Elucidation of the Biological Function of the RPC Family Ubiquitin Ligase Asr1

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LIST OF ABBREVIATIONS

DNA-	Deoxyribonucleic acid
RNA-	Ribonucleic acid
PIC-	Pre-initiation complex
GTFs-	General transcription factors
RNAPII-	RNA polymerase II
CTD-	C-terminal domain of Rpb1
Ser5P-	Serine 5 phosphorylated CTD
Ser2P-	Serine 2 phosphorylated CTD
E1-	Ubiquitin activating enzyme
E2-	Ubiquitin conjugating enzyme
E3-	Ubiquitin ligating enzyme
E4-	Ubiquitin elongating enzyme
Asr1-	Alcohol sensitive ring finger 1
RING-	Really interesting new gene
PHD-	Plant homeodomain
CDB-	C-terminal binding domain
RPC-	RING/PHD/CBD containing protein
Sir-	Silent information regulator
TPE-	Telomere position effect
UPS-	Ubiquitin proteasome system
WT-	Wild type
Ubp3-	Ubiquitin specific protease 3
∆2KTM Rpb1-	Mutation in Rpb1 (*rpb1*::K1452R/K1458R/K1487R ∆1720-1734) that has removed sites of ubiquitylation by Asr1
GOI-	Gene of interest
HRP-	Horseradish peroxidase
PEG-	Polyethylene glycol
5-FOA-	5-Fluoroorotic acid

6-AU-	6-Azauracil
MMS-	Methyl Methanesulfonate
qRT-PCR-	Quantitative reverse transcription PCR
co-IP-	co-Immunoprecipitation
IB-	Immunoblot
RNA-seq-	RNA-sequencing
DUB-	Deubiquitylase
DAM-ID-	DNA adenine methylase identification

DISSERTATION OVERVIEW

Proper transcriptional control is absolutely vital for productive cellular function. Perturbations in the regulation of this process are detrimental, and in some cases lead to disease, developmental defects, as well as cellular death. For this reason, cells have evolved a multitude of ways to manage the process of transcription. Chromatin modifiers have been shown to alter the state of DNA-histone interactions in certain areas of the genome in order to affect access of transcriptional machinery to the gene promoter. Transcriptional activators and co-activators, which have a permissive role in active transcription, are regulated through their access to transcriptional machinery via control of steady state levels and localization of the protein within the cell. Transcriptional machinery is also regulated by post-translational modification, the result of which is the alteration of the binding properties of these proteins to other accessory factors. In the Tansey laboratory, we are particularly interested in how the ubiquitin proteasome system (UPS) regulates transcription through the signaling molecule ubiquitin or other UPS machinery.

Ubiquitin (Ub) is a versatile signaling molecule that can lead to a multitude of biological endpoints depending upon the nature of its attachment to proteins. Polyubiquitylation, attachment of multiple ubiquitin molecules in a chain, is usually associated with the process of ubiquitin-mediated destruction by the proteasome. While some polyubiquitin linkages do lead to protein turnover, this represents only a portion of the diversity of ubiquitin-mediated signaling. Because both proteolytic and non-proteolytic ubiquitin signaling is such a multifaceted operation, this specific post-translational modification is intimately involved in nearly every biological process, including transcription. Through the further characterization of the UPS over the last few decades, it is becoming increasingly clear that both ubiquitin-mediated signaling and the proteasome itself play integral roles in the regulation of transcription.

Close to 10 years ago, the Tansey laboratory discovered an evolutionarily conserved set of proteins that contain a RING finger, a PHD finger, and a domain that binds the C-terminal repeat region of RNA polymerase II (RNAPII), therefore called the RPC protein family. Prior to this, the RPC family of proteins had not been well-studied, however they appeared to be an ideal protein

family to investigate, as they have protein domains that clearly connect the processes of the ubiquitin proteasome system (because of the presence of a RING finger) and transcription (because of the presence of a C-terminal binding domain). The *Saccharomyces cerevisiae* protein Asr1 is the defining member of the RPC proteins, and in a role identified by our lab, is recruited to RNAPII in response to hyperphosphorylation of its regulatory C-terminal tail. Once bound, Asr1 oligoubiquitylates at least two subunits of RNAPII, leading to the ejection of the dissociable RNAPII subunits Rpb4 and Rpb7, as well as the transcriptional inactivation of the polymerase complex. The molecular action of Asr1 in budding yeast demonstrates a striking example of the non-proteolytic action of the Ub-proteasome system on the transcriptional apparatus.

Despite the molecular effect of Asr1 on RNAPII and its conservation throughout eukaryotic life, deletion of the *ASR1* gene results in no overt phenotypes in budding yeast. To reveal the physiological role of Asr1, I have pursued parallel genetic, molecular, and biochemical approaches. These studies led to the discovery that the ubiquitin ligase activity of Asr1 is required for the proper silencing of subtelomeric chromatin. Gene silencing at telomeres is a well-studied process in budding yeast. The canonical method for the establishment of silenced chromatin, or heterochromatin, occurs via removal of an acetyl group on lysine 16 of histone H4 (H4K16) by the silent information regulator, or Sir, complex. However, I found that mutation of the RING finger of Asr1 (which is responsible for its ubiquitin ligase activity) causes an induction of subtelomeric genes while acetylation of H4K16 remains unperturbed. This finding suggests that Asr1 represents an independent mode of gene silencing at telomeres that can occur alongside the Sir complex.

It seemed likely that there could be other proteins that work with Asr1 to regulate gene silencing at telomeres, and when searching for protein binding partners for Asr1, Ubp3 was identified as a potential Asr1 associating factor. Ubp3 is a deubiquitylase (DUB) that has been implicated in a multitude of different biological processes. Importantly, Ubp3 appeared to play a role in the regulation of various steps of transcription, including transcriptional regulation of subtelomeric genes. Additionally, like Asr1, Ubp3 is a known Rpb1 interacting protein. I was able to confirm that

Ubp3, as well as the obligate cofactor of Ubp3 (Bre5), associate with Asr1. Mutational analyses of Ubp3 showed that the N-terminus of Ubp3 was both necessary and sufficient for association to Asr1. Furthermore, Ubp3 required both the presence of and association to Asr1 in order to bind to RNAPII, indicating that Asr1 mediates the associated of Ubp3 to RNAPII.

Through the use of telomeric reporter strains, I was able to confirm that Ubp3 has an anti-silencing effect on telomere proximal genes. Additionally, a double *asr1*/*ubp3* mutant dramatically reduces the phenotype seen by a *ubp3* mutant alone, suggesting that Asr1 and Ubp3 may have opposing roles in the process of telomeric silencing. When measuring transcription of genes at native silenced subtelomeric regions, the induction seen by mutation of the RING finger of Asr1 is completely reversed by mutation of *UBP3*, further confirming antagonistic roles for Asr1 and Ubp3 in telomeric silencing. Asr1 and Ubp3 have opposing biochemical properties, as Asr1 directs the addition of ubiquitin and Ubp3 directs the removal of ubiquitin. When looking at the ubiquitylation status of RNAPII, as in the instance of telomeric gene silencing, Asr1 and Ubp3 have antagonistic roles.

These results led to a model for a Sir-independent mode of the regulation of transcription of telomere proximal genes. Upon initiation of transcription of subtelomeric genes, RNAPII becomes hyperphosphorylated on its CTD. These phosphorylation events allow for the association of Asr1, and subsequently Ubp3 and Bre5. Asr1 is able to ubiquitylate RNAPII in a manner that ejects Rpb4/7 from the complex, the result of which is inhibition of transcription and release from chromatin. Ubp3 is able to deubiquitylate RNAPII, allowing Rpb4/7 to bind, and reform a full 12 subunit RNAPII complex, and reinitiate transcription elsewhere. This model allows for a more rapidly reversible, and subtler silencing of telomere proximal genes that is independent of the silenced state caused by the Sir complex.

Although significant progress has been made in understanding the biological role of Asr1 within budding yeast, there are still many opportunities for discovery. It currently remains unclear how Asr1 is able to specifically target RNAPII that has initiated transcription at telomere proximal genes. One possibility could be that Asr1 recognizes some specific combination of the CTD code

that Asr1 would be able to recognize through its C-terminal binding domain. Another possibility is that Asr1 gets recruited to subtelomeric regions through another protein. I have confirmed that Asr1 associates with Ubp3, and Ubp3 is a known Sir complex interacting protein (through its interaction with Sir4). Therefore it seems plausible that Ubp3 could recruit Asr1 to telomere proximal genes through the Sir complex, which makes direct contact with subtelomeric chromatin. It would also be interesting to know whether the role of Asr1 as a telomeric transcription silencing protein is conserved in other RPC proteins. Silenced chromatin at subtelomeric regions has been observed in organisms as diverse as fission yeast, fruit flies, and humans, all of which have a representative RPC protein family member. If the other RPC family members do not also regulate the transcription of telomere proximal genes, it would be informative to understand why they do not, as this could further reveal the mechanism in which Asr1 works in cells.

CHAPTER 1

INTRODUCTION

1.1 Transcription

One of the most important cellular tasks is to maintain the proper integrity and expression of its genetic information. Alterations to the genome or the transcriptome can lead to cell death or disease, and as such, cells possess a battery of processes to ensure that DNA is appropriately packaged, expressed, repaired, and duplicated. There are three enzymes that execute the process of transcription: RNA polymerase I, RNA polymerase II and RNA polymerase III. The scope of my thesis will focus around the regulation of RNA polymerase II (RNAPII), which is responsible for the transcription of a multitude of different RNA species including all protein coding RNA or messenger RNA (mRNA), certain species of small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), long noncoding RNA (lncRNA), cryptic unstable transcripts (CUTs), Xrn1-dependent unstable transcripts (XUTs), stable unannotated transcripts (SUTs), and in some organisms micro RNA (microRNA) (Srivastava and Ahn 2015).

The transcription cycle

Transcription is a tightly regulated process that occurs in a sequential manner. The first step in transcription is the formation of the pre-initiation complex or PIC. PIC formation occurs through the binding of activators to specific enhancer DNA elements which recruit the general transcription factors (GTFs) and RNAPII (Thomas and Chiang 2006). Once the PIC is formed, transcription is fully initiated and RNAPII progresses into the coding region, called promoter escape. In the early stages of most eukaryotic transcription elongation (~25 nucleotides into the gene), there is a high frequency of abortive transcription, which occurs as another layer of regulation (Saunders, Core et al. 2006). The 5’ capping enzyme (Abd1 in yeast) is incorporated at this stage, and modifies the 5’ region of the nascent transcript to protect it from degradation (Mao, Schwer et al. 1995). RNAPII then recruits a variety of regulatory proteins called transcription elongation factors, such as P-TEFb (positive transcription elongation factor b) in humans or CTD-K1 and TFIIF in yeast, which enable the shift to productive elongating transcription (Sims, Belotserkovskaya et al. 2004, Bres, Yoh et al. 2008, Zhou, Li et al. 2012). RNAPII continues to

transcribe the DNA until it reaches a termination signal, which could range from a few base pairs to many kilobases past the end of the mature mRNA molecule, depending on the species and gene being transcribed (Proudfoot 1989). The termination of transcription transpires concurrently to the processing of the 3’ end of the RNA molecule, encompassing cleavage of the pre-mRNA and polyadenylation of the 3’ end of the transcript (Richard and Manley 2009). Once transcription has been terminated, the mature mRNA molecule is exported out of the nucleus with the help of RNA export proteins, so that the transcript can initiate translation at ribosomes (Kohler and Hurt 2007).

The C-terminal domain (CTD) code

The cell employs a variety of post-translational modifications in order to precisely control the timing and fidelity of transcription, one of the most pervasive being phosphorylation. The goal of regulation by phosphorylation is often to stabilize the interaction between two different proteins, which allows cells to quickly modulate the interactions of proteins without involving transcription and translation. An important example of regulation by phosphorylation is in the differential phosphorylation of the C-terminal domain (CTD) of Rpb1, which is the largest subunit of RNAPII (Srivastava and Ahn 2015). The C-terminal domain is a largely unstructured, conserved, region of Rpb1 that contains a heptapeptide repeating consensus sequence of $Tyrosine_1$, $Serine_2$, $Proline_3$, $Threonine_4$, $Serine_5$, $Proline_6$, and $Serine_7$. This consensus sequence is present in the CTD of Rpb1 in all eukaryotic organisms, however there are varying amounts of degenerate residues within these repeats. The number of CTD repeats is also highly variable between organisms, generally increasing as you move up in organism complexity (budding yeast have 26, while humans have 52) (Srivastava and Ahn 2015). Five of the seven residues of the consensus have the capability to be post-translationally modified. Tyr, Ser, and Thr residues can be phosphorylated as well as glycosylated (Egloff and Murphy 2008, Heidemann, Hintermair et al. 2013). Pro residues are not modified by the covalent addition of a molecule, but can be isomerized to adopt the cis or trans conformation (Morris, Phatnani et al. 1999, Srivastava and Ahn 2015) (**Figure 1.1**). The combination of different modifications allows the cell to write a specific “CTD code” used to signal for the association of various cellular machinery depending on the discrete step in transcription. Although all residues have the ability to be modified in some

way, the majority of research performed has been focused on the regulation by differential phosphorylation of serine 2 (Ser2P) and serine 5 phosphorylation (Ser5P).

Ser2P and Ser5P are two well characterized modifications of the CTD of Rpb1, and allow RNAPII to specifically interact with specific transcriptional accessory factors depending upon the stage of transcription. Prior to the initiation of transcription, the CTD of Rpb1 is hypophosphorylated, which allows for the association of RNAPII with the mediator complex (Myers, Gustafsson et al. 1998). During initiation of transcription, serine 5 within the CTD repeats of Rpb1 becomes hyperphosphorylated, which allows for the binding of several different enzymes integral to the initiation of the transcriptional process such as 5' mRNA capping enzyme (Fabrega, Shen et al. 2003), the histone methyl transferase Set1 which helps establish the boundaries of transcription (Ng, Robert et al. 2003), as well as the Serine 2 specific kinase Bur1 (Cho, Kobor et al. 2001). As RNAPII progresses through the gene, Ser5P levels begin to decline through dephosphorylation by the phosphatases Ssu72 (Krishnamurthy, He et al. 2004) and Rtr1 (Mosley, Pattenden et al. 2009), however low levels of Ser5P remain constant throughout the transcriptional process. At the same time that Ser5P levels decrease, Ser2P levels begin to rise, allowing enzymes important for the later stages of transcription to specifically associate with the elongating polymerase, such as the transcriptional elongation factor Spt6 (Yoh, Cho et al. 2007), splicing factor U2AF65 (David, Boyne et al. 2011), and the polyadenylating factor Pcf11 (Licatalosi, Geiger et al. 2002). The post-translational modifications of the CTD repeats of Rpb1 represent an elegant method through which all eukaryotic organisms can effectively recruit the specific transcriptional machinery required for the various steps in transcription.

The importance of the RNAPII subunits Rpb4 and Rpb7

RNAPII is a multi-protein complex made up of 12 subunits, largely conserved throughout eukaryotic organisms. In budding yeast, the subunits are named by size starting with the largest subunit (Rpb1) to the smallest (Rpb12) (**Figure 1.2**) (Young 1991). The core of RNAPII is made up of 10 subunits, whereas Rpb4 and Rpb7 protrude from the main core (Armache, Kettenberger et al. 2003). The Rpb4/7 subunits form a heterodimer that binds to the RNAPII core subunits through specific interactions of Rpb7 with core subunits of RNAPII, an organization that is

conserved in all eukaryotes (Choder 2004) (**Figure 1.2**). In budding yeast (unlike other organisms such as fission yeast and humans) Rpb4 is non-essential under normal conditions, and along with Rpb7, forma a stable dissociable complex outside of the RNAPII core complex (Choder and Young 1993). However, the Rpb4/7 heterodimer has been shown to be required for transcription under conditions such as temperature and growth in stationary phase (Choder 2004). In *in vitro* chain elongation experiments, the Rpb4/7 complex is required for promoter specific binding of RNAPII, but is dispensable for the elongation of transcription (Edwards, Kane et al. 1991). More recent studies have highlighted the important roles Rpb4/7 play in transcription and mRNA synthesis *in vivo*. There is evidence in budding yeast as well as in archaea that mutation or loss of the Rpb4/7 heterodimer (or its archaeal homolog), negatively affects the processivity of RNAPII, impairing its ability to transcribe genes at an optimal level (Runner, Podolny et al. 2008, Hirtreiter, Grohmann et al. 2010). Deletion of *RPB4* has been shown to cause defects in mRNA metabolism, leading to lower rates of mRNA synthesis as well as lower rates of mRNA degradation (Schulz, Pirkl et al. 2014). This study also corroborated previous findings that Rpb4/7 associate at similar levels with genes as the RNAPII core subunit Rpb3, indicating that the 12 subunit form of RNAPII is the true chromatin bound, transcriptionally active complex (Jasiak, Hartmann et al. 2008, Schulz, Pirkl et al. 2014). Therefore, despite the assertion that the Rpb4/7 heterodimer are dispensable for later stages of RNAPII directed transcription *in vitro*, there is mounting evidence that Rpb4/7 play important roles in many steps of transcription and assist RNAPII to perform at its full capacity.

1.2 Chromatin and the Sir complex

A challenge the typical mammalian cell faces is how to effectively package roughly two meters of DNA—billions of base-pairs—within a nucleus less than 20 micrometers in diameter. Cells solve this problem through the hierarchical compaction of DNA with histones. First, DNA associates with two copies each of the four core histones (H2A, H2B, H3, and H4) to form the nucleosome, then between nucleosomes and histone H1 to form the 30 nm fiber, and then further compacted between 30 nm chromatin fibers in successive iterations to form the chromosome (Woodcock and Ghosh 2010). Packaging of DNA into chromatin not only allows the genetic information to fit within the nucleus, but is essential for the passage of replicated DNA to daughter cells, for

coordinating critical events in genome maintenance and repair, and for proper control of gene expression (Margueron and Reinberg 2010).

The regulatory impact of chromatin has come into sharp focus over the last twenty years, hand-in-hand with a deeper understanding of the epigenetic mechanisms that preside over gene expression (Gardner, Allis et al. 2011). The founding principle connecting the processes of transcriptional regulation and chromatin dynamics is the notion that the packaging of DNA with histones lies on a continuum between two states. On one side of the spectrum is tightly packed chromatin, termed heterochromatin, which restricts the access of transcriptional machinery to DNA, requiring that chromatin be decondensed and nucleosomes dismantled or reorganized to form a more accessible form of chromatin on the opposite side of the spectrum, called euchromatin. Additionally, modification of the histone tails within chromatin allows the cell to integrate a myriad of signaling processes to control access to the DNA, or to signal to the cell that a specific piece of DNA is damaged, recently transcribed, or available for transcription in the future (Bannister and Kouzarides 2011). The potential for a particular gene to be transcribed not only relies upon the state of chromatin compaction, but a variety of other factors, including access of transcriptional machinery to DNA elements and the surrounding chromatin environment of the gene.

Post-translational modification of histones

Another essential component in the regulation of transcription occurs through the post-translational modification of histone tails. It has been known since the mid 1960s that histones are post-translationally modified, and that certain histone modifications potentially impact the level of RNA synthesis (Allfrey, Faulkner et al. 1964). All 4 types of histones in each nucleosome have the potential to be post-translationally modified via their N-terminal tail (and in some cases, within the globular region) including but not limited to methylation, acetylation, phosphorylation, ubiquitylation, and sumoylation, however the most pervasive modifications are methylation and acetylation.

Post-translational modification of histones by methylation occurs on lysine and arginine residues, most prominently on the amino-terminal tails, by histone methyltransferases (HMTs) and is removed by histone demethylases (Greer and Shi 2012). Modification by methylation tends to be more complex than other modifications because lysines can be mono-, di-, or tri-methylated, which have the capacity to lead to a different signaling endpoint (Ng, Yue et al. 2009). Methylation of histones has been shown to confer a diverse array of functions. For example histone H3 lysine 4 tri-methylation (H3K4me3) has been associated with active chromatin (Flanagan, Mi et al. 2005), histone H3 lysine 9 di- and tri-methylation (H3K9me2/3) and histone H3 lysine 27 tri-methylation (H3K27) has been shown to signal silenced chromatin (Lehnertz, Ueda et al. 2003, Rougeulle, Chaumeil et al. 2004), whereas histone H3 lysine 9 mono-methylation (H3K9me) and histone H3 lysine 27 mono-methylation (H3K27me) are associated with gene activation (Barski, Cuddapah et al. 2007).

Histone acetylation occurs on lysine residues through the action of histone acetyl transferases (HATs) and is opposed by histone deacetylases (HDACs). Lysine residues are basic, and positively charged, which causes them to be attracted to the negatively charged DNA, resulting in the compaction of nucleosomes. But upon acetylation of lysine residues, the positive charge becomes neutralized, impairing the electrochemical interactions of lysines with DNA, thereby resulting in loosening of the nucleosome. Acetylation of histones also impairs the higher order folding of nucleosomes, which increases the availability of DNA to transcriptional machinery (Dorigo, Schalch et al. 2003, Shogren-Knaak, Ishii et al. 2006). Because of the anti-silencing effect histone acetylation has, the establishment of heterochromatin is predicated on histone acetylation dynamics, particularly through the deacetylation of histone H4 lysine 16 (H4K16) and in some cases histone H3 lysine 56 (H3K56) acetylation (Xu, Zhang et al. 2007, Oppikofer, Kueng et al. 2011).

Advances in the study of transcriptomics have allowed researchers to further study the telomere position effect. In budding yeast, there are 267 genes located within 20 kb of telomeres, and at steady state these transcripts are found at about 0.5 molecules per cell, much lower than levels of non-telomeric genes (Wyrick, Holstege et al. 1999). The majority of telomere-proximal genes are

involved in the stress response of particular organisms, suggesting that TPE represents an adaptive method of transcriptional regulation important for the response of cells to external stimuli.

The silent information regulator (Sir) complex

The molecular method through which cells control the transcriptional dynamics of heterochromatic regions has been well studied, and the model organism *Saccharomyces cerevisiae* has been an integral tool for uncovering the proteins involved in this process. In this model system, there are three main sites of silent chromatin: rDNA repeats, mating type cassettes (HMR and HML), and subtelomeric regions (Rusche, Kirchmaier et al. 2003). The complex known as the **S**ilenced **I**nformation **R**egulator or Sir complex is responsible for the establishment of these heterochromatic genes (Kueng, Oppikofer et al. 2013).

