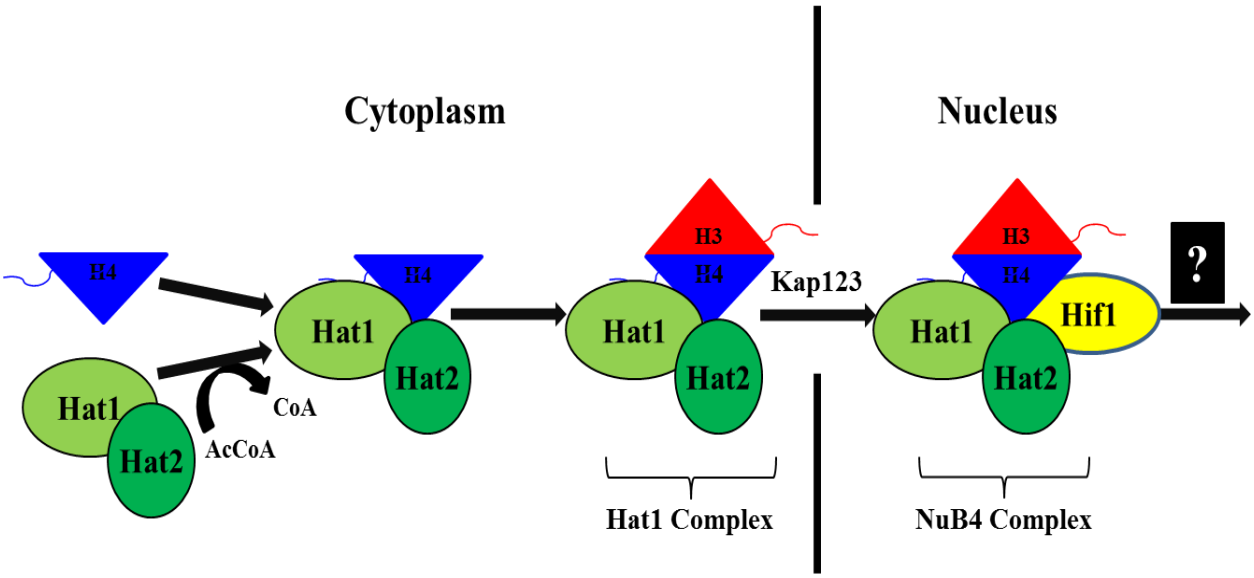


Figure 4: A proposed model of Hat1-complex function in the cytoplasm and the nucleus.

The mechanism begins with the association of Hat1-complex (Hat1/Hat2 complex) with the newly synthesized histone H4 in the cytoplasm, where histone H4 is acetylated on the lysine residues 5 and 12. Following this step, newly synthesized histone H3 forms a complex with acetylated histone H4 and the complex is then imported into the nucleus with the assistance of karyopherins (Kap123). Within the nucleus, Hat1-complex interacts with Hif1 to form NuB4 complex. The NuB4 complex then deposits the H3/H4 complex onto DNA.

1.12.1. Histone Acetyltransferase 1 (Hat1)

Member of type B histone acetyltransferase, Hat1 is an evolutionary conserved protein and has been identified as a member of the GNAT family of HATs. The Hat1 protein has been shown to interact with Asf1 protein in yeast (Fillingham et al., 2008). It is also a member of the HAT-1 and NuB4 complexes in the company of Hat2, which stimulates Hat1 catalytic activity, and Hif1, which is a chromatin assembly factor (Ai and Parthun, 2004; Poveda et al., 2004). The Hat1 homologs have been identified in maize, *Xenopus laevis*, yeast, chicken and humans, and *T.thermophila* (Eberharter et al., 1996; Parthun et al., 1996 and Ahmad et al., 2000; Shah and Fillingham, unpublished).

Even though Hat1 is a highly conserved protein, its expression is not necessary for maintaining yeast and vertebrate cell viability, just like its target lysines in histone H4 (H4K5/12) (Ai and Parthun, 2004; Barman et al., 2006). Furthermore, Hat1 was observed to be required for telomeric silencing, which requires catalytic enzyme activity and proper sub-cellular localization (Kelly et al., 2000; Parthun, 2007). Although Hif1 is non-essential protein, its deletion, as well as Hat2, results in a telomeric silencing defect (Poveda et al., 2004).

1.12.2. Hat1p-Interacting Factor 1 (Hif1)

NuB4 member, Hif1 has been found in the nucleus and was identified as a histone chaperone that specifically interacts with the acetylated H3 and H4 (Ai and Parthun, 2004). Hif1 is a relatively well conserved protein among other budding yeast (Poveda et al., 2004), and is functionally and evolutionarily relevant to the human NASP (Wang et al., 2011). A deletion in *NASP* can result in prostate cancer (Alekseev et al., 2011). The assembly of chromatin involves the Hif1 protein in yeast (Fillingham et al., 2008), and NASP human cells (Campos et al., 2010) which suggest that Hif1/NASP functions are greatly conserved among eukaryotes.

Beyond the clear fact that the Hif1 protein is a well-defined member of the NuB4 complex, it can also participate in the deposition of acetylated histones onto DNA (Ai and Parthun, 2004). Functionally, biochemical data suggest that Hif1 plays a role in chromatin assembly that is independent on its interactions with the Hat1-complex (Wang, 2011; Poveda et al., 2004).

1.13. Suppressor of Ty's 2 (Spt2)

In addition to histones, many non-histone proteins play an essential role in histone regulation chromatin assembly. In *S. cerevisiae*, Spt2 is one of the non-histone proteins that have

shown an indication of regulating chromatin structure during gene expression (Thebault et al., 2011). Spt2 was first discovered as a suppressor of transposition; however, its actual biological function is poorly understood. The Spt2 protein as a consequence of its initial identification as a transposon suppressor, though the mechanism by which it functions remains unknown. Transposons are sequences of DNA which can transfer and incorporate to different loci in the genome (Fontana, 2010), and were discovered by Barbara McClintock in 1950. They also are involved in the control of gene expression, and influence recombination rates and chromosomal rearrangements (Biemont, 2010).

Spt2 has two high mobility group-like domains that can bind DNA and is frequently found in proteins involved in chromatin structure and transcription (Nourani et al., 2006). High mobility group proteins (HMG) function in remodeling chromatin and regulating gene transcription (Zhang and Wang, 2010). Some HMG proteins are associated with common benign tumors (Bjork, 2002 and Dolde et al., 2002). For example, in human cells, the gene encoding one of the HMG proteins, HMGI-C, is overexpressed or rearranged via chromosomal translocations in benign mesenchymal tumors such as leiomyomas and lipomas (Hess, 1998).

Despite the presence of the HMG domain the mechanism by which Spt2 functions remains unclear. A large-scale protein-protein interaction analysis in *S. cerevisiae* by Krogen et al., (2006) suggested that Spt2 physically interacts with Hat2 and HIf1, members of NuB4. This thesis will show confirmation of this physical interaction.

1.14. Yeast as a Model Organism

The budding yeast *Saccharomyces cerevisiae* has been confirmed to be a suitable model for understanding the chromatin assembly and the regulation of gene expression (Karathia et al.,

2011). Yeast is considered one of the simplest eukaryotic organisms, but still shares many cellular processes with other animals, such as *Homo sapiens* (Baudin et al., 1993). Also, although it is a single cell eukaryotic organism, its cell division occurs in a manner similar to that of a mammal. The genome size of *S. cerevisiae* is approximately 12 million base pairs and it contains approximately 6600 genes compared to human genome that includes around 25,000-30,000 genes (Goffeau et al., 1996). Yeast growth is fast in comparison to other organisms, and is inexpensive to grow in laboratory. Exact gene replacement by homologous recombination is easily achieved and molecular genetic reagents are readily available for genetic modifications (e.g. gene deletion) (Baudin et al., 1993).

1.15. Histone Gene Structure in Yeast

In yeast, there are four major core histones which are encoded by two genes each organized in four separate gene loci, each consisting of two histone genes arranged in a head to tail manner, H2A/H2B encoded by *HTA1-HTB1* and *HTA2-HTB2*, respectively, and H3/H4 core histones are encoded by *HHT1-HHF1* and *HHT2-HHF2*, respectively (Osley MA, 1991).

The RI histone genes also present are H2A.Z encoded by *HTZ.1*, centromeric H3 encoded by CSE4, and H1 encoded by *HHO1*. The core histone genes are transcribed from a central promoter, which copy two histones genes within each of the four loci (Osley et al., 1986). The *HHT-HHF* locus is necessary for cell survival, suggesting that H3 and H4 are essential for life (Smith and Stirling 1988). Previously, *HTA1-HTB1* and *HTA2-HTB2* were thought to be identical but research has shown that deletion of *HTA1-HTB1* produces a significant growth phenotype whereas *HTA2-HTB2* is negligible (Norris and Osley, 1987). The *CSE4* gene is essential for kinetochore formation (Wysocki et al., 1999), the deletion of *HTZ1* gene caused the

mutant to grow slowly (Jackson and Gorovsky, 2000), and the deletion of *HHO1* produced no growth phenotype (Patterton, 1998). Therefore, all core histones and centromeric H3 are necessary for life whereas *H2AZ* and *HHO1* are not.

All eight core histone genes are expressed at low levels outside of S-phase except in late G1. Histone gene transcripts accumulate before mid-S-phase and decline until S-phase exit and enter into G2- and M-phases (Hereford et al., 1981). As discussed above, it is essential for the cell to have a regulatory system to repress histone gene expression outside of S-phase.

1.16. Regulation of histone gene expression

The regulation of histone gene expression occurs by several mechanisms. Virtually every stage of histone gene expression is regulated, from transcription to post-transcriptional levels, to processing of mRNA, and to the post-translational modification of proteins (Kurat et al., 2013). The following is a list of regulatory mechanisms where histone gene expression is regulated considering that the most comprehensively utilized stage is transcription initiation.

1.16.1. Transcriptional level

The transcriptional regulation of RD histone genes can be regulated by promoter sequences described as *cis*-acting elements. The transcriptional regulation of RD histone genes depend on two classes of *cis*-acting sequences both of which are found in the yeast *HTA1-HTB1* locus (Osley et al., 1986).

The first class of *cis*-acting sequence is the histone upstream activating sequence (UAS) and it functions in during S-phase specific transcription activation. In *S. cerevisiae,* the UASs have been also found in the non-coding DNA sequences of each one of the four array of the RD

core histone genes (Breeden, 1988), and are sufficient to provide cell cycle expression regulation of a constitutive expressed reporter gene (Osley et al., 1986).

The second class of *cis*-acting sequence is known as CCR (cell cycle control region). Interestingly, the deletion of CCR (also known to as NEG, negatively regulated element) leads to constitutive reporter gene expression as well as a loss of cell cycle control of *HTA1-LacZ* expression (Lycan et al., 1987). Therefore, deletion of the CCR/NEG indicates that CCR negatively affects transcription of RD histone genes (Osley et al., 1986).

In *S. cerevisiae,* four proteins known as Hir1, Hir2, Hir3 (histone transcription regulators) and histone promoter control (HPC) were originally found through genetic screens (Osley and Lycan, 1987; Sherwood et al., 1993). HIR proteins are conserved among various fugal species including the budding yeast *Saccharomyces cerevisiae*. The four HIR proteins form the HIR complex. The human HIRA histone chaperone protein mentioned earlier is a homolog of a fusion of yeast Hir1 and Hir2. HIR complex binds chromatin at the NEG site where it assembles chromatin (H3/H4) onto the promoter of *HAT1-HTB1*. That means NEG acts through the HIR complex to repress histone transcription by assembling the promoter into repressive chromatin. This is then antagonized in S-phase for activation by proteins such as UAS-binding Spt10 (Kurat et al., 2013). The absence of genes encoding one of the four HIR proteins resulted in losing the ability to repress of histone gene pairs outside of S-phase (Osley and Lycan, 1987).

HIR-mediated repression of RD core histone transcription involves additional H3-H4 histone chaperones such as Asf1 and Rtt106 (regulator of ty1 transposition 6) (Osley and Lcayn, 1987; Osley et al., 1986 and Fillingham et al., 2009). Yeast two-hybrid assays and affinity purification experiments showed that Asf1 interacts with the HIR complex through physical

interaction with the Hir1 protein (Green et al., 2005, Sutton et al., 2001). This interaction appears to be evolutionary conserved and the deletion of the *ASF1* gene revealed a similar phenotype to those seen in HIR subunit deletions with respect to RD histone expression (Sutton et al., 2001 and Fillingham et al., 2009). The *RTT106* gene, in silencing, is linked to *ASF1* and *HIR1*; nevertheless it is independent of the chromatin assembly factor CAF-1 (Huang et al., 2005). The deletion of *RTT106* decreased histone density in the regulatory region of NEG-dependent gene pairs (Fillingham et al., 2009).

1.16.2. Post-transcriptional level

In the cell, major exoribonucleic enzymes activities regulate the levels of core histone mRNA accumulation. In *S. cerevisiae,* two significant pathways are involved in the regulation of mRNA degradation either the 3'-5' or 5'-3' pathways. The Xrn1 exoribonuclease plays a critical role in degradation of the mRNA from the 5' – 3' pathway following an initial de-capping of the mRNA by (Dcp1/Dcp2) (Larimer et al., 1992; Beelman et al., 1996). While in the 3'- 5' pathway, the Exosome complex can degrade mRNA (Mitchell et al., 1997). In fact, the primary step in mRNA degradation is the reduction of the polyadenylated tail of the mRNA, or deadenylation which ultimately produces an oligoadenylated mRNA. The oligoadenylated mRNA can then be degradaed via any of two riboexonucleolytic pathways (e.g. Xrn1 or Exosome). The Lsm1-7-Pat1 complex promotes de-capping by Dcp1/Dcp2 and subsequent degradation by Xrn1 by recognizing oligoadenylated mRNAs (Herrero and Moreno, 2011).

Because of this, the Lsm1 (Like Sm) protein plays a crucial role to promote the genome stability in *S. cerevisiae*. The lack of Lsm1 in budding yeast cells leads to a defect in recovery from replication-fork arrest and demonstrates DNA damage sensitivity (Herrero and Moreno,

2011). The HU-sensitive phenotype of an *lsm1Δ* strain can be suppressed by lowering histone dosage through deletion of one of the two respective H2A–H2B or H3–H4 encoding gene pairs (Herrero and Moreno, 2011). This hypersensitivity to DNA damaging drugs, such as hydroxurea, in *Δlsm1* mutant cells highlights the relationship between regulating histone mRNA degradation and genomic stability (Herrero and Moreno, 2011).

The post-transcriptional modification event of histone mRNAs is essential to prevent the toxic effects of an excess of histone levels in *S. cerevisiae*. To ensure a balanced of histone levels after replication reduction incorporation of histone gene suppression histone degradation should occur (Gunjan et al., 2005).

An unidentified S-phase protein has been found to specifically interact with *HTB1* mRNA (Campbell et al., 2002). This protein may prevent the interaction of the Lsm1-7-Pat1 complex (Herrero and Moreno, 2011; Kurat et al., 2013).

1.16.3. Translational level

Regulation of RD core histone translation during the cell cycle remains largely unclear in *S. cerevisiae*. It has recently been identified that La-motif-containing proteins such as Sro9 and Slf1 were co-purified with RD core histones mRNA (Schenk et al., 2012). Sro9 shuttles between the nucleus and the cytoplasm and cross-links effectively transcribed genes (Rother et al., 2010). More research is needed to determine whether Sro9 and/or Slf1 companion S-phase histone mRNA to the ribosome.

1.16.4. Post-translational level

The negative feedback model for post-transcriptional levels monitors histone protein levels in which high levels are repressed by transcription. In *S. cerevisiae*, histone expression can also be regulated at the post-translational level. For instance, a Rad53-dependent mechanism degrades excess soluble histone proteins in *S. cerevisiae* (Gunjan and Verreault, 2003; Gunjan et al., 2006). Moreover, unphosphorylated and unbound soluble histones are ubiquitinated and degraded by the proteasome (Singh et al., 2009).

1.16. Rationale and Objectives

The assembly of the chromatin clearly requires a precise order of the core histone assembly within DNA. This fundamental process is needed for eukaryotic genomes to be packaged within the nucleus to ensure that all transcriptional levels are properly regulated.

Histone chaperones are proteins that play an important role in the assembly of chromatin. They can associate with other histones and participate in assembling histones (Ransom et al., 2010; Das. et al., 2010). Hif1 is a good example of histone chaperones that can bind histones H3 and H4 and deposit them into chromatin during the cell division (Parthun, 2012).

Hif1 shares the domain structure of the NASP family of proteins that contain a large acidic patch lined by four tetratricopeptide repeat (TPR) motifs (Ai and Parthun, 2004 and Wang et al., 2011). Hif1 must possess unique binding sites in order to form a complex with Hat1. To understand this exclusive specificity, I have generated a series of deletion mutations of Hif1 to identify the particular domains responsible for binding to Hat1. I showed that the acidic patch within TPR2 of Hif1 is required for Hat1-Hif1 interaction.

Furthermore, The Spt2 protein may contribute to the regulation of DNA transcription. Models of Spt2 function suggest that it coordinates histone deposition behind RNAPII. However, the mechanism of how Spt2 could coordinate histone deposition remains unknown. It may function significantly in chromatin remodeling and gene transcription regulation (Thebault et al., 2011). A protein-protein interaction between Spt2 and Hat2, and Spt2 and Hif1 has been found in a large scale analysis of protein-protein interactions in yeast (Krogen et al., 2006). Nevertheless, the raw proteomic data, this interaction is not accurate and unconfirmed.

One step in determining the main biological function of Spt2 would be to investigate if Spt2 physically interacts with proteins that function in assembling chromatin and regulating its transcription. I showed that Spt2 interacts with Hif1, Hat1 and Hat2.

Chapter 2: Materials and Methods

2.1. Equipment

Each one of the small volume centrifugations (1.5ml micro-centrifuge tube, Eppendorf tube) at room temperature (RT) were accomplished using an Eppendorf 5424 centrifuge, whereas large volume centrifugations at room temperature were accomplished using an Eppendorf 5702 centrifuge. Micro- centrifugations at 4°C were performed in a Microfuge® 22R refrigerated micro-centrifuge (Beckman Coulter). Centrifugations of both 15ml and 50ml Falcon tubes at room temperature and refrigerated spins were accomplished with an Allegra® X-15R refrigerated centrifuge (Beckman Coulter). Small-scale cultures were grown in a MaxQ 4450 incubating and floor shaker (Thermo Scientific), while large-scale cultural cells were grown in a MaxQ 5000 incubating and refrigerating floor shaker (Thermo Scientific). Polymerase chain reactions (PCR) were accomplished using a GeneAmp PCR System 9600 (Perkin Elmer). All plates were incubated at both 30°C and 37°C using a Mini Incubator (VWR). All agarose gels were made and then electrophoresed in a Mini-Sub Cell GT Cell (Bio-Rad). All SDS-PAGE gels were made in a Mini-PROTEAN Tetra Cell Casting Module and were then electrophoresed in a Mini-PROTEAN Tetra Cell (Bio-Rad). DNA gels and plates were photographed using a Kodak Gel Logic 2200 UV/Visible Imaging and Documentation (GelDoc) System whereas both DNA gels, and plates, at some points, plus blots were observed by a Molecular Imager® Gel Doc™ XR+ System with Image Lab™ Software (Bio-Rad).

2.2. Media, buffers and solutions

The composition of all culture mediums, buffers and solutions used are listed in Appendix C.1.

2.3. Cell cultural conditions

2.3.1. Bacterial strains

Transformation of *Escherichia coli* with the pRB415-12MYC, pYES2, or pYES-H3 vectors were grown overnight on LB plates supplemented with the proper antibiotic at 37°C and stored at 4°C. For preparation of plasmid, 5ml cultures were grown overnight by shaking at 250rpm at 37°C in LB medium supplemented with ampicillin (50μg/ml). For long-term storage, glycerol stocks were prepared by adding 0.9ml of overnight medium culture to 0.9ml of sterile 50% glycerol in a 1.8ml CryoPure cryovial (Sarstedt) and stored at -80°C (Appendix C.2). All bacterial strains used in this study are listed in Appendix C.2.

2.3.2. Yeast strains

The transformation of *Saccharomyces cerevisiae* strains with the pRB415-12MYC, both pYES2, or pYES-H3 vectors were grown on minimal medium plates without leucine or uracil (YNB -LEU or YNB -URA, respectively) at 30°C and stored at 4°C. 5ml cultures were grown overnight in the proper medium and were shaking at 225rpm at 30°C for genomic isolation. For long-term storage, glycerol stocks were prepared as directed above. All yeast strains are listed in Appendix C.2.

2.4. Manual *S. cerevisiae* genomic DNA extraction

Genomic DNA from *S. cerevisiae* was isolated from wild-type BY4742. A single colony of a yeast strain was grown overnight at 30°C in 5ml Yeast Peptone Dextrose (YPD). Next day, yeast cultures were then harvested by centrifuging at 4000rpm for two minutes at room temperature (~20- 23°C) and the supernatant was aspirated off. The pellet was re-suspended in 1ml of ddH_2O and transferred into a screw-cap conical tube (Sarstedt), followed by

centrifugation at 2000rpm for two minutes at room temperature and supernatant was discarded. 250µl of each of (breaking buffer (see Appendix C.1), phenol-chloroform pH= 8, and glass beads) was then added to break down the cells. The mixture was then vortexed hard for two minutes. The sample was then centrifuged at maximum speed for one minute at room temperature. On top of the mixture, 250µl of ddH_2O was then added, and the mixture was vortexed again for one minute followed by centrifugation at maximum speed for two minutes at room temperature. The supernatant, which contains the DNA, was then transferred to a new Eppendorf tube. To precipitate DNA, 2.5 volumes of 100% ethanol was mixed gently with the lysate by inversion and allowed to stand for two minutes at room temperature. DNA was then pelleted by centrifugation at a maximum speed for five minutes at room temperature. The supernatant was aspirated off, and the DNA pellet was then washed by flicking with 500µl 70% ethanol. The DNA was then left in a fume-hood for a few hours to ensure a complete removal of the residual ethanol. Finally, the dried DNA pellet was re-suspended in 200µl of ddH_2O for solubilization and left at 4°C.

2.5. Polymerase chain reaction (PCR)

The final volume of reagents in PCR reactions was 50µl including 2.5 units of Phusion Polymerase (Thermo Scientific-Phusion High-Fidelity, F-503). Forward as well as reverse primers were obtained from ACGT Corporation, The Centre for Applied Genomics (MaRS, The Hospital for Sick Children). The sequences of primers used in this study are all listed in Appendix C.8. PCR was performed for 35 cycles using the following thermal cycler program.

Table 1: Thermal cycling conditions used for PCR.

Stage	Temperature	Time	Cycles
Initial denaturation	98°C	2 minute	1 cycle
Denaturation	98°C	10 seconds	35 cycles
Annealing	60°C	20 seconds	
Elongation	72°C	1 minute	
Final Extension	72°C	2 minute	1 cycle

2.6. DNA gel electrophoresis

Roughly 0.8% to 1.5% agarose gels (wt/vol) were electrophoresed at 95-110 with 1X TBE. Gels were stained with either ethidium bromide for visualization under UV light. To determine DNA sizes, 3µ of either one kilobase (kb) or 100 base pairs (bp) DNA Ladders (GeneDirex RTU (ready-to-use); Appendix C.3). Before loading into an agarose gels, DNA samples were mixed with 6x DNA loading dye. Detection of DNA bands was performed under UV light using a Bio-Rad XRS+ Imager at Ryerson University.

2.7. DNA purifications

A clean-up system for PCR products or following enzymatic reactions was performed using a DF Spin Column DNA purification Kit (Geneaid). A high level of purification was achieved for all downstream applications. Clean-up of PCR products or restriction digests requiring DNA agarose gel extraction was performed using a DF Spin Column DNA purification

Kit (Geneaid). The handling steps of DNA clean-up were performed according to the manufacturer specifications.

2.8. Purification of plasmid DNA and DNA isolation for sequencing

One single colony of cells was inoculated into 5ml of LB and 100 μg/ml ampicillin medium and grown overnight at 37ºC. The overnight was subjected to further DNA analysis or DNA sequencing. Isolation of DNAs was performed using PD spin column High-Speed Plasmid Mini kit (Geneaid) as described by manufacturer's instructions.

2.9. Restriction enzyme digests (linearization)

Enzymatic restriction digests of plasmid DNA, inserted DNA (PCR product DNAs) were digested by adding one unit of restriction enzyme per one microgram of DNA according to supplier specifications (Thermo Scientific). As considered experimentally by my own analysis, the plasmid and the PCR inserts were digested with both *BamH1* and *Pst1* separately and sequentially.

2.9.1. Restriction digestion of plasmid

In order to linearize the plasmid and facilitate the cloning of PCR inserts two restriction enzymes were used which are *BamH1* and *Pst1*. The total volume of the enzymatic reaction was 100μl. The enzymatic reaction for *BamH1* was performed on ice by adding 10μl of plasmid DNA, 75μl of ddH_2O, 10μl of 10x *BamH1* reaction buffer, and 5μl of *BamH1* restriction enzyme. The reaction then was immediately placed at 37°C for either four hours or overnight. To check for a successful recovery, the sample was electrophoresed on 0.8% agarose gel. The enzymatic reaction for Pst1 was performed according to an identical procedure to that used for

BamH1 digestion. To confirm an effective digestion, the sample was electrophoresed on 0.8% agarose gel.

2.9.2. Restriction Digestion of PCR products

The successful products of the PCR-amplifications can be inserted into a plasmid victor. In order to do so, all inserted DNAs were digested by *BamH1* and *Pst1*. The final volume of this enzymatic reaction was 100µl. The restriction digestion reaction for *BamH1* was performed on ice by adding 45µl of PCR products, 40µl of ddH_2O, 10µl of 10x *BamH1* reaction buffer, and 5µl of *BamH1* restriction enzyme. The reaction then was directly moved in a 37°C incubator for either four hours or overnight. The samples were electrophoresed on 0.8% agarose gel to ensure the correct sizes of the bands were visualized. The second enzymatic reaction for *Pst1* was accomplished accordingly. After the last incubation, the samples were electrophoresed on 0.8% agarose gel to ensure a positive reaction.

2.10. Ligation

All vector plasmids used contain an ampicillin resistance gene, and were therefore grown in LB + AMP (100µg/ml). Ligation reactions were performed in total volume of 20µl by adding 1µl of T4 DNA Ligase (Thermo Scientific) for each reaction. The ligation reaction was performed by adding 3µl of plasmid DNA, 5µl of PCR products, 9µl of ddH_2O, 2µl of ligase buffer, and 1µl of T4 ligase enzyme. The mixture was then incubated for two hours at room temperature, followed by either directed transformation into high-efficiency competent *E. coli* cells (DH5α cells) or kept at -20°C.

2.11. *E. coli* transformation

After ligation, 5µl of DNA were transformed into DH5α "High Efficiency competent cells" and were then incubated on ice for thirty minutes, followed by heat-shock at 42°C for 20 seconds and immediately placed on ice once more for two minutes. 950µl of pre-warmed (LB+ Amp) medium was added to the reaction. Cells were then shaken at 250rpm for one hour at 37°C. 200 µl of transformed cells were spread onto (LB+ Amp) plates, which contained 100µg/ml of ampicillin, and grown overnight at 37ºC. To confirm that competent cells were not usually an ampicillin resistance, ddH_2O was added into the transformation reaction instead of DNA.

2.12. Sequencing

All DNA sequencing samples were mini-prepped, and sent to ACGT Corporation, The Centre for Applied Genomics (MaRS, The Hospital for Sick Children). Sequencing was conducted on bacterial clones (listed in Appendix C.2). Several primers were used to sequence Hif1 truncation mutants (Appendix C.7).

2.13. Yeast transformation

Either gene replacement or transformation of a plasmid DNA into a particular yeast strain was performed as following. Yeast cells were inoculated into 5ml of YPD overnight at 30°C with steady shaking at 225rpm. Next morning, yeast cultures were diluted to an optical density (OD_{600}) of 0.2 in 100ml of YPD and were then grown, for 6-8 hours, until an OD_{600} was reached approximately 1.0 at 30°C with steady shaking at 225rpm. About 50 ml of the yeast cells were pelleted at 4,000rpm for four minutes at 4°C. The pellet was washed with 10ml of cold ddH_2O and centrifuged once more as described above. After removing all of the supernatant, yeast cultures were washed in 1ml of 0.1M lithium acetate (LiOAc) and were then transferred to an

Eppendorf tube, and centrifuged at 13,200 for ten seconds. The supernatant was discarded, and cells were then re-suspended into 400µl of 0.1M (LiOAc) to make a 4:1 ratio of (LiOAc:cell) pellet. 50µl of that was transferred in a separate Eppendorf tube for each transformation reaction, and centrifuged at 13,200rpm for ten seconds.

Directly to pellets and in the following order was added: 240µl of 50% polyethylene glycol (PEG) to increase the cells permeability, 36µl of 1M (LiOAc) pH 7.2, layered by 50µl of Salmon Sperm DNA (2mg/ml), a carrier molecule, (10mg/ml, Invitrogen) which was pre-boiled for five minutes. Then, 5µl of plasmid DNA was diluted in 45µl of ddH_2O and was then added to the mixture, in different circumstances 50µl of PCR products were added when being transformed. As an alternative, 50µl of ddH_2O was added to act as negative control. All tubes were vortexed hard for one minute until the suspension was homogenous, and placed at 30°C for no longer than twenty minutes. After incubation, 50µl of 10% DMSO was gently added to increase the transformation 5-10 fold. Cells were then heat shocked at 42°C for ten minutes. Following the second incubation cells were spinned down at 13,200rpm for ten seconds at room temperature. As a final step, cell pellets were re-suspended in 300µl of ddH_2O and was then spared on appropriate selective medium, and incubated at 30 ºC for 72 hours.

When cells were being transformed with the pRb415-12MYC plasmid, the entire volume of cells was plated onto a minimal medium missing leucine (YNB –LEU). When PCR products were being transformed, the cells were spread onto YPD (non-selective medium).

2.14. Spot test analysis

In order to set up a yeast spotting assay, a single colony of a yeast strain was inoculated in 5ml of an appropriate culture medium such as YPD. Cells were grown overnight and shackn at

225rpm at 30°C. The following morning, cell cultures were diluted to an optical density (OD_{600}= 0.2) and were grown to a desired optical density. The serial requested of dilutions were performed for each strain. 5µl drop of each dilution was then spotted onto plates and then incubated at 30 ºC for 48 hours to 72 hours in total.

2.14.1. Spot test assay for analyzing histone overexpression

Strains were transformed with either a vector encoding galactose inducible, tagged histone H3 or the empty vector (pYES2). Following transformation, they were grown overnight in 5ml minimal medium (YNB) either minus uracil or minus uracil/minus leucine (to select for the plasmids) and containing 2% raffinose as a carbon source. The next morning they were diluted to about 0.1 OD_{600}. When they reached 0.5 OD_{600}, expression of H3 was then induced by addition of 2% galactose for about four hours, and cultures were then grown to approximately OD_{600}= 0.7- 0.8. After that, six-fold serial dilutions of each strain were performed using 96-well plates. They were then plated on minimal media either lacking uracil or lacking both uracil and leucine, with either glucose (H3= OFF) or galactose (H3= ON) as carbon source. The plates were incubated for 3-4 days at 30°C.

2.14.2. Spot test assay for analyzing sensitivity for DNA-damaging agent

To determine whether mutant strains were sensitive to DNA-damaging drugs, cells were plated on plates containing 50mM concentrations of HU. Hydroxurea (HU) is a genotoxic drug that blocks the DNA replication and results in defects in the activation of DNA damage checkpoints (Gunjan and Verreault, 2003).

Yeast strains were inoculated in 5ml YPD medium and were grown overnight at 30°C within shaking. The following day, the optical density of each culture was measured and adjusted

to 0.2 OD_{600}. Cells were then grown to approximately OD_{600}= 0.5, and then 1/4 serial dilutions were performed of each cell culture using 96-well plates. From each one of these dilutions 5µl drops were then spotted onto YPD plates, 50mM Hydroxurea plates and 100mM Hydroxurea plates. Plates were incubated at 30°C for three days (YPD plates) and four days (Hydroxurea plates). Colonies were photographed after 3-4 days.

2.15. Yeast harvesting for protein detection

A single colony of the yeast cell was grown overnight in 5ml of a selective culture medium and was shaken at 225rpm at 30°C. The overnight culture was harvested by centrifugation at 3000rpm for two minutes at room temperature. Following discarding the supernatant, cell tubes were placed on ice and were then washed once with 1ml of cold ddH_2O and centrifuged once more at 3000rpm for two minutes, followed by aspirating off the water. Cell pellets were then re-suspended in 200µl of 1x SDS buffer. The samples were boiled for 5 minutes for protein extraction, and immediately centrifuged for ten seconds. After spinning, the samples were directly placed on ice and the supernatant of each sample was then transferred to a fresh 1.5 ml Eppendorf tube. Tubes were kept on ice until gel loading or stored on -80 for later experiments.

2.16. SDS-PAGE and Western blotting analysis

SDS-PAGE (Sodium Dodecyl Sulfate Poly-Acrylamide Gel Electrophoresis) and western blotting were performed for assessing protein expression. The whole cell extract (WCE) from *Saccharomyces cerevisiae* was electrophoresed and separated through a sodium dodecyl sulfate (SDS) polyacrylamide gel based on the size. Subsequently, proteins were transferred to a

nitrocellulose membrane followed by probing antibodies in wo stages of the protein detection technique.

2.16.1. Preparation of polyacrylamide gel electrophoresis (SDS-PAGE)

In order to identify proteins based on size, the proteins were separated on SDS-polyacrylamide gels. 10– 15 µl of prepared protein samples were loaded onto 1ml of 5% stacking and 5ml of 10% running SDS-polyacrylamide gel. A protein standard, PiNK Plus Prestained Protein Ladder (Genedirex; Appendix C.4), consisted of reference bands in the range of 10- 175 kDa was loaded to measure the relative protein sizes. An electric field of 75V (volts) was operated for the first 30 minutes and was then raise to 100V for the separation of proteins.

2.16.2. Proteins transfer

The initial step of proteins transfer is to soak a piece of blotting membrane, (Nitrocellulose membrane; Bio-Rad), in 1x Western transfer buffer. For the wet transfer system, a transfer stack "sandwich" was built up in an electrophoretic transfer cassette considering that the electric current flows from cathode (-ve) to anode (+ve), and according to the following order: a wet sponge, four pieces of wet Whatman filter paper, the SDS-PAGE gel, one wet nitrocellulose membrane, four more pieces of wet Whatman paper, finally followed by an extra wet sponge. The tank was filled with 1x Western transfer buffer (cold buffer) and was run at 78V for 45 minutes to one hour or 15V overnight. The transfer tank should be run in a cold atmosphere to dissipate the heat produced by the electric filed.

2.16.3. Ponceau stain

In order to visualize proteins transferred to nitrocellulose membrane, the membrane was placed into 0.1% Ponceau stain, and shaken gently on The Belly Dancer shaker for five minutes. Following, the membrane was then de-stained with three (five-minute) washes in 1x Tris buffered saline (TBS) at room temperature.

2.16.4. Blocking

To eliminate non-specific binding of antibodies, the membrane was blocked in 5% fat free skim milk solution (BLOTTO) for either one hour at room temperature or overnight at 4°C. Following, three (five-minute) washes were performed in 1x Tris buffered saline (TBS) at room temperature to wash off milk protein.

2.16.5. Immunoblotting

After blocking, blots were incubated within variable concentration of primary antibodies diluted in 3% Bovine Serum Albumin (BSA). Mouse monoclonal IgG α-MYC (Santa Cruz Biotechnology) primary antibody was diluted at 1:7500, or rabbit monoclonal α-TAP (Thermo scientific) primary antibody was diluted at 1:7500. Membranes were probed with primary antibodies for one hour at room temperature or overnight at 4°C. Subsequently, blots were then washed three times in 1x PBS for five minutes each at room temperature to remove non-specific-binding. Blots then were incubated with Horseradish peroxidase-linked (HRP) secondary goat α-mouse antibodies at 1:5000 dilution or HRPO goat α -rabbit IgG 1:5000 diluted in 1x TBS with 5% skim milk and incubated for 45 minutes to one hour at room temperature. Next, three (five-minute) washes were performed in 1x TBS. To visualize the proteins, the blots were incubated in three milliliters of Chemiluminescent Substrate (Cyanagen; Westar® Nova 2011 - High

Sensitivity) or Enhanced Chemiluminescence (ECL) for 5 minutes and manually shackn to generate the best Chemiluminescent signals. Detection was achieved by using Molecular Imager® Gel Doc™ XR+ System with Image Lab™ Software (Bio-Rad).

2.17. Tandem affinity purification

Tandem affinity purification (TAP) was accomplished to isolate MYC-tagged proteins alongside their associating partners in *Saccharomyces cerevisiae*.

Affinity purification (AP) was accomplished by a one-step affinity purification scheme based on Keogh et al. (2006). Purification depends on the interaction between the TAP epitope tag with immunoglobin G (IgG). The TAP tag consists of three segments: the N-terminal calmodulin binding peptide (CBP), a tobacco etch virus (TEV) protease cleavage site, as well as protein A which originally identified in the cell wall of *Staphylococcus aureus*. Protein A recognizes and attaches tightly to IgG during affinity purification in which providing a "pull down" performance of the fusion protein to TAP tag alongside with all associating partners.

2.17.1. Co-Immunoprecipitation (Co-IP)

Yeast cells were grown overnight in 5ml of appropriate culture medium and shaken at 225rpm at 30°C. Next day, cell cultures were diluted to an optical density (OD_{600}= 0.2) in 150ml of the same medium that was used previously. Cells were grown until an OD_{600} of about 0.8 was achieved by shaking at 225rpm at 30°C. Then, each cell culture was transferred to a 50ml Falcon tube and was then harvested by centrifugation at 4,000rpm for five minutes at 4°C. The supernatant was removed and cells were then washed twice with 25ml of cold ddH_2O and centrifuged at 4,000rpm for five minutes at 4°C. Lastly, cells were washed once with 10ml of

ice cold IP wash buffer and centrifuged at 4,000rpm for five minutes at 4°C. The supernatant was aspirated away, and precipitates (cells) were frozen and stored at -80°C.

IgG-sepharose beads (GE Healthcare) were supplemented with 20% ethanol (1:1) ratio and because each purification requires 25µl of IgG-sepharose beads, 50µl of slurry was added to each purification. To remove the ethanol, beads were placed into 1.5ml Eppendorf tubes by gently pipetting using a cut P-1000 tip and then spun down at 2,000rpm for two minutes at 4°C. Beads were washed prior to use. Three washes were performed by adding 750µl of cold IP wash buffer and then mixed gently by inversion followed by centrifugation, at 2,000rpm for two minutes at 4°C. In one of the three washes, buffer including beads was transferred to a fresh Eppendorf tube. After the last wash, supernatant was discarded and IP wash buffer was added in (1:1) ratio with beads. 50µl of the mixed slurry was aliquoted into a fresh Eppendorf tube by using a cut P-200 tip and kept on ice.

The Frozen cell pellets were thawed on ice, and then re-suspended in one ml of IP lysis buffer containing a protease inhibitor tablet (Roche) to block proteolytic degradation of proteins, and moved into a conical tube including 1ml of glass. To lyse the cells, bead beating was performed in a Mini-BeadBeater-16 (BioSpec) using a series of beating bursts for twenty seconds at room temperature followed by two minutes cooling on ice for 10 cycles, as reasoned to be the most experimentally optimal conditions for *S. cerevisiae* according to my own analysis. Following the last burst, tubes were cooled down on ice for two minutes.

In order to filter the whole cell extracts (WCE), the conical tube was flipped upside-down to poke a hole through the bottom of the tube by using a 25Gx5/8 Needle and then placed into

PrecisionGlide syringe (BD). The WCE was precipitated into a 15ml Falcon tube by centrifugation at 4,000rpm for one minute at 4°C.

Cell pellets were re-suspended and then transferred to a new Eppendorf tube, followed by centrifugation at maximum speed (14,000- 15,000rpm) for twenty minutes at 4°C. All supernatant was collected and transferred to a fresh Eppendorf tube. 50µl was isolated into a separate Eppendorf tube and stored at -80°C to use it as an input control. Approximately 850µl of the supernatant remained and it was transferred to the tube that contains a mixed slurry of the IgG-sepharose beads and IP wash buffer. These tubes were rotated for two hours at 4°C. Subsequently, three sets of bead washing were performed by adding 750µl of cold IP wash buffer, rotating for five minutes at 4°C, over at 2,000rpm for two minutes at 4°C, and then aspirating the supernatant away. Through one of the three washes, buffer including beads were moved to a fresh 1.5ml Eppendorf tube to avoid beads binding to tubes.

At this stage and after the final wash, all IP buffer was aspirated away and 80µl of 1x SDS loading dye was added to beads, whereas 50µl of 1x SDS loading dye was added to input samples. Samples were then boiled for five minutes and immediately spun down for ten seconds. Samples then were either stored at -80°C or kept on ice to be loaded onto SDS-PAGE gels.

Chapter 3: Results

3.1. Molecular Analysis of Hif1 Mutants

3.1.1. General strategy for plasmid DNA construction

The full length of the Hif1 gene in *Saccharomyces cerevisiae* consists of 1158 nucleotides that encode a protein of 385 amino acids (Figure5). I obtained the Hif1 nucleotide sequences of *S.cerevisiae* from the *Saccharomyces* genome database (SGD, http://www.yeastgenome.org/) (Appendix C.6). The TPR domains of full length Hif1 were identified by multiple sequence alignments of Hif1. Twelve truncation mutants of Hif1 were generated by either a conventional PCR or overlapping PCR techniques.

Figure 5: Full length of Hif1 consists of 385 amino acids "1158 nucleotides".

The predicted protein domain structure of Hif1 has four tetratricopeptide repeats (TPR) that mediate protein-protein interactions, and a C- terminus which is lysine/arginine-rich (++) that fits the consensus sequence for a monopartite nuclear localization sequence (NLS).

I have constructed six C- terminus truncation mutants predicted of the Hif1 protein, or external deletions, in which I used a conventional PCR strategy (Figure 6). I have constructed another six internal truncation mutants in which I used an overlapping PCR technique (Figure 7). I designed primers to be complementary to the DNA sequence flanking the regions that were targeted for the deletions. PCR products (conventional for external and overlapping for internal) amplified initially from *S. cerevisiae* genomic DNA were then purified, cloned into pRB415-12MYC, and proliferated in *Escherichia coli* DH5α. After PCR amplification, both the PCR

products and plasmid were digested with *BamH1* and *Pst1* that are found in the plasmid polylinker (MCS, multiple cloning site).

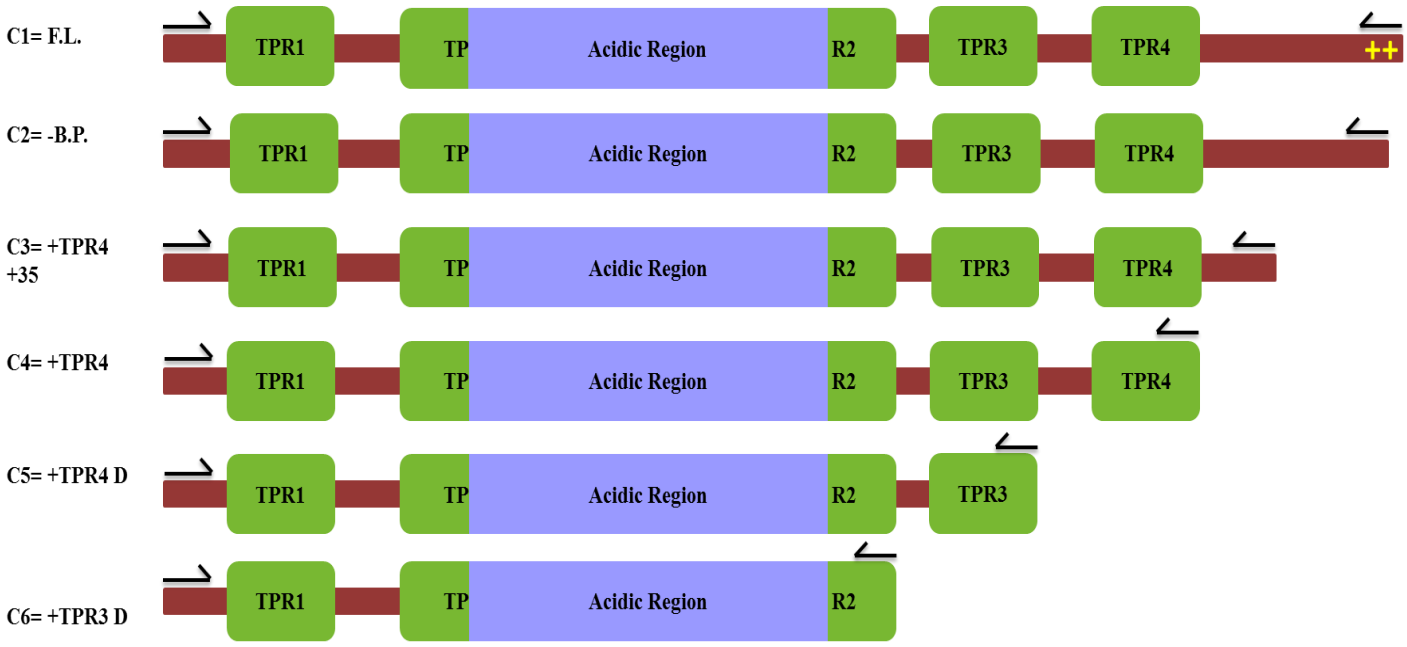


Figure 6: External Deletions of HIF1.

Positions of external deletions HIF1 mutants. Hif1 constructs generated containing external deletions.

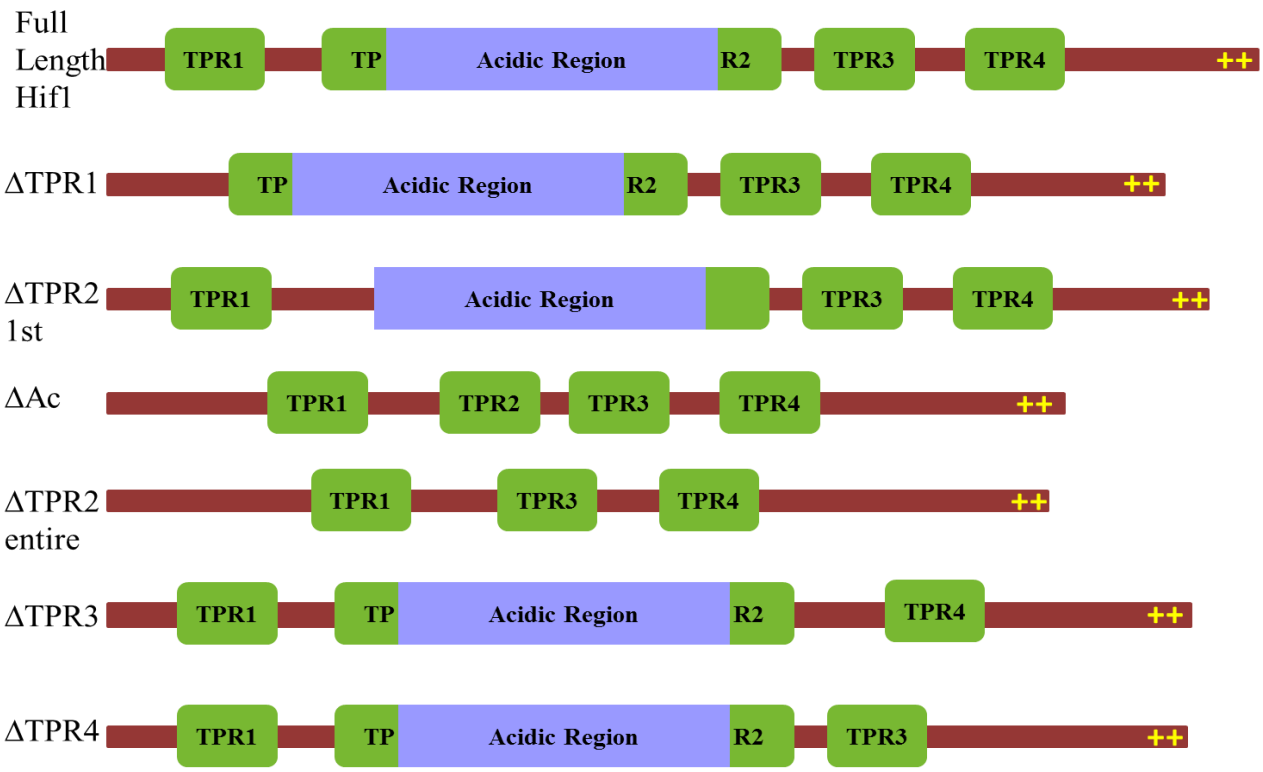


Figure 7: Internal Deletions of HIF1.

Positions of internal deletions HIF1 mutants. Hif1 constructs generated containing internal deletions.

Therewith, all the truncated mutants were inserted into the yeast expression vector pRB415ADH1-12MYC at its MCS. This vector allows selection of transformed yeast cells by conferring ability to grow in –LEU medium because of plasmid *LEU2* gene (for -LEU2 host cells). This vector also contains the DNA sequence encoding an N-terminal 12-MYC epitope tag. The tag allows detection of the 12XMYC-Hif1 protein by anti-MYC antibodies for immunoprecipitation and immunoblotting assays. Positive clones were sequenced to verify the mutations.

3.1.2. Amplification and molecular cloning of *Saccharomyces cerevisiae* Hif1 mutants

There two collections of Hif1 mutants are called C-terminal or external deletions, or internal deletions were used (Figure 6). The first clone the of C-terminal or external deletions is called F.L. which is full length Hif1 that contains all domains including all four TPR domains and the C-terminus domain in addition to basic patch (B.P.). The (-B.P.) clone is an interesting domain called basic patch. The (+TPR4 +35) clone has TPR4 and 35 additional amino acids. The (+TPR4) clone lacks the whole C-terminus tail including the basic patch. The (+TPR4 D) clone lacks the whole C-terminus including TPR4 domain. The (+TPR3 D) clone is also lacks the whole C-terminus including TPR3 domain.

An interesting domain of Hif1 is a basic patch, a cluster of lysine/arginine amino acids which is located right at the C-terminus (Figure 5). This lysine/arginine-rich sequence (++) (Figure 5) fits the consensus sequence for a monopartite nuclear localization sequence (NLS). The consensus sequence has four positions (K-K/R-X-K/R). The Hif1-C is K-K-P-R-R-H-STOP that matches the NLS consensus.

The second set of internal deletion clones (Figure 7) start with ΔTPR1 that is a deletion of TPR1 domain. ΔTPR2- 1st half stands for a clone lacking the first half of the TPR2 domain, while the ΔAcd refers to a clone missing only the acidic region that interrupts the TPR2 domain. ΔTPR2 clone lacks the entire TPR2 including the first half and acidic region. ΔTPR3 clone lacks the TPR3 domain. ΔTPR4 lacks the TPR4 domain.

As shown earlier, I have designed a series of Hif1 constructs that deleted specific regions in HIF1 (Figure 7). The full length Hif1 includes four tetratricopeptide (TPR) repeats that are predicted to mediate protein-protein interactions. One of these TPR domains, which is the second TPR domain, is interrupted with an acidic region (Figure 5).

3.1.2.1. Successful amplification of C- terminal truncation mutants (External Deletions)

In order to engineer the Hif1 deletion mutants (Figure 6), I used a conventional PCR strategy using a forward (F) and a reverse (R) primer (See Appendix C.8). Each primer used in this technique was designed with an appropriate restriction site on their 5' ends (*BamH1*-forwared) and (*Pst1*-reverse) to facilitate cloning of PCR product in frame with DNA encoding 12-MYC in pRB415-12MYC.

Results in Figure 8 indicate that the amplification of the C-terminal deletion HIF1 mutants was successfully completed. The PCR products were gel purified and cloned into pRB415-12MYC.

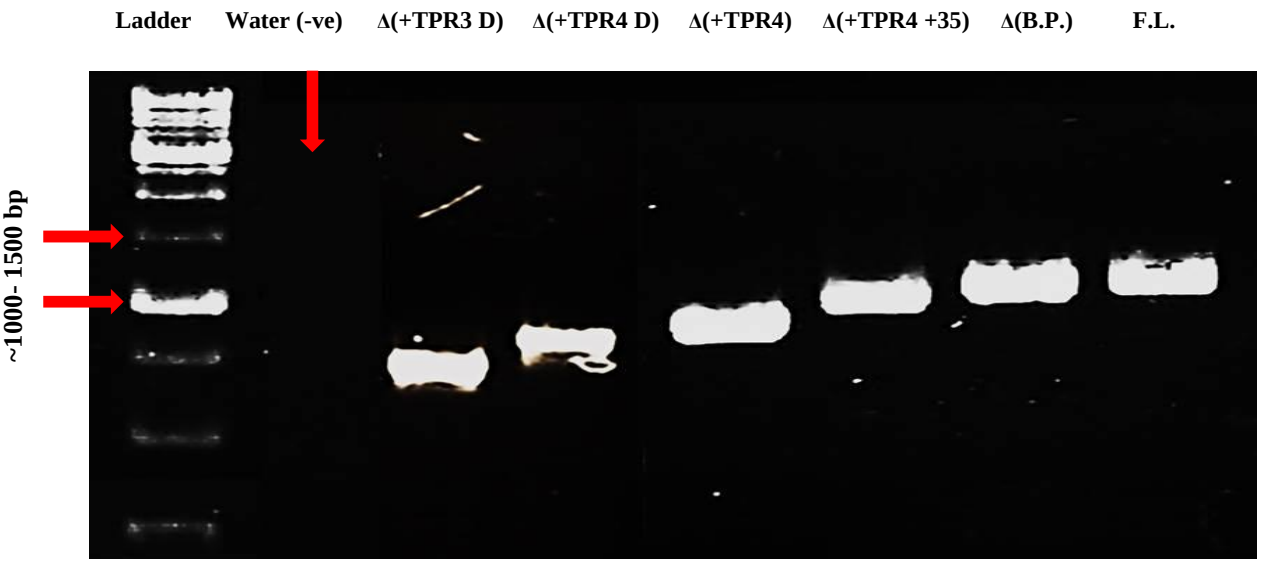


Figure 8: Production of amplification of the C-terminal HIF1 deletion mutants.