The Sir complex is a stable, stoichiometric trimeric complex (Cubizolles, Martino et al. 2006) made up of three proteins first identified in a screen for factors that control the expression of genes at the budding yeast mating locus: Sir2, Sir3, and Sir4 (Rine and Herskowitz 1987). Sir3 binds to chromatin to form a scaffold that aides in the spreading of the complex across chromatin (Wang, Li et al. 2013). The recruitment of Sir complex proteins is bolstered by the association of Sir4 with other chromatin associated factors such as Rap1 (Moretti, Freeman et al. 1994), which binds silencing elements in DNA (Shore and Nasmyth 1987, Buchman, Kimmerly et al. 1988), and the Ku heterodimer (Roy, Meier et al. 2004), which binds to telomere ends (Gravel, Larrivee et al. 1998). Sir2 is a NAD-dependent deacetylase (Landry, Sutton et al. 2000, Smith, Brachmann et al. 2000) that is guided to heterochromatic regions through the interaction with Sir4 (Moazed, Kistler et al. 1997).

Spreading of the Sir complex on chromatin

The method through which the Sir complex disperses across chromatin has become more controversial in recent years. A popular model contends that Sir3 preferentially binds to unacetylated H4K16 and H3K79 (Hecht, Laroche et al. 1995, Altaf, Utley et al. 2007), and then specifically to sites of silencing through Rap1 and the Ku complex. Sir3 subsequently attracts the

Sir4/Sir2 heterodimer to form a trimer. Chromatin-bound Sir2 can then deacetylate adjacent H4K16ac, producing the by-product O-acetyl-ADP-ribose, which itself has been shown to attract more Sir3 (Liou, Tanny et al. 2005). This establishes a cycle which leads to the coating of nucleosomes with the Sir complex (**Figure 1.3**). There are some opponents of this model that point out that the Sir complex does not appear to load sequentially onto silenced chromatin, but instead they load all at once (Radman-Livaja, Ruben et al. 2011). Despite this observation, the model of the Sir complex sequentially loading on to chromatin is the most predominant in the field.

Silencing by the Sir complex

The current scientific consensus is that the silencing of chromatin by the Sir complex occurs via steric hindrance through hypoacetylation of histones directed by Sir2, particularly at H4K16 (Johnson, Kayne et al. 1990). This deacetylation by the Sir complex is predicted to block the access of transcriptional machinery to the silenced gene, preventing transcription. However, pre-initiation complexes and serine 5 phosphorylated RNAPII have both been found to be located at areas of chromatin silenced by the Sir complex (Sekinger and Gross 2001), without the presence of transcriptional factors involved in later stages such as the 5' capping enzyme and other elongation factors (Gao and Gross 2008). This observation has lead some researchers to speculate that transcriptional silencing by the Sir complex occurs between the steps of initiation and elongation, by preventing the access of mediator and other general transcription factors, stalling RNAPII at promoter proximal regions. Transcriptional silencing at heterochromatic regions has also been shown to occur independent of histone deacetylation, indicating that there may be alternative modes of gene silencing at telomeres besides through the Sir complex (Koch and Pillus 2009).

Telomere position effect

Transcriptional silencing at subtelomeric regions has been the most prominent area of study in the field of heterochromatin dynamics, and one of the reasons is because of the discovery of the telomere position effect (TPE). TPE is a phenomenon first described in *S. cerevisiae* during an analysis of telomere dynamics, wherein researchers observed different effects on transcription of

reporter genes depending upon their placement in proximity to telomeric regions (Gottschling, Aparicio et al. 1990). The silencing effect seen at subtelomeric regions is widely variable between telomeres, and appears to be dependent upon the elements that make up the specific telomere (Pryde and Louis 1999). Not only has this phenomena been observed in budding yeast, but in the fission yeast *Schizosaccharomyces pombe* (Tong, Keller et al. 2012), *Drosophila melanogaster* (Cryderman, Morris et al. 1999, Mason, Haoudi et al. 2000), and humans (Baur, Zou et al. 2001). TPE is a variegated phenomenon, which can be seen in *S. cerevisiae* when *ADE2* is ectopically placed in silenced subtelomeric regions. When *ADE2* is being expressed under otherwise wild type conditions, cells are white, however when *ADE2* is mutated or silenced, the adenine biosynthetic intermediate aminoimidazoleribotide (AIR) accumulates, which when oxidized turns yeast cells a deep pinkish-red color (Dorfman 1969). The variegation effect leads to red and white sectored colony formation depending on whether a particular cell (and its progeny) silences *ADE2* or not (Park and Lustig 2000).

1.3 Transcription and the Ubiquitin-Proteasome System (UPS)

Another post-translational modification featured prominently in the regulation of not only transcription, but a whole host of cellular processes, is ubiquitylation. Ubiquitin is a 76 amino acid protein first discovered as a polypeptide linked to histone H2A (Goldknopf and Busch 1977), however the field of ubiquitin-proteasomal regulation has substantially progressed since this discovery, and the diversity of ubiquitin signaling processes may be most apparent in the regulation of transcription.

Substrate ubiquitylation

Ubiquitin conjugation occurs through a process that is conserved throughout all eukaryotic organisms. Ubiquitin becomes covalently linked to substrate proteins through a cascade of enzymes, starting with the ubiquitin activating enzyme, or E1 (**Figure 1.4**). ATP is expended to “charge” the ubiquitin molecule which forms a thioester linkage via its C-terminus to a lysine residue on the E1. The charged ubiquitin moiety is then transferred to an ubiquitin conjugating enzyme, or E2. This E2 is then directed to a substrate by a ubiquitin ligase, or E3. The transfer of ubiquitin from the E2 to the substrate can occur two different ways depending on the whether the

E3 is a HECT ubiquitin ligase or a RING ubiquitin ligase. In the case of HECT ligases, the ubiquitin thioester linkage is physically transferred to the E3, before eventual deposition onto a lysine residue on the substrate (Metzger, Hristova et al. 2012). In the case of RING ubiquitin ligases, the E2 and E3 bind via the RING domain of the E3 which appears to "activate" the E2 (Deshaies and Joazeiro 2009), linkage to the substrate. The process of ubiquitin linkage to substrate proteins is reversible via the action of de-ubiquitylating enzymes called DUBs. Furthermore, ubiquitin can form 8 different kinds of polyubiquitin chains, either via the seven unique lysines contained within ubiquitin (K6, K11, K27, K29, K33, K48, and K63), or as linear chains that link through the N-terminus of ubiquitin. These polyubiquitin chains are formed by ligases that add additional ubiquitin onto an already ubiquitylated substrate and are known as ubiquitin elongating enzymes or E4s. Polyubiquitin chains have been shown to be formed on all 7 lysines within the ubiquitin molecule (Zhang, Lv et al. 2013, Durcan, Tang et al. 2014, Michel, Elliott et al. 2015, Palicharla and Maddika 2015), however the most biologically relevant chains appear to be K48 and K63. Polyubiquitin chains are normally associated with proteasomal degradation, as is seen with K48 linkages. However, K63 linkages are typically seen in signaling (Wang, Yang et al. 2015) and trafficking (Erpapazoglou, Walker et al. 2014) and generally do not signal degradation by the proteasome. To further complicate this process, ubiquitin chains can be branched (multiple linkages from one ubiquitin residue) or mixed (multiple lysine linkages within a chain) (Nakasone, Livnat-Levanon et al. 2013, Meyer and Rape 2014). Additionally, monoubiquitylation, or ligation of a singular ubiquitin residue onto a substrate, can also be used for regulation via adapters called ubiquitin binding domains (UBDs) (Harper and Schulman 2006) or to prevent the association of proteins (Daulny, Geng et al. 2008).

Ubiquitylation in the control of transcription

One of the reasons the ubiquitin proteasome system (UPS) is such an intriguing network for the control of transcription is its versatility. The UPS has a diversity of tools to implement, the most evident is through the control of steady state levels of transcriptional activators and co-activators through the polyubiquitylation of substrates via K48 chains and destruction by the proteasome. Prominent examples of regulation by this process include the mammalian transcription factors β-catenin of the Wnt pathway (Stamos and Weis 2013), the oncoprotein Myc (Thomas and Tansey

2011), and the tumor suppressor p53 (Pant and Lozano 2014). However, the regulation of the proteolytic stability of a protein is only one layer of regulation by ubiquitylation. In addition, ubiquitylation has the ability to control the form and function of transcription factors through proteolytic and non-proteolytic methods as well. The sonic hedgehog pathway component Gli3 is a transcriptional repressor in its full length form, but upon ubiquitylation, it is sent to the proteasome for partial degradation (Wang, McMahon et al. 2007). This processed fragment opposes the function of full length Gli3. In the case of the transcription factor FOXO4, upon the sensing of oxidative stress, FOXO4 is monoubiquitylated (van der Horst, de Vries-Smits et al. 2006). This ubiquitylation directs FOXO4 to the nucleus where it is in its transcriptionally active form. This process can also be reversed through the deubiquitylation by USP7.

Ubiquitin can also affect the binding capacity of transcription factors. Gal4 is a transcriptional activator in *S. cerevisiae* that induces the transcription of genes for the metabolism of galactose in the absence glucose (Traven, Jelicic et al. 2006). Unmodified Gal4 is actively stripped from chromatin by the 19S base of the proteasome, preventing transcription, however upon monoubiquitylation, Gal4 appears to "lock" in place on chromatin (Archer, Delahodde et al. 2008). Conversely, ubiquitin has also been shown to induce the removal of chromatin bound substrates through a poly-ubiquitin-selective, ATP-dependent, segregase complex known as Cdc48 (p97 in mammalian cells) to extract substrates prior to degradation. Cdc48-dependent extraction of ubiquitylated substrates from chromatin has been shown to occur with the yeast MAT2 repressor (Wilcox and Laney 2009), RNAPII after DNA damage (Verma, Oania et al. 2011), and with both natural and synthetic transcriptional regulators (Ndoja, Cohen et al. 2014). This suggests that many, if not all, polyubiquitylated DNA-bound substrates need to be passed through Cdc48 complexes to reach the proteasome.

Transcriptional regulation through ubiquitin signaling on histones

The non-proteolytic functions of regulation by ubiquitylation work in a similar manner to other post-translational modifications. One of the most established areas of ubiquitin signaling in transcription is histone ubiquitylation. All histones have been shown to be ubiquitylated (Weake and Workman 2008), however the best studied examples are monoubiquitylation of H2A and

H2B. Ubiquitylation of H2A in humans is coordinated by at least two ubiquitin ligases, Ring1B and 2A-HUB (Cao, Tsukada et al. 2005, Zhou, Zhu et al. 2008). This ubiquitylation mark results in transcriptional silencing, and appears to act downstream of other silencing marks such as H3K27me and H3K9me (Cao, Tsukada et al. 2005). H2A ubiquitylation has been shown to stimulate the association of core histones with the linker histone H1, leading researchers to hypothesize that the transcriptional repression seen in H2A monoubiquitylation results from the compaction of histones (Jason, Finn et al. 2005, Zhu, Zhou et al. 2007).

While H2A ubiquitylation has been shown to be repressive to transcription, H2B ubiquitylation at lysine 123 is a mark of gene activation. This ubiquitylation event is not only responsible for direct physical modifications of the histone core, by relaxing chromatin to allow access to transcriptional machinery, but it is responsible for signaling to other chromatin modifiers (Geng, Wenzel et al. 2012). H2B ubiquitylation primarily occurs at lysine 123, and was one of the first examples of “histone crosstalk” when it was discovered that ubiquitylation at this mark is required for H3K4 and H3K79 di- and tri-methylation (Briggs, Xiao et al. 2002, Sun and Allis 2002).

Ubiquitylation of RNA polymerase II

Ubiquitylation is involved in the regulation of almost every step of transcription, yet direct regulation of RNAPII by ubiquitylation under normal conditions has yet to be discovered despite that fact that it has been known for nearly 20 years that RNAPII is ubiquitylated (Bregman, Halaban et al. 1996). In the event of DNA damage via UV irradiation or through a chemical agent such as cisplatin, RNAPII becomes ubiquitylated in a manner that requires the phosphorylation of the CTD of Rpb1 (Bregman, Halaban et al. 1996, Ratner, Balasubramanian et al. 1998, Mitsui and Sharp 1999). This ubiquitylation was thought to only occur during DNA damage, but has since been shown to occur during other instances of transcriptional arrest in what is believed to be a way to clear the RNA polymerase machinery from chromatin as a “last resort” (Somesh, Reid et al. 2005). The identity of the E3 that ubiquitylates RNAPII has previously been a point of contention; however the process has since been elucidated by the Svejstrup lab. They found that in budding yeast, the HECT ubiquitin ligase Rsp5 targets stalled RNAPII and polyubiquitylates Rpb1 via a lysine 63 (K63) chain (Harreman, Taschner et al. 2009). This K63 chain is trimmed to

a single ubiquitin molecule via Ubp2, a DUB that associates with Rsp5. The Elc1/Cul3 complex, acting as an E4, can then extend the monoubiquitin into K48 linked chains. The process of ubiquitylating RNAPII is conserved in Human cells, with the Rsp5 homolog Nedd4 acting as the E3 and the Elc1/Cul3 cognate ElonginA/B/C-Cullin 5 complex as the E4.

1.4 RPC family of proteins

One of the goals of the Tansey laboratory is to characterize proteins that intersect the two processes of transcription and the UPS. While searching the literature to find proteins that fit this criteria, a group of proteins were discovered that not only had the ability to associate with Rpb1 through a newly discovered RNAPII interacting domain, they also contained a RING finger domain (allowing for potential ubiquitin ligase activity) and a PHD finger domain (which possesses the potential of chromatin binding activity) (Yuryev, Patturajan et al. 1996). To utilize the genetic tractability of yeast, a BLAST search was performed to identify a homologous protein in budding yeast (*Saccharomyces cerevisiae*). Although no truly homologous protein was found, there were a number of proteins with significant homology to the C-terminal repeat binding domain (CBD). Some of these RPC family members also contained serine/arginine rich regions called SR domains that have been implicated in mRNA splicing (Shepard and Hertel 2009). Among these proteins, there was a large subset that contained either a RING/PHD finger at the N-terminal domain of the protein, which were called RPC (RING/PHD/CBD) proteins. RPC proteins are found in a wide range of organisms, from unicellular organisms like budding yeast to more complex metazoans. (**Figure 1.5**).

RING finger domain

As mentioned above, the three domains that define the RPC family of proteins are an N-terminal RING finger, a PHD finger, and a CBD that binds to the C-terminal repeats of Rpb1. RING finger containing proteins make up one of the two main classes of ubiquitin ligases (the other being HECT ubiquitin ligases). The canonical RING finger consensus sequence is Cys-X_2-Cys-$X_{(9\text{-}39)}$-Cys-$X_{(1\text{-}3)}$-His-$X_{(2\text{-}3)}$-Cys-X_2-Cys-$X_{(4\text{-}48)}$-Cys-X_2-Cys, where X is any amino acid (Deshaies and Joazeiro 2009). The cysteine and histidine residues are buried within the core of RING finger proteins and coordinate the binding of two zinc residues. This coordination of zinc binding is

essential for the activity of RING finger proteins, and point mutations affecting two or more of the Cysteine/Histidine residues are often used to disrupt the E3 activity of the protein. Higher metazoans of the RPC family of proteins have a near identical RING finger domain, while lower metazoans and yeast are quite diverse, yet there are certain areas of high homology (**Figure 1.6**). When comparing the sequences of the RPC family proteins of budding yeast (Asr1) and humans (PHRF1) with the RING domain of other budding yeast proteins (Tul1, Hel1, Uls1, and Bre1), Asr1 and PHRF1 are more closely related to each other than to the other budding yeast RING finger proteins (**Figure 1.7 A and B**).

PHD finger domain

PHD fingers are a class of chromatin binding domains that recognize the N-terminal tail of histone H3 (Musselman and Kutateladze 2011). Proteins that contain a PHD finger have been shown to associate with both unmodified H3 (Lan, Collins et al. 2007) and modified forms of H3 (Lange, Kaynak et al. 2008, Otani, Nankumo et al. 2009). PHD fingers are often confused with RING fingers because they both are able to chelate zinc ions through a similar coordination of cysteine and histidine residues, however PHD domains have been shown to fold differently and have not been shown to possess E3 ligase activity (Scheel and Hofmann 2003, Bottomley, Stier et al. 2005). A sequence comparison of RPC family PHD fingers shows a large amount of conservation (**Figure 1.8**). An alignment of the RPC family members PHRF1 and Asr1 with other budding yeast PHD finger proteins (Jhd2, Bye1, Cti6, and Set3) shows close relation between Asr1 and PHRF1, as well as the the histone H3K4 demethylase Jhd2, which binds to chromatin independently of methylated H3K4 (**Figure 1.9**) (Huang, Ramakrishnan et al. 2015). Of note is the lack of a conserved tryptophan residue in PHRF1, Asr1 and Jhd2 that is seen in other PHD domains. This aromatic residue is required for the association of methylated H3 tails (**Figure 1.9**; *boxed region*), suggesting RPC proteins, like Jhd2, do not possess the ability to bind H3K4 methylated histones (Sanchez and Zhou 2011).

C-terminal binding domain

The most C-terminally located domain of RPC proteins is the CBD, which binds the C-terminal repeats of Rpb1. This particular domain of RPC proteins has not been well studied since its

discovery nearly 20 years ago (Yuryev, Patturajan et al. 1996). An alignment of RPC family proteins reveals that metazoans have a nearly identical CBD whereas the three yeast species analyzed have a high degree of divergence, yet there are small sites of significant homology (**Figure 1.10**). Despite the divergence in sequence, the CBD of Asr1 has been shown to directly associate with the C-terminal repeats of Rpb1 experimentally, demonstrating that these domains function similarly within the cell (Daulny, Geng et al. 2008).

The human RPC family member PHRF1

Until recently, the human member of the RPC family, PHRF1, has not been extensively studied. While the function of this protein has remained unclear, PHRF1 has been identified in multiple genome-wide association studies (GWAS) for systemic lupus erythematosus (SLE) (International Consortium for Systemic Lupus Erythematosus, Harley et al. 2008, Suarez-Gestal, Calaza et al. 2009, Salloum, Franek et al. 2010, Chung, Taylor et al. 2011, Sanchez, Nadig et al. 2011, Jarvinen, Hellquist et al. 2012). The frequency with which PHRF1 has been identified in SLE GWA studies suggests that it does play a role in the progression of this disease, but with the current studies, it is unclear how PHRF1 affects the progression of the disease.

There is also evidence that PHRF1 acts in the regulation of DNA end-joining in response to double strand DNA breaks (Chang, Chu et al. 2015). A role for PHRF1 in the DNA damage response was first proposed in 2007, when PHRF1 was identified in a large scale screen for proteins phosphorylated by DNA damage kinases ATM and ATR (Matsuoka, Ballif et al. 2007). Cells also stall in M phase when they are treated with irradiation and PHRF1 siRNA concurrently, suggesting that in the absence of PHRF1, DNA damage machinery is not able to sense damage. Recently PHRF1 was characterized as a tumor suppressor (Ettahar, Ferrigno et al. 2013, Prunier, Zhang et al. 2015). In these studies, the researchers show that PHRF1 binds and ubiquitylates TGIF, which frees up cPML (another tumor suppressor) to activate SMAD signaling. The scope of these studies do not consider any role PHRF1 may play in the regulation of RNAPII directly.

The *Saccharomyces cerevisiae* protein Asr1

Asr1 or **A**lcohol **S**ensitive **R**ING/PHD finger is a ~35kDa protein originally characterized as an alcohol stress sensing protein in budding yeast, although this finding has not been reproduced by our laboratory (Daulny, Geng et al. 2008) or others (Izawa, Ikeda et al. 2006). Biochemical analysis of Asr1 shows that it is a bona fide E3 ubiquitin ligase that specifically associates with the serine 5 hyperphosphorylated form of Rpb1, seen in recently initiated RNAPII complexes. Once Asr1 is bound to Rpb1, it is able to oligoubiquitylate at least 2 subunits of RNAPII (Rpb1 and Rpb2). The result of these ubiquitylation events is a transcriptionally inactive RNAPII complex that is devoid of the Rpb4/7 heterodimer (Daulny, Geng et al. 2008). Despite the explicit molecular action of Asr1 on RNAPII, it is unclear the biological context in which Asr1 acts. Since its initial characterization, Asr1 has been suggested to be involved in stress sensing via the targeting of calmodulin (Fries, Frank et al. 2011) as well as having a role in cell cycle progression (Zou, Yan et al. 2015), however neither of these processes consider the direct role Asr1 has on the modification of RNAPII.

1.5 Summary of thesis

The RPC family of proteins are well conserved throughout eukaryotes, featuring members from budding yeast to humans. Despite the well characterized biochemical actions of the *Saccharomyces cerevisiae* member Asr1 in the negative regulation of RNA polymerase II, the biological role of this protein remains unclear. The purpose of my thesis is to uncover the cellular actions of Asr1 in the regulation of RNAPII, using a multidimensional approach, and to provide additional insight into the purpose of RPC proteins in eukaryotes.

Through the work in this thesis, I show that Asr1 specifically interacts with the deubiquitylase Ubp3 and its obligate cofactor Bre5. I also found that Ubp3 requires the association of Asr1 for its association with RNA polymerase II (RNAPII). Ubp3 has been implicated in many different biological process, but I have shown that the only genetic interaction shown between Asr1 and Ubp3 is in the process of telomeric silencing and ubiquitylation of RNAPII. I show that loss of ubiquitylation activity by Asr1 induces transcription of at least 2 telomeric reporters and many different endogenous subtelomeric genes. Point mutations of lysine residues on *RPB1* that

prevent the ubiquitylation by Asr1 also result in the induction of native telomeric genes. Loss of *UBP3* expression within the context of Asr1 mutation reverses the induction of subtelomeric genes, suggesting an opposing role for Ubp3 in telomeric silencing. Asr1 physically associates with silenced subtelomeric genes, independent of its RING activity, suggesting a direct role in telomeric silencing. Mutation of *ASR1* or *RPB1* do not affect the acetylation status of H4K16 compared to wild type (WT) at areas of telomeric silencing, suggesting that Asr1/Ubp3 regulate telomeric expression via an alternative method than the silent information regulator (Sir) complex. In all, this thesis uncovers a unique method in which an E3 ligase regulates the transcriptional activity of RNAPII via non-proteolytic ubiquitylation.