The figure represents the results of successful PCR amplification of each of C-terminal deletions. 3µl of the PCR products were analyzed on an 0.8% agarose gel embedded with RedSafe. The first lane represents 1kb DNA ladder, and the second was a (-ve) control since water was used as template. The predicted size of DNAs should be ~1158- 630 base pairs as shown in table1.

The names of clones are as shown in Figure 6.

3.1.2.2. Successful amplification of Hif1 internal deletion mutants

I generated a number of internal deletions in the genes utilizing the overlapping PCR strategy (described in Figure 9 for ΔTPR3). Briefly, two separate conventional PCRs were conducted for each deletion followed by a third PCR (Figure 9). The first PCR was performed to amplify a segment of DNA just prior to the start of sequence of the region to be deleted, while the second PCR was performed to amplify the DNA sequence just after the region to be deleted (Figure 9).

Figure 10 shows 5μg of each of these two PCR reactions used in the internal deletion mutants separated on a 1.5% agarose gel. The DNA was intact and thus appropriate for use as a template for a third round PCR (Figure 9). The predicted molecular weights of the Hif1 internal deletion amplifications were calculated (Table 2), and found to match with the sizes of the detectable bands on the agarose gel (Figure 10). For more details and further comparison see attached DNA ladder in appendix C.3.

Next, the two PCR products were gel purified, denatured, hybridized to allow then to bind each other at the point that they overlapped (Figure 9). Then, dNTPs, Taq polymerase and forward and reserve primers were added and a third conventional PCR was conducted to generate and amplify the internal deletion constructs using the new overlap product as template (Figure 11). Again, all primers were designed with an appropriate restriction site on their 5' ends (*BamH1*-forwared) and (*Pst1*-reverse) to enable cloning of PCR product into *BamH1/Pst1* site on pRB415-12MYC.

Table 2: The expected number of nucleotide base pairs of internal deletion PCR products.

Domain	PCR1	PCR2
TPR1	63bp	993bp
TPR2- 1st half	180bp	906bp
Acd (Acidic Region)	252bp	594bp
TPR2 entire	180bp	540bp
TPR3	705bp	315bp
TPR4	837bp	219bp

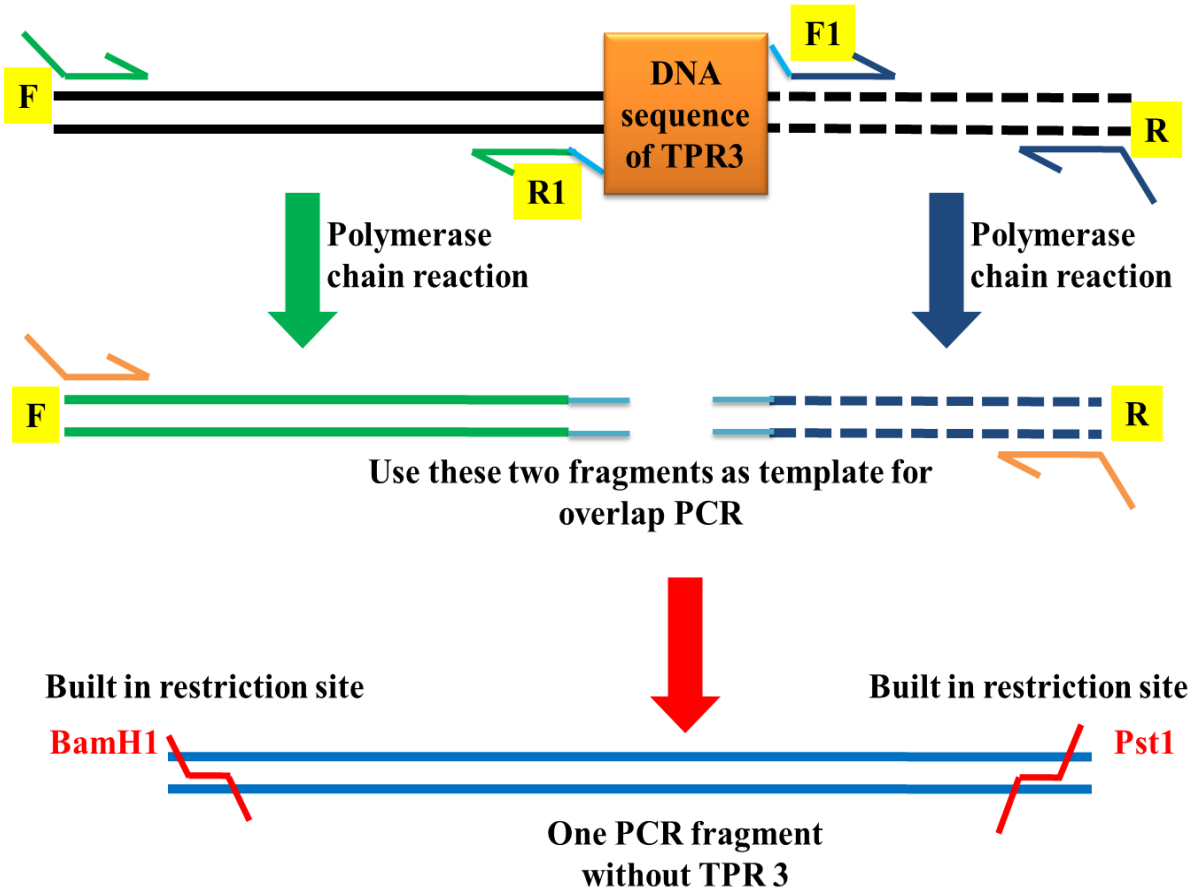


Figure 9: Overlapping PCR strategy to engineer deletion of TPR3.

Two separate conventional PCRs were used to generate all internal deletions. This figure illustrates an example of deletion of TPR3. As observed in the figure, the first PCR was performed using F + R1 and the second PCR was performed using F1 + R. Note that R1 and F1 have additional ~20 bases of complementarity to each other (light blue). Yeast genomic DNA was used as a template for both PCR1 and PCR2.

The third PCR was performed using F + R primers. At this point, I used my PCR products (PCR1 and PCR2) as a template to amplify one PCR fragment without TPR3. Also, the primers that were used have restriction enzyme sites as their 5' ends (*BamH1*-forwared) and (*Pst1*-reverse) to facilitate cloning of PCR products.

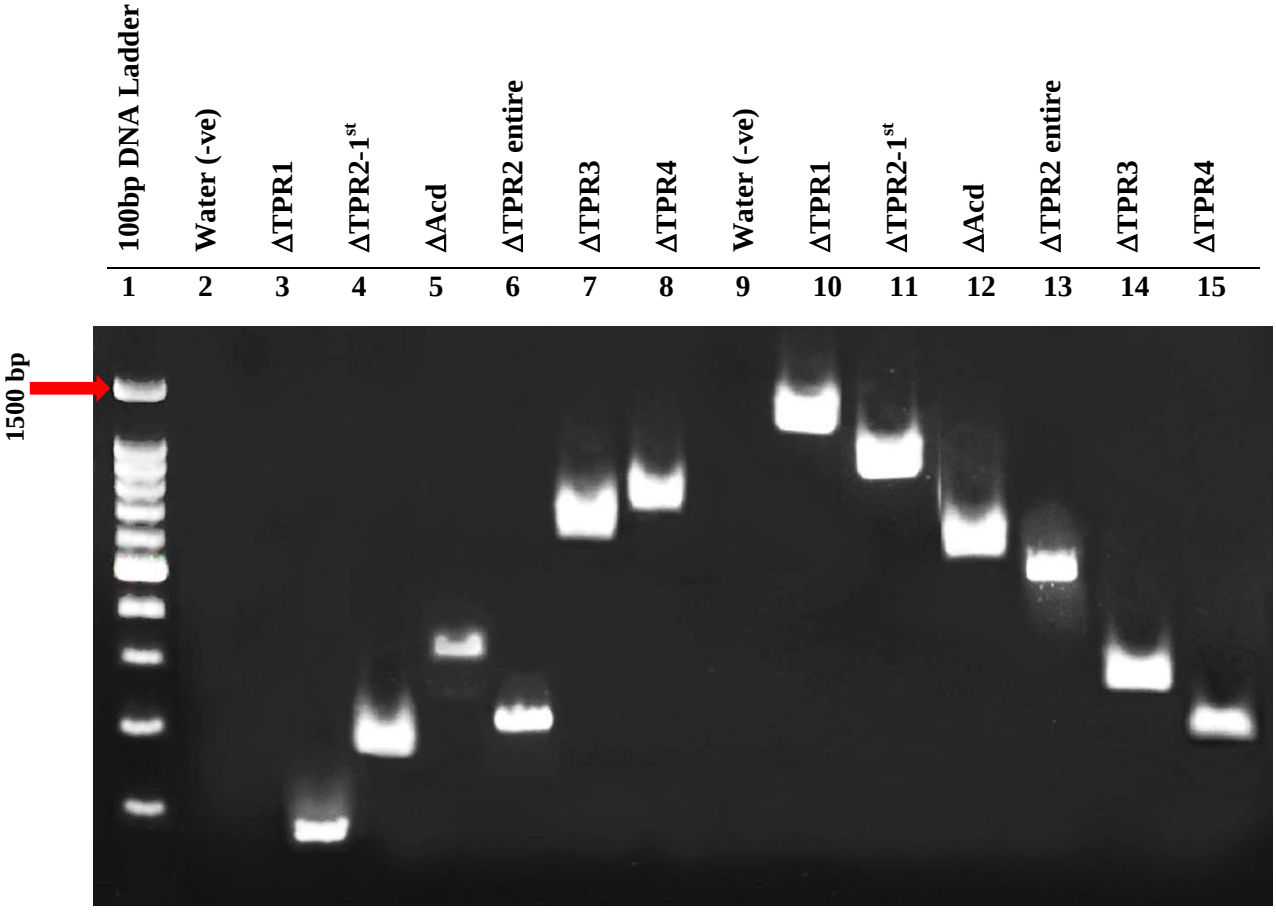


Figure 10: Production of PCR1 and PCR2 of Hif1 internal mutants.

The figure represents the results of successful amplification of each one of the first two PCR reactions for Hif1 internal mutants. 5µg of PCR products were electrophoresed on a 1.5% agarose gel. The first lane represents 100bp DNA ladder, and the second was a (-ve) control as well as lane 9 since water was used as a primer. Lanes 3 to 8 showed the successful amplifications of the DNA sequence right before the targeted TPR domain, while lanes 10 to 15 showed the successful amplifications of the DNA sequence right after the targeted TPR domain. According to the calculated M.W. of DNA, sizes were compared to the DNA ladder to be around the correct size that expected (See Table 2 and Appendix C.3).

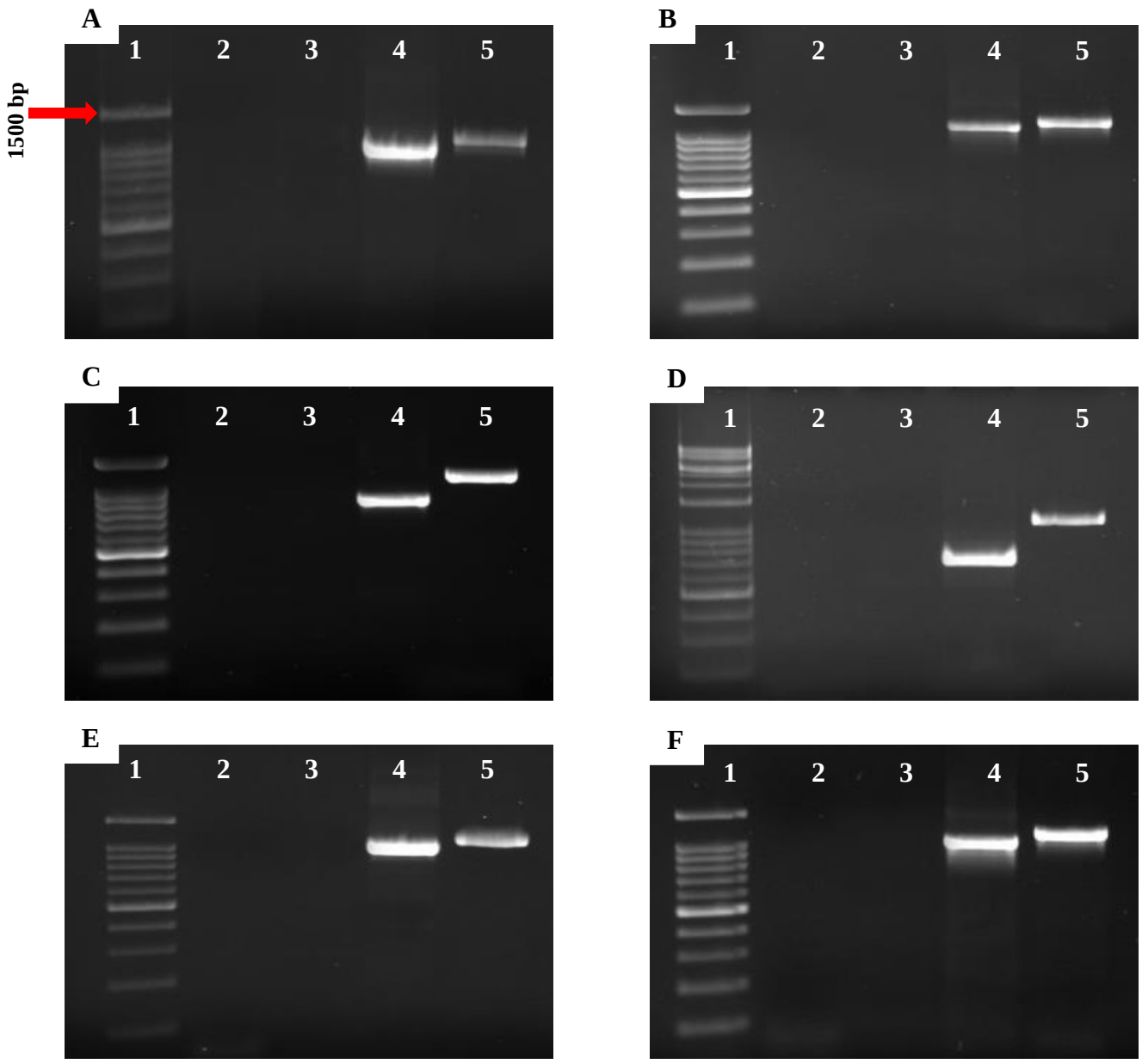


Figure 11: PCR3 of HIF1 internal deletions.

The figure represents agarose gel electrophoresis of internal deletions of TPR1, TPR2-1st, Acd, TPR2 entire, TPR3 and TPR4 (Figure A, B, C, D, E and F, respectively). 100 bp DNA ladder was loaded on lane 1 on the six agarose gels. A negative control (ddH_2O) was loaded on lane 2 and 3 (as a template and primers, respectively). Lane 4 shows a complete deletion of a particular TPR Domain, and lane 5 is the Hif1 wild type background which should be ~1158bp in size. Please see Table 2 and Appendix C.3 to compare sizes with the DNA ladder.

Figure 12 shows purification of PCR products in Figure 11 (A-F, lane 4) and, indicate that amplification of all internal deletions of HIF1 were successfully amplified. The PCR products were gel purified, and cloned into pRB415-12MYC as described.

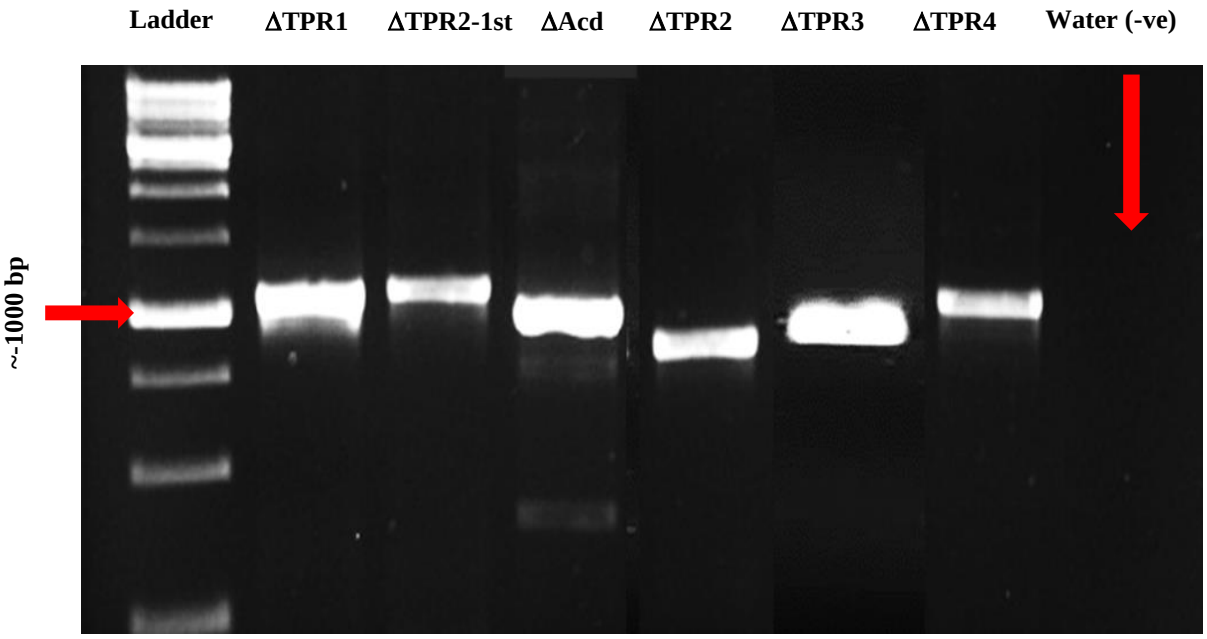


Figure 12: Production of internal deletion constructed mutants of HIF1.

The figure represents the results of successful amplification of each of internal deletions. 3µg of PCR products were electrophoresed on a 0.8% agarose gel. The first lane represents 1kb DNA ladder, and the last was a (-ve) control since water was used as template. The predicted size of DNAs should be ~1086- 658 base pairs as shown in Table1.

3.2. Molecular cloning of Hif1 truncated mutants

Each deletion clone (external and internal) was cloned into pRB415-12MYC, a CEN-based expression vector contains a DNA sequence in frame with *BamH1* in MSC that will provide them an N-terminal 12-MYC epitope tag (12xMYC). Epitope tagged Hif1 clones are then expressed under the yeast (ADH1) alcohol dehydrogenase 1 promoter that permits constitutive expression of cloned genes in *S. cerevisiae* (Figure 13). Since *BamH1*and *Pst1* can flank all inserts, all primers were designed with an appropriate restriction site on their 5` ends (*BamH1*-forwared) and (*Pst1*-reverse) for cloning purposes. A stop codon was included in the reverse primers. Ligated plasmids were then transformed into *E.coli* DH5α before being re-purified and digested with appropriate restriction enzymes. Those with positive inserts of the appropriate predicted size, (Figure 14), were sequenced at (ACGT, University of Toronto) to confirm 100% match to templates.

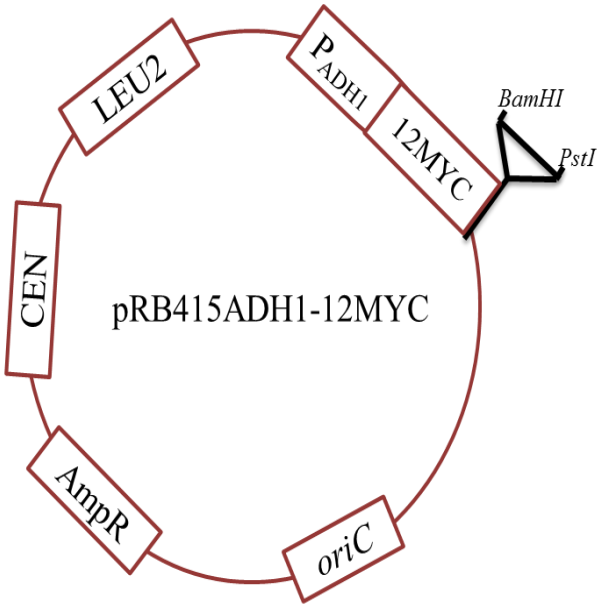


Figure 13: Figure represents the cloning vector pRB415-12MYC.

The vector has DNA sequence encoding a 12-MYC epitope tag in frame with *BamH1* that imparts 12-MYC tag on target proteins. It is a CEN- based vector that allows chromosome-like segregation of the plasmid in Yeast. Also, the ADH1 promoter was used to drive expression of the *HIF1* clones. The *LEU2* biosynthetic gene marker allows plasmid selection in yeast by growth of a LEU- host in –Leu media. An origin of replication that facilitates the replication in the host cell (*E.coli*.) was present along with ampicillin-resistance gene (AmpR).

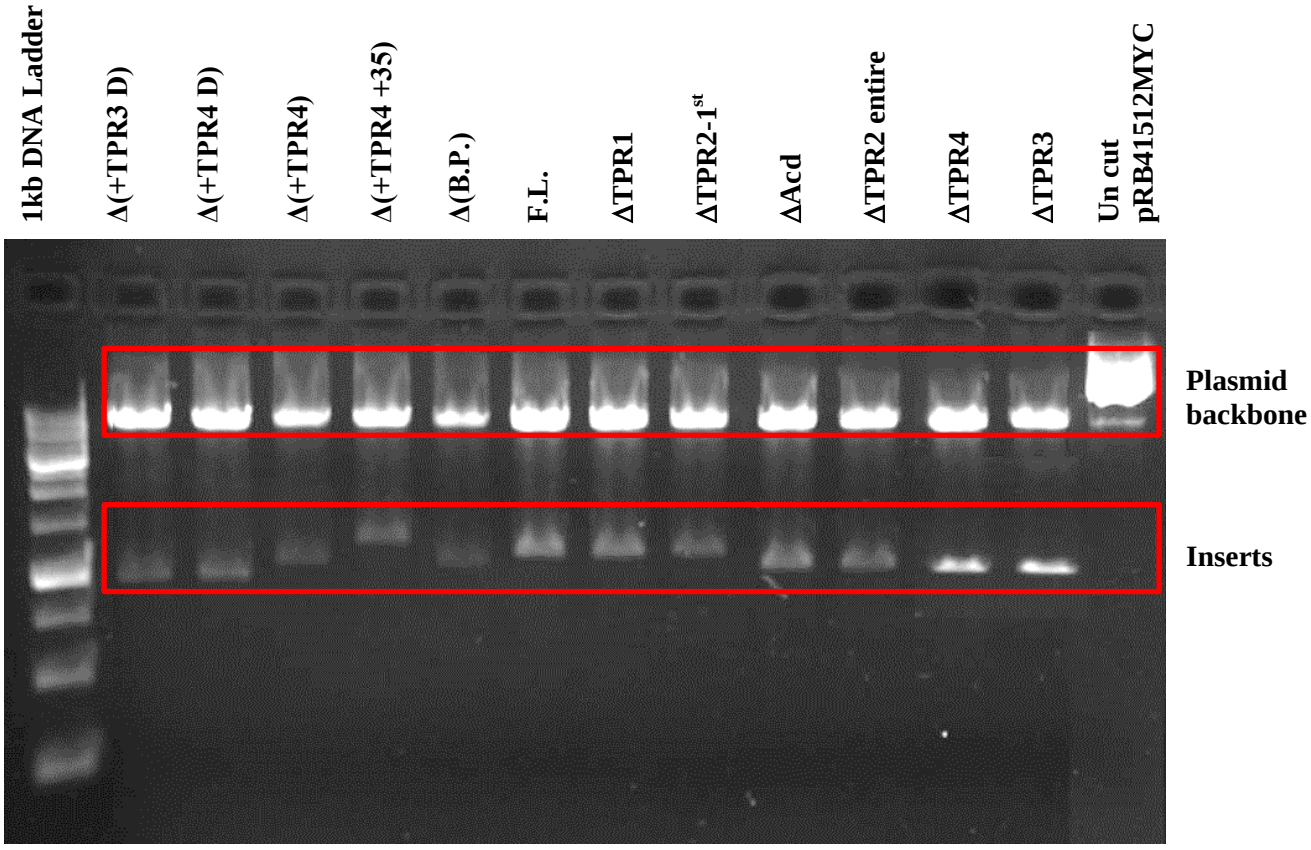


Figure 14: Verification of molecular cloning of Hif1 deletion clones by restriction enzyme digestions of constructs.

This experiment represents successful cloning of all deletions into pRB14512MYC where digestion with *BamH1* and *Pst1* released a fragment of the expected size of inserts from Figure 8 and Figure 12. 10µg of digested plasmids were electrophoresed on an 0.8% agarose gel at 85V. The first lane represents 1kb DNA ladder, and the last was uncut plasmid. Digestion was done with first, *BamH1* that left O/N. The following day, *Pst1* was left four hours before running the gel.

3.3. Expression and purification of Hif1 truncated mutants in *S.cerevisiae*

Having generated an expression vector for Hif1 clones, the next step was to determine if the proteins were expressed. Plasmids encoding full length and Hif1 deletion mutants were transformed into a strain of *Saccharomyces cerevisiae* that is also Hat1-TAP, and is deleted for both *HIF1* and *LEU2*. The LEU2 selectable marker encoded on pRB415-12MYC allows transformants to grow on -LEU media. The empty plasmid and full length 12MYC-Hif1 were transformed into both wild-type, and Hat1-TAP. The C-terminal TAP epitope tag on Hat1 includes the Protein A epitope tag that has affinity for mammalian IgG (See section 3.4). The expected total number of nucleotides and predicted molecular weight sizes for all Hif1 clones were calculated as shown in Table3.

Table 3: The total number of nucleotide base pairs and predicted molecular weight of Hif1 mutants without 12-MYC.