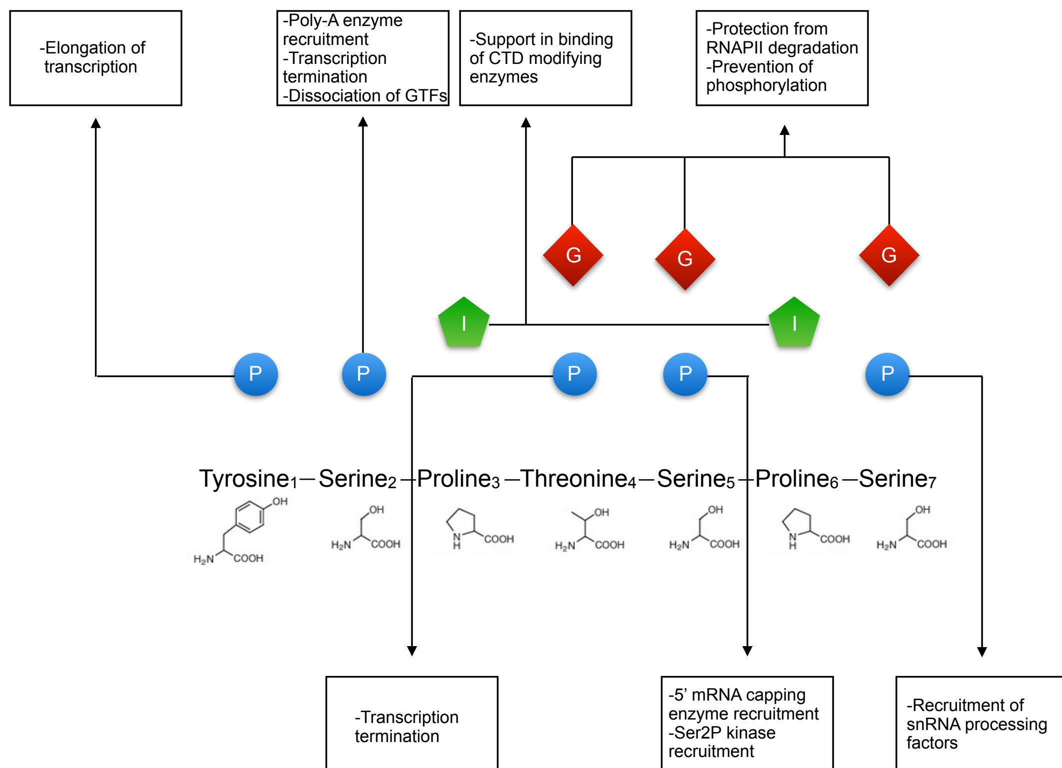

Figure 1.1 The C-terminal domain of Rpb1. Residues that make up the consensus C-terminal repeat region of Rpb1 are shown, with the possible post-translation modifications that affect the activity of Rpb1. P=phosphorylation, I=isomerization of proline between *cis* and *trans* conformations, G=glycosylation.

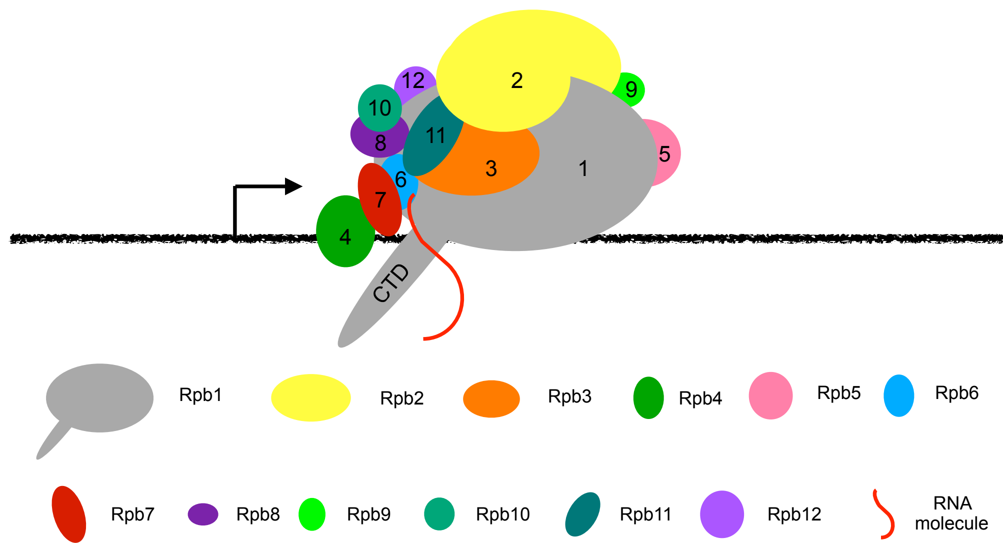

Figure 1.2 Subunit composition of RNA polymerase II (RNAPII) holoenzyme. Cartoon depiction of budding yeast RNAPII transcribing a gene with its base subunits. Individual subunits are differentially color-coded and labelled with a number according to their protein name. The C-terminal domain (CTD) of Rpb1 is depicted as an extension from the core Rpb1 molecule.

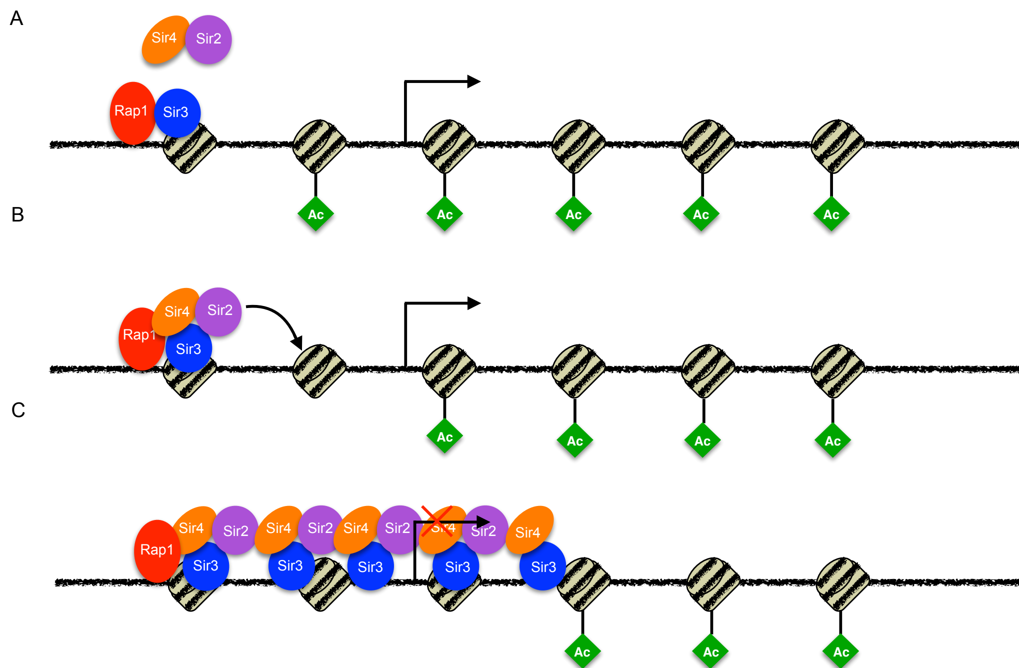

Figure 1.3 Model of Sir complex spreading on chromatin. (*A*) Rap1 binds and recruits Sir3 to unmodified histones surrounding area of heterochromatin. (*B*) Sir2/Sir4 dimer is recruited and binds to Sir3 forming a trimeric complex. Once chromatin bound, Sir2 deacetylates adjacent histones at H4K16. (*C*) Deacetylation attracts additional Sir complex subunits, which continues the recruitment cycle, spreading the Sir complex across the gene, deacetylating additional H4K16 residues and establishing an area of heterochromatin.

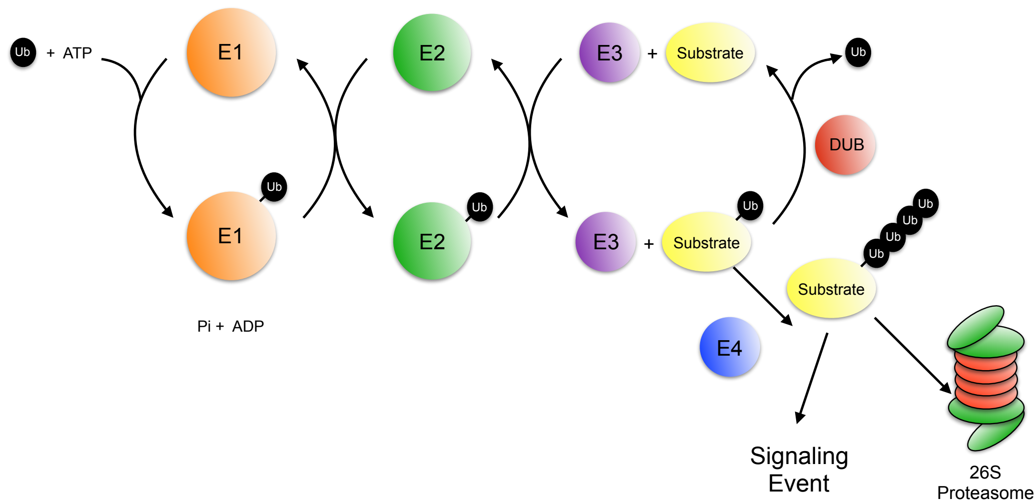

Figure 1.4 Enzymatic cascade of ubiquitylation in cells. The E1 activates ubiquitin in an ATP dependent reaction that forms a thioester bond at the C-terminal end of ubiquitin on the E1. Ubiquitin is then transferred to an E2 conjugating enzyme. The E3 ligase recruits the substrate to the E2 and stimulates its activity. Ubiquitin is then either transferred directly to the substrate (in RING ligases) or to the E3 then the substrate (in HECT ligases). The process of ubiquitylation is reversible through the action of a deubiquitylating enzyme (DUB). Ubiquitin chains can be extended through the action of an E4. Polyubiquitylated substrates are then directed to the proteasome for destruction or some other signaling event depending upon the composition of the chain.

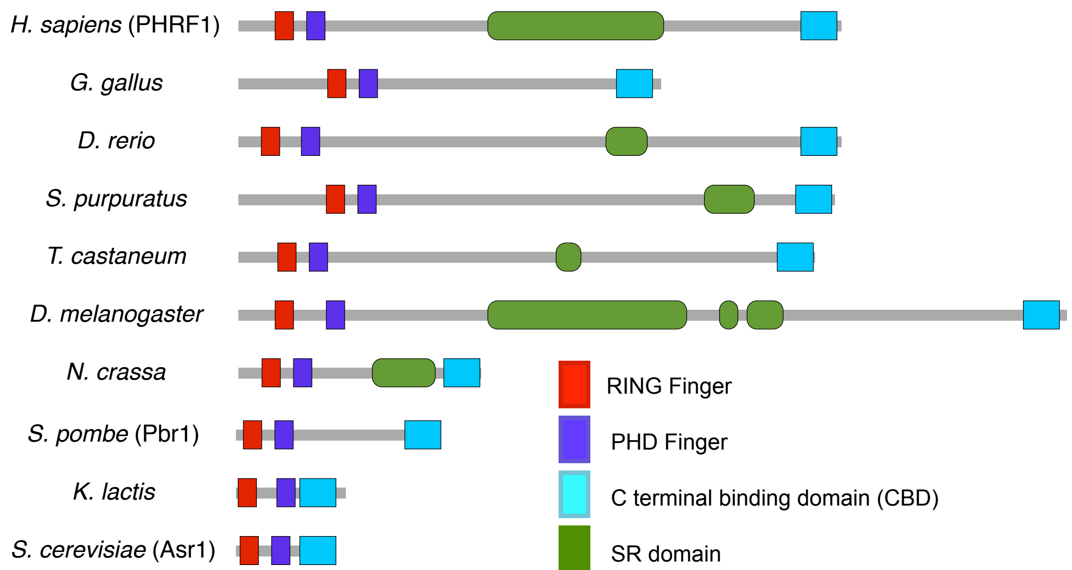

Figure 1.5 The RPC family of proteins. Cartoon representation of RPC proteins, which are found in a multitude of eukaryotic organisms. Proteins depicted to scale. The RING finger is represented in red, the PHD finger is represented in purple, SR domains are represented in green, and the CBD is represented in cyan.

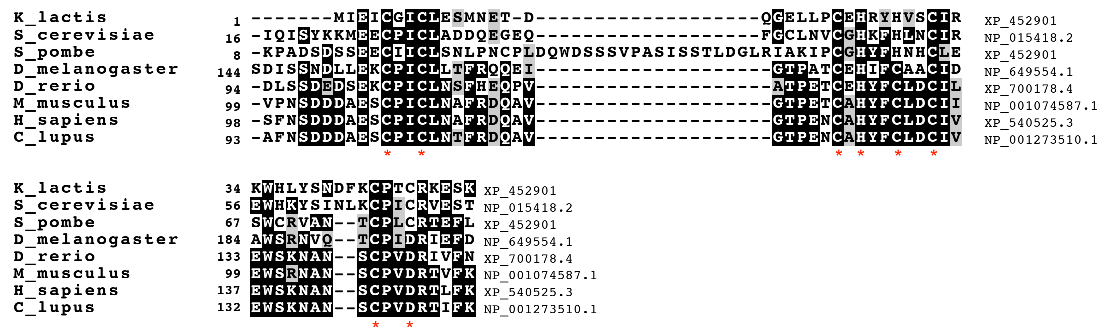

Figure 1.6 Sequence alignment of RPC family RING finger domains. Sequences of domains compiled from the NCBI database (*www.ncbi.nlm.nih.gov/*), and labeled with their gene accession numbers. Alignments were done using Clustal Omega (*http://www.ebi.ac.uk/Tools/msa/clustalo/*). Identical residues highlighted in black and similar residues highlighted in grey using Boxshade (*http://www.ch.embnet.org/software/BOX_form.html*). Zinc coordinating residues identified with a red asterisk.

A

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S_cerevisiae_TUL1    689 --GGGTAEHTVDCAICMSDVPIYIEEIPETHKVDQHSYMVTPCNHVFHTSCLENWMNYKL NP_012890.1
H_sapiens_PHRF1       98 --SFNSDDDAESCPICLNAF-----------R-DQAVGTPENCAHYFCLDCIVEWSKNAN NP_001273510.1
S_cerevisiae_ASR1     16 --IQISYKKMEECPICLADD-----------QEGEQFGCLNVCGHKFHLNCIREWHKYSI NP_015418.2
S_cerevisiae_HEL1    169 --REVEFKNDFTCIICCDKK------------DTET--FALECGHEYCINCYRHYIKDKL NP_012942.3
S_cerevisiae_ULS1   1320 --QVITSMNSMTCFWCMEQL------------EPEAMSVLTGCGHLICDTCIEPFIEESS NP_014834.1
S_cerevisiae_BRE1    636 VEELANFRTLVYCSLCSKNW------------K---NMAIKTCGHVFCENCCKERLAARM NP_010209.1

S_cerevisiae_TUL1    747 Q---------------CPVCRSPLPPL----- NP_012890.1
H_sapiens_PHRF1      137 S---------------CPVDRTLFKCICIRAQ NP_001273510.1
S_cerevisiae_ASR1     56 N-------------LKCPICRVESTHLEVGEG NP_015418.2
S_cerevisiae_HEL1    213 H---------EGNIITCMDCSLALKNEDID-- NP_012942.3
S_cerevisiae_ULS1   1366 MLPQAKKTKGGAFAIPCKDCQRLTNEKDIVS- NP_014834.1
S_cerevisiae_BRE1    681 --------------RKCPTCNKAFSSNDLLTV NP_010209.1
```

B

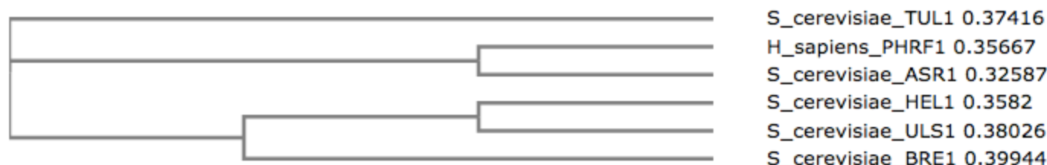


Figure 1.7 Sequence alignment of Asr1 and PHRF1 with budding yeast RING finger domains. (*A*) Sequences of domains compiled from the NCBI database (*www.ncbi.nlm.nih.gov/*), and labeled with their gene accession numbers. Alignments were done using Clustal Omega (*http://www.ebi.ac.uk/Tools/msa/clustalo/*). Identical residues highlighted in black and similar residues highlighted in grey using Boxshade (http://www.ch.embnet.org/software/BOX_form.html). (*B*) Phylogram showing the evolutionary relationships of RING domains, constructed using the “Phylogenetic Tree” tool on Clustal Omega.

```
S_cerevisiae     137 -TIKIIQCSICGDTDVSRLSLYCQDCEAIYHETCLRGLACEVGDRNTWQECTDCRSNALL NP_015418.2
K_lactis         136 QGVELLQCALCGEID-DDITLYCESCETLFHSSCLNELLCEVGEKEWC--CIECDGTLCR XP_452901
S_pombe          118 EGSETCRCVICGRSDHAEVLLLCDGCDDAYHTYCLN--MDAVPIEEFY--CPNCVLLNYQ NP_588450.1
D_melanogaster   240 SEEEVTNCEICESPDREDVMLLCDSCNQGYHMDCLDPPLYEIPAGSWY--CDNCIDSDDE NP_649554.1
D_rerio          180 VDLDQTSCEICGGRDREDRLLLCDGCDAGYHMECLTPPLDAVPVEEWF--CPECIANNRT XP_700178.4
M_musculus       181 EEEDPTFCEVCGRSDREDRLLLCDGCDAGYHMECLDPPLQEVPVDEWF--CPECTVPGVD NP_001074587.1
H_sapiens        179 EEEDPTFCEVCGRSDREDRLLLCDGCDAGYHMECLDPPLQEVPVDEWF--CPECAAPGVV XP_540525.3
C_lupus          175 EEEDPTFCEVCGRSDREDRLLLCDGCDAGYHMECLDPPLQEVPVDEWF--CPECAAPGAA NP_001273510.1
                            *  *              *  *     *  *              *  *
```

Figure 1.8 Sequence alignment of RPC family PHD finger domains. Sequences of domains compiled from the NCBI database (*www.ncbi.nlm.nih.gov/*), and labeled with their gene accession numbers. Alignments were done using Clustal Omega (*http://www.ebi.ac.uk/Tools/msa/clustalo/*). Identical residues highlighted in black and similar residues highlighted in grey using Boxshade (*http://www.ch.embnet.org/software/BOX_form.html*). Zinc coordinating residues identified with a red asterisk.

```
S_cerevisiae_ASR1   142 QCSICGDTDV--------SRLSLYCQDCEAIYHETCLRGLACEV---GDRNTWQECTDCR NP_015418.2
H_sapiens_PHRF1     185 FCEVCGRSDR--------EDRLLLCDGCDAGYHMECLDPPLQEV---PVD-EWF-CPECA NP_001273510.1
S_cerevisiae_JHD2   237 ACIVCRKTND--------PKRTILCDSCDKPFHIYCLSPPLERV---PSG-DWI-CNTCI NP_012653.1
S_cerevisiae_BYE1    74 -RCLCGANNENYDAAEYSHGDMVQCDGCDTWQHIKCMTDGKDTIDGLMSEDSKYYCELCD NP_012921.3
S_cerevisiae_CTI6    74 -RCICGELDTPD-----DSGFFIQCEQCSSWQHGYCVSI-----TQ-DNAPDKYWCEQCR NP_015144.1
S_cerevisiae_SET3   119 -TCIC---DLND-----DDGFTIQCDHCNRWQHAICYGI-----KDIGMAPDDYLCNSCD NP_012954.3
```

1.9 **Sequence alignment of Asr1 and PHRF1 with budding yeast PHD finger domains.** Sequences of domains compiled from the NCBI database (*www.ncbi.nlm.nih.gov/*), and labeled with their gene accession numbers. Alignments were done using Clustal Omega (*http://www.ebi.ac.uk/Tools/msa/clustalo/*). Identical residues highlighted in black and similar residues highlighted in grey using Boxshade (http://www.ch.embnet.org/software/BOX_form.html). Conserved tryptophan necessary to form an “aromatic cage” in the binding of H3K4 methylated histones is boxed.