C-terminus mutants External deletions	Nucleotides	MW	**N-terminus mutants Internal deletions**	Nucleotides	MW
C1= Δ(B.P.)	1134bp	41.96kDa	**N1=** ΔTPR1	1056bp	39.07kDa
C2= Δ(+ TPR4 +35)	1050bp	38.85kDa	**N2=** ΔTPR2- 1st	1086bp	40.18kDa
C3= Δ(+ TPPR4)	945bp	34.96kDa	**N3=** ΔAcd	833bp	30.82kDa
C4= Δ(+ TPR4 Domain)	837bp	30.97kDa	**N4=** ΔTPR2- entire	658bp	24.35kDa
C5= Δ(+ TPR3 Domain)	631bp	23.35kDa	**N5=** ΔTPR3	1056bp	39.07kDa
C6= (F.L.)	1158bp	42.85kDa	**N6=** ΔTPR4	1056bp	39.07kDa

After transformation, I verified proper protein expression of the MYC-tagged Hif1 clones, and compared to controls. To this end, the whole cell lysate was obtained from yeast

strains grown in –LEU media plus glucose as a source of carbon expressing the Hif1 truncated mutants and controls. Whole cell extracts were loaded onto 10% SDS-PAGE gels at 100V, followed by protein transfer to nitrocellulose. When immunoblotting was performed, I blotted with α-TAP (Thermo scientific) antibody that recognizes the TAP epitope tag on Hat1. Similarly, I blotted with α-MYC (Santa Cruz Biotechnology) antibody which recognizes the N-terminal 12xMYC tag to verify that the Hif1 clones, which were transformed into pRB415-12MYC, were actually being expressed in *S. cerevisiae*.

I examined the cloned external and internal Hif1 mutants, and they all were effectively expressed at sizes that matched the predicted molecular weight (Figure 15 and 16 compare to Table 3). These results directed a further step of affinity purification to determine whether a particular HIF1 mutant remained able to physically interact with Hat1.

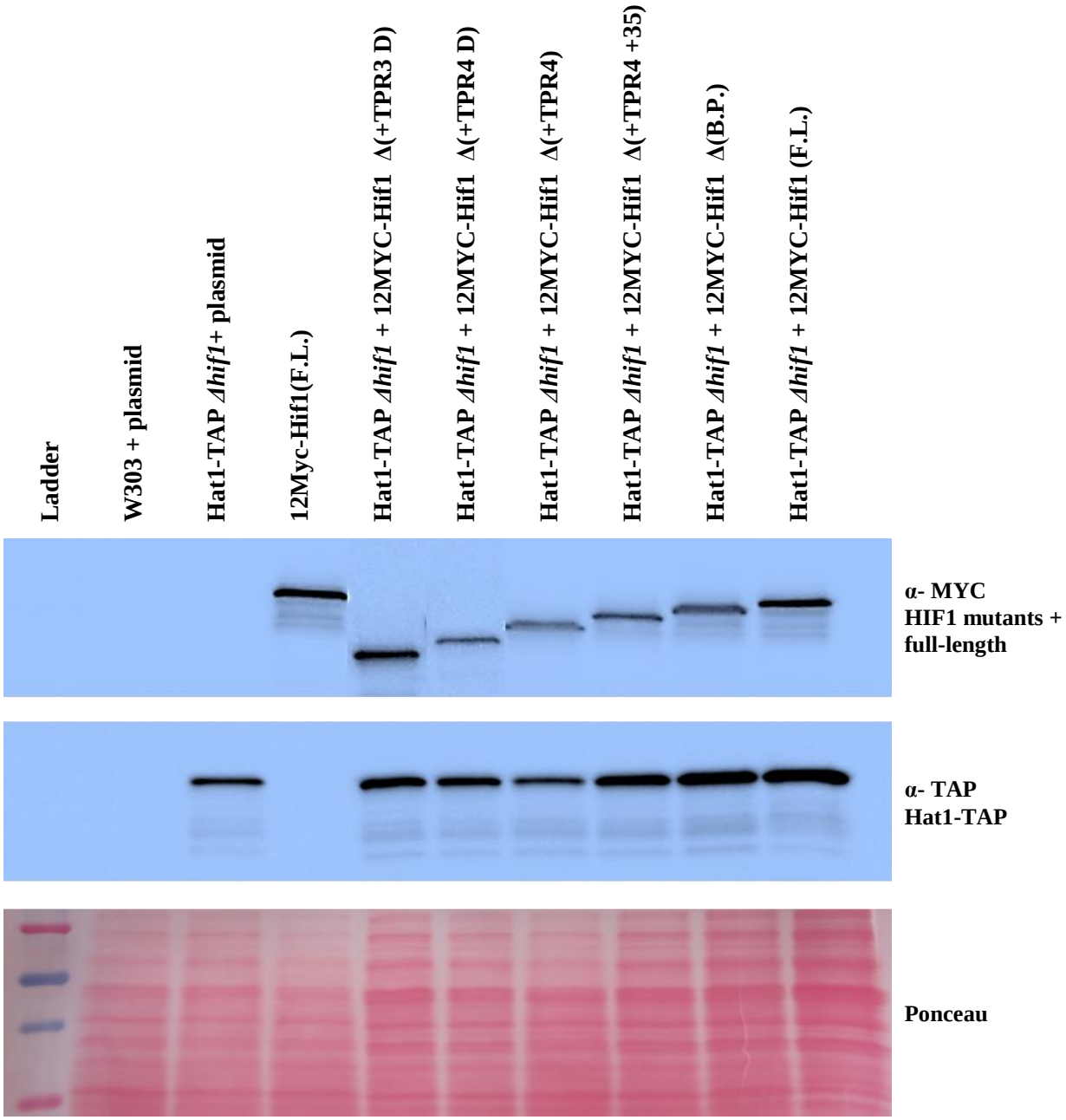


Figure 15: Successful protein expression in yeast of constructed external deletion mutants of Hif1.

WCEs from indicated strains were separated by 10% SDS/PAGE, and transferred to a nitrocellulose membrane. The membrane was stained with Ponceau stain to ensure equal loading of WCE (lower panel) and analyzed by immunoblotting with the antibodies shown to right of each panel.

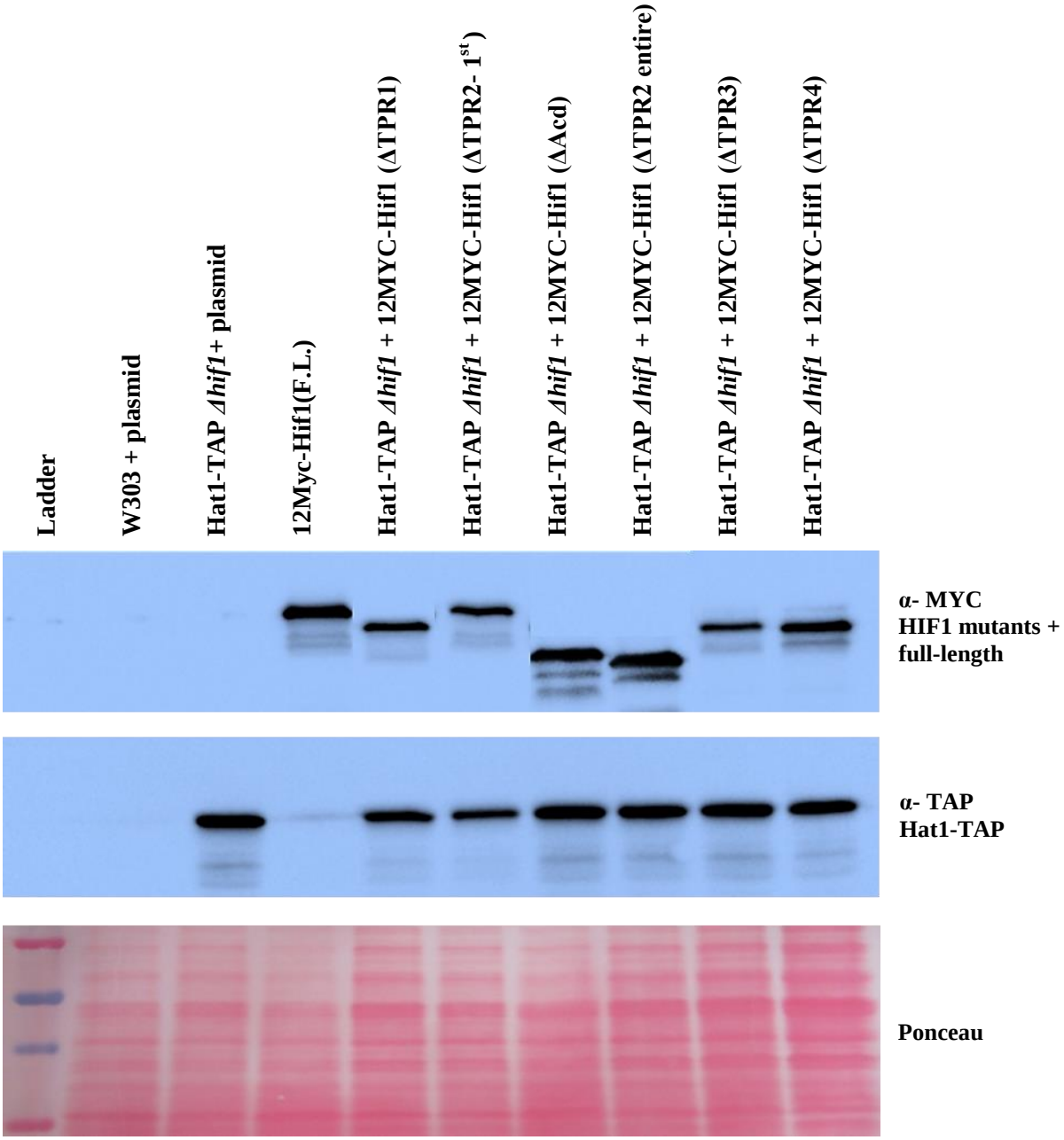


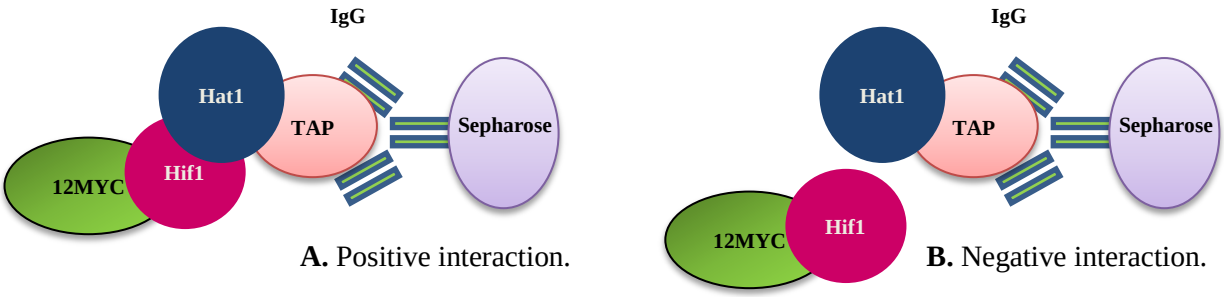
Figure 16: Successful protein expression of constructed internal deletion mutants of Hif1.

WCEs from indicated strains were separated by 10% SDS/PAGE, and transferred to a nitrocellulose membrane. The membrane was stained with Ponceau stain (lower panel) and analyzed by immunoblotting with the antibodies shown to right of each panel.

3.4. Co-Immunoprecipitation strategy

As a first step toward identifying which Hif1 truncation mutants physically interact with the Hat1 complex, I performed immunoprecipitation experiments with soluble extracts from yeast strains that express, MYC-tagged forms of each of the Hif1 truncation mutants, as well as Hat1-TAP from it's the genomic locus. As mentioned previously, all Hif1 truncation mutants including full length were tagged at the N- terminus with 12XMYC whereas Hat1 was tagged at C-terminus with TAP. Hat1-TAP should cross-react with anti-TAP antibodies. The chromatography matrix IgG sepharose beads have affinity for protein A of the TAP tag. Subsequently, TAP pulls down any interacting partners like HIF1 with Hat1-TAP while any proteins not "precipitated" by Protein A will be washed away (Figure 17). I predict Hif1 would not interact with Hat1 if one of the deletions I made removed the Hat1-integrating domain.

To determine what part of HIF1 interacts with Hat1, IgG-Sepharose, immunoprecipitates (IPs) were examined along with input WCE by immunoblotting with antibodies against MYC and TAP to test the effect of Hif1 deletions on its ability to interact with Hat1.



A. Positive interaction. **B.** Negative interaction.

Figure 17: One-Step Affinity Purification Scheme.

A. Hif1-MYC interacts with Hat1 as an example of Hif1 truncation mutants; interaction is revealed by immunoprecipitation with Anti-TAP antibodies followed by western blotting.
B. No interaction is shown between Hif1-MYC and Hat1 due to removal of Hat1-interacting domain.

3.5. Co-Immunoprecipitation studies of internal and deletion mutants of HIF1

After optimizing protein expression of deletions constructed, I moved on to investigate which domain of Hif1 was essential for Hat1 complex. To solve this problem, I performed immunoprecipitation analysis with soluble extracts collected from both external and internal constructed mutants in two separate sets.

3.5.1. C-terminal tail of Hif1 is not needed for Hat1 interaction

Initially, co-immunoprecipitation of Hif1 external deletions from Hif1 C-terminus were performed to investigate whether the Hif1 C-terminus is required for Hat1 interaction. To confirm that the experimental conditions were appropriate I used full-length 12XMYC Hif1 as a positive control and empty plasmid as a negative control. To confirm the specificity of the antibodies, wild-type+ full length Hif1 was used to test for MYC cross-reactivity and the Hat1-TAP Δ*hif1+* plasmid was used to examine for TAP cross-reactivity.

Immunoprecipitates and input materials were analyzed by immunoblotting with antibodies against MYC and TAP. In order to confirm the expression of controls and truncated mutants, 15% of the entire materials were saved as “inputs” (Figure 18). The input results provided evidence that the experiment was working in that expected proteins were expressed. Furthermore, every single one of C-terminal MYC-tagged truncation mutants were immunoprecipitated by Hat1-TAP (Figure 19), suggesting that C-terminal fragments of Hif1 which includes TPR3, TPR4, and the basic patch at C-terminus are dispensable for Hif1 interaction with the Hat1 complex. Not that full length Hif1 was only co-purified if Hat1-TAP was present showing my experimental conditions were appropriate.

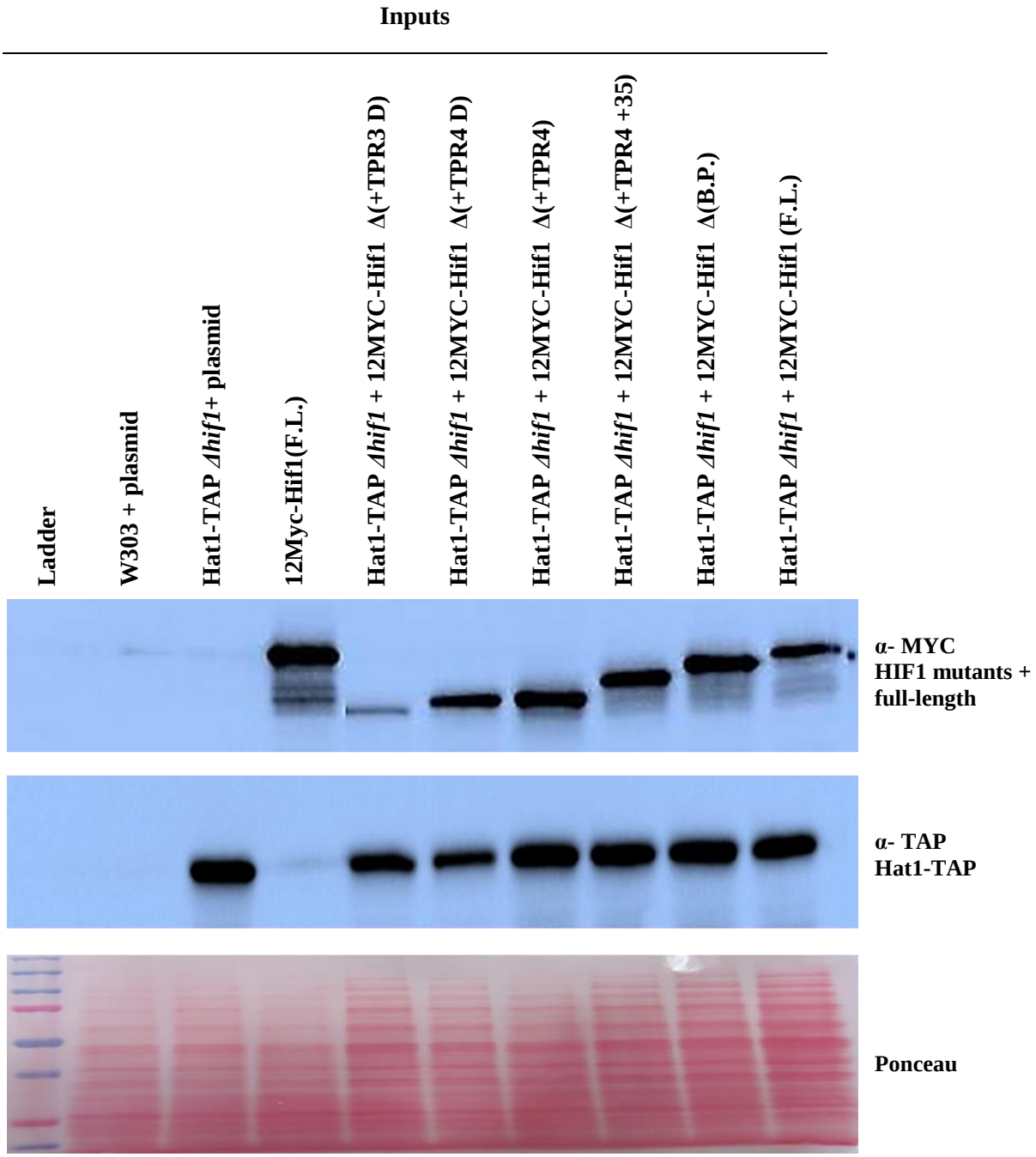


Figure 18: Western blotting of input fractions of Hif1 C-terminal (external) deletions constructs.

Soluble cell extracts of yeast expressing Hif1 C-terminal deletions and controls strains. Inputs were separated by electrophoresis on 10% SDS/PAGE, and transferred to a nitrocellulose membrane. The membrane was stained with Ponceau stain (lower panel). The blot was then probed by immunoblotting with antibodies shown to right of each panel.

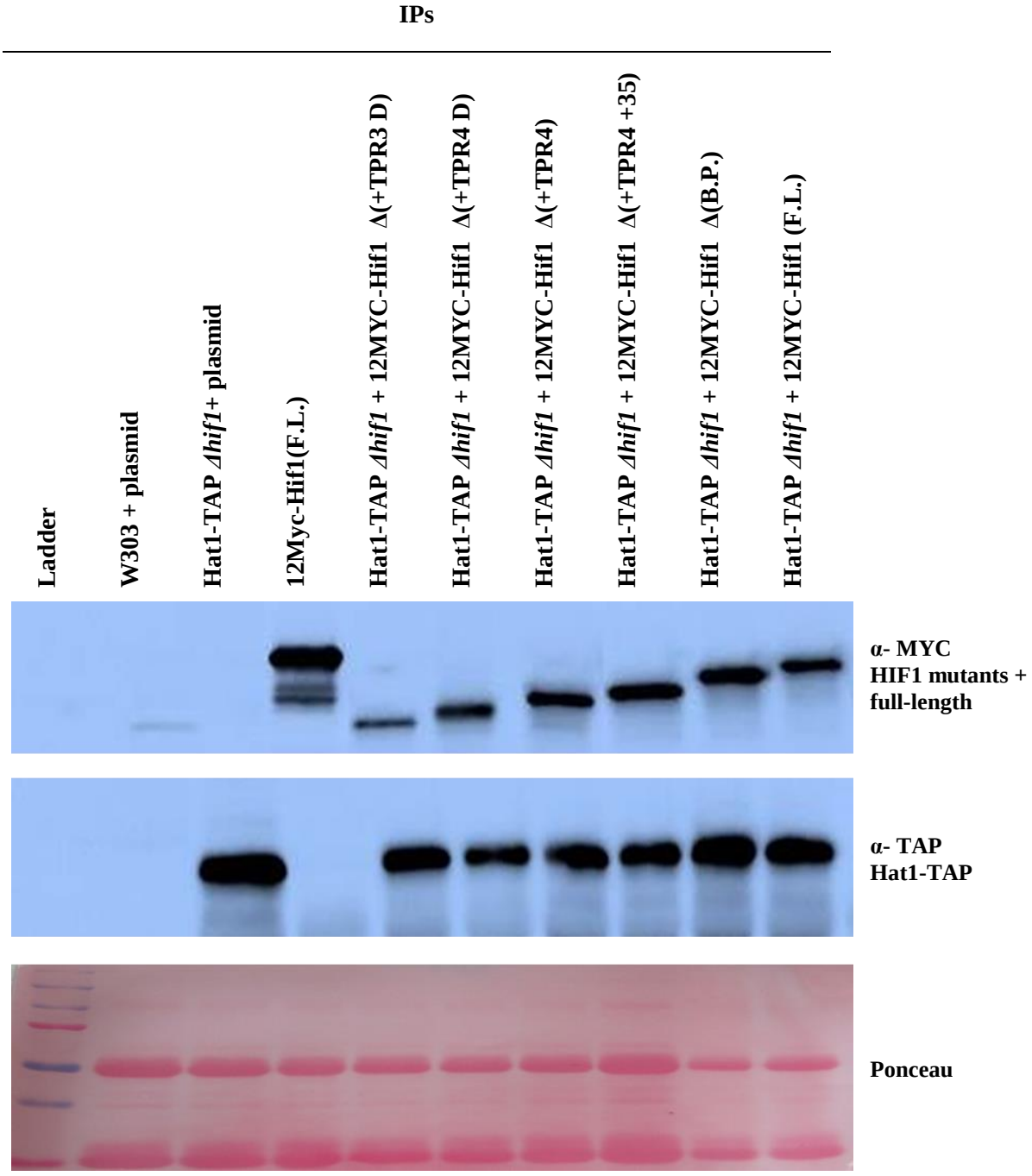


Figure 19: Western blotting of IP fractions of Hif1 C-terminal (external) deletions constructs.

Soluble fractions from each cell line were immunoprecipitated were separated by 10% SDS/PAGE, and transferred to a nitrocellulose membrane. The membrane was stained with Ponceau stain (lower panel). IPs were probed with indicated antibodies.

3.5.2. Deletion of the acidic region within TPR2 resulted in the loss of Hif1-Hat1 interaction

Investigating which domain from Hif1 is interacting with Hat1 complex became even more interesting since my previous data thus far does not show any remarkable finding. The second blot was performed as described previously. To assess whether any of the internal mutants is physically associated with Hat1, I analyzed the immunoprecipitated soluble fractions of Hif1 mutants by immunoblotting with antibodies against MYC and TAP.

Consistent with the previous result, an internal deletion of TPR3 or TPR4 did not prevent Hat1 interaction, as well as deletion of TPR1. However, deletion of the TPR2 acidic patch or the whole domain did prevent Hat1 interaction (Figure 21). Note that deletion of the first part of TPR2 did not affect the Hif1-Hat1 interaction emphasizing the importance of the acidic patch (Figure 21).

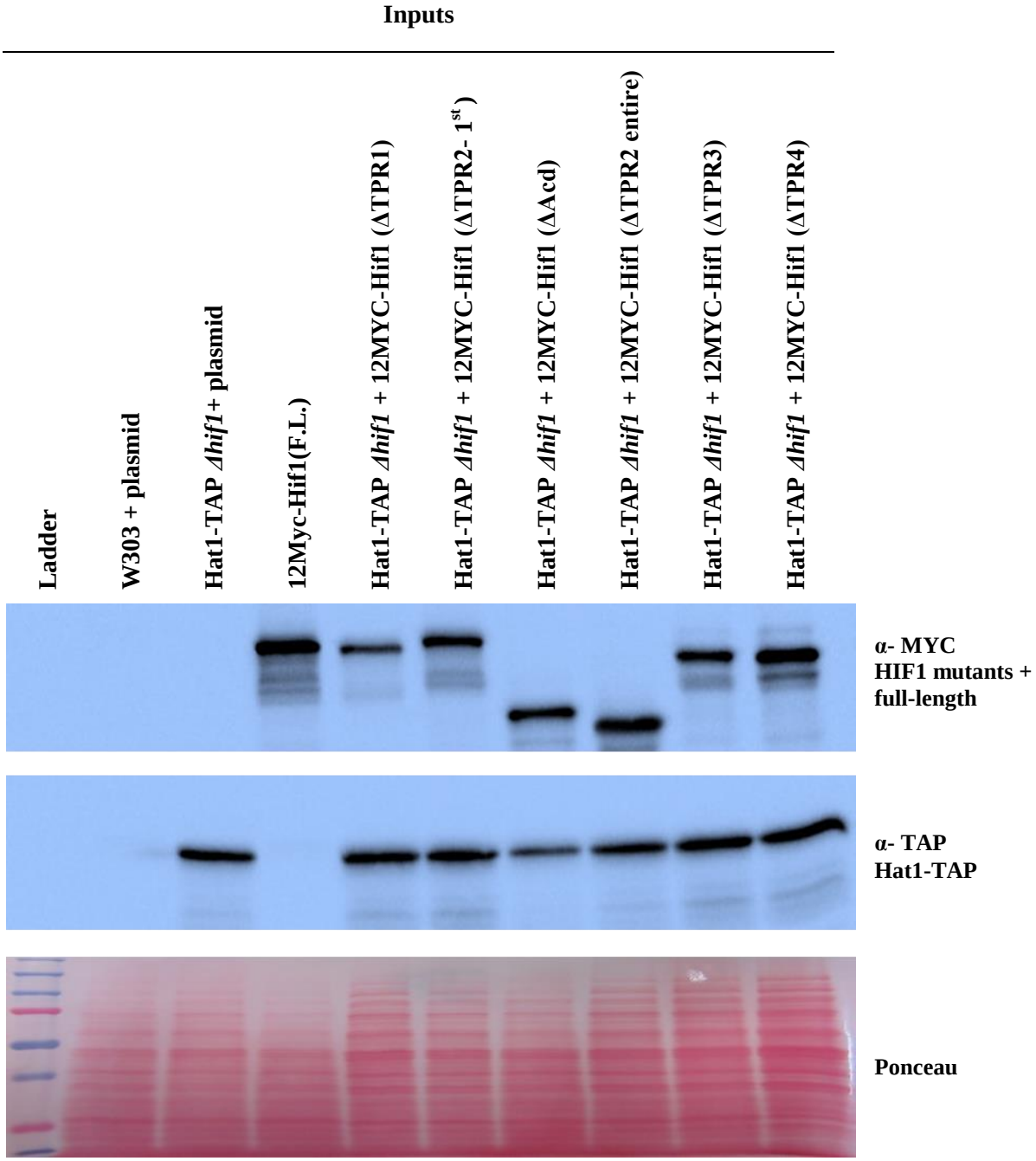


Figure 20: Western blotting of input fractions of Hif1 internal deletions constructs.

Soluble cell extracts of yeast cells expressing Hif1 C-terminal deletions and control strains were separated by 10% SDS/PAGE, and transferred to a nitrocellulose membrane. The membrane was stained with Ponceau stain (lower panel). Inputs were probed by immunoblotting with antibodies shown to right of each panel.