```
D_melanogaster  2202 LRKLNRQERVVEEVKLVLKPYFNKKAITKDDYKDIMRRAVPKICHSRSGEINPHKIKNLI NP_649554.1
D_rerio         1576 MKKLHMQERAIEEVKLAIKPFYQKRDITKEEYKEILRKAVQKVCHSKSGEINPVKVANLV XP_700178.4
H_sapiens       1561 MKKLHMQERAVEEVKLAIKPFYQKREVTKEEYKDILRKAVQKICHSKSGEINPVKVANLV NP_001273510.1
M_musculus      1594 MKKLHMQERAVEEVKLAIKPFYQKREVTKEEYKDILRKAVQKICHSKSGEINPVKVANLV NP_001074587.1
C_lupus         1538 MKKLHMQERAVEEVKLAIKPFYQKREVTKDEYKDILRKAVQKICHSKSGEINPVKVGNLV XP_540525.3
S_pombe          494 --SYETKYRIERLVNAALKPYYREAKISKDQFALFNKNICRSVYTALSDGTLSLEGPQQH NP_588450.1
K_lactis         235 TISYEDKCRIQGYVRTELDKFYHRGSLSKDRYIAINKMVSRKLYGLSSTGFDPSRINYEE XP_452901
S_cerevisiae     234 EQIRNAKHKIQMHVRRALDRYPLPLLRFKDAYKHVNKQVSRKLYRLSDNKYLPDQYDYD- NP_015418.2
```

1.10 **Sequence alignment of RPC family CBD.** Sequences of domains compiled from the NCBI database (*www.ncbi.nlm.nih.gov/*), and labeled with their gene accession numbers. Alignments were done using Clustal Omega (*http://www.ebi.ac.uk/Tools/msa/clustalo/*). Identical residues highlighted in black and similar residues highlighted in grey using Boxshade (http://www.ch.embnet.org/software/BOX_form.html).

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Yeast strains

The strains used in this thesis are listed in **Table 2.1**. The deletion mutants used were either collected from the *MATa* yeast knockout library (*Thermo Fischer*) or deleted using homologous recombination of the *URA3*, *HIS3MX6*, *kanMX6*, or *natMX4* cassettes. Proteins were epitope tagged by using homologous recombination of epitope and antibiotic or auxotrophic cassettes (Knop, Siegers et al. 1999, Funakoshi and Hochstrasser 2009). In both cases, primers were designed with 50 bp of homology to either side of desired insertion (the cassette replaced the entire ORF in the case of deletions and the epitope was inserted just before the STOP codon in the case of protein tagging) and 18-22 bp homologous with insertion cassette. Polymerase Chain Reaction (PCR) was used to amplify the cassette using the Expand Long Template PCR system (Roche) as indicated by the provided protocol. PCR products were transformed into cells using the High Efficiency LiAc/ss-DNA/PEG protocol (Gietz and Schiestl 2007). Overnight cultures were diluted to 50 ml at 0.2 OD_{600} and allowed to grow to log phase (~0.8 OD_{600}) at 30 °C. The culture was spun down and washed with 0.1M LiAC. Transformation reactions were as follows: 1/10th of the total yeast cells, 240 µl PEG 3500 (50% w/v), 36 µl 1M LiAC, 50 µl salmon sperm DNA (2 mg/ml), 5 µl PCR reaction (or 50 ng plasmid) and 29µl H_20. Reaction was vortexed and heat shocked in a 42 °C water bath for 40 minutes. Reactions were spun down and the transformation mix was removed. If the cassette is an antibiotic resistance, the cells are resuspended in 1 ml YPAD and recovered for 1 hour at 30 °C, then spread on appropriate selective agar plate. Genetic integrations were validated by PCR.

2.2 Plasmids

Plasmids used in this study are described in **Table 2.2.** pRS415 Ubp3–3MYC was made by PCR-amplification of coding sequences of MYC-epitope tagged *UBP3* from genomic DNA of strain YTM41 (**Table 2.1**) and cloning into the *XhoI* and *SpeI* sites of pRS415 GPD (Mumberg, Muller et al. 1995). Amino-terminal Ubp3 deletion mutants N45∆, N90∆, N145∆, and N180∆ as well as internal Ubp3 deletions 91-115∆, 116-145∆, 141-160∆, and 161-180∆ were made using the

pRS415 Ubp3–3MYC plasmid via whole plasmid PCR. pRS415 N180 Ubp3-GFP-3MYC was constructed using Gibson assembly with eGFP (pKT0127) (Sheff and Thorn 2004) and pRS415 Ubp3-3MYC as a template. pRS415 GFP-3MYC plasmid was made using the pRS415 N180-Ubp3-GFP-3MYC plasmid via whole plasmid PCR using primers designed to delete Ubp3-sequences. pRS415 HA–Asr1, pRS415 HA–Asr1 RING, and pRS415 HA–Asr1 PHD plasmids were made using Gibson assembly by PCR-amplification of the coding sequences of HA–Asr1 from the relevant pYES2 HA–Asr1 vectors (Daulny, Geng et al. 2008) and cloning into pRS415 GPD. The integrity of all recombinant plasmids was confirmed by DNA sequencing.

2.3 Primers

All primers used in this study can be found in **Table 2.3**.

2.4 Antibodies

The following antibodies and antibody conjugates were usedin this study: anti-FLAG: M2-HRP (*Sigma*; A8592), M2 affinity gel (*Sigma*; A2220); anti-MYC: 9E10 (Vanderbilt Molecular Biology Core), anti-MYC-HRP (*Roche*; 11 814 150 001); anti-HA: 12CA5 (Cold Spring Harbor Monoclonal Shared Resource, anti-HA-HRP (*Roche*; 3F10); anti-H4K16ac (*Millipore*; 07-329); anti-RNA polymerase II subunit B1 (phospho-CTD Ser-5) (*Millipore*; 04-1572); anti-Act1 (*Abcam*; ab8224); Rabbit anti-Rat IgG HRP (*Thermo Fisher Scientific*; PA128573); Goat anti-Mouse IgG HRP (*Thermo Fisher Scientific*; 32430), and anti-Rpb1 CTD *(Cell Signaling Technology*; 2629S).

Table 2.1: Yeast strains used in this thesis

Strain	Genotype	Source
BY4741	MATa, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0*	Open Biosystems
BY4742	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0*	Open Biosystems
YTM1	BY4741 but *UBP3*-3HA::*KanMX6*	This study
YTM2	YTM1 but *ASR1*-3FLAG::*HIS3MX6*	This study
YTM3	BY4741 but *BRE5*-3FLAG::*HIS3MX6*	This study
YTM4	YTM3 but *ASR1*-3HA::Kan*MX6*	This study
YTM5	BY4741 but *asr1Δ*::*NatMX4*	This study
ΔUbp3	BY4741 but *ubp3Δ*::*KanMX6*	Thermo Scientific MATa deletion library
ΔSir2	BY4741 but *sir2Δ*::*KanMX6*	Thermo Scientific MATa deletion library
YTM6	Ubp3Δ but *asr1Δ*::*NatMX4*	This study
YTM7	YTM6 but *BRE5*-3FLAG::*HIS3MX6*	This study
YTM8	YTM6 but *RPB3*-3FLAG::*HIS3MX6*	This study
YTM9	YTM7 but +[pRS415 GPD] +[pYES2 HA-Asr1]	This study
YTM10	YTM7 but +[pRS415 GPD] +[pYES2 HA-Asr1 RING]	This study
YTM11	YTM7 but +[pRS415 GPD] +[pYES2 HA-Asr1 PHD]	This study
YTM12	YTM7 but +[pRS415 GPD] +[pYES2 HA-CBD]	This study
YTM13	YTM7 but +[pRS415 Ubp3-3MYC] +[pYES2 HA-Asr1]	This study
YTM14	YTM7 but +[pRS415 Ubp3-3MYC] +[pYES2 HA-Asr1 RING]	This study
YTM15	YTM7 but +[pRS415 Ubp3-3MYC] +[pYES2 HA-Asr1 PHD]	This study
YTM16	YTM7 but +pRS415 Ubp3-3MYC] +[pYES2 HA-CBD]	This study
YTM17	YTM7 but +[pRS415 Ubp3-3MYC N180Δ] + [pYES2 HA-Asr1]	This study
YTM18	YTM7 but +[pRS415 Ubp3-3MYC N145Δ] + [pYES2 HA-Asr1]	This study
YTM19	YTM7 but +[pRS415 Ubp3-3MYC N90Δ] + [pYES2 HA-Asr1]	This study
YTM20	YTM7 but +[pRS415 Ubp3-3MYC N45Δ] +pYES2 HA-Asr1]	This study
YTM21	YTM8 but +[pRS415 GPD] +[pYES2]	This study
YTM22	YTM8 but +[pRS415 GPD] +[pYES2 HA-Asr1]	This study
YTM23	YTM8 but +[pRS415 Ubp3-3MYC] +[pYES2]	This study
YTM24	YTM8 but +[pRS415 Ubp3-3MYC] +[pYES2 HA-Asr1]	This study

Table 2.1: Yeast strains used in this thesis		
YTM25	YTM8 but +[pRS415 Ubp3-3MYC N180∆] + [pYES2]	This study
YTM26	YTM8 but +[pRS415 Ubp3-3MYC N180∆] + [pYES2 HA-Asr1]	This study
YTM27	BY4742 but *asr1*::C26A/C29A	Daulny *et. al* 2008
YTM28	YTM27 but *ubp3∆*::*KanMX6*	This study
YTM29	BY4742 but *rpb1*::K1452R/K1458R/K1487R ∆1720-1734	Daulny *et. al* 2008
LPY4819	MATα *hmr*::*TRP1* rDNA::*ADE2-CAN1* TELVR::*URA3*	Clarke *et. al* 2006
LPY4819 ∆Asr1	LPY4819 but *asr1∆*::*NatMX4*	This study
LPY4819 ∆Ubp3	LPY4819 but *ubp3∆*::*KanMX6*	This study
LPY4819 ∆Asr1 ∆Ubp3	LPY4819 but *asr1∆*::*NatMX4* *∆ubp3*::*KanMX6*	This study
LPY4977	LPY4819 but *sir2∆*::*HIS3MX6*	Clarke *et. al* 2006
YPH499UTAT	MATa *ura3-52, lys2-801, ade2-101, trp1- 63, his3- 200, leu2- 1*, TELVR::*ADE2*, TELVII-L::*URA3*	Monson *et. al* 1997
UTAT TM1	YPH499UTAT but *asr1∆*::*NatMX4*	This study
UTAT TM2	YPH499UTAT but *ubp3∆*::*KanMX6*	This study
UTAT TM3	UTAT TM1 but *ubp3∆*::*KanMX6*	This study
UTAT TM4	YPH499UTAT +[pRS415 GPD]	This study
UTAT TM5	UTAT TM1 +[pRS415 GPD]	This study
UTAT TM6	UTAT TM1 +[pRS415 HA-Asr1]	This study
UTAT TM7	UTAT TM1 +[pRS415 HA-Asr1 RING]	This study
UTAT TM8	UTAT TM1 +[pRS415 HA-Asr1 PHD]	This study
UTAT TM9	UTAT TM2 +[pRS415 GPD]	This study
UTAT TM10	UTAT TM2 +[pRS415 Ubp3-3MYC]	This study
UTAT TM11	UTAT TM2 +[pRS415 Ubp3-3MYC N180∆]	This study
UTAT TM12	YPH499UTAT but *sir2∆*::*KanMX6*	This study
YTM31	BY4741 +[pRS316]	This study
YTM32	BY4741 +[pUB221]	This study
YTM33	YTM5 +[pUB221]	This study
YTM34	BY4742 but asr*1*::C26A/C29A/C66A/C69A + [pUB221]	This study
YTM35	∆Ubp3 but +[pUB221]	This study
YTM36	YTM6 but +[pUB221]	This study
YTM37	YTM34 but *ubp3∆*::*KanMX6*	This study
Asr1-TAP	BY4742 but *ASR1*-TAP::*KlURA3*	Daulny *et. al* 2008

Table 2.1: Yeast strains used in this thesis		
Rpb1-TAP	BY4741 but *RPB1*-TAP::*HIS3MX6*	Open Biosystems
YTM38	BY4742 but *ASR1*-DAM::*KanMX6*	This study
YTM39	BY4742 but *asr1::C26A/C29A*-DAM*::KanMX6*	This study
YTM40	BY4742 but *ASR1pr*-DAM::*KanMX6*	This study
YTM41	BY4741 but *UBP3*-3MYC::*HIS3MX6*	This study
YTM42	YTM8 +[pYES2 HA-ASR1] +[pRS415 N180Ubp3-GFP-3MYC]	This study
YTM43	YTM8 +[pYES2 HA-ASR1] +[pRS415 GFP-3MYC]	This study
YTM44	BY4741 +[pRS415 GPD]	This study
YTM45	ΔUbp3 +[pRS415 GPD]	This study
YTM46	ΔUbp3 +[pRS415 Ubp3-3MYC]	This study
YTM47	ΔUbp3 +[pRS415 Ubp3-3MYC N180Δ]	This study
YTM48	ΔUbp3 +[pRS415 Ubp3-3MYC N145Δ]	This study
YTM49	YTM8 but +[pRS415 Ubp3-3MYC 91-115Δ] + [pYES2 HA-Asr1]	This study
YTM50	YTM8 but +[pRS415 Ubp3-3MYC 116-145Δ] + [pYES2 HA-Asr1]	This study
YTM51	YTM8 but +[pRS415 Ubp3-3MYC 141-160Δ] + [pYES2 HA-Asr1]	This study
YTM52	YTM8 but +[pRS415 Ubp3-3MYC 161-180Δ] + [pYES2 HA-Asr1]	This study

Table 2.2: Plasmids used in this thesis		
Plasmid	**Description**	**Source**
pYES2	*GAL1* promoter with *URA3* selectable marker and a 2µ origin of replication	Daulny *et. al* 2008
pYES2 HA-Asr1	pYES2 but HA-Asr1	Daulny *et. al* 2008
pYES2 HA-Asr1 RING mutant	pYES2 but HA-Asr1 C26A/C29A/C66A/C69A	Daulny *et. al* 2008
pYES2 HA-Asr1 PHD mutant	pYES2 but HA-Asr1 C143A/C146A/C186A/C189A	Daulny *et. al* 2008
pYES2 HA-Asr1 CBD	pYES2 but HA-Asr1 N198∆	Daulny *et. al* 2008
pRS415 GPD	GPD promoter with *LEU2* as a selectable marker and a CEN origin of replication	ATCC #87358
pRS415 GPD Ubp3-3MYC	Ubp3-3Myc	This study
pRS415 GPD Ubp3-3MYC N45∆	Ubp3-3Myc N45∆	This study
pRS415 GPD Ubp3-3MYC N90∆	Ubp3-3Myc N90∆	This study
pRS415 GPD Ubp3-3MYC N145∆	Ubp3-3Myc N145∆	This study
pRS415 GPD Ubp3-3MYC N180∆	Ubp3-3Myc N180∆	This study
pRS415 GPD Ubp3-3MYC 91-115∆	Ubp3-3MYC 91-115∆	This study
pRS415 GPD Ubp3-3MYC 116-145∆	Ubp3-3MYC 116-145∆	This study
pRS415 GPD Ubp3-3MYC 141-160∆	Ubp3-3MYC 141-160∆	This study
pRS415 GPD Ubp3-3MYC 161-180∆	Ubp3-3MYC 161-180∆	This study
pRS415 GPD HA-Asr1	HA-Asr1	This study
pRS415 GPD HA-Asr1 RING mutant	HA-Asr1 C26A/C29A/C66A/C69A	This study
pRS415 GPD HA-Asr1 PHD mutant	HA-Asr1 C143A/C146A/C186A/C189A	This study
pRS316	URA3 as a selectable marker and a CEN origin of replication	ATCC #77145
pUB 221	6xHIS-MYC-Ubiquitin under *CUP1* promoter with *TRP* and *URA* selectable markers and a 2µ origin of replication	Yaglom *et. al* 1995

Table 2.3: Primers used in this thesis.		
Name	**Sequence**	**Purpose**
ASR1del_NatR_F	TAATGCATATATGGAAGAACAGAAAGTGAGAAAAAAAAAATAGAAAAAAAGacatggaggcccagaataccct	Deletion of *ASR1*
ASR1del_NatR_R	TACTAGAGTATTATACTGTTTATATCTTGTTTTAATGTTATATTGCATTAcagtatagcgaccagcattcac	Deletion of *ASR1*
ASR1del_confirm_F	GTTGGAGGCTTGTATGTGTGTG	Deletion of *ASR1*
UBP3del_KANR_F	GTAAGTCAGACTCGTCTGCTACCATCATCCAGGTACCGCTTTCCTTTCCATCCCAGCGtgggcctccatgtcgctgg	Deletion of *UBP3*
UBP3del_KANR_R	TGTATTGTATTATTGCTATATTATTTTTTATGTATTTTGTCTATAATACCatcgatgaattcgagctcg	Deletion of *UBP3*
UBP3del_confirm_F	GGATCACTCTCCCACCAGC	Deletion of *UBP3*
SIR2del_KANR_F	TCGGTAGACACATTCAAACCATTTTTCCCTCATCGGCACATTAAAGCTGGtgggcctccatgtcgctgg	Deletion of *SIR2*
SIR2del_KANR_R	TGTAAATTGATATTAATTTGGCACTTTTAAATTATTAAATTGCCTTCTACatcgatgaattcgagctcg	Deletion of *SIR2*
SIR2del_confirm_F	CCCTTGGAGCCTCACTCCC	Deletion of *SIR2*
KAN/HIS_R	ATCGCGAGCCCATTTATACC	KAN/HIS/NAT cassette check
Bre5_pFA6atag_F	TACTAATGGAACACGTTCTCATAGAAAGCAACCCCTAAAAAGAAAGGACgggggaggcgggggtgga	Tagging of Bre5 with FLAG
Bre5_pFA6atag_R	ACTAATCATACAGTTTCTTGTTTCAATTTTTTAGATTTTAATTAGCGGgaattcgagctcgtttaaac	Tagging of Bre5 with FLAG
Bre5tag_confirm_F	GATCACGAAGAATACTACAAAACC	Tagging of Bre5
Ubp3_pYMtag_F	GAAGCTTCTGATTCGAGGACTGCCTATATTTTAATGTATCAAAAGAGAAATcgtacgctgcaggtcgac	Tagging of Ubp3 with HA/MYC
Ubp3_pYMtag_R	AATATGCCAAGCATAAAGTGTAACTCTGTTTCTCTGTCTGTCTCTATTTCatcgatgaattcgagctcg	Tagging of Ubp3 with HA/MYC
Ubp3tag_confirm_F	GTTTTGAAAGGTGGCGAAGA	Tagging of Ubp3
Asr1_pYMtag_F	TTAGCCCGAACTGGAGTGCACACAGAACTCCTAATTTACTGTCATGATGAGcgtacgctgcaggtcgac	Tagging of Asr1 with HA/MYC
Asr1_pYMtag_R	CGAGAGATGGGTCACCCGCTTCTGAGGGTTTTTTTTAGGGCTCATACTAGatcgatgaattcgagctcg	Tagging of Asr1 with HA/MYC
Asr1tag_confirm_F	GATTAAGCCTGTATTGCCAAG	Tagging of Asr1
Asr1-pFA6atag_F	AGCCCGAACTGGAGTGCACACAGAACTCCTAATTTACTGTCATGATGAGgggggaggcgggggtgga	Tagging of Asr1 with FLAG

Table 2.3: Primers used in this thesis.		
Asr1-pFA6atag_R	CTACTCATCATGACAGTAAATTAGGAG TTCTGTGTGCACTCCAGTTCGGGgaatt cgagctcgtttaaac	Tagging of Asr1 with FLAG
Rpb3-pFA6atag-F	CAATGCATCTCAAATGGGTAATACTGG ATCAGGAGGGTATGATAATGCTTGGggg ggaggcgggggtgga	Tagging of Rpb3 with FLAG
Rbp3-pFA6atag-R	ACTAATCATAATGATACATACATGCATAT AAAGCTTTTTTTCTCTTATTAgaattcgagct cgtttaaac	Tagging of Rpb3 with FLAG
Rbp3-confirm-F	GATCAGGAGGGTATGATAATGC	Tagging of Rpb3
Asr1_DAM_F	TAGCCCGAACTGGAGTGCACACAGAA CTCCTAATTTACTGTCATGATGAGtctggt tccggtgaacagaaac	DAM tag of Asr1
Asr1_DAM_R	TATGGGTCAAACTTGTGCTTTTATACG TCGTACGAGAGATGGGTCACCCGctgg atggcggcgttagtatc	DAM tag of Asr1
Ubp3_DAM_F	AAGCTTCTGATTCGAGGACTGCCTATA TTTTAATGTATCAAAAGAGAAATtctggttc cggtgaacagaaac	DAM tag of Ubp3
Ubp3_DAM_R	AATATGCCAAGCATAAAGTGTAACTCT GTTTCTCTGTCTGTCTCTATTTCctggatg gcggcgttagtatc	DAM tag of Ubp3
Sir2_DAM_F	AGGATAAGGGCGTGTATGTCGTTACAT CAGATGAACATCCCAAAACCCTCtctggt tccggtgaacagaaac	DAM tag of Sir2
Sir2_DAM_R	CGGTACATGTAATATTTCACCCGGTAC AATGAAAATAGCAGAATGTAAATctggatg gcggcgttagtatc	DAM tag of Sir2
Asr1_DAM_control_F	TGCATATATGGAAGAACAGAAAGTGAG AAAAAAAAATAGAAAAAAAGATGtctggtt ccggtgaacagaaac	DAM tag of Asr1
Asr1_DAM_control_R	TTAATGGTATCCATCACCAGCGTACCT CTAAGCCTTTCGCTGAGAAATTCctggat ggcggcgttagtatc	DAM tag of Asr1
Ubp3_DAM_control_F	CTACCATCATCCAGGTACCGCTTTCCT TTCCATCATCATTAAAAAAAATGtctggttc cggtgaacagaaac	DAM tag of Ubp3
Ubp3_DAM_control_R	GCGCCTTGATTACCGCTGCTTCCATT GTTATTGGTAATGCCATTAGAGTGctgga tggcggcgttagtatc	DAM tag of Ubp3
Sir2_DAM_control_F	GTAGACACATTCAAACCATTTTTCCCT CATCGGCACATTAAAGCTGGATGtctggtt ccggtgaacagaaac	DAM tag of Sir2
Sir2_DAM_control_R	AGATAGTAAATGTATAACGAGTTCAAAT CCTCGGGGAGGTAAGTGTCTAActggat ggcggcgttagtatc	DAM tag of Sir2
Sir2_confirm_F	CCCGGACTTCAGATCTTCTGAG	DAM tag of Sir2
Asr1_415_Coding_Gibson_F	ACTCGAGGTCGACGGTATCGATAAGA CCATGGGGCCATACCCATAC	Cloning of Asr1

Table 2.3: Primers used in this thesis.		
Asr1_415_Coding_Gibson_R	CGGGCTGCAGGAATTCGATATCAAGCT ACTCATCATGACAGTAAATTAGGAGTT C	Cloning of Asr1
Asr1_415_Plasmid_Gibson_F	GAACTCCTAATTTACTGTCATGATGAGT AGCTTGATATCGAATTCCTGCAGCCCG	Cloning of Asr1
Asr1_415_Plasmid_Gibson_R	GTATGGGTATGGCCCCATGGTCTTATC GATACCGTCGACCTCGAGT	Cloning of Asr1
Ubp3_SalI_F	GCGCGCGTCGACCTACTCATCATGAC AGTAAATT	Cloning of Ubp3
pYM1-Xho1	CCGGCGCTCGAGCATAAATCATAAGA AATTCGC	Cloning of Ubp3
ACT1_F	GACGCTCCTCGTGCTGTCTT	QPCR
ACT1_R	GTCTTTTTGACCCATACCGACC	QPCR
YFR057W_F	GCCAAGCTTCCAATATCACGA	QPCR
YFR057W_R	GGAATGATCTTGGAAATCGATCA	QPCR
YNR077C_F	GCGGCCCCAAATATTGTAT	QPCR
YNR077C_R	TGGTGGTGATTTTGTGGGTA	QPCR
YCL074W_F	CAGATGGACGTTGACACTGC	QPCR
YCL074W_R	AACCCGGGTGGTTGTTTTAC	QPCR
PHO84_F	GACCGCTTTGTTCTGTGTCA	QPCR
PHO84_R	TTGGACCGAAGTTTTGGAAG	QPCR
PHO12_F	GGTGGTTCTGGGCCATACTA	QPCR
PHO12_R	TTCACCGTGTCTACCAACCA	QPCR
PHO89_F	TTGCATTTTTGGATGCCTTT	QPCR
PHO89_R	GGGTCGTTGGTAAAAATGGA	QPCR
PRM7_F	ACCAGACCAAGTGGTCCAAC	QPCR
PRM7_R	ATCTTGGCTGGTTGAAGTGG	QPCR
SPL2_F	GGTCACCAGCATAAGGGAAG	QPCR
SPL2_R	ACGCTGCGCTCTACTTGAAT	QPCR
YBR056C-B_F	CCAGCGCAGCTACAACATAG	QPCR
YBR056C-B_R	TTGTAACTGTGGCGATTGCT	QPCR
PHO84_-199_DAM_F	CAGCATGATGCAACCACATT	DAM-ID QPCR
PHO84_-199_DAM_R	CGAGCCACAATAGTAAGTGGA	DAM-ID QPCR
PHO84_+71_DAM_F	GTGGTAACATGGCCTTCCAC	DAM-ID QPCR

Table 2.3: Primers used in this thesis.		
PHO84_+71_DAM_R	TGGACTCCAAAGCCAATCTT	DAM-ID QPCR
PHO84_+1584_DAM_F	CGCCTTATTCATGTTGTTGG	DAM-ID QPCR
PHO84_+1584_DAM_R	AGGATCGATTTCATCGTGGT	DAM-ID QPCR
PHO84_+1996_DAM_F	ATCATTTCTCGAGCCTCTGG	DAM-ID QPCR
PHO84_+1996_DAM_R	TCAAGTCGCTTGCTTAGTCG	DAM-ID QPCR
PHO89_DAMID_-164_F	TAAGCCCGGTTTTCGATATG	DAM-ID QPCR
PHO89_DAMID_-164_R	TGTCACTCATTCTATGACAATTCA	DAM-ID QPCR
PHO89_DAMID_+47_F	TTGCATTTTTGGATGCCTTT	DAM-ID QPCR
PHO89_DAMID_+47_R	CCATGGCTTGCCAGTATTTT	DAM-ID QPCR
PHO89_DAMID_+1143_F	TCTGGTGACCTGAAAGGAATG	DAM-ID QPCR
PHO89_DAMID_+1143_R	TGCAGTAATGGCTTGGAGAA	DAM-ID QPCR
PHO89_DAMID_+1974_F	GGTAGAACTTTTATTGCTCAGTGACTT	DAM-ID QPCR
PHO89_DAMID_+1974_R	CACATAGCCATGCCAGGTAA	DAM-ID QPCR
ALD6_DAMID_-367_F	ACTTTACCGTTTTGGGCATC	DAM-ID QPCR
ALD6_DAMID_-367_R	TGCTATATCGCATTCGTTGC	DAM-ID QPCR
ALD6_DAMID_+18_F	TGACACTGCTGAACCAGTCA	DAM-ID QPCR
ALD6_DAMID_+18_R	TGAATAGACCGGTTGGTTGC	DAM-ID QPCR
Ubp3-1-180del-Spe1-F	GGCCGCACTAGTTACAACATGTCACA GTATGATTTATACAAG	Mutagenesis of Ubp3
Ubp3_145del_Spe1-F	GGCCGCACTAGTAAAAAAAATGTCTCC AAACAGTGGCAGCAATG	Mutagenesis of Ubp3
Ubp3_90del_F_Spe1-F	GGCCGCACTAGTAAAAAAAATGAATAA CAACAACATTAACAAG	Mutagenesis of Ubp3
Ubp3_45del_F_Spe1-F	GGCCGCACTAGTAAAAAAAATGGCCC CATATCTATACCCCAC	Mutagenesis of Ubp3
Ubp3_91-145_R	GGTAGTGCTTCCGCC	Mutagenesis of Ubp3
Ubp3_91-145_F	TCTCCAAACAGTGGCAG	Mutagenesis of Ubp3
Ubp3_91-115_F	GGCGCCAACTCTAGC	Mutagenesis of Ubp3
Ubp3_115-145_R	TTGATTACCGCTGCTTCCAT	Mutagenesis of Ubp3
Ubp3_141-180_R	ATGATTGTTAGAGTAATTGTGATGG	Mutagenesis of Ubp3

Table 2.3: Primers used in this thesis.		
Ubp3_141-180_F	TCACAGTATGATTTATACAAGTTTG	Mutagenesis of Ubp3
Ubp3_141-160_F	TCTTCCAACGGCAACG	Mutagenesis of Ubp3
Ubp3_161-180_R	GTTGGTCTGTTTTTTCATGC	Mutagenesis of Ubp3
Gibson_415Emptycontrol_R	TTTTTTAGATCCGTCGAAACTAAGTT	Mutagenesis of Ubp3
Gibson_GFP_control_F	ATGAAAGGAGAAGAACTTTTCAC	Mutagenesis of Ubp3

CHAPTER 3

GENOMIC AND PROTEOMIC APPROACHES TO DISCOVER THE BIOLOGICAL ROLE OF ASR1

3.1 Introduction

Despite the clear biochemical effects Asr1 has on the regulation RNAPII, the biological role that Asr1 plays within a cell remains unclear (Daulny, Geng et al. 2008). Other studies have attempted to uncover the function of Asr1, however none of the biological roles suggested for Asr1 have been able to connect the molecular impact Asr1 has on RNAPII to a function within the cell (Betz, Schlenstedt et al. 2004, Fries, Frank et al. 2011, Zou, Yan et al. 2015). Therefore, I have undertaken a multi-pronged approach to uncover the negative regulatory role Asr1 exerts on RNAPII based on its biochemistry. The first approach taken was to investigate the global physical interactions of Asr1 through a tandem affinity purification and mass spectrometry approach. The purpose of this proteomic approach was to identify the proteins with which Asr1 physically associates with, then focus in on specific interactions to determine how Asr1 is involved in the regulation of transcription, using the established roles of these interacting proteins as a guide. The second approach was to assess the effect that mutation of *ASR1* has on the cellular transcriptome. An *ASR1* deletion strain has previously been used in the laboratory to perform both microarray and RNA-seq experiments to measure the global effect on transcription caused by *ASR1* deletion. However, this deletion mutant resulted in little to no change in transcription compared to a congenic WT strain (unpublished). Therefore, I have focused specifically on a point mutant of *ASR1* that inhibits the RING activity of Asr1 without perturbing other domains of Asr1 that may be involved in other functions in the cell.

Asr1 interacting proteins

Previous proteomic studies of Asr1 revealed an association with 10 of the 12 subunits of RNAPII, excluding the heterodimer Rpb4/7 (Daulny, Geng et al. 2008). Asr1 was shown to directly bind the CTD of Rpb1, and specifically required Ser5P for association, whereas Ser2P was inconsequential for its interaction. Aside from these RNAPII subunits, there are very few other confirmed Asr1 interacting proteins. The Bailer lab found that Asr1 associates with a group of

importins/exportins via a proposed nuclear localization sequence (NLS) that overlaps with the C-terminal binding domain (CBD) of Asr1 (Fries, Betz et al. 2007). Our laboratory has confirmed that Asr1 is able to shuttle between the cytoplasm and nucleus (unpublished data), which would fit with the observation that Asr1 associates with importins/exportins. In a separate study, the Bailer lab showed that Asr1 associates with calmodulin (Cmd1) in a manner that is dependent upon Ca^{2+} concentration (Fries, Frank et al. 2011). Upon binding Cmd1, it is ubiquitylated by Asr1. However, this observation does not give an indication as to the role of Asr1 in the context of RNAPII regulation.