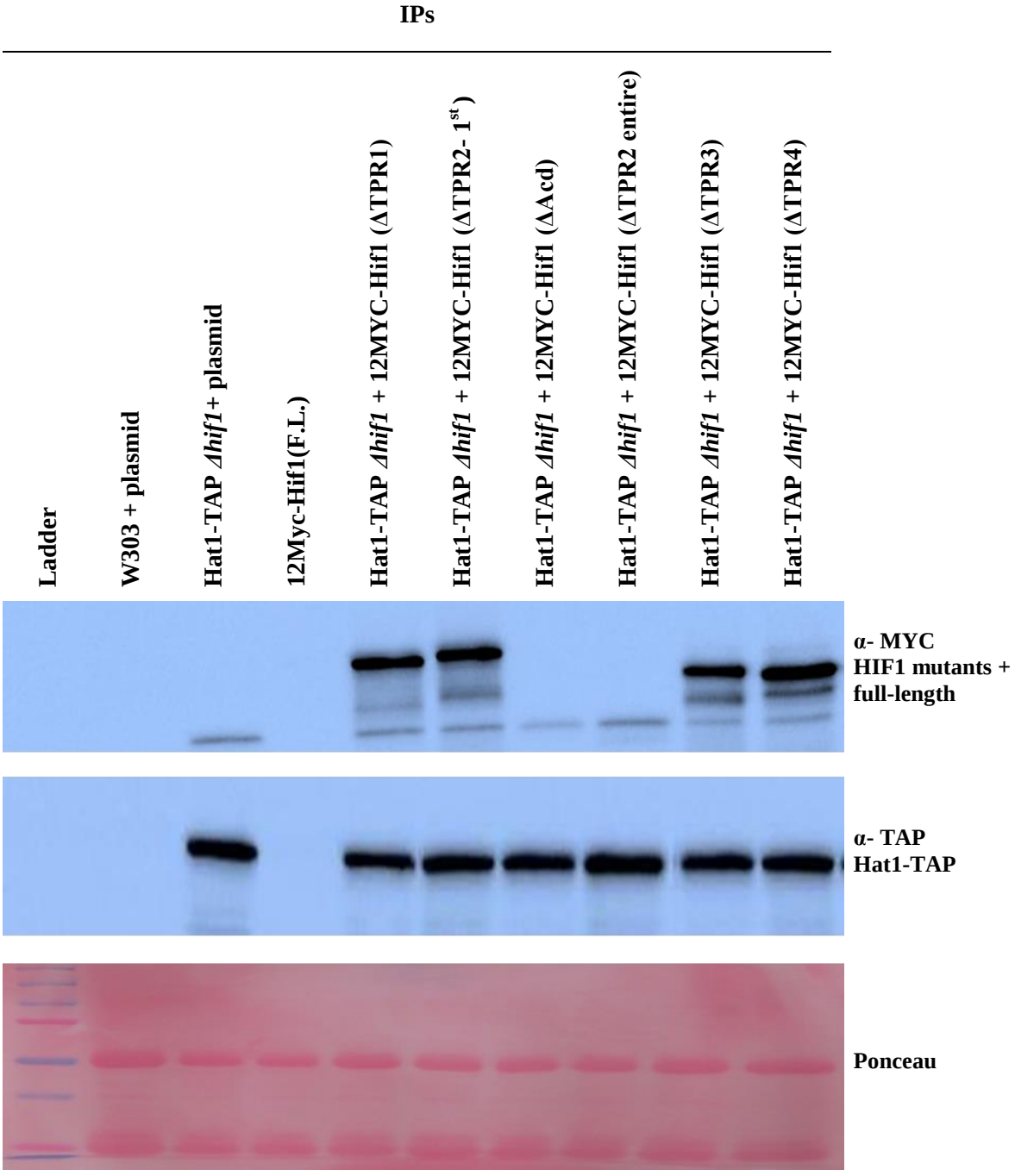


Figure 21: Western blotting of IPs fractions of Hif1 internal deletions constructs.

Soluble fractions from each cell line were immunoprecipitated, and were separated by 10% SDS/PAGE, and transferred to a nitrocellulose membrane. The membrane was stained with Ponceau stain (lower panel). IPs were probed with indicating antibodies.

Altogether, this experiment can be summarized as following:

1. C-terminal truncation mutants (external deletions):

In the co-immunoprecipitation assay, all of the C-terminal deletions do not show any significant loss of the interaction (Figure 20). Therefore, the entire C-terminus tail including TPR3 and TPR4 is dispensable for Hif1-Hat1 interaction.

2. Internal deletions:

In the co-immunoprecipitation assay, most of the internal deletions do not affect the interaction. However, my closing data showed that Hif1 and Hat1 interaction was not recovered with ΔAcd and ΔTPR2 'entire' which together suggests that the deletion of the acidic region resulted in disrupting the interaction with Hat1 (Figure 21). Therefore, the acidic region is crucial for the Hif1-Hat1 interaction.

3.6. Functional Analysis of Hif1

3.6.1. *hif1* and *hat2* mutants are more sensitive to histone overexpression than hat1 mutants.

Our lab is interested in the regulation of gene expression of histone genes. It has previously been shown that over-expression of core histone genes are deleterious for yeast (Gunjan and Verreault, 2003). It has also been shown that over-expressing histones in strains carrying deletions of genes whose protein functions in the regulation of gene histone expression causes the strain to be quite sick. Therefore, to determine whether Hif1, Hat1 and/or Hat2 have a role in histone metabolism, I tested the phenotype of histone overexpression on wild-type and the mutant strains.

Strains carrying either an empty vector or a plasmid encoding H3 under the control of a galactose inducible promoter were plated on both glucose and galactose media in 5-fold serial dilutions (See Materials and Methods). These plates were as well lacking uracil (YNB, -URA) to enable continual selection of the plasmids in yeast (See Materials and Methods). For a negative control, I transformed all strains including wild-type into empty plasmid to compare the differences and similarities of the final outcome.

Results presented in Figure 22 indicate that there was no variance detected in the growth of wild-type cells on glucose and galactose. On the other hand, *hif1Δ* and *hat2Δ* mutant cells were more sensitive to histone over expression by their reduced ability to grow on galactose. This effect was not observed for empty plasmid on galactose. Furthermore, *hat1Δ* mutant cells showed moderate growth defect on galactose in presence of H3 plasmid, but was not as slow as for *hif1Δ* and *hat2Δ* mutant cells.

Moreover, *lsm1Δ* mutant cells have been shown previously to be hypersensitive to histone over expression and were used as a positive control in this experiment (Herrero and Moreno, 2011). The *lsm1Δ* mutant cells, as expected, showed an extreme hypersensitivity to histone over expression, which demonstrated that the experiment was performed successfully (Figure 22).

Results suggest that Hif1 and Hat2 are involved in the regulation of histone levels possibly to buffer of excess soluble histones. That means within *hif1Δ* and *hat2Δ* mutant cells, the increased level of excess histones in the cell resulted in cell toxicity.

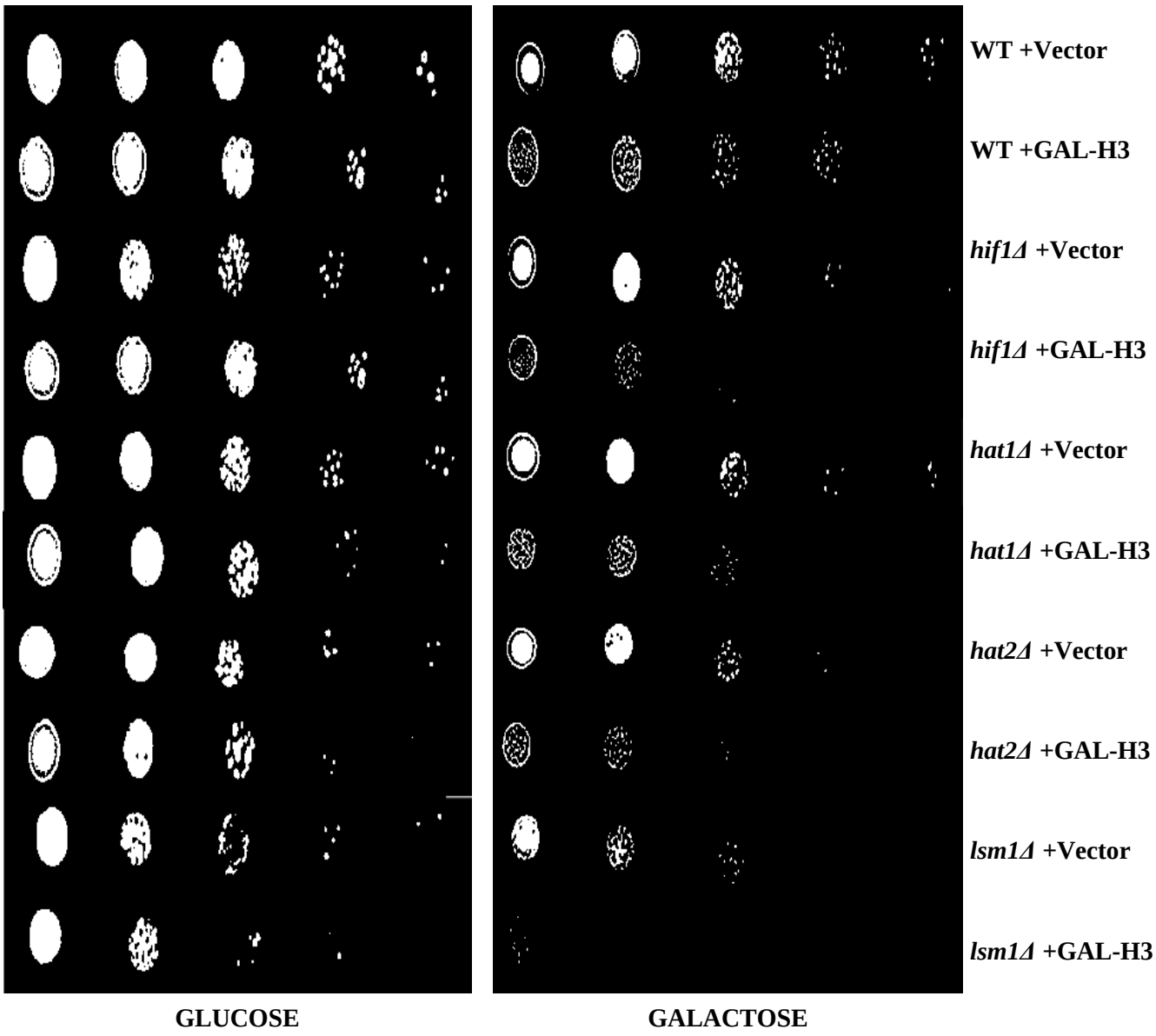


Figure 22: hif1 and hat2 mutants are sensitive to histone overexpression.

Strains were transformed to an empty vector (pYES2) and a vector encoding galactose inducible H3. Cells were grown in medium minus uracil. 2% raffinose was added as a carbon source. H3 expression was induced by adding 2% of galactose for 4 hours. When they reached 0.7 – 0.8 OD_{600}, 5µl drop of 5-fold serial dilutions of each strain were spotted on media without uracil containing either glucose or galactose. Plates were incubated at 30°C and photographed after 3-4 days of growth.

3.6.2. Hat1-complex members exhibit very mild sensitivity to growth on medium containing Hydroxurea (HU)

Hypersensitivity to genotoxic agents such as hydroxurea (HU) can cause defects in the activation of DNA damage checkpoints which leads to defects in DNA repair (Gunjan and Verreault, 2003). I therefore examined *hif1Δ*, *hat1Δ* and *hat2Δ* sensitivity to HU.

In this assays, wild-type cells were used as a negative control as well. Furthermore, Lsm1, which plays a role in replication-dependent histone mRNA degradation and previously showed hypersensitivity to DNA-damaging drugs (Herrero and Moreno, 2011), was used as positive control for these experiments. Plates used in this experiment were treated with 50mM Hydroxurea (HU) and lacking uracil (YNB, -URA) to allow selection of plasmids in yeast (See Materials and Methods). Finally, mutant cells were plated on glucose and galactose media in 5-fold serial dilutions (See Materials and Methods).

In this case, deletion of *HIF1*, *HAT1* and *HAT2* resulted in a very mild sensitivity to the DNA-damaging agent. Data suggests that all Hat1-complex members could be important for the activation of DNA damaging checkpoints (Figure 23).

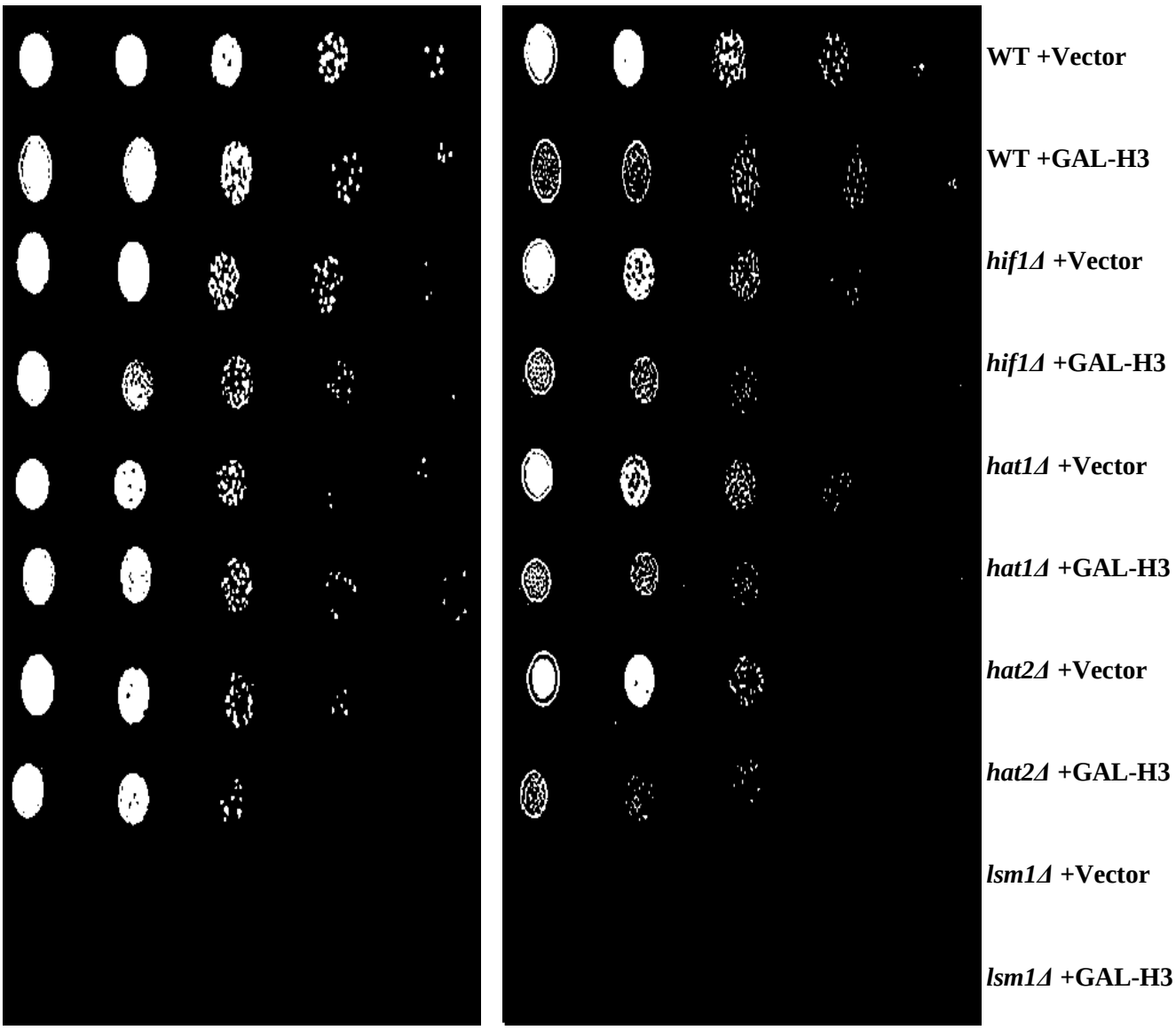


Figure 23: Hat1-complex members show mild sensitivity to the DNA-damaging drug.

Mutant strains were used to check sensitivity to growth in HU-containing medium. Cells were grown in medium minus uracil. 2% raffinose was added as a carbon source. When the cultures reached 0.7 – 0.8 OD_{600}, 5µl drop of 5-fold serial dilutions of each strain were spotted on media without uracil containing glucose or galactose plus 50 mM HU. Plates were incubated at 30°C and photographed after 3-4 days of growth.

3.6.3. Most of the internal and external Hif1 deletions are sensitive to histone overexpression.

To determine whether the ability to bind Hat1 is important for resistance to histone overexpression, I collected all my internal and external deletion Hif1 mutants, as well as full-length Hif1, and transformed them along with a plasmid encoding H3 under the control of a galactose inducible promoter and transformed then into *hif1Δ* yeast. Subsequently, they were plated on glucose and galactose media in 5-fold serial dilutions (See Materials and Methods). The absence of both uracil and leucine (YNB, -URA -LEU) was to permit selection of both plasmids in yeast (See Materials and Methods). I did not transform empty plasmid for this round of expression as already showed the GAL defect in *hif1Δ* is specific to H3 overexpression.

Results observed in this expression with internal and external Hif1 deletion mutants do not correlate with a simple model where ability to bind Hat1 is affected or causes the defect in resistance to histone overexpression (Figure 24). If that were the case, I would expect to see the same phenotype as *hif1Δ* only for the deletion of the acidic region and the complete deletion of TPR2. Instead I observed the same phenotype as *hif1Δ* for the most of the mutants. The only mutant that rescued the *hif1Δ* phenotype (along with full-length Hif1) is ΔTPR4 the internal deletion of TPR4 (Figure 24). Thus I can say TPR4 is not required for resistance to histone overexpression.

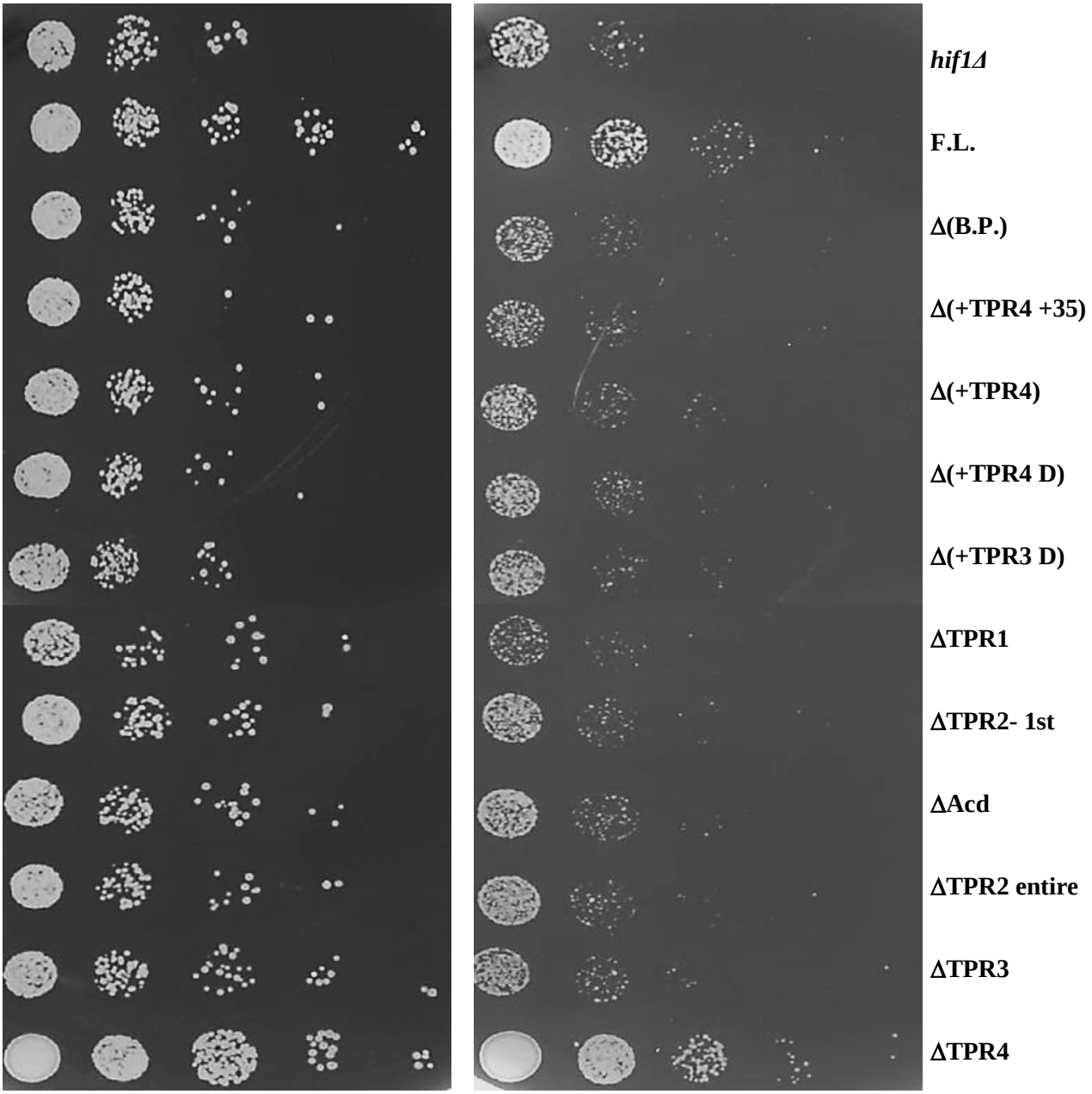


Figure 24: hif1 internal and external deletion mutants are sensitive to histone overexpression.

Strains were transformed with a vector encoding galactose inducible histone H3. Cells were grown in medium minus both uracil and leucine containing 2% raffinose as a carbon source. H3 overexpression was induced by adding 2% of galactose for 4 hours. When they reached 0.7 – 0.8 OD_{600}, 5µl drop of 5-fold serial dilutions of each strain were spotted on media without uracil and leucine contains either glucose or galactose. Plates were incubated at 30°C and photographed after 3-4 days of growth.

3.7. Spt2 interacts with Hif1 and other members of NuB4

The global landscape of protein complexes in yeast published by Krogen et al., (2006) showed a possible physical protein-protein interaction exists between Spt2 and two of the subunits of NuB4 (Hat2 and Hif1) in *S.cerevisiae*. To confirm this result, I used PCR and yeast molecular genetic methods to separately add directly to their chromosomal locus a 13xMYC C-terminal epitope tag to the *HAT1*, *HAT2* and *HIF1* genes in a strain of yeast already carrying the tandem affinity purification (TAP) epitope tag on Spt2.

The next step to confirm the Spt2-Hif1 physical interaction and determine whether other members of NuB4 (Hat1 and Hat2) also interact. I again used co-immunoprecipitation (Co-IP) (Figure 26). I generated whole cell extracts from the appropriate strains of yeast grown in YPD, purified Spt2-TAP on IgG-Sepharose, and used SDS-PAGE combined with western blotting with anti-MYC antibody to monitor possible co-purification of MYC-tagged Hif1, Hat2 or Hat1.

In order to further confirm the precise present of Spt2-TAP results, I also employed anti-TAP antibody. A positive control, Asf1-TAP Hif1-13myc (Fillingham et al., 2008), was used in this experiment. To confirm the specificity of the antibodies, Hif1-13MYC alone was used to test for MYC cross-reactivity and Spt2-TAP alone was used to examine for TAP cross-reactivity. Untagged wild-type (W303) was used as negative control.

Detection of proteins was conducted by western blot analysis. All necessary proteins were presented and expressed at the correct molecular weights corresponding to the protein plus epitope tag. Figure 25 represents a western blot analysis of starting material for the Co-IP. This was used to confirm controls and the strains proteins expression.

Conclusive data implies that not only Hif1 physically interacts with the Spt2 but also Hat1 and Hat2 as well (Figure 26). This suggests the possibility that the Spt2 protein coordinates chromatin reassembly behind RNA polymerase II via Hif1.

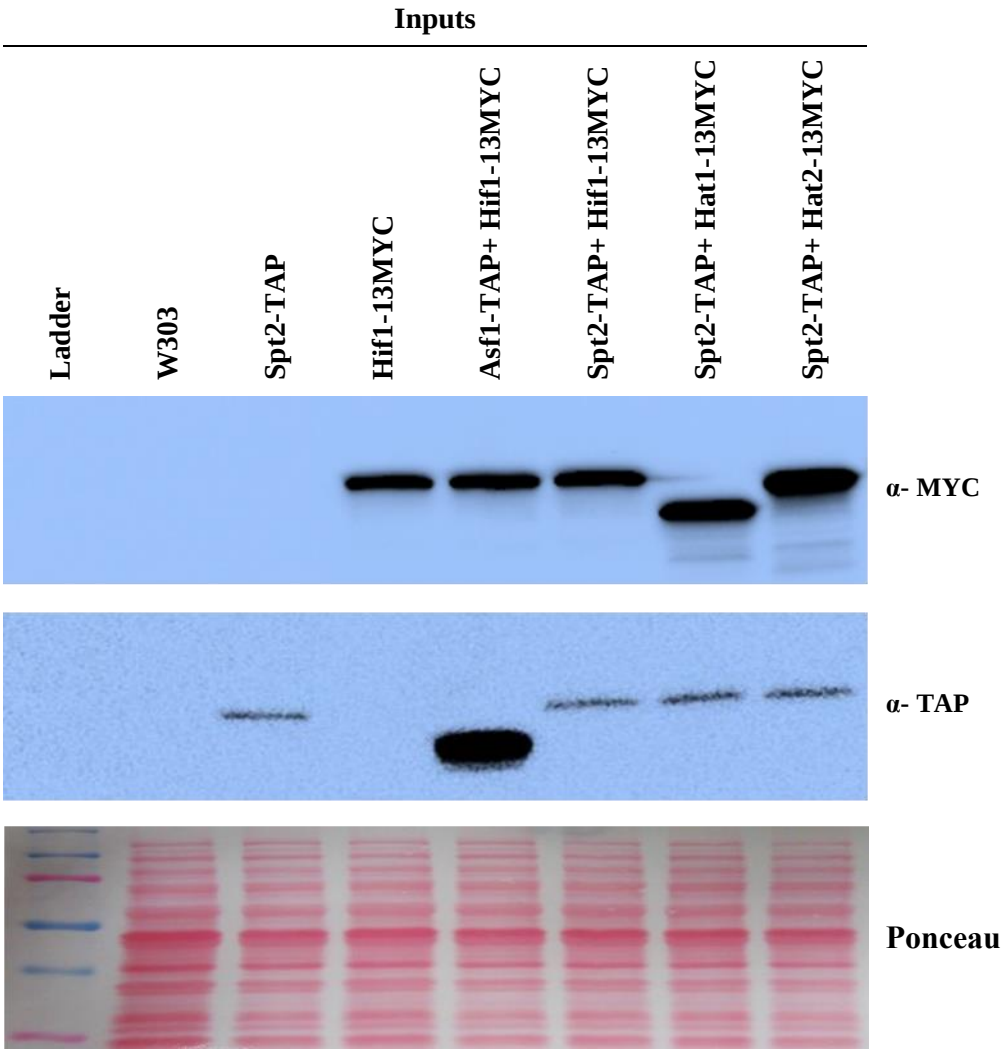


Figure 25: Input materials of Spt2-TAP and other members of Hat1-complex strains.

Soluble cell extracts of yeast cells expressing Hif1, Hat1, Hat2 and control strains were separated by 10% SDS/PAGE, and transferred to a nitrocellulose membrane. The membrane was stained with Ponceau stain (lower panel). Inputs were probed by immunoblotting with antibodies shown to right of each panel.

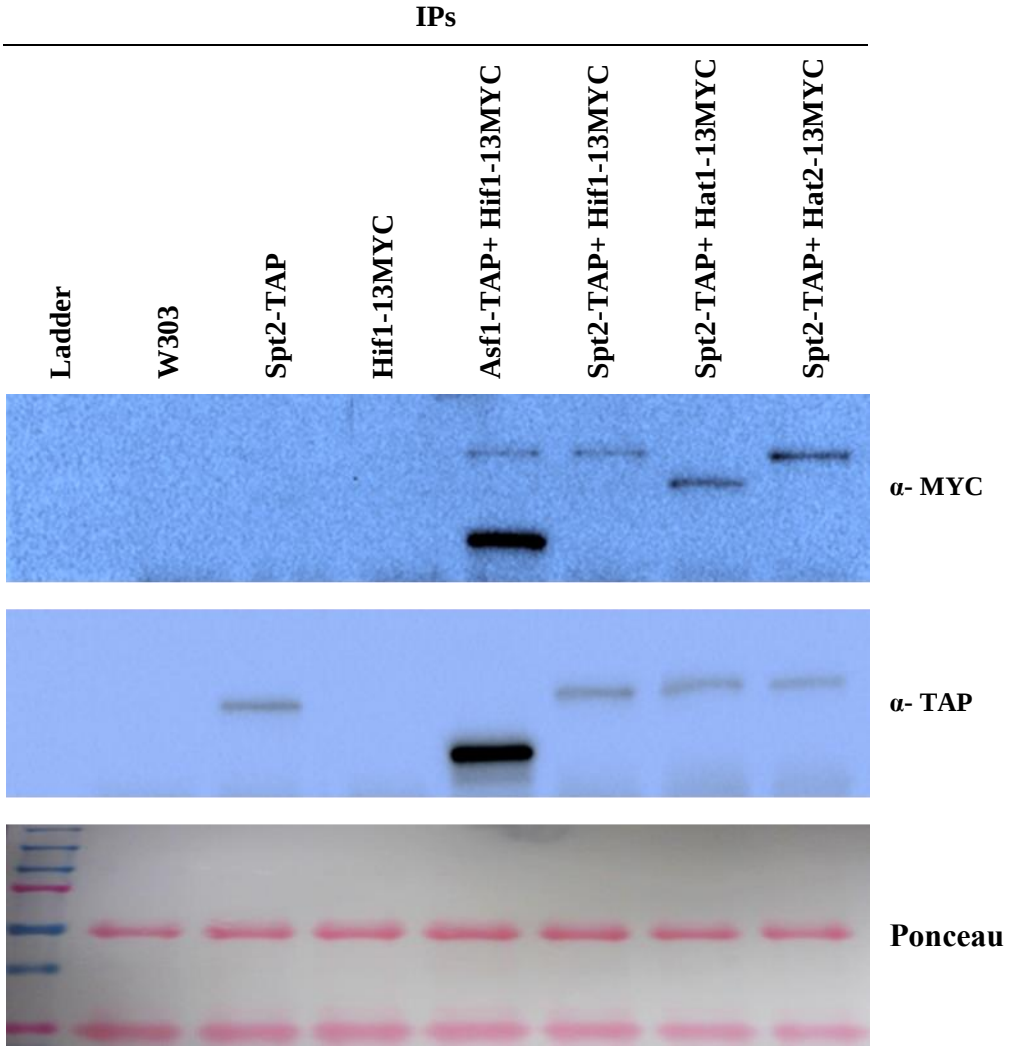


Figure 26: IPs materials of Spt2-TAP and other members of Hat1-complex strains.

Soluble cell extracts after immunoprecipitation were separated by 10% SDS/PAGE, and transferred to a nitrocellulose membrane. The membrane was stained with Ponceau stain (lower panel). Inputs were probed by immunoblotting with antibodies shown to right of each panel.

3.8. *spt2Δ* cells are minimally affected by H3 overexpression whereas the deletion of *SPT2* results in hypersensitivity to DNA-damaging drugs.

Spt2 is known to function in regulating chromatin assembly and gene expression (Nourani et al., 2006). Although in what manner this is accomplished and what is the actual role of Spt2 remains unknown. Consequently, I hypothesized that Spt2, like Hif1 and Hat2, could be also required for degradation of excess free histones that unpackaged into chromatin.

Once again, I analyzed the phenotype of histone overexpression on wild-type and *spt2Δ*. As I did with *hif1Δ*, I transformed *spt2Δ* cells into both an empty vector and a plasmid encoding H3 under the control of a galactose inducible promoter. Then, they were plated on glucose and galactose (Figure 27). Furthermore, I checked whether or not *spt2Δ* cells are sensitive to DNA-damaging drugs by adding 50 mM hydroxurea to the medium (Figure 28).

The *spt2Δ* strain was not sensitive to histone overexpression as shown by its ability to grow on galactose. In presence of H3 the same as with no H3, this implies Spt2 is not necessary for degradation of excess soluble histones. In contrast, cells lacking Spt2 displayed hypersensitivity to DNA-damaging drugs which means that Spt2 may have a role in the regulation of genome stability.

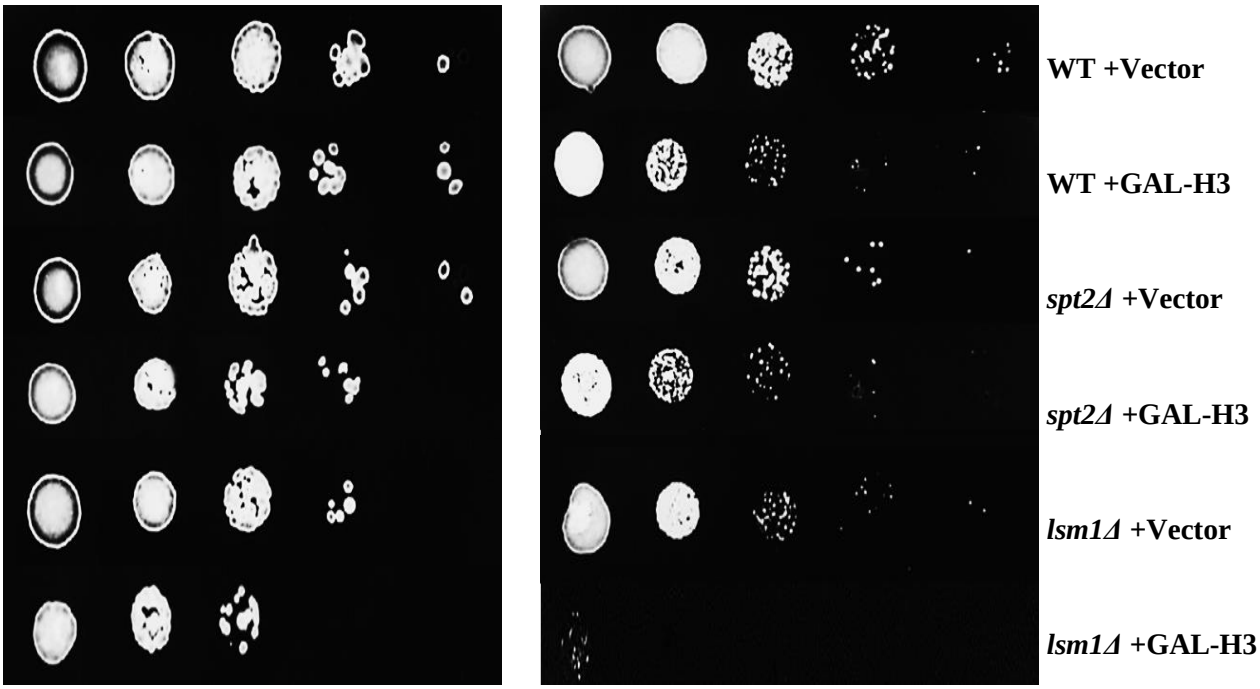


Figure 27: spt2Δ cells are slightly affected by histone overexpression.

Strains were transformed to an empty vector (pYES2) and a vector encoding a galactose inducible promoter. Cells were grown in medium minus uracil containing 2% raffinose as a carbon source. H3 induced by adding 2% of galactose for 4 hours. When they reached 0.7 – 0.8 OD_{600}, 5µl drop of 5-fold serial dilutions of each strain were spotted on media without uracil containing either glucose or galactose. Plates were incubated at 30°C and photographed after 3-4 days of growth.

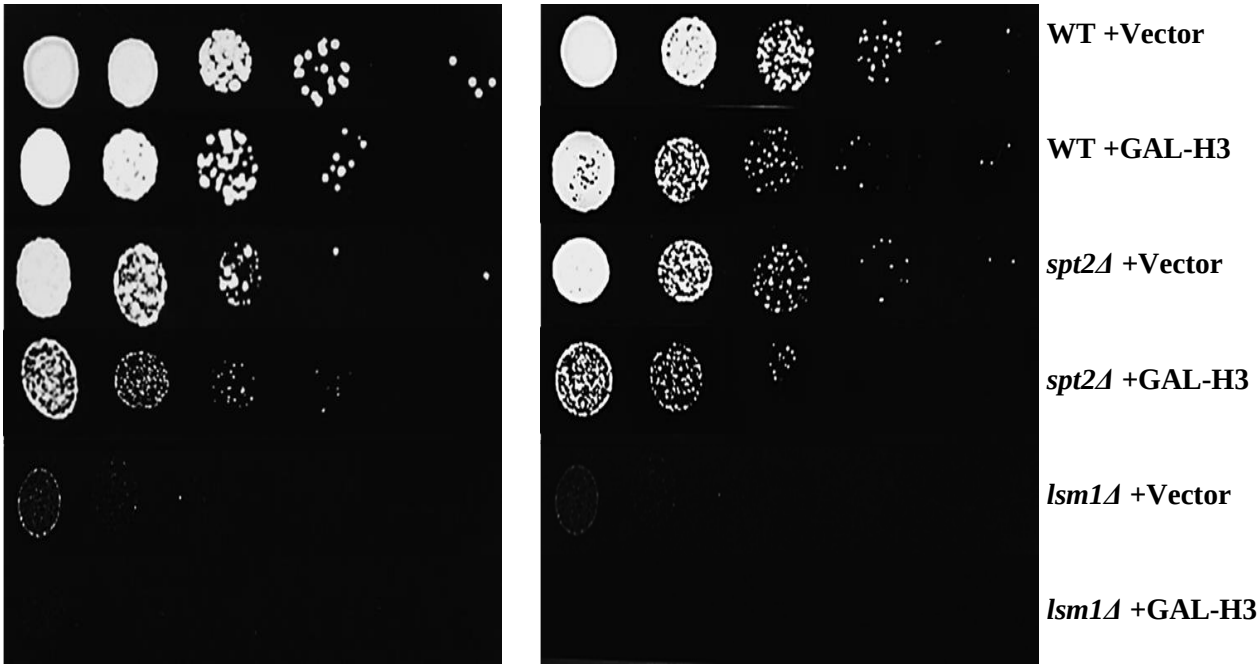


Figure 28: Deletion of SPT2 results in hypersensitivity to DNA-damaging drug.

Mutant strains were used to check sensitivity. Cells were grown in medium minus uracil containing 2% raffinose as a carbon source. When they reached 0.7 – 0.8 OD_{600}, 5µl drop of 5-fold serial dilutions of each strain were spotted on media without uracil contains glucose or galactose plus 50 mM HU. Plates were incubated at 30°C and photographed after 3-4 days of growth.

Chapter 4: Discussion

For several decades, chromatin assembly has been of great interest to many researchers for several reasons. First, through understanding the histone genes which encode major proteins involved in packaging DNA into chromatin in the nucleus. Additionally, through understanding of a series of mechanisms in which nucleosome is assembled to eventually form a chromosome. Finally, through understanding of that the dynamic modification of chromatin can tightly control histone gene expression. Histone modifiers, histone variants, chromatin remodelers and histone chaperones all have critical roles in this genetic network. In this study, I have shed light on the structural and the functional role of a histone chaperone Hif1 in the model organism, *Saccharomyces cerevisiae*.

4.1. Hif1 interacts with the Hat1 complex via acidic domain of TPR2

Although Hif1 is known to be one of NuB4 components (the nuclear Hat1-complex) (Poveda et al., 2004), the domain in Hif1 responsible for Hat1 physical interaction remains unknown. Recently, it has been shown that a deletion of the acidic patch in the TPR2 domain of NASP, the human homolog of Hif1, prevented interaction with the linker histone H1 (Wang et al., 2012). In addition, the fourth domain of NASP was shown to be essential for the physical interaction of NASP with core histone H3/H4.

In order to determine the role of TPR domains in Hif1, I first asked which domain could be essential to interact with the Hat1-complex. Interestingly, the results of the co-immunoprecipitation analysis of the expressed Hif1 mutants with full length Hif1 demonstrated a difference to NASP. None of the C-terminus truncated mutants were important for Hif1-Hat1 interaction suggesting no role for TPR3 or TPR4. In addition, of the internal deletion mutants,

only the acidic region deletion (ΔAcd) of TPR2 interfered with Hat1-binding. Thus the acidic region, ΔAcd (residues 252-564) has been shown in this study to have an essential role for Hif1 interaction with the Hat1 complex. Based on the previous studies and my results, there are many potential domains of Hif1 that can interact with the core histone H3/H4 (e.g. TPR1, TPR2 including the acidic patch, TPR3 and TPR4).

With regard to my results, the binding site target of the acidic patch domain with the Hat1-complex remains an open-ended question. As known, the Hat1-complex consists of four protein subunits including Hif1, Hat1, Hat2 and the core histones H3 and H4. This suggests four possible models for the acidic patch biding site which could be Hat1, Hat2 or the core histones H3 and H4. To determine whether the acidic region of the TPR2 domain binds H3 or H4, future studies should focus on the expression of full-length Hif1 or Hif1 ΔAcd which then can be incubated with core histones H3 and H4 to see if Hif1-ΔAcd can bind H3 and H4 compared to full length Hif1 which should bind H3-H4. In addition, a study similar to the one described in this thesis can determine what domain of Hif1 is potentially responsible for the interaction with Hat2.

4.2. Hif1 and Hat2 are sensitive to histone overexpression (O/E)

In order to ensure precise chromatin assembly, histones synthesis and degradation should be tightly controlled and in balance. The rate of replication fork elongation is decreased when DNA damage occurs during S phase (Tercero and Diffley, 2001). As a result, cell cycle progression involving DNA synthesis slows down until the damage has been repaired (Paulovich and Hartwell, 1995). Consequently, excess histones accumulate directing for an essential repression of histone gene expression or degradation of histone mRNA (Sutton et al., 2001 and

Dominski et al., 2001). Otherwise, the cell will suffer from increased toxicity as result of an excess of histones (Marzluff and Duronio, 2002).

In yeast cells, a protein kinase, Rad53, is required to excess histone degradation, and involved in DNA damaging response as its deletion demonstrated excess histones accumulation and chromosome loss (Gunjan and Verreault, 2003). Moreover, a promoter of histone mRNA degradation, Lsm1, was identified to play a pivotal role in genome stability in *S. cerevisiae* (Herrero and Moreno, 2011). Significantly, Deletion of *LSM1* results in hypersensitivity to histone overexpression which leads to accumulation of free histones as well as a hypersensitivity to DNA damaging agents. Also, Lsm1 is needed to regulate histone mRNA decay (Herrero and Moreno, 2011).

Upon these observations, related work by my colleague, Tanja Durbic, who showed that *Δlsm1* is synthetic sick to synthetic lethal (SS/SL) with *Δhif1* (Durbic and Fillingham, unpublished). I thought that Hif1 might have a dynamic role, in a parallel pathway with Lsm1, in regulation of histone expression. Thus, I examined Hif1 sensitivity to histone overexpression as well as to a DNA damaging drugs such as hydroxurea (HU). Since Hif1 interacts with both Hat1 and Hat2, I included Hat1 and Hat2 to be tested in this assay. Also, because I showed an interaction between Hif1 and Spt2, examination of Spt2 had been considered.

Results of *hif1Δ* and *hat2Δ* mutant cells showed hypersensitivity to histone overexpression, not *hat1Δ* or spt2*Δ*, while mild sensitivity was observed to hydroxurea for all of them except for spt2*Δ* mutant cells which were sensitivity to the DNA-damaging drug suggesting that Spt2 may play a role in the regulation of genome stability.

Taken together, data are consistent with several possibilities that Hif1 and Hat2 are playing a role in any of several potential histone metabolism mechanisms: (1) Function in a pathway upstream of the Rad53 kinase to phosphorylate excess histones and target them for proteolysis. (2) Hif1 and Hat2 could be essential cellular buffers for histones. (3) Important proteins that normally repress histone gene expression such as Lsm1 or Hir1.

In correlation with Hif1-Hat1 complex interaction I initially expected that only the deletion of the acidic patch will only show a growth defect and sensitivity to histone overexpression. Surprisingly, all Hif1 truncated mutants, except ΔTPR4, displayed hypersensitivity to histone overexpression (Figure 24). This data suggests that there are some functions of Hif1 that require all its domains except for the TPR4 domain. I speculate that these domains may be essential for interacting with Hat2 or the core histone H3/H4.

4.3. Spt2 protein interacts with Hat1-complex members, Hif1, Hat1 and Hat2.

Earlier studies have suggested that Spt2, the mammalian HMG-like protein, plays a negative role in transcription regulation and also coordinates histone deposition behind RNA polymerase (Nourani et al., 2006). In this study, I showed that Hif1 interacts with Spt2, and thus I also hypothesized that Hif1 functions in coordinating chromatin re-assembly behind RNA polymerase II and following transcription (Figure 29).

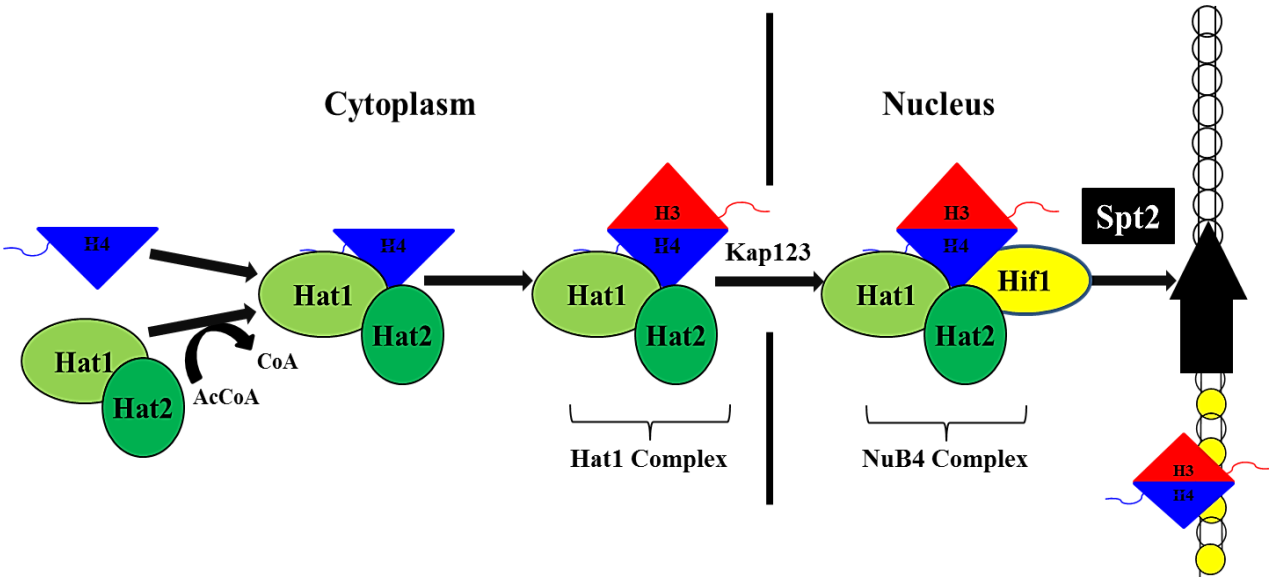


Figure 29: A hypothetical model for Hif1-Spt2 interaction.

This model suggests that Spt2 may direct Hif1to mediated chromatin assembly behind RNA polymerase II and after transcription.

Hif1 interacts with Spt2 behind RNA polymerase to coordinate chromatin assembly through an unknown mechanism. Additionally, Spt2 is also known to localize at the *GAL1* promoter gene under inducing conditions in yeast (Nourani et al. 2006). Furthermore, it regulates the levels of histone H3 over transcribed regions. Hence, Hif1 participates in transcription elongation, chromatin dynamics, and genome stability (Nourani et al., 2006).

To investigate the hypothesis of whether Hif1 is precisely located on the *GAL1* chromatin under inducing conditions, galactose, of transcription, chromatin immunoprecipitation (ChIP) can be used to co-purify DNA sequences with Hif1 protein. If Hif1 localizes to the *GAL1* gene, the *SPT2* gene should be deleted to investigate the theory of *SPT2* recruitment of Hif1 to *GAL1*.

Besides that, via ChIP I can further identify which Hif1 domain is required for the recruitment to GAL1.

4.4. Proposed directions and possible approaches

1. In order to determine what subunit of Hif1p is involved within the H3/H4 histones interaction, Co-IPs should be done to test mutant collections for the ability to bind histones.

2. Investigation of the acidic region internal deletion mutant for the ability to bind with Hat2 by Co-immunoprecipitation of the yeast strain that is Hat2-TAP *hif1Δ* and pRB415-12MYC *hif1Δ*.

3. Since the deletions of HIF1 and HAT2 have shown a phenotype in H3 overexpression, I hypothesize that either they have a direct role in regulation of histone gene expression or they act as histone chaperons. If direct role, Hif1 and Hat2 should specially localize to the *HTA1* gene. This can be assessed using ChIP.

4. Test which domain of the Hif1 protein is involved within the Spt2 interaction.

5. Test the Spt2-Hif1 physical interaction by deletion of Hat1 or/and Hat2 to see whether Hat1 or Hat2 is required for Spt2 interaction.

6. Test F.L. and –B.P. mutants as GFP tagged proteins for nuclear vs. cytoplasmic localization to examine the precise role of the basic patch at C-terminus whether is it an NLS or not.

Appendix A: Sro9

A.1. Introduction

In *Saccharomyces cerevisiae*, Sro9, Suppressor of rho3, was originally found in the cytoplasm and believed to function in transcription and translation events (Tsukada and Gallwitz, 1996 and Rother et al., 2010). In addition, Sro9 also plays a role in gene expression processes by traveling between the nucleus and the cytoplasm. With regards to that, Sro9 is also found to interact with protein complexes involved in the nuclear and the cytoplasmic processes of gene expression which means that Sro9 is associated with actively transcribed genes (Rother et al., 2010).

Moreover, Sro9 shares with Slf1 approximately 29.8% similarity throughout their amino acid sequence (Wolfe and Shields, 1997), and both Sro9 and Slf1 proteins are RNA-binding proteins which belong to a highly conserved La motif-containing proteins family that are found in all eukaryotes (Yu et al., 1994; Yoo and Wolin, 1994 and Wolin and Cedervall, 2002).

Rtt109 is a specific histone acetyltransferas (HAT) that is expressed in S-phase, and has been found to acetylate H3-K56 and H3-K9 (Fillingham et al., 2008). Acetylation of H3-K9 suggest that Rtt109 has an important role in transcription of S-phase specific genes and likely H3 genes, which has variants H3.1 and H3.3 that are different in their expression (Gunjan et al., 2005). The synthetic genetic interaction of the *rtt109Δ* was observed with deletions of the transcription-related genes (Fillingham et al., 2008).

Synthetic lethality was observed with a double mutant of *rtt109Δ* and *sro9Δ* (Fillingham et al., 2008). Therefore, Sro9 is thought to be a candidate in gene expression. If transcription is observed with Sro9 and *rtt109Δ*, then Sro9 is responsible for gene expression. If synthetic

lethality is present with *sro9Δ* and *rtt109Δ*, then it can be postulated that both Sro9 and Rtt109 are likely involved in histone gene expression during S-phase.

A.2. Hypothesis

Due to the diverse role of Sro9 in gene regulation processes of transcription, translation and mRNA stability (Rother et al., 2010) it is probable that Sro9 activates histone gene expression. The purpose of this study is to understand whether Sro9 activates and is responsible for histone gene expression. Another objective of the study is to understand whether Sro9 protects histone mRNA from LSM1 or from the exosome during S-phase.

To understand how Sro9 could prevent mRNA from degradation, a deeper understanding of gene repressors and inhibitors must be considered. LSM1 is an essential protein that is involved in mRNA degradation of replication-dependent histones. LSM1 acts as a histone repressor and antagonizes histone gene expression (Herrero and Moreno; 2011). A deletion of Lsm1 increases the level of histone transcripts and leads to accumulation of histone mRNA (Herrero and Moreno; 2011). Thus, it can be hypothesized that if Sro9 is deleted – even though Lsm1 is still present –the phenotype would be reduced in order to inhibit any antagonizing action among Sro9 and Lsm1. This can further strengthen my hypothesis where Sro9 is involved in histone gene expression.

A.3. Methodology

As stated previously, when histone mRNA expression is changed then the DNA becomes more sensitive to damage. In order to determine this, it must be understood if *sro9Δ* is HU sensitive. A spot test was performed to analyze for these changes.

Yeast strains were inoculated in YPD medium and were grown over-night at 30°C within shaking. The following day, the concentration of each culture was measured and adjusted to about 0.2 OD_{600}. 1/4 serial dilutions were performed of each cell culture. From each one of these dilutions 5 µl drops were spotted onto YPD plates, 50 mM Hydroxurea plates and 100 mM Hydroxurea plates. Plates were incubated at 30°C for 2 days (YPD plates) and 4 days (Hydroxurea plates).

Strains also were transformed with both a vector encoding galactose inducible, tagged histone H3 and the empty vector (pYES2). After transformation, cells were grown overnight in 5ml minimal medium (YNB minus uracil for plasmid selection. 2% raffinose was added as a carbon source. The next morning cells were diluted to about 0.1 OD_{600}. Expression of H3 was then induced when cells reached 0.5 OD_{600} by addition of 2% galactose for about four hours, and were then grown to approximately OD_{600}= 0.7- 0.8. Following that, four-fold serial dilutions of each strain were performed using 96-well plates. Cells were then plated on minimal media lacking uracil on both glucose (H3= OFF) and galactose (H3= ON) as carbon source. The plates were incubated for 3-4 days at 30°C.