Transcriptional effect of Asr1

In addition to the effect Asr1 has on the subunit composition of RNAPII, RNAPII purified using Asr1 as a bait exhibited little to no activity compared to RNAPII purified using Rpb1 as bait (Daulny, Geng et al. 2008). Asr1 has also been shown to associate with the housekeeping gene *PMA1*, as well as the inducible genes *RPL33a* and *HSP104* in an activity dependent manner (Daulny, Geng et al. 2008). Despite the compelling molecular effect Asr1 has on RNAPII, deletion of *ASR1* from cells has resulted in little to no observable transcriptional defect in whole transcriptome analyses (unpublished). Because of this paradox, I wanted to investigate whether a different Asr1 mutant may have a more profound effect on transcription. Therefore, I used an Asr1 RING finger mutant ($Asr1^{RINGmut}$) to assess the effect Asr1 has on transcription. This mutant contains two point mutations (C26A/C29A) preventing the coordination of zinc ions by the RING domain, thereby blocking the ubiquitin ligase activity of Asr1. The rationale behind the use of this mutant is that the $Asr1^{RINGmut}$ would prevent the ubiquitylation of Rpb1 by Asr1 without perturbation of other Asr1 domains that could coordinate the binding of additional protein partners. If the absence of Asr1 affected the cellular localization or association of other proteins with a potential target of Asr1, the effect that loss of *ASR1* had on a cell could be masked, however I may still be able to see an effect with the more subtle $Asr1^{RINGmut}$. Therefore I predicted that I will be able to observe a transcriptional defect in the $Asr1^{RINGmut}$ where I could not in the *ASR1* delete cells.

3.2 Materials and Methods

Purification and proteomic analysis of Asr1-associated proteins

The tandem affinity purification (TAP) protocol used in this study was adapted from the Seraphin laboratory (Puig, Caspary et al. 2001). Overnight cultures were grown in YPAD, and diluted to 4 L at 0.2 OD_{600}. Yeast cultures were grown to ~2 OD_{600}, spun down, and flash frozen in a 50 ml conical tube. Frozen pellets were placed in bead beating chambers with glass beads and ~100 ml lysis buffer (0.1% NP-40 (*Calbiochem*), 10mM phosphate buffer pH 8, 150mM NaCl (*RPI*), 2mM EDTA (*Sigma*), 50mM NaF (*Sigma*), 0.1mM Na_3VO_4 (*Sigma*), and protease inhibitors added fresh: 1 Complete tablet/50 ml buffer (*Roche*), 1.3mM benzamidine (*Sigma*), and 1mM PMSF (*Life Technologies*)). Pellets were subjected to bead beating for 30 seconds on, 1 minute off on ice until ~90% cell lysis (estimated under DIC microscopy). Lysates were spun on a table top centrifuge at 4 °C and 3500 rcf to clear cellular material. Lysate was then incubated with IgG agarose for 2 hours at 4 °C. After washes and equilibration with TEV cleavage buffer (10mM Tris-HCl, pH 8.0, 150mM NaCl, 0.1% NP-40, 0.5mM EDTA, and 1.0mM DTT), the beads were incubated with TEV (500U; *Sigma*). Flow-through was collected in a new tube and the beads were washed with calmodulin binding buffer (10mM Tris-HCl, pH 8.0, 150mM NaCl, 1mM Mg^{2+} acetate, 1mM Imidazole, 2mM $CaCl_2$, 10mM BME) and the flow-through was collected and combined with the flow-through after TEV cleavage. Calmodulin beads were added to the combined flow-through fractions and incubated 2 hours at 4 °C. The beads were washed and then eluted with calmodulin elution buffer (10mM Tris-HCl, pH 8.0, 150mM NaCl, 0.02% NP-40, 1mM Mg^{2+} acetate, 1mM Imidazole, 20mM EGTA, 10mM BME). Following the final elution step from calmodulin beads, proteins were concentrated by TCA precipitation, and half the sample was analyzed by MudPIT as described (MacCoss, McDonald et al. 2002, Martinez, Emfinger et al. 2012). Peptide MS/MS spectral data were searched against a protein database using Sequest (Yates, Eng et al. 1995), and the resulting identifications collated and filtered using Scaffold (*Proteome Software*). Protein descriptions in **Table 3.1 and 3.2** were obtained from http://www.yeastgenome.org/.

Co-immunoprecipitation of Asr1 tagged at the endogenous locus

Asr1, Ubp3, and Bre5 were tagged at their endogenous locus with MYC, HA, or FLAG tags using homologous recombination (Knop, Siegers et al. 1999, Funakoshi and Hochstrasser 2009). Yeast cultures were grown from a single colony overnight in 5 ml YPAD at 30 °C (2% dextrose, 2% peptone, 1% yeast extract, 40 mg/L adenine). The next day, 100 ml cultures were diluted to 0.2 OD_{600} and grown to log phase (~0.8-1.0 OD_{600}), and cell pellets were collected. Lysates were prepared by bead beating using 1 ml yeast lysis buffer (see **Purification and proteomic analysis of Asr1-associated proteins** for recipe). Lysates were incubated with 10 µg HA antibody for 2 hours and 35 µl (1:1 beads to buffer) equilibrated protein G sepharose beads (*Sigma*) for 1 hour at 4 °C for HA IP or 35 µl (1:1 beads to buffer) equilibrated M2 agarose beads (*Sigma*) for 3 hours at 4 °C for FLAG IP. Beads were washed 3x with lysis buffer and resuspended in 100 µl 2x Laemmli buffer. Immunoblotting was performed using appropriate antibodies (anti-FLAG; M2-HRP, anti-HA; 12CA5-HRP) together with Supersignal West Pico (*Pierce*) or Supersignal West Femto (*Pierce*).

RNA-sequencing (RNA-seq)

Overnight cultures were grown at 30 °C in YPAD. The next day 25 ml cultures were diluted to 0.2 OD_{600} and grown to log phase (~0.8 OD_{600}). RNA was collected using the Hot Acid Phenol protocol (Collart and Oliviero 2001). Ribosomal RNA reduction was performed using the Ribo-Zero Gold (Yeast) Kit (Epicenter), followed by RNA fragmentation and conversion to cDNA using the NEBNext First Strand, and Second Strand, Synthesis Modules (NEB). Library preparation was performed using the NEBNext DNA Library Prep Master Mix Set for Illumina (NEB). 50 million single-end reads were obtained for each sample on an Illumina HiSeq2500 Sequencer 3 (Illumina). Ribosomal RNA reduction, library preparation, and sequencing were performed by The Genomic Services Lab at Hudson Alpha (Huntsville, AL). Two distinct biological replicates were analyzed. Data were quality controlled at multiple stages (Guo, Ye et al. 2014) during processing using QC3 (Guo, Zhao et al. 2014). TopHat2 (Kim, Pertea et al. 2013) was used to align reads to the sacCer2 reference genome by Dr. Yan Guo of the Department of Cancer Biology. Cufflinks (Trapnell, Roberts et al. 2012) was used to quantify gene expression and perform differential gene expression analysis. A false discovery rate of greater than 0.05 was used as a significance

threshold. RNA-seq data are deposited at GEO with accession number GSE72740. Primary RNA-seq data available at: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=ghwjooiotdevvqp&acc=GSE72740. Gene descriptions in **Table 3.3** were obtained from http://www.yeastgenome.org/.

3.3 Results

A multitude of proteins copurify with Asr1

To gain insight into the biological role of Asr1, I performed a large scale purification of TAP tagged Asr1 cells and subsequent mass spectrometry analysis of purified proteins. The purpose of this analysis was to identify potential protein interactors of Asr1 that could connect a biological function for Asr1 to the regulation of RNAPII. It is important to note that the particular mass spectrometry analysis performed here was not quantitative, although it was intended to provide a general idea of the interaction partners of Asr1. I have manually curated a list grouped by biological processes of potential Asr1 interacting proteins that have spectral counts in the Asr1-TAP experiment over four fold than that of the untagged control strain (**Table 3.1**).

There were a multitude of proteins that were present above background levels in the Asr1-TAP purification. A tremendous amount of ribosomal proteins and ribosome associated factors as well as cytoskeletal proteins were present in the this list. These proteins are very common contaminants of tandem affinity purification/mass spectrometry experiments because of the abundance of ribosomes and cytoskeletal proteins in the cell (Gingras, Gstaiger et al. 2007). There was a considerable amount of general transcription factors, including 5 subunits of the TFIID complex. Because Asr1 associates with Ser5P Rpb1, it would make sense that Asr1-TAP co-purifies with so many proteins that are bound to or associated with RNAPII at the initiation of transcription. There were also six proteasomal subunits detected in the Asr1-TAP purification. Asr1 is highly ubiquitylated and has a short half life, which could explain why it associates with the proteasome (Daulny, Geng et al. 2008).

Asr1 associates with Ubp3/Bre5

There were an abundance of various proteins identified as potential Asr1 interacting proteins, so I needed a way to narrow my focus of possible physical interactors of Asr1. Because I knew that RNAPII physically associates with a multitude of different factors and that Asr1 is a Rpb1 interacting protein, I was concerned that I would potentially encounter a large number of proteins that don't specifically interact with Asr1, but rather associate with Asr1 through their own interaction with RNAPII. Therefore an Rpb1-TAP purification was performed side-by-side with the Asr1-TAP purification, and the spectral counts for specific interacting proteins were compared between the two purifications. I looked particularly for proteins that were both over 2-fold enriched in the Asr1-TAP purification compared to the Rpb1-TAP purification and that could connect the processes of transcription regulation and the UPS. Several proteasome subunits potentially fit this category (Rpn1, Rpn2, Rpt4, and Rpt5) and several proteins that affect transcription (Tbf1, Taf5, and Wtm1), however, two proteins (Ubp3 and Bre5) that were specifically enriched in the Asr1-TAP purification compared to Rpb1-TAP particularly stood out as interesting (**Table 3.2**). There are several reasons I decided to focus on these two candidate proteins. First, Bre5 is an obligate co-factor of Ubp3 (a deubiquitylating enzyme; (Baker, Tobias et al. 1992)), which requires the association of Bre5 to be enzymatically active (Cohen, Stutz et al. 2003) and both were enriched in Asr1-TAP purifications compared to Rpb1-TAP purifications, suggesting that there is some level of specificity to the interaction. Secondly, Ubp3 and Bre5 fit perfectly into the previously defined criteria of proteins that intersect both the transcription and the ubiquitin proteasome systems. Ubp3, along with Bre5, have been shown to associate with RNAPII (Kvint, Uhler et al. 2008). Ubp3 has also been implicated in a multitude of different cellular functions including but not limited to the silencing of chromatin (Moazed and Johnson 1996), retrograde transport in the Golgi (Cohen, Stutz et al. 2003), deubiquitylation of RNAPII (Kvint, Uhler et al. 2008), and ribophagy (Ossareh-Nazari, Bonizec et al. 2010, Ossareh-Nazari, Cohen et al. 2010). To confirm the results of the TAP/mass spectrometry experiment, I co-immunoprecipitated Asr1 in the presence of endogenously tagged Ubp3 and Bre5 and subjected them to Western blotting (**Figure 3.1 A and B**). This result further confirms the claim that Ubp3 and Bre5 are Asr1 interacting proteins.

Transcriptome analysis

As mentioned before, a microarray and an RNA-seq experiment had already been completed to identify the difference in the transcriptome of WT versus *asr1*Δ cells, and both resulted with essentially no changes in transcription. Therefore I submitted RNA from WT and $Asr1^{RINGmut}$ cells for comparative transcriptomics by RNA-seq. There were 278 genes significantly differentially expressed in the $Asr1^{RINGmut}$ compared to WT, 150 induced and 128 repressed. I decided to set a threshold of a 2 fold change in expression compared to WT, which resulted in the identification of 56 genes differentially expressed in the $Asr1^{RINGmut}$, 27 induced and 29 repressed (**Table 3.3**). There did not appear to be any correlation between these genes in terms of gene ontology, although, ~33% of the induced genes lie within 50 kb of telomere ends, whereas only ~7% of the repressed genes are within 50 kb of telomere ends. These results suggest that the ubiquitin ligase activity of Asr1 could be important for the regulation of telomeric silencing in budding yeast.

3.4 Discussion

Proteomic analysis of Asr1

Through proteomic analysis of Asr1-TAP purified cells, I identified a multitude of potential Asr1 interacting proteins. To focus in on potential binding factors, I compiled a list of proteins that were specifically enriched in an Asr1-TAP purification over an Rpb1-TAP purification, resulting in the identification of several intriguing potential Asr1 interacting proteins. One protein that was identified as being enriched in the Asr1-TAP purification compared to Rpb1-TAP is Kap123, a budding yeast importin that has already been shown to associate with Asr1 (Fries, Betz et al. 2007). Kap123 is responsible for the import of both histones as well as ribosomes (Mosammaparast, Guo et al. 2002, Sydorskyy, Dilworth et al. 2003). If Asr1 and ribosomal proteins are both cargo for the same importin, this could be one explanation for the large amount of ribosomal proteins and associated factors found in the Asr1-TAP purification.

One of the more compelling results from the proteomic analysis, and the one that I will focus on for the rest of this thesis, is the identification of Ubp3 and its cofactor Bre5 in the Asr1-TAP enriched pool, as well as *bona fide* Asr1 interacting proteins. Ubp3 and Bre5 have been

implicated in numerous different biological processes and the association between these two proteins and Asr1 provide a compelling starting point for the discovery of a biological role for Asr1.

Transcriptome analysis

In total, $Asr1^{RINGmut}$ significantly affected the transcription of 278 genes compared to WT congenic cells. I set a cutoff of genes found to be significantly misregulated over 2 fold in the $Asr1^{RINGmut}$ cells compared to WT and found 56 total genes. There was a near even split between these genes, with 29 being repressed and 27 being induced. I chose to focus on the induced genes for this analysis because I was interested in the direct regulatory impact that Asr1 has on RNAPII, which based on previous biochemical data should be a negative role (Daulny, Geng et al. 2008), however there are some notable trends within the repressed set of genes. Of the 29 repressed genes, only 1 is a verified protein with a known function, the plasma membrane transporter *AZR1*. Of the rest of the repressed genes, 16 are snoRNAs, 8 are putative or predicted proteins of unknown function, 3 are tRNAs, and 1 is a retrotransposon. It seems unlikely that Asr1 directly represses the transcription of these genes because of its inhibitory affect on RNAPII, however I cannot rule out a secondary effect in which Asr1 targets a negative regulator of any of these genes. Of the repressed genes, a large percentage were small nucleolar RNAs (snoRNAs). This class of transcripts encompass a group of non-coding RNAs that are involved in the editing of ribosomal RNA (rRNA) and are required for ribosome biogenesis (Dieci, Preti et al. 2009). There are 76 snoRNAs in budding yeast, so $Asr1^{RINGmut}$ represses the expression of ~24% of snoRNAs. In yeast, the vast majority of snoRNAs are monocistronic, being transcribed from individual promoters, indicating that the repression of snoRNAs could not have resulted from the repression of a few loci resulting in the repression of many snoRNAs (Dieci, Preti et al. 2009). One of the proteins specifically enriched in the Asr1-TAP purification is Tbf1, which is a telobox containing regulatory factor. Interestingly, this protein is required for the full expression of snoRNAs (Preti, Ribeyre et al. 2010). It is possible that Asr1 somehow controls the dynamics of Tbf1 binding to chromatin, which could in turn affect global snoRNA expression, however it remains unclear as to whether the repression of snoRNA transcripts upon Asr1 RING finger mutation is biologically significant, therefore further investigation is required.

Of the genes that were identified to have been induced over 2 fold in the Asr1^{RINGmut} cells compared to congenic WT cells, they did not appear to group to any one particular cellular process. Although, I did notice that ~33% of these genes were located within 50 kb of their respective telomere, whereas only ~7% of the repressed genes were proximal to the telomeres. Therefore, Asr1 does not seem to affect the transcription of genes based on a particular cellular process, but rather by their location in the genome. The specific induction of telomere proximal genes is consistent with the fact that Asr1 associates with Ubp3, which has been shown to be an subtelomeric gene anti-silencing protein, however the method through which Ubp3 regulates transcription is unknown. There are a multitude of different subtelomeric genes that were not affected by Asr1^{RINGmut} cells, but because the robustness of transcription of genes in subtelomeric regions is so varied based both on location within the chromosomal arm as the specific elements that make up the particular telomere, it is possible that the induction of other silenced genes was not able to be detected by RNA-seq. The fact that Asr1^{RINGmut} causes a global induction of subtelomeric genes along with the fact that Asr1 interacts with Ubp3, a known anti-silencing protein, presents the regulation of subtelomeric transcription as a compelling potential biological role for Asr1.

Table 3.1: Potential Asr1 interacting proteins				
Category	**Protein**	**Untagged**	**Asr1–TAP**	**Description**
Transcription	CAT8	1	16	Zinc cluster transcriptional activator; necessary for derepression of a variety of genes under non-fermentative growth conditions, active after diauxic shift, binds carbon source responsive elements; relative distribution to the nucleus increases upon DNA replication stress.
Transcription	TAF3	0	9	TFIID subunit (47 kDa); involved in promoter binding and RNA polymerase II transcription initiation.
Transcription	TAF4	0	4	TFIID subunit (48 kDa); involved in RNA polymerase II transcription initiation; potential Cdc28p substrate.
Transcription	TAF5	0	11	Subunit (90 kDa) of TFIID and SAGA complexes; involved in RNA polymerase II transcription initiation and in chromatin modification.
Transcription	TAF6	0	8	Subunit (60 kDa) of TFIID and SAGA complexes; involved in transcription initiation of RNA polymerase II and in chromatin modification, similar to histone H4; relocalizes to the cytosol in response to hypoxia.
Transcription	TAF12	0	7	Subunit (61/68 kDa) of TFIID and SAGA complexes; involved in RNA polymerase II transcription initiation and in chromatin modification, similar to histone H2A.
Transcription	IWS1	0	12	Protein involved in RNA polymerase II transcription; is constitutively recruited to the CYC1 promoter and is required for recruitment of chromatin remodeling factors for the expression of CYC1 gene; interacts genetically or physically with RNAP II, TBP, TFIIS, and chromatin remodelling factors; central domain highly conserved throughout eukaryotes; mutations confer an Spt- phenotype.
Transcription	SNF2	1	9	Catalytic subunit of the SWI/SNF chromatin remodeling complex; involved in transcriptional regulation; contains DNA-stimulated ATPase activity; functions interdependently in transcriptional activation with Snf5p and Snf6p.

Table 3.1: Potential Asr1 interacting proteins				
Category	**Protein**	**Untagged**	**Asr1–TAP**	**Description**
Transcription	TRA1	0	11	Subunit of SAGA and NuA4 histone acetyltransferase complexes; interacts with acidic activators (e.g., Gal4p) which leads to transcription activation; similar to human TRRAP, which is a cofactor for c-Myc mediated oncogenic transformation.
Transcription	TFG1	1	10	TFIIF (Transcription Factor II) largest subunit; involved in both transcription initiation and elongation of RNA polymerase II; homologous to human RAP74.