A.4. Results

A.4.1. *sro9Δ* mutant cells are sensitive to Hydroxurea.

The spot test demonstrated that *sro9Δ* is in fact HU sensitive, yet *slf1Δ* was not HU sensitive (Figure A.1). This is an interesting observation since both proteins are La-domain containing proteins as well as these proteins belong to a new set of the La motif proteins (Sobel and Wolin, 1999), as their functionalities were thought to be very similar, yet the results are contrary to this belief.

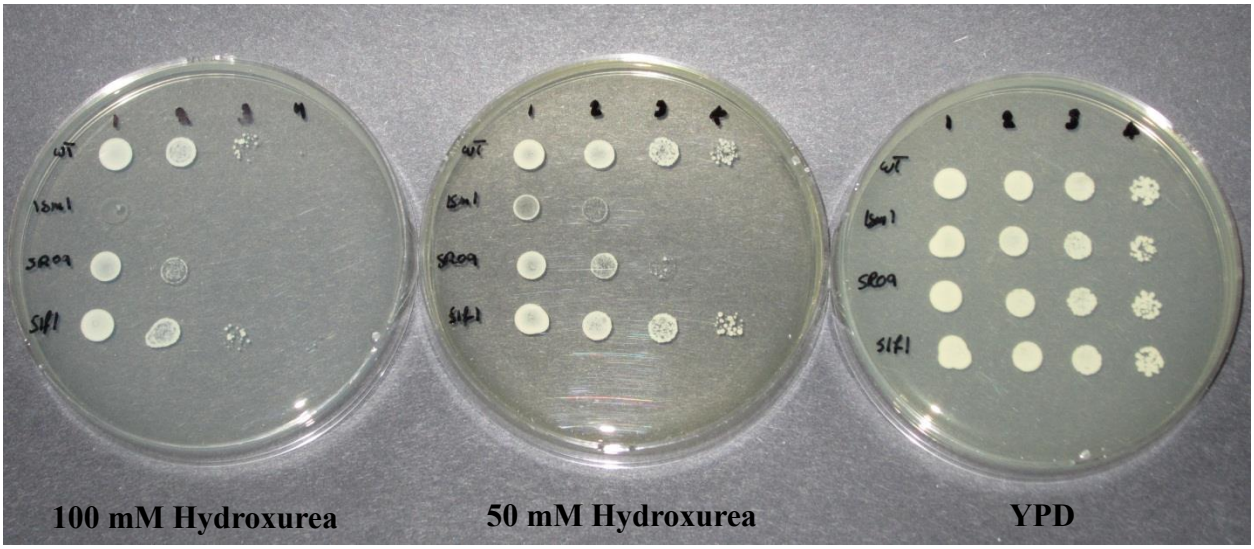


Figure A.1: Deletion of *sro9* is sensitive to DNA-damaging drug (HU).

A spot test was performed in the presence of Hydroxurea 50 mM (low concentration) and 100 mM (high concentration) to examine the sensitivity of different mutants to DNA damage. The sensitivity of *lsm1Δ* cells to HU was very high compared to *sro9Δ*. In contrast, *slf1Δ* cells showed less sensitivity to the DNA damaging drug compared to the wild-type strain.

A.4.2. *sro9Δ/ lsm1Δ* double mutants are hypersensitive to HU

I examined possible growth defect of *sro9Δ/ lsm1Δ* double mutants in comparison to the single mutant strains by performing spot test analysis on glucose as a control and on HU.

The *sro9Δ* single mutant showed intermediate growth defect in contrast to wild type while the *lsm1Δ* mutant cells, as shown previously, displayed a great hypersensitivity to HU. The *sro9Δ/ lsm1Δ* double mutant cells showed an extreme hypersensitivity to HU indicating that Sro9 might have a role, in a similar pathway with Lsm1, in the regulation of histone gene expression.

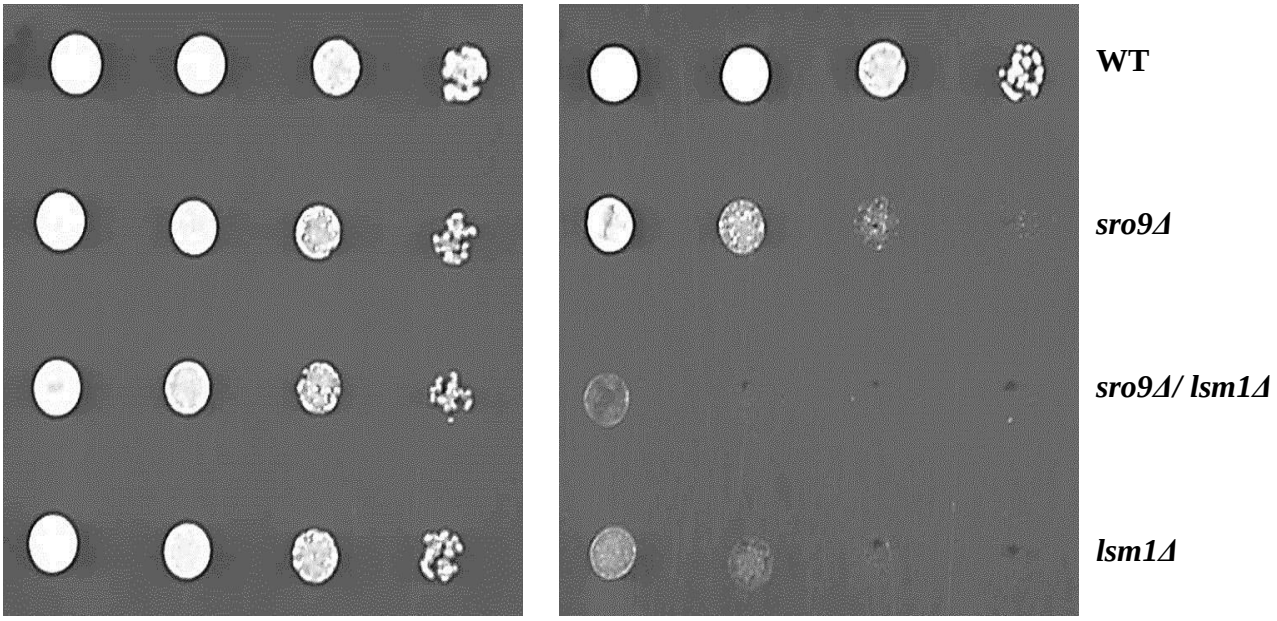


Figure A.2: Deletion of *sro9+ lsm1* resulted in hypersensitivity to DNA-damaging drug (HU).

A spot test was performed in the presence of Hydroxurea 50 mM to examine the sensitivity of different mutants to DNA damage. The sensitivity of *lsm1Δ* cells to HU was very high compared to *sro9Δ*. However, *sro9Δ/ lsm1Δ* cells showed an extreme sensitivity to the DNA damaging drug compared to the wild-type strain.

A.4.3. The growth defect phenotype of *lsm1Δ* mutant is suppressed by Sro9 overexpression

To determine and understand the effects of Sro9 overexpression it is hypothesized that if Sro9 is overexpressed then it would lead to higher activation of the Sro9 gene and lead to a higher expression of histone mRNA (Rother *et al*., 2010). It is expected that if Sro9 is an activator, a higher level of histone mRNA present within these strains. Therefore, if Sro9 is deleted there should be less histone mRNA present.

In *S. cerevisiae*, I was looking for mutations that suppress the toxicity of LSM1 deletion. Interestingly, I found that SRO9 gene is sensitive to histone overexpression. Based on these data, I examined possible defective activation phenotype of *lsm1Δ* mutant by performing spot test analysis on glucose and galactose to determine the phenotype of the histone overexpressed Sro9. I performed this experiment on *sro9Δ* single mutant, *lsm1Δ* single mutant and *sro9Δ/ lsm1Δ* double mutant cells.

Results indicate that due to the role of Sro9 in the activation of gene expression, its deletion lead to a suppression of the Lsm1 phenotype.

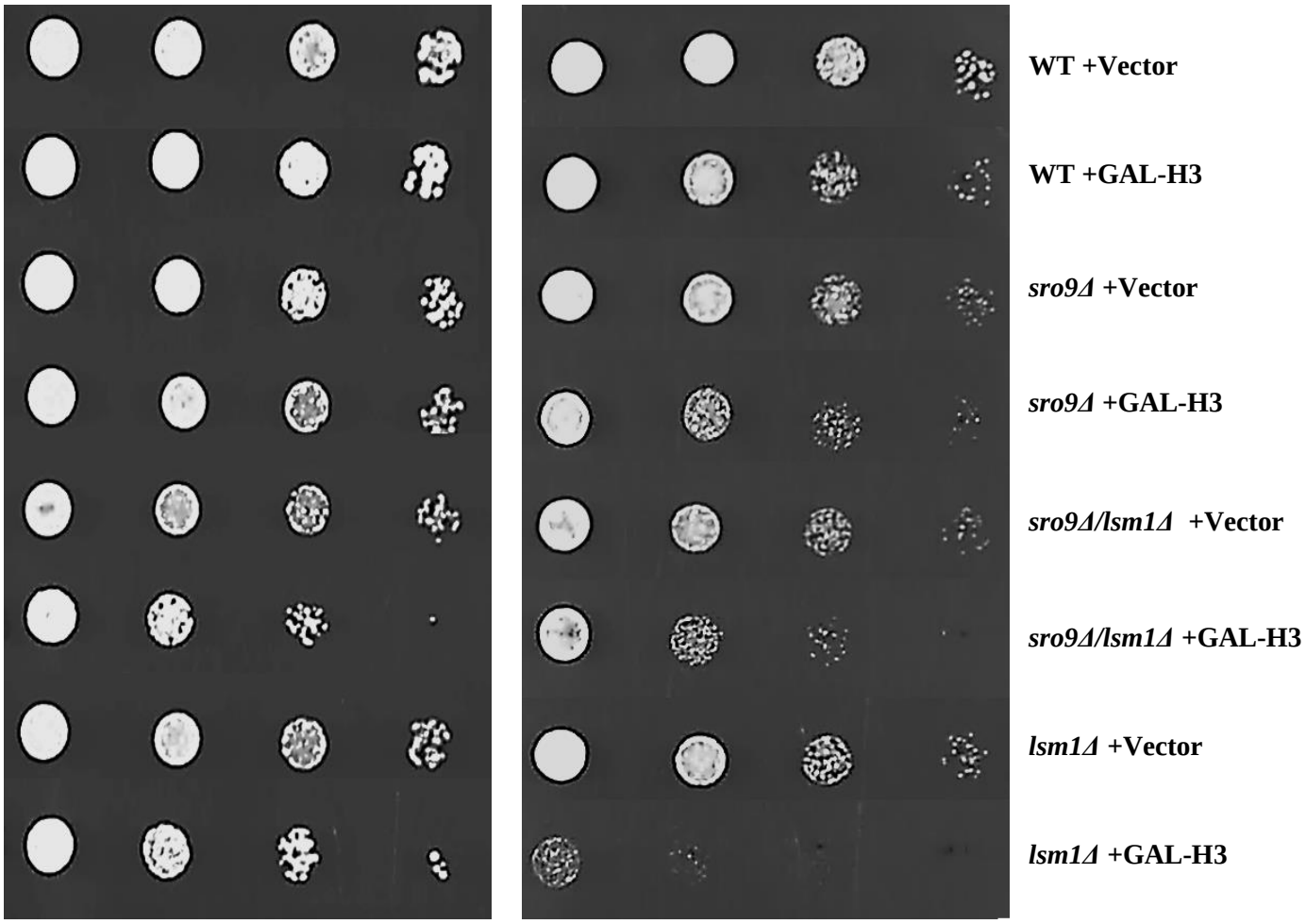


Figure A.3: The toxicity of LSM1 deletion is suppressed by SRO9 overexpression.

Strains were transformed to an empty vector (pYES2) and a vector encoding galactose inducible H3. Cells were grown in medium minus uracil. 2% raffinose was added as a carbon source. H3 expression was induced by adding 2% of galactose for 4 hours. When they reached 0.7 – 0.8 OD_{600}, 5µl drop of 4-fold serial dilutions of each strain were spotted on media without uracil containing either glucose or galactose. Plates were incubated at 30°C and photographed after 3-4 days of growth.

Appendix B: Crn1

B.1. Introduction

CRN1 gene encodes the yeast homologue of coronins which are known to be an evolutionarily conserved family of WD repeat proteins which function in organization of the actin cytoskeleton in eukaryotes (Moseley and Goode, 2006). Crn1, CoRoNin, is an actin and microtubule-associated protein (Heil-Chapdelaine et al., 1998), and consists of five amino-terminal WD repeats (Rybakin and Clemen, 2005 and Moseley and Goode, 2006).

Crn1p was found to bind actin filaments (F-actin) and cross-link actin filaments to build up long actin filament bundles structures (Heil-Chapdelaine et al., 1998). Crn1 protein also controls the actin filament nucleation factors and branching off activity of the Arp2/3 complex through association with the Arc35p subunit (Humphries et al., 2002). The Arp2/3 complex catalyzes actin polymerization by forming a new nucleation core; however this complex has low actin nucleation activity and is triggered by binding to the actin filament or by association with activator proteins such as Las17p and Abp1p (Kreishman-Deitrick and Rosen, 2002).

B.2. Hypothesis

A previous work from Fillingham et al. (2008) provided preliminary evidence that Hif1 interacts with several additional proteins, CRN1 and YPL108W.

At this point I started with Crn1 and used the same strategy outlined in Materials and Methods section to determine if the CRN1 yeast protein interacts with Hif1 through an affinity purification scheme. In addition to that Crn1 is a cortical actin cytoskeletal component that regulates Arp2p/Arp3p complex activity and actin patch assembly. The human NASP is connected to autophagy by defending the soluble H3-H4 against degradation by chaperone-

mediated autophagy, thereby it would be interesting to determine if an interaction between Hif1 and Crn1 occurs, as well, understanding the involvement of Crn1 in the response to DNA damage in yeast.

A.3. Methodology

To confirm and characterize this protein-protein interaction, I used PCR and yeast molecular genetic methods to separately add a 13xMYC epitope tag to the *HIF1* gene in a strain of yeast already carrying the tandem affinity purification (TAP) epitope tag (See Materials and Methods). I used co-immunoprecipitation (Co-IP) methods to confirm the Crn1-Hif1 physical. To accomplish this I generated whole cell extracts from an appropriate strain of yeast, purified Crn1-TAP on IgG-Sepharose, and used SDS-PAGE combined with western blotting with anti-MYC antibody to monitor co-purification of MYC-tagged Hif1. In order to further confirm the results, I also employed anti-TAP antibody (See Materials and Methods). A positive control, Asf1-TAP Hif1-13myc (Fillingham et al., 2008), was used in this experiment.

B.4. Results

B.4.1. A potential interaction between Crn1 and Hif1 proteins

Unfortunately, the Western blot results indicated the same band size for (Crn1-TAP Hif1-MYC) strain in both input material and affinity purification when I probed against α-MYC and α-TAP (Figure B.1). In fact, the calculation of both showed different fragment sizes (Crn1-TAP= 92.5kDa and Hif1-13MYC= 62kDa), as determined by proteins detection. However, that there was not a specific signal to the appropriate strain corresponding to Hif1-13MYC in the affinity purification samples that can be compared with a specific signal to the appropriate strain corresponding to input material of Crn1-TAP.

Aside from that, all controls worked perfectly as no signal was observed on wild-type (untagged strain) as well as no MYC cross reactivity corresponding to Hif1 in the affinity purification as it should be in input material (Figure B.1). Additionally, the positive loading control Asf1-TAP Hif1-13myc that was obtained from (Fillingham et al., 2008) was included to signify that lack of signals for both antibodies in wild-type sample was not due to absence of sample.

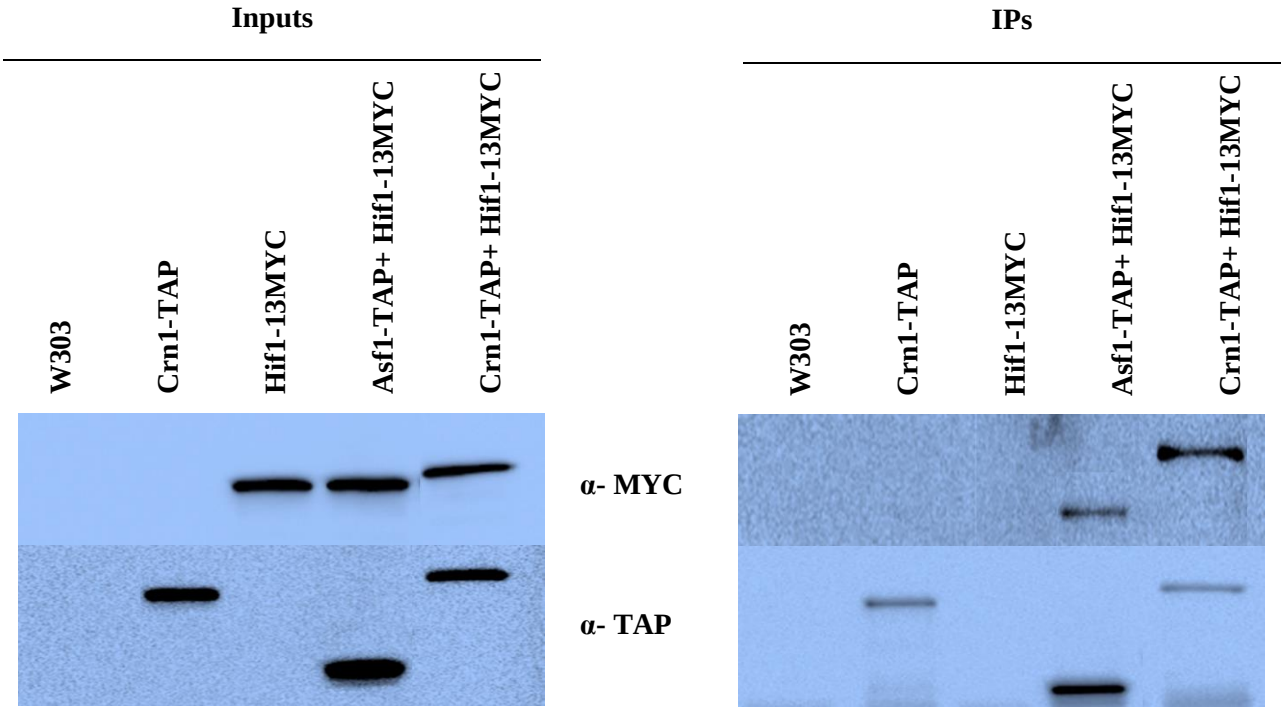


Figure B.1: Input and IP materials of Crn1 show that Crn1 might interact with Hif1.

Soluble cell extracts of yeast cells expressing the Crn1-TAP Hif1-13MYC strain and control strains. 50 µl were isolated for inputs, and 850µl of IP samples were separated by 10% SDS/PAGE, and transferred to a nitrocellulose membrane. The membrane was stained with Ponceau stain. Inputs and IPs were probed by immunoblotting with antibodies shown in the center.

This interaction can be certainly confirmed by improving the quality of western blot. Due to undistinguished sizes of proteins, samples should be run on 8% SDS-PAGE gels at 80V with importantly increasing the separation time to two hours and a half instead of one hour and half as I do usually. Following the above procedure, proteins separation can be significantly improved and results can be confirmed.

Appendix C: Supplementary Material

Appendix C.1. The composition of all media, buffers and solutions used

Item	Composition
0.8% Agarose Gel (wt/vol) (50ml)	0.8g Agarose 100ml 1xTBE 3µl Ethidium Bromide (EtBr) (10mg/ml) or 5µl Red Safe (RedSafe)
0.5M Ammonium Hydroxide (NH_4OH)	1ml 14.5M NH_4OH 28ml ddH_2O
10% APS (Ammonium Persulfate) (wt/vol)	0.5g Ammonium Persulfate 5ml ddH_2O
Breaking Buffer (50ml)	10ml 10% Triton™ X-100 5ml 10% SDS 1ml 100mM NaCl 0.5ml 1M Tris, pH8 0.1µl 1mM EDTA 33.4ml ddH_2O
3% Bovine Serum Albumin (BSA) (wt/vol)	1.5g BSA 50 ml 1x TBS
50% Glycerol (vol/vol) (100ml)	50ml Glycerol 100ml ddH_2O
IP Buffer for Washing Cells (50 ml)	0.5ml 1M Tris pH8 1.5ml 5M NaCl 0.5ml 10% NP-40 47.5ml ddH_2O
LB Broth (1L)	10g Tryptone 5g Yeast Extract 10g NaCl 15g Agar for Plates

	1ml Ampicillin (10mg/ml) ddH_2O to 1L
10x Lithium Acetate Solution (LiOAc) (wt/vol) (500ml)	52g LiOAc 50mL 1M Tris, pH 7.5-8.0 10mL 0.5M EDTA ddH2O to 500ml
Saccharomyces Lysis Solution (50ml)	2.5ml 1M Tris pH 8 1.5ml 5M NaCl 75µl 1M MgOAc 500µl 10% NP-4O 500µl 0.5 M EDTA ddH2O to 50ml
5% Milk Solution (BLOTTO) (wt/vol) (100ml)	10g Skim Milk powder 200ml PBS
1M MgCl2 (M.W.=203.3g/mol) (100ml)	20.33g MgCl2 ddH2O to 100ml
5M NaCl (500ml)	146.1g NaCl ddH_2O to 500ml
10% NP-40 (vol/vol)	2.5ml NP-40 22.5ml ddH_2O
50% Poly Ethylene Glycol (PEG) (wt/vol) (1L)	500g PEG ddH_2O to 1L
100mM PMSF (10ml)	0.174g PMSF 10ml Isopropanol
0.1% Ponceau (wt/vol) (500ml)	0.5g Ponceau S 25ml Acetic Acid ddH_2O to 500ml
1x Running Buffer (1L)	100ml 10x Transfer Buffer 10ml 10% SDS 890ml ddH_2O

1x SDS Laemmli Sample Buffer (10ml)	9.2 ml 10% SDS 500µl β-mercaptoethanol 300µl 2M Tris pH 6.8 50mg Bromophenol Blue ddH_2O to 10ml
5% Stacking Gel (16ml)	2.4ml Acrylamide 29:1 4ml 10% SDS 20µl TEMED 160µl 10% APS 2.4ml ddH2O
1x Transfer Buffer (4L)	400ml 10x Running Buffer 800ml Methanol 10ml 10% SDS ddH_2O to 4L
10x Tris borate EDTA (TBE) pH 8.0 (4L)	10g Tris Base 5.5 Boric Acid 0.93g EDTA ddH_2O to 4L Titrate with HCl until pH=8.0
5x Tris Buffered Saline (TBS) (4L)	48.44 g Tris Base 584.4g NaCl ddH_2O to 4L Titrate with HCl until pH=8.0
1M Tris-HCl pH 8.0 (500ml)	60.55g Tris Base ddH_2O to 500ml, pH to 8.0
10mM Tris pH 7.4 (500ml)	0.61g Tris Base ddH_2O to 500ml, pH to 7.4
10x Western Running Buffer (4L)	120.2g Tris Base 576.1g Glycine ddH_2O to 4L

YNB -LEU Liquid Media (1L)	20g Glucose 6.74 YNB 2.0g -LEU powder 15g Agar for Plates ddH_2O to 1L
YNB –URA Liquid Media (1L)	20g Glucose 6.74 YNB 2.0g -URA powder 15g Agar for Plates ddH_2O to 1L
YPD Liquid Media (1L)	10g Yeast Extract 20g Peptone 15g Agar for Plates 20g Dextrose ddH_2O to 1L
YPD+ G418 (300µg/ml)	1L YPD 300mg Geneticin (G418) Sulfate
YPD+ Hydroxurea (HU)	1L YPD + Agar 3.8g 50 mM HU 7.6g 100mM HU

Appendix C.2. All strains generated in this study

#	Strain code	Strain name	Marker	Strain version
1	JF087	pRB415ADH1-12MYC+ Δ(+TPR3Domain)	Amp	Bacterial strain
2	JF086	pRB415ADH1-12MYC+ Δ(+TPR4Domain)	Amp	Bacterial strain
3	JF076	pRB415ADH1-12MYC+ Δ(+TPR4)	Amp	Bacterial strain
4	JF080	pRB415ADH1-12MYC+ Δ(+TPR4 +35)	Amp	Bacterial strain
5	JF077	pRB415ADH1-12MYC+ Δ(Basic Patch)	Amp	Bacterial strain
6	JF078	pRB415ADH1-12MYC+ Full Length F.L.	Amp	Bacterial strain
7	JF079	pRB415ADH1-12MYC+ ΔTPR1	Amp	Bacterial strain
8	JF082	pRB415ADH1-12MYC+ ΔTPR2- 1st	Amp	Bacterial strain
9	JF084	pRB415ADH1-12MYC+ ΔAcd (Acidic region)	Amp	Bacterial strain
10	JF088	pRB415ADH1-12MYC+ ΔTPR2 entire	Amp	Bacterial strain
11	JF083	pRB415ADH1-12MYC+ ΔTPR3	Amp	Bacterial strain
12	JF085	pRB415ADH1-12MYC+ ΔTPR4	Amp	Bacterial strain
13	JF200	W303+ plasmid	-LEU	Yeast strain
14	JF201	Hat1-TAP Δhif1+ plasmid	-LEU	Yeast strain
15	JF202	12MYC-Hif1(F.L.)	-LEU	Yeast strain
16	JF243	Hat1-TAP *Δhif1*+ 12MYC-Hif1 Δ(+TPR3Domain)	-LEU	Yeast strain
17	JF242	Hat1-TAP *Δhif1*+ 12MYC-Hif1 Δ(+TPR4Domain)	-LEU	Yeast strain
18	JF203	Hat1-TAP *Δhif1*+ 12MYC-Hif1 Δ(+TPR4)	-LEU	Yeast strain
19	JF204	Hat1-TAP *Δhif1*+ 12MYC-Hif1 Δ(+TPR4 +35)	-LEU	Yeast strain
20	JF206	Hat1-TAP *Δhif1*+ 12MYC-Hif1 ΔB.P(Basic Patch)	-LEU	Yeast strain

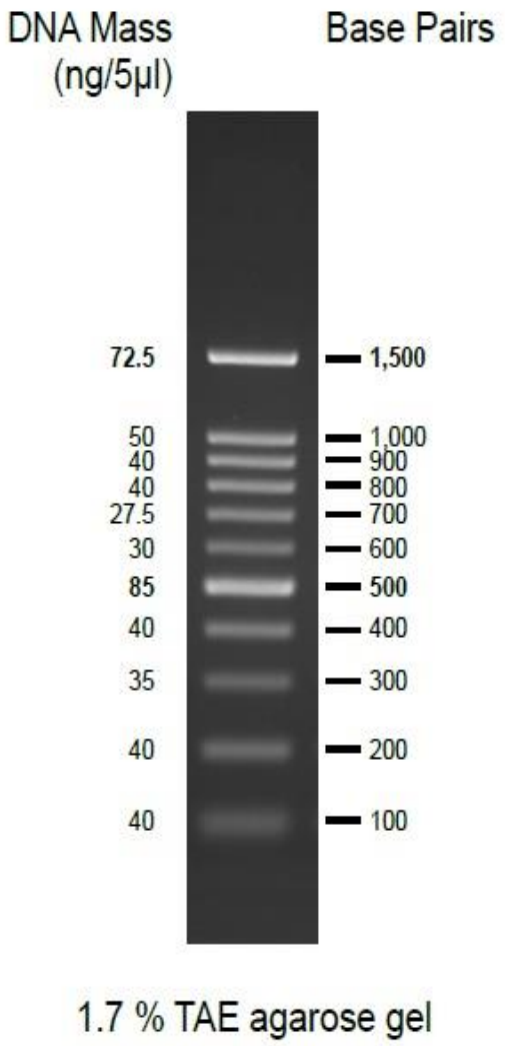
21	JF205	Hat1-TAP *Δhif1*+ 12MYC-Hif1 +Full Length F.L.	-LEU	Yeast strain
22	JF207	Hat1-TAP *Δhif1*+ 12MYC-Hif1 ΔTPR1	-LEU	Yeast strain
23	JF208	Hat1-TAP *Δhif1*+ 12MYC-Hif1 ΔTPR2- 1st	-LEU	Yeast strain
24	JF209	Hat1-TAP *Δhif1*+ 12MYC-Hif1 ΔAcd	-LEU	Yeast strain
25	JF241	Hat1-TAP *Δhif1*+ 12MYC-Hif1 ΔTPR2 entire	-LEU	Yeast strain
26	JF210	Hat1-TAP *Δhif1*+ 12MYC-Hif1 ΔTPR3	-LEU	Yeast strain
27	JF211	Hat1-TAP *Δhif1*+ 12MYC-Hif1 ΔTPR4	-LEU	Yeast strain
28	JF167	pYES+ WT	-URA	Yeast strain
29	JF169	pYES+ *hif1*Δ::KAN	-URA	Yeast strain
30	JF175	pYES+ *ha1*Δ::KAN	-URA	Yeast strain
31	JF177	pYES+ *hat2*Δ::KAN	-URA	Yeast strain
32	JF257	pYES+ *spt2*Δ::KAN	-URA	Yeast strain
33	JF259	pYES+ *crn1*Δ::KAN	-URA	Yeast strain
34	JF185	pYES+ *npl3*Δ::KAN	-URA	Yeast strain
35	JF261	pYES+ *ctf4*Δ::KAN	-URA	Yeast strain
36	JF263	pYES+ *dcc1*Δ::KAN	-URA	Yeast strain
37	JF179	pYES+ *sro9*Δ::KAN	-URA	Yeast strain
38	JF183	pYES+ *sro9*Δ::NAT, *lsm1*Δ::KAN	-URA	Yeast strain
39	JF171	pYES+ *Δlsm1*	-URA	Yeast strain
40	JF168	pYES+ H3+ WT	-URA	Yeast strain
41	JF170	pYES+ H3+ *hif1*Δ::KAN	-URA	Yeast strain
42	JF176	pYES+ H3+ *ha1*Δ::KAN	-URA	Yeast strain

43	JF178	pYES+ H3+ *hat2*Δ::KAN	-URA	Yeast strain
44	JF258	pYES+ H3+ *spt2*Δ::KAN	-URA	Yeast strain
45	JF260	pYES+ H3+ *crn1*Δ::KAN	-URA	Yeast strain
46	JF186	pYES+ H3+ *npl3*Δ::KAN	-URA	Yeast strain
47	JF262	pYES+ H3+ *ctf4*Δ::KAN	-URA	Yeast strain
48	JF264	pYES+ H3+ *dcc1*Δ::KAN	-URA	Yeast strain
49	JF180	pYES+ H3+ *sro9*Δ::KAN	-URA	Yeast strain
50	JF184	pYES+ H3+ *sro9*Δ::NAT, *lsm1*Δ::KAN	-URA	Yeast strain
51	JF172	pYES+ H3+ *lsm1*Δ::KAN	-URA	Yeast strain
52	JF248	pYES+ H3+ 12MYC-Hif1 Δ(+TPR3Domain)	-LEU -URA	Yeast strain
53	JF247	pYES+ H3+ 12MYC-Hif1 Δ(+TPR4Domain)	-LEU -URA	Yeast strain
54	JF246	pYES+ H3+ 12MYC-Hif1 Δ(+TPR4)	-LEU -URA	Yeast strain
55	JF245	pYES+ H3+ 12MYC-Hif1 Δ(+TPR4 +35)	-LEU -URA	Yeast strain
56	JF244	pYES+ H3+ 12MYC-Hif1 ΔB.P.	-LEU -URA	Yeast strain
57	JF250	pYES+ H3+ 12MYC-Hif1 +Full Length (F.L.)	-LEU -URA	Yeast strain
58	JF251	pYES+ H3+ 12MYC-Hif1 ΔTPR1	-LEU -URA	Yeast strain
59	JF252	pYES+ H3+ 12MYC-Hif1 ΔTPR2- 1st	-LEU -URA	Yeast strain
60	JF253	pYES+ H3+ 12MYC-Hif1 ΔAcd	-LEU -URA	Yeast strain
61	JF254	pYES+ H3+ 12MYC-Hif1 ΔTPR2 entire	-LEU -URA	Yeast strain
62	JF255	pYES+ H3+ 12MYC-Hif1 ΔTPR3	-LEU -URA	Yeast strain
63	JF256	pYES+ H3+ 12MYC-Hif1 ΔTPR4	-LEU -URA	Yeast strain
64	JF249	pYES+ H3+ 12MYC-*Δhif1*	-LEU -URA	Yeast strain

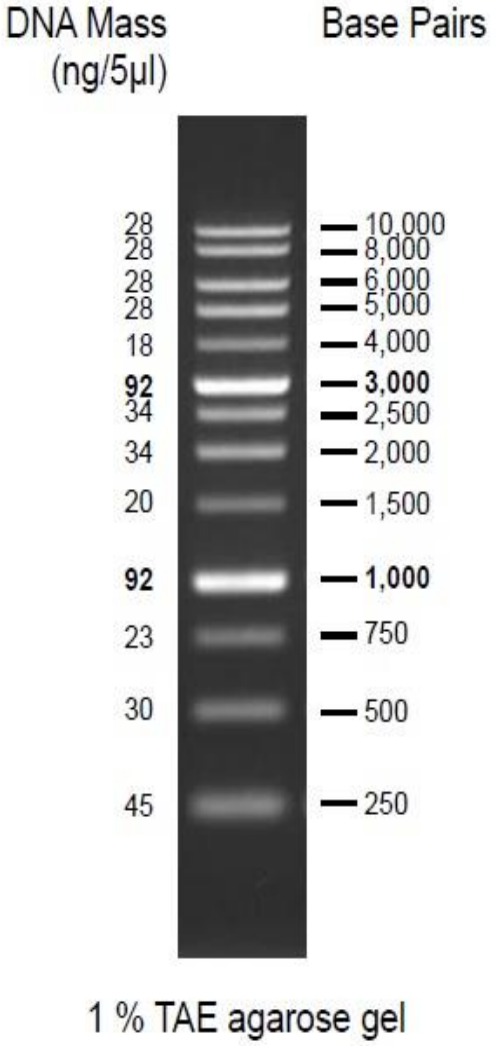
65	JF187	BY4742, *sro9*Δ::NAT	-	Yeast strain
66	JF189	BY4742, *sro9*Δ::NAT, *rtt109*Δ::KAN	-	Yeast strain
67	JF190	BY4742, *sro9*Δ::NAT, *lsm1*Δ::KAN	-	Yeast strain
68	JF188	BY4742, *sro9*Δ::NAT, *slf1*Δ::KAN	-	Yeast strain
69	JF110	Spt2-TAP+ Hif1-13MYC	-	Yeast strain
70	JF102	Spt2-TAP+ Hat1-13MYC	-	Yeast strain
71	JF111	Spt2-TAP+ Hat2-13MYC	-	Yeast strain
72	JF143	Crn1-TAP+ Hif1-13MYC	-	Yeast strain

Appendix C.3. DNA ladders used in this study

100bp DNA Ladder RTU (Ready-to-Use)

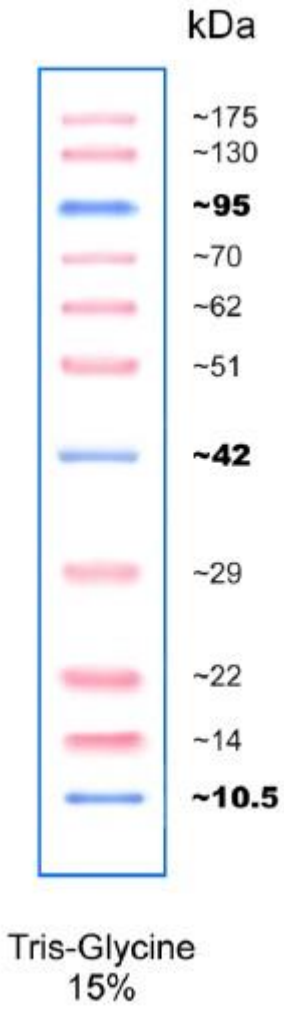


1kp DNA Ladder RTU (Ready-to-Use)

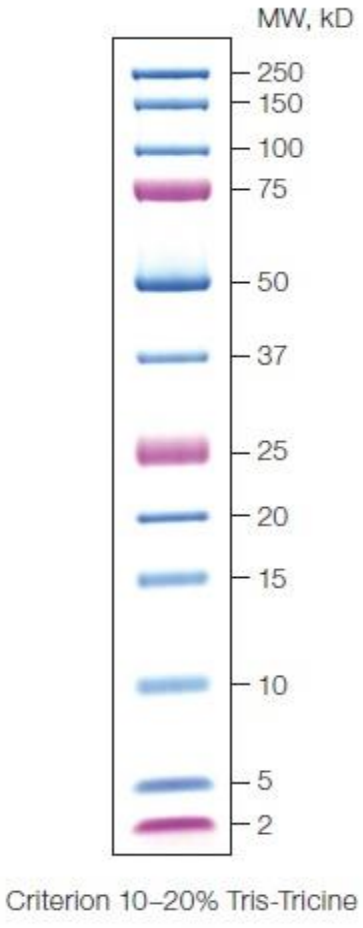


Appendix C.4. Protein ladders used in this study

PiNK Plus Prestained Protein Ladder



Precision Plus Protein™ Dual Color



Appendix C.5. The coding sequence of the *Saccharomyces cerevisiae* Hif1

YLL022C Chr 12

Hif1, 1158 base pairs, reverse complement.

ATGAAACTAAGGGCAGAAGACGTGTTGGCAAACGGAACTAGCAGACATAAAGTTCA
AATTGATATGGAAAGACAGGTTCAAATTGCAAAAGATTTACTCGCACAGAAAAAGT
TCTTAGAAGCTGCTAAACGCTGCCAACAAACTTTGGACTCTCTCCCAAAAGATGGTT
TGTTGCCTGATCCTGAACTGTTTACTATATTCGCACAAGCTGTATACAACATGGAAG
TACAGAATTCTGGTAATCTGTTTGGGGACGCTCTTCTGGCAGGTGACGATGGATCTG
GGTCTGAGTCTGAGTCTGAGCCTGAGTCTGATGTGAGCAATGGAGAAGAGGGGAAC
GAGAACGGTCAAACAGAAATTCCTAATTCTAGGATGTTCCAATTCGACCAAGAAGA
GGAAGACTTGACTGGTGATGTCGACAGTGGTGATAGTGAGGACAGTGGCGAAGGTA
GCGAAGAAGAGGAAGAAAACGTGGAGAAAGAGGAAGAACGCTTAGCTTTGCACGA
ATTGGCTAACTTCAGTCCAGCGAATGAACATGACGATGAAATTGAAGACGTATCGC
AACTTCGCAAGTCTGGTTTCCACATTTACTTTGAAAATGATCTGTATGAGAATGCTTT
GGACCTACTGGCGCAGGCCTTGATGCTGTTGGGCCGCCCTACAGCAGATGGCCAATC
TCTAACCGAGAACAGCAGATTGCGCATCGGTGATGTGTATATCCTGATGGGTGACAT
CGAAAGGGAGGCGGAGATGTTCAGTAGAGCCATTCATCATTACTTGAAGGCGCTTG
GCTATTACAAGACCTTGAAACCCGCAGAACAAGTAACTGAGAAGGTGATACAAGCA
GAATTTTTGGTGTGTGATGCTTTAAGGTGGGTTGATCAGGTGCCGGCTAAGGACAAA
CTAAAAAGATTCAAGCATGCCAAGGCTCTGCTTGAAAAACACATGACTACAAGACC
CAAGGACAGCGAGTTACAGCAGGCAAGGCTCGCACAGATTCAAGACGATATTGATG
AGGTGCAAGAAAATCAGCAGCACGGCTCCAAGAGGCCTCTTTCGCAGCCCACGACC
TCCATCGGCTTCCCCGCCCTCGAAAAGCCCCTTGGTGACTTCAATGATCTCTCTCAAC
TGGTCAAGAAGAAGCCTAGAAGGCATTGA

Appendix C.6. The protein translation of the *Saccharomyces cerevisiae* Hif1

YLL022C Chr 12

Hif1, 385 Amino Acids, reverse complement

MKLRAEDVLANGTSRHKVQIDMERQVQIAKDLLAQKKFLEAAKRCQQTLDSLPKDGLL
PDPELFTIFAQAVYNMEVQNSGNLFGDALLAGDDGSGSESESEPESDVSNGEEGNENGQ
TEIPNSRMFQFDQEEEDLTGDVDSGDSEDSGEGSEEEEENVEKEEERLALHELANFSPAN
EHDDEIEDVSQLRKSGFHIYFENDLYENALDLLAQALMLLGRPTADGQSLTENSRLRIGD
VYILMGDIEREAEMFSRAIHHYLKALGYYKTLKPAEQVTEKVIQAEFLVCDALRWVDQ
VPAKDKLKRFKHAKALLEKHMTTRPKDSELQQARLAQIQDDIDEVQENQQHGSKRPLS
QPTTSIGFPALEKPLGDFNDLSQLVKKKPRRH*

Appendix C.6.1 The protein translation of Hif1 (external deletions)

C.6.1.1

C1= Full length Hif1

MKLRAEDVLANGTSRHKVQIDMERQVQIAKDLLAQKKFLEAAKRCQQTLDSLPKDGLL
PDPELFTIFAQAVYNMEVQNSGNLFGDALLAGDDGSGSESESEPESDVSNGEEGNENGQ
TEIPNSRMFQFDQEEEDLTGDVDSGDSEDSGEGSEEEEENVEKEEERLALHELANFSPAN
EHDDEIEDVSQLRKSGFHIYFENDLYENALDLLAQALMLLGRPTADGQSLTENSRLRIGD
VYILMGDIEREAEMFSRAIHHYLKALGYYKTLKPAEQVTEKVIQAEFLVCDALRWVDQ
VPAKDKLKRFKHAKALLEKHMTTRPKDSELQQARLAQIQDDIDEVQENQQHGSKRPLS
QPTTSIGFPALEKPLGDFNDLSQLVKKKPRRH*

C.6.1.2

C2= Δ (B.P.)

MKLRAEDVLANGTSRHKVQIDMERQVQIAKDLLAQKKFLEAAKRCQQTLDSLPKDGLL
PDPELFTIFAQAVYNMEVQNSGNLFGDALLAGDDGSGSESESEPESDVSNGEEGNENGQ
TEIPNSRMFQFDQEEEDLTGDVDSGDSEDSGEGSEEEEENVEKEEERLALHELANFSPAN
EHDDEIEDVSQLRKSGFHIYFENDLYENALDLLAQALMLLGRPTADGQSLTENSRLRIGD
VYILMGDIEREAEMFSRAIHHYLKALGYYKTLKPAEQVTEKVIQAEFLVCDALRWVDQ
VPAKDKLKRFKHAKALLEKHMTTRPKDSELQQARLAQIQDDIDEVQENQQHGSKRPLS
QPTTSIGFPALEKPLGDFNDLSQLV*

C.6.1.3

C3= Δ (+TPR4 +35)

MKLRAEDVLANGTSRHKVQIDMERQVQIAKDLLAQKKFLEAAKRCQQTLDSLPKDGLL
PDPELFTIFAQAVYNMEVQNSGNLFGDALLAGDDGSGSESESEPESDVSNGEEGNENGQ
TEIPNSRMFQFDQEEEDLTGDVDSGDSEDSGEGSEEEEENVEKEEERLALHELANFSPAN
EHDDEIEDVSQLRKSGFHIYFENDLYENALDLLAQALMLLGRPTADGQSLTENSRLRIGD
VYILMGDIEREAEMFSRAIHHYLKALGYYKTLKPAEQVTEKVIQAEFLVCDALRWVDQ
VPAKDKLKRFKHAKALLEKHMTTRPKDSELQQARLAQIQDDIDEVQEN*

C.6.1.4

C4= Δ (+TPR4)

MKLRAEDVLANGTSRHKVQIDMERQVQIAKDLLAQKKFLEAAKRCQQTLDSLPKDGLL
PDPELFTIFAQAVYNMEVQNSGNLFGDALLAGDDGSGSESESEPESDVSNGEEGNENGQ

TEIPNSRMFQFDQEEEDLTGDVDSGDSEDSGEGSEEEEENVEKEEERLALHELANFSPAN
EHDDEIEDVSQLRKSGFHIYFENDLYENALDLLAQALMLLGRPTADGQSLTENSRLRIGD
VYILMGDIEREAEMFSRAIHHYLKALGYYKTLKPAEQVTEKVIQAEFLVCDALRWVDQ
VPAKDKLKRFKHAKALLEKH*

C.6.1.5

C5= Δ (+TPR4 domain)

MKLRAEDVLANGTSRHKVQIDMERQVQIAKDLLAQKKFLEAAKRCQQTLDSLPKDGLL
PDPELFTIFAQAVYNMEVQNSGNLFGDALLAGDDGSGSESESEPESDVSNGEEGNENGQ
TEIPNSRMFQFDQEEEDLTGDVDSGDSEDSGEGSEEEEENVEKEEERLALHELANFSPAN
EHDDEIEDVSQLRKSGFHIYFENDLYENALDLLAQALMLLGRPTADGQSLTENSRLRIGD
VYILMGDIEREAEMFSRAIHHYLKALGYYKTLKPAEQVTEKV*

C.6.1.6

C6= Δ (+TPR3 domain)

MKLRAEDVLANGTSRHKVQIDMERQVQIAKDLLAQKKFLEAAKRCQQTLDSLPKDGLL
PDPELFTIFAQAVYNMEVQNSGNLFGDALLAGDDGSGSESESEPESDVSNGEEGNENGQ
TEIPNSRMFQFDQEEEDLTGDVDSGDSEDSGEGSEEEEENVEKEEERLALHELANFSPAN
EHDDEIEDVSQLRKSGFHIYFENDLYENALDLLAQALMLLGRPTADGQSLTENSRLRI*

Appendix C.6.2 The protein translation of Hif1 (internal deletions)

C.6.2.1

N1= ΔTPR1

MKLRAEDVLANGTSRHKVQID==GLLPDPELFTIFAQAVYNMEVQNSGNLFGDALLAG
DDGSGSESESEPESDVSNGEEGNENGQTEIPNSRMFQFDQEEEDLTGDVDSGDSEDSGEG
SEEEEENVEKEEERLALHELANFSPANEHDDEIEDVSQLRKSGFHIYFENDLYENALDLL
AQALMLLGRPTADGQSLTENSRLRIGDVYILMGDIEREAEMFSRAIHHYLKALGYYKTL
KPAEQVTEKVIQAEFLVCDALRWVDQVPAKDKLKRFKHAKALLEKHMTTRPKDSELQ
QARLAQIQDDIDEVQENQQHGSKRPLSQPTTSIGFPALEKPLGDFNDLSQLVKKKPRRH*

C.6.2.2

N2= ΔTPR2- 1st half

MKLRAEDVLANGTSRHKVQIDMERQVQIAKDLLAQKKFLEAAKRCQQTLDSLPKDGLL
PD==DALLAGDDGSGSESESEPESDVSNGEEGNENGQTEIPNSRMFQFDQEEEDLTGDVD
SGDSEDSGEGSEEEEENVEKEEERLALHELANFSPANEHDDEIEDVSQLRKSGFHIYFEN
DLYENALDLLAQALMLLGRPTADGQSLTENSRLRIGDVYILMGDIEREAEMFSRAIHHY
LKALGYYKTLKPAEQVTEKVIQAEFLVCDALRWVDQVPAKDKLKRFKHAKALLEKHM
TTRPKDSELQQARLAQIQDDIDEVQENQQHGSKRPLSQPTTSIGFPALEKPLGDFNDLSQ
LVKKKPRRH*

C.6.2.3

N3= ΔAcd

MKLRAEDVLANGTSRHKVQIDMERQVQIAKDLLAQKKFLEAAKRCQQTLDSLPKDGLL
PDPELFTIFAQAVYNMEVQNSGNLFG==LRKSGFHIYFENDLYENALDLLAQALMLLGR
PTADGQSLTENSRLRIGDVYILMGDIEREAEMFSRAIHHYLKALGYYKTLKPAEQVTEK
VIQAEFLVCDALRWVDQVPAKDKLKRFKHAKALLEKHMTTRPKDSELQQARLAQIQD
DIDEVQENQQHGSKRPLSQPTTSIGFPALEKPLGDFNDLSQLVKKKPRRH*

C.6.2.4

N4= ΔTPR2 entire

MKLRAEDVLANGTSRHKVQIDMERQVQIAKDLLAQKKFLEAAKRCQQTLDSLPKDGLL
PD==LDLLAQALMLLGRPTADGQSLTENSRLRIGDVYILMGDIEREAEMFSRAIHHYLKA
LGYYKTLKPAEQVTEKVIQAEFLVCDALRWVDQVPAKDKLKRFKHAKALLEKHMTTR

PKDSELQQARLAQIQDDIDEVQENQQHGSKRPLSQPTTSIGFPALEKPLGDFNDLSQLVK
KKPRRH*

C.6.2.5

N5= ΔTPR3

MKLRAEDVLANGTSRHKVQIDMERQVQIAKDLLAQKKFLEAAKRCQQTLDSLPKDGLL
PDPELFTIFAQAVYNMEVQNSGNLFGDALLAGDDGSGSESESEPESDVSNGEEGNENGQ
TEIPNSRMFQFDQEEEDLTGDVDSGDSEDSGEGSEEEEENVEKEEERLALHELANFSPAN
EHDDEIEDVSQLRKSGFHIYFENDLYENALDLLAQALMLLGRPTADGQSLTENSRLRI==
KPAEQVTEKVIQAEFLVCDALRWVDQVPAKDKLKRFKHAKALLEKHMTTRPKDSELQ
QARLAQIQDDIDEVQENQQHGSKRPLSQPTTSIGFPALEKPLGDFNDLSQLVKKKPRRH*

C.6.2.6

N6= ΔTPR4

MKLRAEDVLANGTSRHKVQIDMERQVQIAKDLLAQKKFLEAAKRCQQTLDSLPKDGLL
PDPELFTIFAQAVYNMEVQNSGNLFGDALLAGDDGSGSESESEPESDVSNGEEGNENGQ
TEIPNSRMFQFDQEEEDLTGDVDSGDSEDSGEGSEEEEENVEKEEERLALHELANFSPAN
EHDDEIEDVSQLRKSGFHIYFENDLYENALDLLAQALMLLGRPTADGQSLTENSRLRIGD
VYILMGDIEREAEMFSRAIHHYLKALGYYKTLKPAEQVTEKV==MTTRPKDSELQQARL
AQIQDDIDEVQENQQHGSKRPLSQPTTSIGFPALEKPLGDFNDLSQLVKKKPRRH*

Appendix C.7. DNA sequencing primers

Sequencing primers	Sequence
HJ559	5'-cgttgtaaaacgacggccag-3'
AJJ296	5'-tggactgaagttagccaattc-3'
AJL320	5'-cagatcattttcaaagtaaat-3'

Appendix C.8. DNA sequencing primers

DNA sequence primers	Sequence
Hif1F(BAMH1)	5'-CCCGGATCCatgaaactaagggcagaagac-3'
Hif1F(TPRd1)	5'-ggtttgttgcctgatcctgaa-3'
Hif1R(TPRd1)	5'-aggatcaggcaacaaaccatcaatttgaactttatg-3'
Hif1F(dAc)	5'-cttcgcaagtctggtttccac-3'
Hif1R(dAc)	5'-gtaaatgtggaaaccagacttgcgaagcccaaacagattaccaga-3'
Hif1F(dTPR2a)	5'-gacgctcttctggcaggt-3'
Hif1R(dTPR2a)	5'-acctgccagaagagcgtcatcaggcaacaaaccatc-3'
Hif1F(dTPR2entire)	5'-ttggacctactggcgcag-3'
Hif1R(dTPR2entire)	5'-gtccgccagtaggtccaaatcaggcaacaaaccatc-3'
Hif1F(dTPR3)	5'-aaacccgcagaacaagta-3'
Hif1R(dTPR3)	5'-tacttgttctgcgggtttgatgcgcaatctgctgtt-3'
Hif1F(dTPR4)	5'-atgactacaagacccaag-3'
Hif1R(dTPR4)	5'-cttgggtcttgtagtcatcaccttctcagttacttg-3'
Hif1R(+TPR3Domain)	5'-CCCCTGCAG**tca**gatgcgcaatctgctgttctc-3'
Hif1R(+TPR4Domain)	5'-CCCCTGCAG**tca**caccttctcagttacttgttc-3'
Hif1R(+TPR4-PSTI)	5'-CCCCTGCAG**tca**gtgtttttcaagcagagcctt-3'
HIf1R(+TRP4+35-PSTI)	5'-CCCCTGCAG**tca**cctcttggagccgtgctg-3'
Hif1R(-BASIC-PSTI)	5'-CCCCTGCAG**tca**gaccagttgagagagatcatt-3'
Hif1R(PSTI)	5'-CCCCTGCAG**tca**atgccttctaggcttctt-3'
yHif1F	5'-aaggacagcgagttacagcaggcaa-3'

yHif1R	5'-gctagtgtttcttgctccttatgaa-3'
ySro9F	5'-ttcacttctggtaggtcaagaa-3'
ySro9R	5'-acagctttgctggaggatgatt-3'
ySro9confF	5'-tgctcatcgcaaatttttcagaaatggtgc-3'
yLsm1confF	5'-gaagtcgtgaatatacaacggtgtt-3'
yRtt109confF	5'-atgtatgtgcatatgtagtc-3'
KanB	5'-ctgcagcgaggagccgtaat-3'
NatR	5'-gaagccgtccccggtggcggtgacgcg-3'

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