Transcription	TFB1	0	8	Subunit of TFIIH and nucleotide excision repair factor 3 complexes; required for nucleotide excision repair, target for transcriptional activators; relocalizes to the cytosol in response to hypoxia.
Transcription	PBP1	0	7	Component of glucose deprivation induced stress granules; involved in P-body-dependent granule assembly; similar to human ataxin-2; interacts with Pab1p to regulate mRNA polyadenylation; interacts with Mkt1p to regulate HO translation; protein increases in abundance and relative distribution to the nucleus increases upon DNA replication stress.
Transcription	PAB1	28	130	Poly(A) binding protein; part of the 3'-end RNA-processing complex, mediates interactions between the 5' cap structure and the 3' mRNA poly(A) tail, involved in control of poly(A) tail length, interacts with translation factor eIF-4G; stimulates, but is not required for the deadenylation activity of the Pan2p-Pan3p poly(A)-ribonuclease complex.
Transcription	REB1	0	8	RNA polymerase I enhancer binding protein; DNA binding protein that binds to genes transcribed by both RNA polymerase I and RNA polymerase II; required for termination of RNA polymerase I transcription; Reb1p bound to DNA acts to block RNA polymerase II readthrough transcription.

Table 3.1: Potential Asr1 interacting proteins				
Category	**Protein**	**Untagged**	**Asr1–TAP**	**Description**
Transcription	TBF1	0	12	Telobox-containing general regulatory factor; binds TTAGGG repeats within subtelomeric anti-silencing regions (STARs), blocking silent chromatin propagation; binds majority of snoRNA gene promoters, required for full snoRNA expression; caps DSB flanked by long T2AG3 repeats and blocks checkpoint activation.
Transcription	SPT5	4	19	Component of the universally conserved Spt4/5 complex (DSIF complex); the complex has multiple roles in concert with RNA polymerases I and II, including regulation of transcription elongation, RNA processing, quality control, and transcription-coupled DNA repair.
Splicing	HSH155	0	8	U2-snRNP associated splicing factor; forms extensive associations with the branch site-3' splice site-3' exon region upon prespliceosome formation; similarity to the mammalian U2 snRNP-associated splicing factor SAP155.
Splicing	NPL3	2	12	RNA-binding protein; promotes elongation, regulates termination, and carries poly(A) mRNA from nucleus to cytoplasm; represses translation initiation by binding eIF4G; required for pre-mRNA splicing; interacts with E3 ubiquitin ligase Bre1p, linking histone ubiquitination to mRNA processing; may have role in telomere maintenance; dissociation from mRNAs promoted by Mtr10p; phosphorylated by Sky1p in cytoplasm; protein abundance increases in response to DNA replication stress.
Splicing	MSL5	0	8	Component of commitment complex; which defines first step in splicing pathway; essential protein that interacts with Mud2p and Prp40p, forming a bridge between the intron ends; also involved in nuclear retention of pre-mRNA; relocalizes to the cytosol in response to hypoxia.

Table 3.1: Potential Asr1 interacting proteins				
Category	**Protein**	**Untagged**	**Asr1–TAP**	**Description**
Ubiquitin/ Proteasome	RPT2	1	14	ATPase of the 19S regulatory particle of the 26S proteasome; one of six ATPases of the regulatory particle; involved in the degradation of ubiquitinated substrates; required for normal peptide hydrolysis by the core 20S particle; N-myristoylation of Rpt2p at Gly2 is involved in regulating the proper intracellular distribution of proteasome activity by controlling the nuclear localization of the 26S proteasome.
Ubiquitin/ Proteasome	RPT3	4	15	ATPase of the 19S regulatory particle of the 26S proteasome; one of ATPases of the regulatory particle; involved in the degradation of ubiquitinated substrates; substrate of N-acetyltransferase B.
Ubiquitin/ Proteasome	RPT5	1	23	ATPase of the 19S regulatory particle of the 26S proteasome; one of six ATPases of the regulatory particle; involved in the degradation of ubiquitinated substrates; recruited to the GAL1-10 promoter region upon induction of transcription; similar to human TBP1.
Ubiquitin/ Proteasome	RPN1	4	23	Non-ATPase base subunit of the 19S RP of the 26S proteasome; may participate in the recognition of several ligands of the proteasome; contains a leucine-rich repeat (LRR) domain, a site for protein-protein interactions; RP is the acronym for regulatory particle.
Ubiquitin/ Proteasome	RPN2	0	27	Subunit of the 26S proteasome; substrate of the N-acetyltransferase Nat1p; protein abundance increases in response to DNA replication stress.
Ubiquitin/ Proteasome	RPN3	0	7	Essential non-ATPase regulatory subunit of the 26S proteasome lid; similar to the p58 subunit of the human 26S proteasome; temperature-sensitive alleles cause metaphase arrest, suggesting a role for the proteasome in cell cycle control.

Table 3.1: Potential Asr1 interacting proteins				
Category	**Protein**	**Untagged**	**Asr1–TAP**	**Description**
Ubiquitin/ Proteasome	BRE1	0	8	E3 ubiquitin ligase; forms heterodimer with Rad6p to regulate K63 polyubiquitination in response to oxidative stress and to monoubiquinate histone H2B-K123, which is required for the subsequent methylation of histone H3-K4 and H3-K79; required for DSBR, transcription, silencing, and checkpoint control; interacts with RNA-binding protein Npl3p, linking histone ubiquitination to mRNA processing; Bre1p-dependent histone ubiquitination promotes pre-mRNA splicing.
Ubiquitin/ Proteasome	BRE5	0	17	Ubiquitin protease cofactor; forms deubiquitination complex with Ubp3p that coregulates anterograde and retrograde transport between the endoplasmic reticulum and Golgi compartments; null is sensitive to brefeldin A.
Ubiquitin/ Proteasome	UBP3	1	62	Ubiquitin-specific protease involved in transport and osmotic response; interacts with Bre5p to co-regulate anterograde and retrograde transport between the ER and Golgi; involved in transcription elongation in response to osmostress through phosphorylation at Ser695 by Hog1p; inhibitor of gene silencing; cleaves ubiquitin fusions but not polyubiquitin; also has mRNA binding activity; protein abundance increases in response to DNA replication stress; role in ribophagy.
Cell Division	CDC3	1	6	Component of the septin ring that is required for cytokinesis; septins are GTP-binding proteins that assemble with other septins into rod-like complexes that can associate with other rods to form filament polymers; septin rings at the mother-bud neck act as scaffolds for recruiting factors needed for cell division and as barriers to prevent diffusion of specific proteins between mother and daughter cells.

Table 3.1: Potential Asr1 interacting proteins				
Category	**Protein**	**Untagged**	**Asr1–TAP**	**Description**
Cell Division	CDC10	0	4	Component of the septin ring, required for cytokinesis; septins are GTP-binding proteins that assemble into rod-like hetero-oligomers that can associate to form filaments; septin rings at the mother-bud neck act as scaffolds for recruiting cell division factors and as barriers to prevent diffusion of specific proteins between mother and daughter cells; N-terminus interacts with phosphatidylinositol-4,5-bisphosphate; protein abundance increases under DNA damage stress.
Cell Division	CDC27	0	12	Subunit of the Anaphase-Promoting Complex/Cyclosome (APC/C); APC/C is a ubiquitin-protein ligase required for degradation of anaphase inhibitors, including mitotic cyclins, during the metaphase/anaphase transition.
Cell Division	CDC16	0	8	Subunit of the anaphase-promoting complex/cyclosome (APC/C); which is a ubiquitin-protein ligase required for degradation of anaphase inhibitors, including mitotic cyclins, during the metaphase/anaphase transition; required for sporulation; relocalizes to the cytosol in response to hypoxia.
Cell Division	CDC23	0	6	Subunit of the Anaphase-Promoting Complex/Cyclosome (APC/C); APC/C is a ubiquitin-protein ligase required for degradation of anaphase inhibitors, including mitotic cyclins, during the metaphase/anaphase transition.
Cell Division	CDC53	0	6	Cullin; structural protein of SCF complexes (which also contain Skp1p, Cdc34p, Hrt1p and an F-box protein) involved in ubiquitination; SCF promotes the G1-S transition by targeting G1 cyclins and the Cln-CDK inhibitor Sic1p for degradation; human homolog CUL1 can complement yeast cdc53 null mutant.

Table 3.1: Potential Asr1 interacting proteins				
Category	**Protein**	**Untagged**	**Asr1–TAP**	**Description**
Cell Division	APC1	0	10	Largest subunit of the Anaphase-Promoting Complex/Cyclosome; APC/C is a ubiquitin-protein ligase required for degradation of anaphase inhibitors, including mitotic cyclins, during the metaphase/anaphase transition; component of the platform domain of the APC/C, based on structural analysis; localizes to nuclear foci that become diffuse upon DNA replication stress.
Nuclear Pore	NUP60	0	15	FG-nucleoporin component of central core of the nuclear pore complex; contributes directly to nucleocytoplasmic transport and maintenance of the nuclear pore complex (NPC) permeability barrier and is involved in gene tethering at the nuclear periphery; relocalizes to the cytosol in response to hypoxia; both NUP1 and NUP60 are homologous to human NUP153
Nuclear Pore	KAP123	5	18	Karyopherin beta; mediates nuclear import of ribosomal proteins prior to assembly into ribosomes and import of histones H3 and H4; localizes to the nuclear pore, nucleus, and cytoplasm; exhibits genetic interactions with RAI1.
Nuclear Pore	KAP95	2	9	Karyopherin beta; forms a complex with Srp1p/Kap60p; interacts with nucleoporins to mediate nuclear import of NLS-containing cargo proteins via the nuclear pore complex; regulates PC biosynthesis; GDP-to-GTP exchange factor for Gsp1p.
Translation	SSD1	1	45	Translational repressor with a role in polar growth and wall integrity; regulated by Cbk1p phosphorylation to effect bud-specific translational control and localization of specific mRNAs; interacts with TOR pathway components; contains a functional N-terminal nuclear localization sequence and nucleocytoplasmic shuttling appears to be critical to Ssd1p function.
Translation	NUG1	1	19	GTPase that associates with nuclear 60S pre-ribosomes; required for export of 60S ribosomal subunits from the nucleus.

Table 3.1: Potential Asr1 interacting proteins				
Category	**Protein**	**Untagged**	**Asr1–TAP**	**Description**
Translation	RPL11A	1	36	Ribosomal 60S subunit protein L11A; expressed at twice the level of Rpl11Bp; involved in ribosomal assembly; depletion causes degradation of 60S proteins and RNA; homologous to mammalian ribosomal protein L11 and bacterial L5; RPL11A has a paralog, RPL11B, that arose from the whole genome duplication.
Translation	RPL13B	2	13	Ribosomal 60S subunit protein L13B; not essential for viability; homologous to mammalian ribosomal protein L13, no bacterial homolog; RPL13B has a paralog, RPL13A, that arose from the whole genome duplication.
Translation	RPL14A	1	17	Ribosomal 60S subunit protein L14A; N-terminally acetylated; homologous to mammalian ribosomal protein L14, no bacterial homolog; RPL14A has a paralog, RPL14B, that arose from the whole genome duplication.
Translation	RPL21A	2	23	Ribosomal 60S subunit protein L21A; homologous to mammalian ribosomal protein L21, no bacterial homolog; RPL21A has a paralog, RPL21B, that arose from the whole genome duplication.
Translation	RPL22A	5	232	Ribosomal 60S subunit protein L22A; required for the oxidative stress response in yeast; homologous to mammalian ribosomal protein L22, no bacterial homolog; RPL22A has a paralog, RPL22B, that arose from the whole genome duplication.
Translation	RPL22B	0	6	Ribosomal 60S subunit protein L22B; homologous to mammalian ribosomal protein L22, no bacterial homolog; RPL22B has a paralog, RPL22A, that arose from the whole genome duplication.
Translation	RPL30	8	43	Ribosomal 60S subunit protein L30; involved in pre-rRNA processing in the nucleolus; autoregulates splicing of its transcript; homologous to mammalian ribosomal protein L30, no bacterial homolog.

Table 3.1: Potential Asr1 interacting proteins				
Category	**Protein**	**Untagged**	**Asr1–TAP**	**Description**
Translation	RPL31A	6	138	Ribosomal 60S subunit protein L31A; associates with karyopherin Sxm1p; loss of both Rpl31p and Rpl39p confers lethality; homologous to mammalian ribosomal protein L31, no bacterial homolog; RPL31A has a paralog, RPL31B, that arose from the whole genome duplication.
Translation	RPL31B	6	137	Ribosomal 60S subunit protein L31B; associates with karyopherin Sxm1p; loss of both Rpl31p and Rpl39p confers lethality; homologous to mammalian ribosomal protein L31, no bacterial homolog; RPL31B has a paralog, RPL31A, that arose from the whole genome duplication.
Translation	RPL32	2	13	Ribosomal 60S subunit protein L32; overexpression disrupts telomeric silencing; homologous to mammalian ribosomal protein L32, no bacterial homolog.
Translation	RPL33B	2	11	Ribosomal 60S subunit protein L33B; rpl33b null mutant exhibits normal growth while rpl33a rpl33b double null mutant is inviable; homologous to mammalian ribosomal protein L35A, no bacterial homolog; RPL33B has a paralog, RPL33A, that arose from the whole genome duplication.
Translation	RPL43A	2	33	Ribosomal 60S subunit protein L43A; null mutation confers a dominant lethal phenotype; homologous to mammalian ribosomal protein L37A, no bacterial homolog; RPL43A has a paralog, RPL43B, that arose from the whole genome duplication.
Translation	RPS1A	14	57	Ribosomal protein 10 (rp10) of the small (40S) subunit; homologous to mammalian ribosomal protein S3A, no bacterial homolog; RPS1A has a paralog, RPS1B, that arose from the whole genome duplication.
Translation	RPS1B	12	62	Ribosomal protein 10 (rp10) of the small (40S) subunit; homologous to mammalian ribosomal protein S3A, no bacterial homolog; RPS1B has a paralog, RPS1A, that arose from the whole genome duplication.

Table 3.1: Potential Asr1 interacting proteins				
Category	**Protein**	**Untagged**	**Asr1–TAP**	**Description**
Translation	RPS3	20	99	Protein component of the small (40S) ribosomal subunit; has apurinic/apyrimidinic (AP) endonuclease activity; essential for viability; nascent Rps3p is bound by specific chaperone Yar1p during translation; homologous to mammalian ribosomal protein S3 and bacterial S3.
Translation	RPS4A	10	40	Protein component of the small (40S) ribosomal subunit; mutation affects 20S pre-rRNA processing; homologous to mammalian ribosomal protein S4, no bacterial homolog; RPS4A has a paralog, RPS4B, that arose from the whole genome duplication.
Translation	RPS5	31	132	Protein component of the small (40S) ribosomal subunit; least basic of non-acidic ribosomal proteins; phosphorylated in vivo; essential for viability; homologous to mammalian ribosomal protein S5 and bacterial S7.
Translation	RPS7A	12	115	Protein component of the small (40S) ribosomal subunit; interacts with Kti11p; deletion causes hypersensitivity to zymocin; homologous to mammalian ribosomal protein S7, no bacterial homolog; RPS7A has a paralog, RPS7B, that arose from the whole genome duplication.
Translation	RPS7B	7	87	Protein component of the small (40S) ribosomal subunit; interacts with Kti11p; deletion causes hypersensitivity to zymocin; homologous to mammalian ribosomal protein S7, no bacterial homolog; RPS7B has a paralog, RPS7A, that arose from the whole genome duplication; protein abundance increases in response to DNA replication stress.
Translation	RPS14A	9	73	Protein component of the small (40S) ribosomal subunit; required for ribosome assembly and 20S pre-rRNA processing; mutations confer cryptopleurine resistance; homologous to mammalian ribosomal protein S14 and bacterial S11; RPS14A has a paralog, RPS14B, that arose from the whole genome duplication.

Table 3.1: Potential Asr1 interacting proteins				
Category	**Protein**	**Untagged**	**Asr1–TAP**	**Description**
Translation	RPS16A	5	55	Protein component of the small (40S) ribosomal subunit; homologous to mammalian ribosomal protein S16 and bacterial S9; RPS16A has a paralog, RPS16B, that arose from the whole genome duplication.
Translation	RPS19A	10	124	Protein component of the small (40S) ribosomal subunit; required for assembly and maturation of pre-40 S particles; homologous to mammalian ribosomal protein S19, no bacterial homolog; mutations in human RPS19 are associated with Diamond Blackfan anemia; RPS19A has a paralog, RPS19B, that arose from the whole genome duplication.
Translation	RPS22A	7	34	Protein component of the small (40S) ribosomal subunit; homologous to mammalian ribosomal protein S15A and bacterial S8; RPS22A has a paralog, RPS22B, that arose from the whole genome duplication.
Translation	RPS24A	9	36	Protein component of the small (40S) ribosomal subunit; homologous to mammalian ribosomal protein S24, no bacterial homolog; RPS24A has a paralog, RPS24B, that arose from the whole genome duplication.
Translation	RPS29B	1	5	Protein component of the small (40S) ribosomal subunit; homologous to mammalian ribosomal protein S29 and bacterial S14; RPS29B has a paralog, RPS29A, that arose from the whole genome duplication.
Translation	LSM12	2	18	Protein of unknown function that may function in RNA processing; interacts with Pbp1p and Pbp4p and associates with ribosomes; contains an RNA-binding LSM domain and an AD domain; GFP-fusion protein is induced by the DNA-damaging agent MMS; relative distribution to the nucleus increases upon DNA replication stress.

Table 3.1: Potential Asr1 interacting proteins				
Category	**Protein**	**Untagged**	**Asr1–TAP**	**Description**
Translation	SUP1	2	14	Polypeptide release factor (eRF1) in translation termination; mutant form acts as a recessive omnipotent suppressor; methylated by Mtq2p-Trm112p in ternary complex eRF1-eRF3-GTP; mutation of methylation site confers resistance to zymocin; has a role in cytokinesis through interaction with Mlc1p.
Translation	TIF32	5	26	eIF3a subunit of the eukaryotic translation initiation factor 3 (eIF3); subunit of the core complex of eIF3; essential for translation; part of a Prt1p-Rpg1p-Nip1p subcomplex that stimulates binding of mRNA and tRNA(i)Met to ribosomes; involved in translation reinitiation; eIF3 is also involved in programmed stop codon readthrough.
Translation	TIF34	2	19	eIF3i subunit of the eukaryotic translation initiation factor 3 (eIF3); subunit of the core complex of eIF3; essential for translation; stimulates rate of ribosomal scanning during translation reinitiation; eIF3 is also involved in programmed stop codon readthrough.
Translation	TIF35	0	11	eIF3g subunit of the eukaryotic translation initiation factor 3 (eIF3); subunit of the core complex of eIF3; is essential for translation; stimulates resumption of ribosomal scanning during translation reinitiation; eIF3 is also involved in programmed stop codon readthrough.
Translation	TIF45	1	6	mRNA cap binding protein and translation initiation factor eIF4E; the eIF4E-cap complex is responsible for mediating cap-dependent mRNA translation via interactions with translation initiation factor eIF4G (Tif4631p or Tif4632p); protein abundance increases in response to DNA replication stress; mutants are defective for adhesion and pseudohyphal growth; human homolog EIF4E can complement yeast cdc33 null mutant.
Translation	TIF4631	2	12	Translation initiation factor eIF4G; subunit of the mRNA cap-binding protein complex (eIF4F) that also contains eIF4E (Cdc33p); interacts with Pab1p and with eIF4A (Tif1p); also has a role in biogenesis of the large ribosomal subunit; TIF4631 has a paralog, TIF4632, that arose from the whole genome duplication.

Table 3.1: Potential Asr1 interacting proteins				
Category	**Protein**	**Untagged**	**Asr1–TAP**	**Description**
Translation	TIF4632	3	16	Translation initiation factor eIF4G; subunit of the mRNA cap-binding protein complex (eIF4F) that also contains eIF4E (Cdc33p); associates with the poly(A)-binding protein Pab1p, also interacts with eIF4A (Tif1p); TIF4632 has a paralog, TIF4631, that arose from the whole genome duplication.
Translation	NUG1	1	19	GTPase that associates with nuclear 60S pre-ribosomes; required for export of 60S ribosomal subunits from the nucleus.
Translation	LSG1	1	15	Putative GTPase involved in 60S ribosomal subunit biogenesis; required for the release of Nmd3p from 60S subunits in the cytoplasm.
Translation	NEW1	0	11	ATP binding cassette protein; cosediments with polysomes and is required for biogenesis of the small ribosomal subunit; Asn/Gln-rich rich region supports [NU+] prion formation and susceptibility to [PSI+] prion induction.
Translation	DHR1	0	24	Essential DEAH-box ATP-dependent RNA helicase specific to U3 snoRNP; predominantly nucleolar in distribution; required for 18S rRNA synthesis.
Translation	TSR1	0	29	Protein required for processing of 20S pre-rRNA in the cytoplasm; associates with pre-40S ribosomal particles; inhibits the premature association of 60S subunits with assembling 40S subunits in the cytoplasm; similar to Bms1p; relocalizes from nucleus to cytoplasm upon DNA replication stress.
Translation	EAP1	0	10	eIF4E-associated protein, competes with eIF4G for binding to eIF4E; accelerates mRNA degradation by promoting decapping, facilitated by interaction with eIF4E; essential for Puf5p mediated repression; associates with Puf5p and Dhh1p; inhibits cap-dependent translation; functions independently of eIF4E to maintain genetic stability; plays a role in cell growth, implicated in the TOR signaling cascade.
Translation	SUI3	0	10	Beta subunit of the translation initiation factor eIF2; involved in the identification of the start codon; proposed to be involved in mRNA binding.

Table 3.1: Potential Asr1 interacting proteins				
Category	**Protein**	**Untagged**	**Asr1–TAP**	**Description**
Translation	PRT1	2	13	eIF3b subunit of the eukaryotic translation initiation factor 3 (eIF3); subunit of the core complex of eIF3; essential for translation; part of a subcomplex (Prt1p-Rpg1p-Nip1p) that stimulates binding of mRNA and tRNA(i)Met to ribosomes; eIF3 is also involved in programmed stop codon readthrough.
Translation	NIP1	3	14	eIF3c subunit of the eukaryotic translation initiation factor 3 (eIF3); involved in the assembly of preinitiation complex and start codon selection; eIF3 is also involved in programmed stop codon readthrough.
Translation	RRP3	0	8	Protein involved in rRNA processing; required for maturation of the 35S primary transcript of pre-rRNA and for cleavage leading to mature 18S rRNA; homologous to eIF-4a, which is a DEAD box RNA-dependent ATPase with helicase activity.
Translation	RRP12	0	11	Protein required for export of the ribosomal subunits; associates with the RNA components of the pre-ribosomes; has a role in nuclear import in association with Pse1p; also plays a role in the cell cycle and the DNA damage response; contains HEAT-repeats.
Translation	RRP8	0	4	Nucleolar S-adenosylmethionine-dependent rRNA methyltransferase; methylates adenine (m1A) of the large subunit (LSU) rRNA at position 645; involved in pre-rRNA cleavage at site A2; mutation is synthetically lethal with a gar1 mutation; deletion disrupts telomere maintenance by influencing the expression of neighboring gene STN1.
Translation	RSM7	0	10	Mitochondrial ribosomal protein of the small subunit; has similarity to E. coli S7 ribosomal protein.
Translation	RSM28	0	8	Mitochondrial ribosomal protein of the small subunit; genetic interactions suggest a possible role in promoting translation initiation.
Actin/Myosin/ Cytoskeleton	MYO1	1	29	Type II myosin heavy chain; required for wild-type cytokinesis and cell separation; localizes to the actomyosin ring; binds to myosin light chains Mlc1p and Mlc2p through its IQ1 and IQ2 motifs respectively.

Table 3.1: Potential Asr1 interacting proteins				
Category	**Protein**	**Untagged**	**Asr1–TAP**	**Description**
Actin/Myosin/ Cytoskeleton	MYO2	1	19	Type V myosin motor involved in actin-based transport of cargos; required for the polarized delivery of secretory vesicles, the vacuole, late Golgi elements, peroxisomes, and the mitotic spindle; MYO2 has a paralog, MYO4, that arose from the whole genome duplication.
Actin/Myosin/ Cytoskeleton	MYO4	0	11	Type V myosin motor involved in actin-based transport of cargos; required for mRNA transport, including ASH1 mRNA, and facilitating the growth and movement of ER tubules into the growing bud along with She3p; MYO4 has a paralog, MYO2, that arose from the whole genome duplication.
Actin/Myosin/ Cytoskeleton	BNI1	0	13	Formin; polarisome component; nucleates the formation of linear actin filaments, involved in cell processes such as budding and mitotic spindle orientation which require the formation of polarized actin cables, functionally redundant with BNR1.
Actin/Myosin/ Cytoskeleton	SYP1	0	11	Negative regulator of WASP-Arp23 complex; involved in endocytic site formation; directly inhibits Las17p stimulation of Arp23 complex-mediated actin assembly in vitro; may regulate assembly and disassembly of the septin ring; colocalizes and interacts with septin subunits; potential role in actin cytoskeletal organization.
Actin/Myosin/ Cytoskeleton	BUD2	0	10	GTPase activating factor for Rsr1p/Bud1p; plays a role in spindle position checkpoint distinct from its role in bud site selection; required for both axial and bipolar budding patterns; mutants exhibit random budding in all cell types.
Actin/Myosin/ Cytoskeleton	BEM2	1	30	Rho GTPase activating protein (RhoGAP); involved in the control of cytoskeleton organization and cellular morphogenesis; required for bud emergence; potential GAP for Rho4p.
Actin/Myosin/ Cytoskeleton	RGA2	0	13	GTPase-activating protein for polarity-establishment protein Cdc42p; implicated in control of septin organization, pheromone response, and haploid invasive growth; regulated by Pho85p and Cdc28p; RGA2 has a paralog, RGA1, that arose from the whole genome duplication.

Table 3.1: Potential Asr1 interacting proteins				
Category	**Protein**	**Untagged**	**Asr1–TAP**	**Description**
Actin/Myosin/ Cytoskeleton	MHP1	0	21	Microtubule-associated protein involved in microtubule organization; involved in assembly and stabilization of microtubules; overproduction results in cell cycle arrest at G2 phase; similar to Drosophila protein MAP and to mammalian MAP4 proteins.
Actin/Myosin/ Cytoskeleton	RVS161	1	19	Amphiphysin-like lipid raft protein; N-BAR domain protein that interacts with Rvs167p and regulates polarization of the actin cytoskeleton, endocytosis, cell polarity, cell fusion and viability following starvation or osmotic stress.
Actin/Myosin/ Cytoskeleton	RVS167	0	21	Actin-associated protein with roles in endocytosis and exocytosis; N-BAR domain protein that interacts with Rvs161p to regulate actin cytoskeleton, endocytosis, and viability following starvation or osmotic stress; recruited to bud tips by Gyl1p and Gyp5p during polarized growth; homolog of mammalian amphiphysin.
Actin/Myosin/ Cytoskeleton	SHS1	0	8	Component of the septin ring that is required for cytokinesis; present at the ends of rod-like septin hetero-oligomers; C-terminal extension is important for recruitment of Bni5p to the mother-bud neck, which in turn is required for Myo1p recruitment and cytokinesis; undergoes sumoylation and phosphorylation during mitosis; protein abundance increases in response to DNA replication stress.
Actin/Myosin/ Cytoskeleton	SLM1	2	8	Phosphoinositide PI4,5P(2) binding protein, forms a complex with Slm2p; acts downstream of Mss4p in a pathway regulating actin cytoskeleton organization in response to stress; TORC2 complex substrate and effector; protein abundance increases in response to DNA replication stress; SLM1 has a paralog, SLM2, that arose from the whole genome duplication.
Secretory	SEC1	2	25	Sm-like protein involved in docking and fusion of exocytic vesicles; binds to assembled SNARE complexes at the membrane and stimulates membrane fusion; localization to sites of secretion (bud neck and bud tip) is dependent on SNARE function; interacts directly with essential exocyst subunit Sec6p.

Table 3.1: Potential Asr1 interacting proteins				
Category	**Protein**	**Untagged**	**Asr1–TAP**	**Description**
Secretory	SEC7	1	6	Guanine nucleotide exchange factor (GEF) for ADP ribosylation factors; involved in proliferation of the Golgi, intra-Golgi transport and ER-to-Golgi transport; found in the cytoplasm and on Golgi-associated coated vesicles.
Secretory	SEC26	1	5	Essential beta-coat protein of the COPI coatomer; involved in ER-to-Golgi protein trafficking and maintenance of normal ER morphology; shares 43% sequence identity with mammalian beta-coat protein (beta-COP).
Secretory	SEC10	0	5	Essential 100kDa subunit of the exocyst complex; the exocyst mediates polarized targeting and tethering of post-Golgi secretory vesicles to active sites of exocytosis at the plasma membrane prior to SNARE-mediated fusion.
Secretory	SEC63	1	5	Essential subunit of Sec63 complex; with Sec61 complex, Kar2p/BiP and Lhs1p forms a channel competent for SRP-dependent and post-translational SRP-independent protein targeting and import into the ER; other members are Sec62p, Sec66p, and Sec72p.
Secretory	KIN1	0	9	Serine/threonine protein kinase involved in regulation of exocytosis; localizes to the cytoplasmic face of the plasma membrane; KIN1 has a paralog, KIN2, that arose from the whole genome duplication.
Secretory	KIN2	0	8	Serine/threonine protein kinase involved in regulation of exocytosis; localizes to the cytoplasmic face of the plasma membrane; KIN2 has a paralog, KIN1, that arose from the whole genome duplication.
Secretory	EXO84	0	16	Exocyst subunit with dual roles in exocytosis and spliceosome assembly; subunit of the the exocyst complex which mediates polarized targeting and tethering of post-Golgi secretory vesicles to active sites of exocytosis at the plasma membrane (PM) prior to SNARE-mediated fusion; required for exocyst assembly and targeting the complex to specific sites on the bud tip PM; associates the U1 snRNP; role in pre-mRNA splicing and prespliceosome formation; possible Cdc28 substrate.

Table 3.1: Potential Asr1 interacting proteins				
Category	**Protein**	**Untagged**	**Asr1–TAP**	**Description**
Secretory	SEC33	2	11	Alpha subunit of COPI vesicle coatomer complex; complex surrounds transport vesicles in the early secretory pathway
Secretory	DRS2	0	8	Trans-golgi network aminophospholipid translocase (flippase); maintains membrane lipid asymmetry in post-Golgi secretory vesicles; contributes to clathrin-coated vesicle formation, endocytosis, protein trafficking between the Golgi and endosomal system and the cellular response to mating pheromone; autoinhibited by its C-terminal tail; localizes to the trans-Golgi network; mutations in human homolog ATP8B1 result in liver disease.
Secretory	KRE2	1	18	Alpha1,2-mannosyltransferase of the Golgi; involved in protein mannosylation; KRE2 has a paralog, KTR6, that arose from the whole genome duplication.
Secretory	YPK1	1	8	Serine/threonine protein kinase; phosphorylates, downregulates flippase activator Fpk1p; inactivates Orm1p, Orm2p inhibitors of serine:palmitoyl-coenzyme A transferase by phosphorylation in response to compromised sphingolipid synthesis; mutations affect receptor-mediated endocytosis and sphingolipid-mediated and cell integrity signaling pathways; human homolog SGK1 can complement yeast null mutant; human homolog SGK2 can complement yeast ypk1 ypk2 double mutant.
Secretory	TCB1	2	14	Lipid-binding ER protein involved in ER-plasma membrane tethering; one of 6 proteins (Ist2p, Scs2p, Scs22p, Tcb1p, Tcb2p, Tcb3p) that connect ER to plasma membrane and regulate PI4P levels by controlling access of Sac1p phosphatase to its substrate PI4P in PM; contains 3 calcium and lipid binding domains; non-tagged protein also localizes to mitochondria; C-termini of Tcb1p, Tcb2p and Tcb3p interact; TCB1 has a paralog, TCB2, that arose from the whole genome duplication.

Table 3.1: Potential Asr1 interacting proteins				
Category	**Protein**	**Untagged**	**Asr1–TAP**	**Description**
Mitochondria	TOM70	1	19	Component of the TOM (translocase of outer membrane) complex; involved in the recognition and initial import steps for all mitochondrially directed proteins; acts as a receptor for incoming precursor proteins; TOM70 has a paralog, TOM71, that arose from the whole genome duplication.
Mitochondria	YHM1	1	9	Mitochondrial GTP/GDP transporter; essential for mitochondrial genome maintenance; has a role in mitochondrial iron transport; member of the mitochondrial carrier family.
Mitochondria	TIM44	2	14	Essential component of the TIM23 complex; tethers the import motor and regulatory factors (PAM complex) to the translocation channel (Tim23p-Tim17p core complex); TIM23 complex is short for the translocase of the inner mitochondrial membrane.
Mitochondria	TIM50	1	6	Essential component of the TIM23 complex; acts as receptor for the translocase of the inner mitochondrial membrane (TIM23) complex guiding incoming precursors from the TOM complex; may control the gating of the Tim23p-Tim17p channel.
Calcium Signalling	CMK1	1	28	Calmodulin-dependent protein kinase; may play a role in stress response, many Ca++/calmodulin dependent phosphorylation substrates demonstrated in vitro, amino acid sequence similar to mammalian Cam Kinase II; CMK1 has a paralog, CMK2, that arose from the whole genome duplication.
Calcium Signalling	CMK2	0	7	Calmodulin-dependent protein kinase; may play a role in stress response, many CA++/calmodulan dependent phosphorylation substrates demonstrated in vitro, amino acid sequence similar to mammalian Cam Kinase II; CMK2 has a paralog, CMK1, that arose from the whole genome duplication.
RNA binding	SRO9	2	36	Cytoplasmic RNA-binding protein; shuttles between nucleus and cytoplasm and is exported from the nucleus in an mRNA export-dependent manner; associates with translating ribosomes; involved in heme regulation of Hap1p as a component of the HMC complex, also involved in the organization of actin filaments; contains a La motif; SRO9 has a paralog, SLF1, that arose from the whole genome duplication.

Table 3.1: Potential Asr1 interacting proteins				
Category	**Protein**	**Untagged**	**Asr1–TAP**	**Description**
RNA binding	DBP1	2	28	Putative ATP-dependent RNA helicase of the DEAD-box protein family; mutants show reduced stability of the 40S ribosomal subunit scanning through 5' untranslated regions of mRNAs; protein abundance increases in response to DNA replication stress; DBP1 has a paralog, DED1, that arose from the whole genome duplication.
RNA binding	RRP6	0	6	Nuclear exosome exonuclease component; has 3'-5' exonuclease activity that is regulated by Lrp1p; involved in RNA processing, maturation, surveillance, degradation, tethering, and export; role in sn/snoRNAs precursor degradation; forms a stable heterodimer with Lrp1p; has similarity to E. coli RNase D and to human PM-Sc1 100 (EXOSC10); mutant displays reduced transcription elongation in the G-less-based.
RNA binding	DED1	21	126	ATP-dependent DEAD (Asp-Glu-Ala-Asp)-box RNA helicase; required for translation initiation of all yeast mRNAs; binds to mRNA cap-associated factors, and binding stimulates Ded1p RNA-dependent ATPase activity; mutation in human homolog DBY is associated with male infertility; human homolog DDX3X complements ded1 null mutation; DED1 has a paralog, DBP1, that arose from the whole genome duplication.

Table 3.2: Proteins enriched in Asr1-TAP vs. Rpb1-TAP				
Protein	**Rpb1–TAP**	**Asr1–TAP**	**Asr1/ Rpb1**	**Description**
Rpb1	2418	70	0.03	RNA polymerase II largest subunit B220; part of central core; phosphorylation of C-terminal heptapeptide repeat domain regulates association with transcription and splicing factors; similar to bacterial beta-prime.
Rpb2	2066	58	0.03	RNA polymerase II second largest subunit B150; part of central core; similar to bacterial beta subunit.
Rpb3	1007	26	0.03	RNA polymerase II third largest subunit B44; part of central core; similar to prokaryotic alpha subunit.
Rpb4	526	4	0.01	RNA polymerase II subunit B32; forms dissociable heterodimer with Rpb7p; Rpb4/7 dissociates from RNAPII as Ser2 CTD phosphorylation increases; Rpb4/7 regulates cellular lifespan via mRNA decay process; involved in recruitment of 3'-end processing factors to transcribing RNAPII complex, export of mRNA to cytoplasm under stress conditions; also involved in translation initiation.
Rpb5	377	10	0.03	RNA polymerase subunit ABC27; common to RNA polymerases I, II, and III; contacts DNA and affects transactivation.
Rpb6	202	3	0.01	RNA polymerase subunit ABC23; common to RNA polymerases I, II, and III; part of central core; similar to bacterial omega subunit.
Rpb7	684	0	0.00	RNA polymerase II subunit B16; forms dissociable heterodimer with Rpb4p; Rpb4/7 dissociates from RNAPII as Ser2 CTD phosphorylation increases; Rpb4/7 regulates cellular lifespan via mRNA decay process; involved in recruitment of 3'-end processing factors to transcribing RNA polymerase II complex, export of mRNA to cytoplasm under stress conditions; also involved in translation initiation.
Rpb8	88	2	0.02	RNA polymerase subunit ABC14.5; common to RNA polymerases I, II, and III.
Rpb9	120	4	0.03	RNA polymerase II subunit B12.6; contacts DNA; mutations affect transcription start site selection and fidelity of transcription.
Rpb10	338	2	0.01	RNA polymerase subunit ABC10-beta; common to RNA polymerases I, II, and III.
Rpb11	256	5	0.02	RNA polymerase II subunit B12.5; part of central core; similar to Rpc19p and bacterial alpha subunit.
Rpb12	34	0	0.00	RNA polymerase subunit ABC10-alpha, found in RNA pol I, II, and III; relocalizes from nucleolus to cytoplasm upon DNA replication stress.

Table 3.2: Proteins enriched in Asr1-TAP vs. Rpb1-TAP				
Protein	**Rpb1–TAP**	**Asr1–TAP**	**Asr1/ Rpb1**	**Description**
Asr1	1	20	20.00	Ubiquitin ligase that modifies and regulates RNA Pol II; involved in a putative alcohol-responsive signaling pathway; accumulates in the nucleus under alcohol stress; has a role in organization of septins and the actin cytoskeleton; contains a Ring/PHD finger domain similar to the mammalian rA9 protein.
Rpn1	1	23	23.00	Non-ATPase base subunit of the 19S RP of the 26S proteasome; may participate in the recognition of several ligands of the proteasome; contains a leucine-rich repeat (LRR) domain, a site for protein-protein interactions; RP is the acronym for regulatory particle.
Kap123	1	18	18.00	Karyopherin beta; mediates nuclear import of ribosomal proteins prior to assembly into ribosomes and import of histones H3 and H4; localizes to the nuclear pore, nucleus, and cytoplasm; exhibits genetic interactions with RAI1.
Cdc27	1	12	12.00	Subunit of the Anaphase-Promoting Complex/Cyclosome (APC/C); APC/C is a ubiquitin-protein ligase required for degradation of anaphase inhibitors, including mitotic cyclins, during the metaphase/anaphase transition.
Tbf1	1	12	12.00	Telobox-containing general regulatory factor; binds TTAGGG repeats within subtelomeric anti-silencing regions (STARs), blocking silent chromatin propagation; binds majority of snoRNA gene promoters, required for full snoRNA expression; caps DSB flanked by long T2AG3 repeats and blocks checkpoint activation.
Rpt5	2	23	11.50	ATPase of the 19S regulatory particle of the 26S proteasome; one of six ATPases of the regulatory particle; involved in the degradation of ubiquitinated substrates; recruited to the GAL1-10 promoter region upon induction of transcription; similar to human TBP1.
Rpt4	1	11	11.00	ATPase of the 19S regulatory particle of the 26S proteasome; one of six ATPases of the regulatory particle; involved in degradation of ubiquitinated substrates; contributes preferentially to ERAD; required for spindle pole body duplication; mainly nuclear localization.
Taf5	1	11	11.00	Subunit (90 kDa) of TFIID and SAGA complexes; involved in RNA polymerase II transcription initiation and in chromatin modification.
Wtm1	2	16	8.00	Transcriptional modulator; involved in regulation of meiosis, silencing, and expression of RNR genes; required for nuclear localization of the ribonucleotide reductase small subunit Rnr2p and Rnr4p; contains WD repeats.

Table 3.2: Proteins enriched in Asr1-TAP vs. Rpb1-TAP				
Protein	**Rpb1–TAP**	**Asr1–TAP**	**Asr1/ Rpb1**	**Description**
Cdc16	1	8	8.00	Subunit of the anaphase-promoting complex/cyclosome (APC/C); which is a ubiquitin-protein ligase required for degradation of anaphase inhibitors, including mitotic cyclins, during the metaphase/anaphase transition; required for sporulation; relocalizes to the cytosol in response to hypoxia
Rpn2	4	27	6.75	Subunit of the 26S proteasome; substrate of the N-acetyltransferase Nat1p; protein abundance increases in response to DNA replication stress.
Ecm29	1	5	5.00	Scaffold protein; assists in association of the proteasome core particle with the regulatory particle; inhibits proteasomal ATPase activity; degraded by the mature proteasome after assembly; contains HEAT-like repeats; protein increases in abundance and relocalizes from nucleus to cytoplasm upon DNA replication stress.
Ubp3	13	62	4.77	Ubiquitin-specific protease involved in transport and osmotic response; interacts with Bre5p to co-regulate anterograde and retrograde transport between the ER and Golgi; involved in transcription elongation in response to osmostress through phosphorylation at Ser695 by Hog1p; inhibitor of gene silencing; cleaves ubiquitin fusions but not polyubiquitin; also has mRNA binding activity; protein abundance increases in response to DNA replication stress; role in ribophagy.
Bre5	6	17	2.83	Ubiquitin protease cofactor; forms deubiquitination complex with Ubp3p that coregulates anterograde and retrograde transport between the endoplasmic reticulum and Golgi compartments; null is sensitive to brefeldin A.

Table 3.3: Genes differentially expressed over 2 fold between WT and Asr1-RING mutant in RNA-seq				
Systematic Name	**Standard Name**	**Log2 Fold change**	**Gene Description**	**Median Distance from Telomere**
tQ(CUG)M	CDC65	-2.0612	Glutamine tRNA (tRNA-Gln), predicted by tRNAscan-SE analysis; can mutate to cause constitutive pseudohyphal growth in homozygous diploids; required for localization of transcription factor Gln3p to the nucleus in response to nitrogen starvation or rapamycin treatment	24,919
YGR224W	AZR1	-2.04648	Plasma membrane transporter of the major facilitator superfamily; involved in resistance to azole drugs such as ketoconazole and fluconazole	9,843
snR72		-1.87679	C/D box small nucleolar RNA (snoRNA); guides 2'-O-methylation of large subunit (LSU) rRNA at position A876	383,033
YLR365W		-1.65871	Dubious open reading frame; unlikely to encode a functional protein, based on available experimental and comparative sequence data; partially overlaps dubious gene YLR364C-A; YLR365W is not an essential gene	187,766
tR(ACG)J		-1.64223	Arginine tRNA (tRNA-Arg), predicted by tRNAscan-SE analysis; one of 6 nuclear tRNA genes containing the tDNA-anticodon ACG (converted to ICG in the mature tRNA), decodes CGU, CGC, and probably CGA codons into arginine, one of 19 nuclear tRNAs for arginine	461,829
YGL263W	COS12	-1.52426	Protein of unknown function; member of the DUP380 subfamily of conserved, often subtelomerically-encoded proteins	86,470
YDR210C-D		-1.49455	Retrotransposon TYA Gag and TYB Pol genes; transcribed/translated as one unit; polyprotein is processed to make a nucleocapsid-like protein (Gag), reverse transcriptase (RT), protease (PR), and integrase (IN); similar to retroviral genes	15,524
snR78		-1.44653	C/D box small nucleolar RNA (snoRNA); guides 2'-O-methylation of large subunit (LSU) rRNA at position U2421	36,492

Table 3.3: Genes differentially expressed over 2 fold between WT and Asr1-RING mutant in RNA-seq				
Systematic Name	**Standard Name**	**Log2 Fold change**	**Gene Description**	**Median Distance from Telomere**
snR74		-1.37259	C/D box small nucleolar RNA (snoRNA); guides 2'-O-methylation of small subunit (SSU) rRNA at position A28	461,812
snR71		-1.33714	C/D box small nucleolar RNA (snoRNA); guides 2'-O-methylation of large subunit (LSU) rRNA at position A2946	515,765
YOR072W-B		-1.32751	Putative protein of unknown function; identified by expression profiling and mass spectrometry	295,449
snR52		-1.3265	C/D box small nucleolar RNA (snoRNA); guides 2'-O-methylation of large subunit (LSU) rRNA at position U2921 and small subunit (SSU) rRNA at position A420	19,101
snR66		-1.32312	C/D box small nucleolar RNA (snoRNA); guides 2'-O-methylation of large subunit (LSU) rRNA at position U2417	25,812
YNL042W-B		-1.27892	Putative protein of unknown function	86,528
YPR108W-A		-1.24543	Putative protein of unknown function; identified by fungal homology and RT-PCR	4,057
snR39B		-1.23423	C/D box small nucleolar RNA (snoRNA); guides 2'-O-methylation of large subunit (LSU) rRNA at position G805	566,958
tL(GAG)G		-1.22698	Leucine tRNA (tRNA-Leu), predicted by tRNAscan-SE analysis; not essential for viability even though this is the only tRNA that decodes GAG codons	41,679
YLL056C		-1.21083	Putative protein of unknown function; transcription is activated by paralogous transcription factors Yrm1p and Yrr1p and genes involved in pleiotropic drug resistance (PDR); expression is induced in cells treated with the mycotoxin patulin	137,075

Table 3.3: Genes differentially expressed over 2 fold between WT and Asr1-RING mutant in RNA-seq				
Systematic Name	**Standard Name**	**Log2 Fold change**	**Gene Description**	**Median Distance from Telomere**
YOR314W		-1.17324	Dubious open reading frame; unlikely to encode a functional protein, based on available experimental and comparative sequence data	306,400
snR79		-1.17201	C/D box small nucleolar RNA (snoRNA); guides 2'-O-methylation of small subunit (SSU) rRNA at position C1007	54,921
snR69		-1.15528	C/D box small nucleolar RNA (snoRNA); guides 2'-O-methylation of large subunit (LSU) rRNA at position C2948	516,882
snR55		-1.14246	C/D box small nucleolar RNA (snoRNA); predicted to guide 2'-O-methylation of small subunit (SSU) rRNA at position U1269	648,163
snR73		-1.12074	C/D box small nucleolar RNA (snoRNA); guides 2'-O-methylation of large subunit (LSU) rRNA at position C2959	133,848
snR161		-1.11256	H/ACA small nucleolar RNA (snoRNA); guides pseudouridylation of small subunit (SSU) rRNA at positions U632 and U766	509,769
snR58		-1.09758	C/D box small nucleolar RNA (snoRNA); guides 2'-O-methylation of large subunit (LSU) rRNA at position C663	635,540
YLR046C		-1.05447	Putative membrane protein; member of the fungal lipid-translocating exporter (LTE) family of proteins; transcription is activated by paralogous transcription factors Yrm1p and Yrr1p along with genes involved in multidrug resistance; YLR046C has a paralog, RTA1, that arose from the whole genome duplication	15,841
snR75		-1.03683	C/D box small nucleolar RNA (snoRNA); guides 2'-O-methylation of large subunit (LSU) rRNA at position G2288	281,916

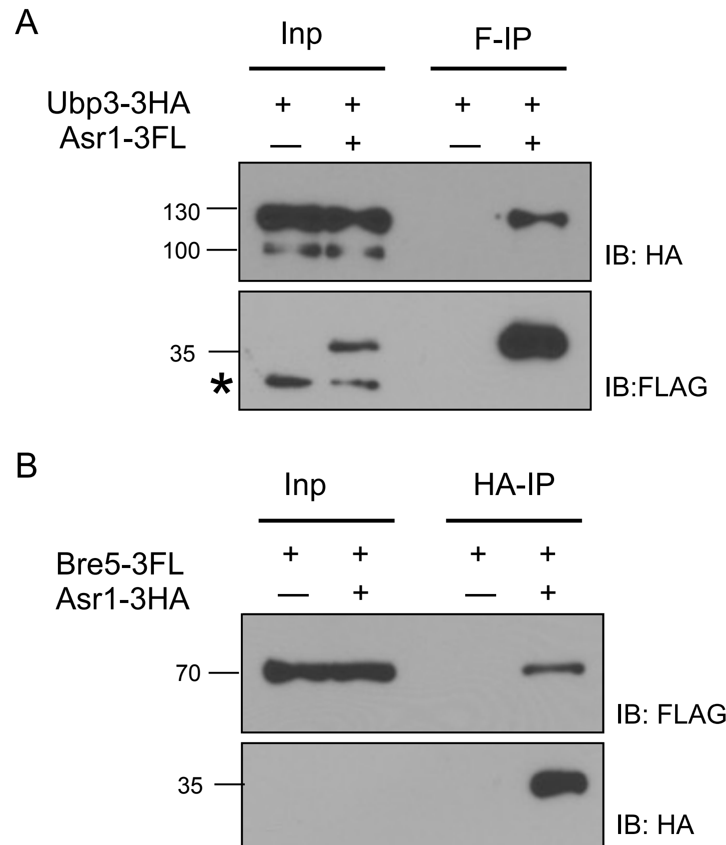
Table 3.3: Genes differentially expressed over 2 fold between WT and Asr1-RING mutant in RNA-seq				
Systematic Name	**Standard Name**	**Log2 Fold change**	**Gene Description**	**Median Distance from Telomere**
snR60		-1.03626	C/D box small nucleolar RNA (snoRNA); guides 2'-O-methylation of large subunit (LSU) rRNA at positions A817 and G908	97,179
snR68		-1.03417	C/D box small nucleolar RNA (snoRNA); guides 2'-O-methylation of large subunit (LSU) rRNA at position A2640	349,182
YGR161C	RTS3	1.01338	Putative component of the protein phosphatase type 2A complex	626,469
YFR053C	HXK1	1.04324	Hexokinase isoenzyme 1; a cytosolic protein that catalyzes phosphorylation of glucose during glucose metabolism; expression is highest during growth on non-glucose carbon sources; glucose-induced repression involves hexokinase Hxk2p; HXK1 has a paralog, HXK2, that arose from the whole genome duplication	839,770
YMR011W	HXT2	1.05848	High-affinity glucose transporter of the major facilitator superfamily; expression is induced by low levels of glucose and repressed by high levels of glucose	136,136
YDR281C	PHM6	1.10192	Protein of unknown function; expression is regulated by phosphate levels	505,919
YOR338W		1.12126	Putative protein of unknown function; YOR338W transcription is regulated by Azf1p and its transcript is a specific target of the G protein effector Scp160p; identified as being required for sporulation in a high-throughput mutant screen; YOR338W has a paralog, FUN19, that arose from the whole genome duplication	626,072
YOR062C		1.12585	Protein of unknown function; similar to Reg1p; expression regulated by glucose and Rgt1p; GFP-fusion protein is induced in response to the DNA-damaging agent MMS; YOR062C has a paralog, YKR075C, that arose from the whole genome duplication	283,432

Table 3.3: Genes differentially expressed over 2 fold between WT and Asr1-RING mutant in RNA-seq				
Systematic Name	**Standard Name**	**Log2 Fold change**	**Gene Description**	**Median Distance from Telomere**
YDR277C	MTH1	1.16894	Negative regulator of the glucose-sensing signal transduction pathway; required for repression of transcription by Rgt1p; interacts with Rgt1p and the Snf3p and Rgt2p glucose sensors; phosphorylated by Yck1p, triggering Mth1p degradation; MTH1 has a paralog, STD1, that arose from the whole genome duplication	364,826
YKR091W	SRL3	1.16936	GTB motif (G1/S transcription factor binding) containing protein; binds SBF-regulated promoters in hydroxyurea-treated cells; when overexpressed, suppresses the lethality of a rad53 null mutation; potential Cdc28p substrate; SRL3 has a paralog, WHI5, that arose from the whole genome duplication	729,709
YGR146C	ECL1	1.19767	Protein of unknown function; mitochondrial-dependent role in the extension of chronological lifespan; overexpression increases oxygen consumption and respiratory activity while deletion results in reduced oxygen consumption under conditions of caloric restriction; induced by iron homeostasis transcription factor Aft2p; multicopy suppressor of temperature sensitive hsf1 mutant; induced by treatment with 8-methoxypsoralen and UVA irradiation	188,084
YBL043W	ECM13	1.20173	Non-essential protein of unknown function; induced by treatment with 8-methoxypsoralen and UVA irradiation; ECM13 has a paralog, YJR115W, that arose from the whole genome duplication	27,857
YOL152W	FRE7	1.2059	Putative ferric reductase with similarity to Fre2p; expression induced by low copper levels	390,225

Table 3.3: Genes differentially expressed over 2 fold between WT and Asr1-RING mutant in RNA-seq				
Systematic Name	**Standard Name**	**Log2 Fold change**	**Gene Description**	**Median Distance from Telomere**
YBR005W	RCR1	1.28636	Protein of the ER membrane involved in cell wall chitin deposition; may function in the endosomal-vacuolar trafficking pathway, helping determine whether plasma membrane proteins are degraded or routed to the plasma membrane; RCR1 has a paralog, RCR2, that arose from the whole genome duplication	366,422
YAR071W	PHO11	1.31843	One of three repressible acid phosphatases; glycoprotein that is transported to the cell surface by the secretory pathway; induced by phosphate starvation and coordinately regulated by PHO4 and PHO2; PHO11 has a paralog, PHO12, that arose from a segmental duplication	203,785
YKR075C		1.38742	Protein of unknown function; similar to Reg1p; expression regulated by glucose and Rgt1p; GFP-fusion protein is induced in response to the DNA-damaging agent MMS; YKR075C has a paralog, YOR062C, that arose from the whole genome duplication	547,242
YOR385W		1.4338	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm; YOR385W is not an essential gene	586,133
YMR316W	DIA1	1.46835	Protein of unknown function; involved in invasive and pseudohyphal growth; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm in a punctate pattern	145,700
YOL016C	CMK2	1.49027	Calmodulin-dependent protein kinase; may play a role in stress response, many CA++/calmodulan dependent phosphorylation substrates demonstrated in vitro, amino acid sequence similar to mammalian Cam Kinase II; CMK2 has a paralog, CMK1, that arose from the whole genome duplication	626,742

Table 3.3: Genes differentially expressed over 2 fold between WT and Asr1-RING mutant in RNA-seq				
Systematic Name	**Standard Name**	**Log2 Fold change**	**Gene Description**	**Median Distance from Telomere**
YPL019C	VTC3	1.52209	Subunit of vacuolar transporter chaperone (VTC) complex; involved in membrane trafficking, vacuolar polyphosphate accumulation, microautophagy and non-autophagic vacuolar fusion; VTC3 has a paralog, VTC2, that arose from the whole genome duplication	151,371
YBR056C-B		1.68571	Dubious open reading frame; unlikely to encode a functional protein, based on available experimental and comparative sequence data; almost completely overlaps the dubious ORF YBR056W-A	626,250
YJR150C	DAN1	1.68879	Cell wall mannoprotein; has similarity to Tir1p, Tir2p, Tir3p, and Tir4p; expressed under anaerobic conditions, completely repressed during aerobic growth	627,110
YBR296C	PHO89	1.9054	Plasma membrane Na+/Pi cotransporter; active in early growth phase; similar to phosphate transporters of Neurospora crassa; transcription regulated by inorganic phosphate concentrations and Pho4p; mutations in related human transporter genes hPit1 and hPit2 are associated with hyperphosphatemia-induced calcification of vascular tissue and familial idiopathic basal ganglia calcification	650,640
YKR075W-A		1.95347	Dubious open reading frame unlikely to encode a protein; completely overlaps the uncharacterized gene YKR075C; identified by gene-trapping, microarray-based expression analysis, and genome-wide homology searching	3,361
YBR056W-A		2.1083	Protein of unknown function; mRNA identified as translated by ribosome profiling data; partially overlaps dubious ORF YBR056C-B; YBR056W-A has a paralog, YDR034W-B, that arose from the whole genome duplication	233,975

Table 3.3: Genes differentially expressed over 2 fold between WT and Asr1-RING mutant in RNA-seq				
Systematic Name	**Standard Name**	**Log2 Fold change**	**Gene Description**	**Median Distance from Telomere**
YHR136C	SPL2	2.27773	Protein with similarity to cyclin-dependent kinase inhibitors; downregulates low-affinity phosphate transport during phosphate limitation by targeting Pho87p to the vacuole; upstream region harbors putative hypoxia response element (HRE) cluster; overproduction suppresses a plc1 null mutation; promoter shows an increase in Snf2p occupancy after heat shock; GFP-fusion protein localizes to the cytoplasm	222,812
YDL039C	PRM7	2.37292	Pheromone-regulated protein; predicted to have one transmembrane segment; promoter contains Gcn4p binding elements; in W303 strain one continuous open reading frame comprising of YDL037C, the intergenic region and YDL039C encodes the IMI1	625,829
YHR215W	PHO12	2.60504	One of three repressible acid phosphatases; glycoprotein that is transported to the cell surface by the secretory pathway; pregulated by phosphate starvation; PHO12 has a paralog, PHO11, that arose from a segmental duplication	147,214
YML123C	PHO84	3.4533	High-affinity inorganic phosphate (Pi) transporter; also low-affinity manganese transporter; regulated by Pho4p and Spt7p; mutation confers resistance to arsenate; exit from the ER during maturation requires Pho86p; cells overexpressing Pho84p accumulate heavy metals but do not develop symptoms of metal toxicity	116,150



3.1 **Asr1 associates with both Ubp3 and Bre5.** (*A*) Asr1 co-precipitates with Ubp3. Extract was prepared from cells expressing HA-tagged Ubp3, either alone (YTM1) or in conjunction with FLAG (FL)-tagged Asr1 (YTM2). Immunoprecipitation (IP) was performed with an anti-FLAG antibody (F-IP), and products subjected to immunoblotting (IB) with anti–HA and –FLAG antibodies. For Ubp3–HA, 1% of the input (Inp) to the IP was also analyzed by IB; for Asr1–FLAG, 7.5% of the input was analyzed.. The * indicates a presumed breakdown product of Ubp3. *(B*) Asr1 co-precipitates with Bre5. Extract was prepared from cells expressing FLAG (FL)-tagged Bre5, either alone (YTM3) or in conjunction with HA–tagged Asr1 (YTM4). IP was performed with an anti-HA antibody (HA-IP), and products subjected to IB with anti–HA and –FLAG antibodies. For Bre5–FL, 1% of the input (inp) to the IP was analyzed by IB; for Asr1–HA, 7.5% of the input was analyzed. Asr1–HA is not visible in the input material due to its low abundance.

CHAPTER 4

ASSOCIATION OF ASR1 AND UBP3

4.1 Introduction

In the previous chapter, I established that Asr1 associates with Ubp3 and its obligate cofactor Bre5. This association provides a starting point with which to probe the biological role of Asr1 in budding yeast. Before examining the possibility of Asr1 functioning in the known biological roles of Ubp3, it is important to establish the nature of their association. By characterizing the association of Asr1 and Ubp3, I will not only be able to gain further insight into how these two proteins interact within the cell but I will also gain valuable biological tools for use in future assays in the mutants that disrupt the association of Asr1 and Ubp3.

Within this chapter, I use mutational analyses coupled with co-immunoprecipitation experiments to further the understanding of how Asr1, Ubp3, and Bre5 interact. There are several questions these experiments are meant to address in order to elucidate the biological function of Asr1. Question 1 is how do Asr1, Ubp3, and Bre5 interact with each other and other surrounding proteins? This question will be explored by elucidating the domains of Ubp3 that are required for association of Asr1 along with using the knowledge of the location of the Bre5 binding domain on Ubp3 (Cohen, Stutz et al. 2003). Question 2 is how does Asr1, Ubp3, and RNAPII associate. It has previously been shown that Asr1 and Ubp3 both associate with RNAPII (Daulny, Geng et al. 2008, Kvint, Uhler et al. 2008), so there are three possibilities as to how these proteins potentially associate with RNAPII: 1) Asr1 could associate directly with RNAPII and direct the binding of Ubp3. 2) Ubp3 could associate directly with RNAPII and direct the binding of Asr1. 3) Asr1 and Ubp3 could associate with RNAPII independently of each other. Of these options, #2 seems unlikely as it has been previously established that Asr1 binds directly to the CTD of Rpb1 (Daulny, Geng et al. 2008).

4.2 Materials and Methods

Co-immunoprecipitation of plasmid expressed Asr1 or Ubp3

Cultures were grown up from a single cell overnight in 5 ml of CSM-Ura/Leu +Raff media (2% raffinose, 6.7 g/ml nitrogenous base w/o amino acids (RPI), 0.79g CSM dropout mix (*Sunrise Science*)). The next day, 100 ml cultures were diluted to 0.2 OD_{600} and grown to ~0.6 OD_{600}, at which time 10 ml of 20% galactose (RPI) was added to induce Asr1 expression. 100 OD of cell pellet was collected after 4 hours of induction (1 ml at 1 OD_{600} = 1 OD of cells). Lysates were prepared by bead beating using 1 ml yeast lysis buffer (see **Chapter 3.3** under **Purification and proteomic analysis of Asr1-associated proteins** for full recipe). Lysates were incubated with 10 µg anti-MYC antibody (9E10; Vanderbilt Molecular Biology Core) for 2 hours, then incubated with protein G sepharose (Sigma) for 1 hour. Beads were washed 3x with lysis buffer and resuspended in 100 µl 2x Laemmli buffer. Immunoblotting was performed using appropriate antibodies (anti-HA; 12CA5-HRP, anti-MYC; 9E10-HRP, anti-Serine 5 phosphorylated Rpb1; 3E8) together with Supersignal West Pico (*Pierce*) or Supersignal West Femto (*Pierce*).

4.3 Results

The RING/PHD fingers of Asr1 are dispensable for Ubp3 association

I used several mutants of Asr1 to ask what, if any domains are required for association with Ubp3. For this assay, I used WT Asr1, which I know associates with Ubp3, as well as a RING and a PHD finger mutant version of Asr1 that each contain cysteine to alanine point mutations that prevent these domains from chelating zinc, which renders them inactive. Also included in the assay is the CBD of Asr1 (**Figure 4.1 A**). I found that interaction of Asr1 with Ubp3 does not depend on the integrity of the Asr1 RING (**Figure 4.1 B**, *compare lanes 13 and 14*) or PHD (**Figure 4.1 B**, *compare lanes 13 and 15*) fingers, but does require the N-terminus of Asr1, as expression of the CBD alone is insufficient to support interaction with Ubp3 (**Figure 4.1 B**, *compare lanes 13 and 16*). Thus Asr1 interacts with Ubp3 via sequences that are dispensable for interaction with Rpb1, further indicating that the Asr1/Ubp3 interaction is not coordinated through association with RNAPII.

Asr1 associates with the first 180 amino acids of Ubp3

To determine what domains of Ubp3 are required for association of Asr1, I performed co-immunoprecipitation experiments with N-terminal deletion constructs of Ubp3 (**Figure 4.2 A**). I found that deletion of the first 180 or 145 amino acids of Ubp3 prevents the association of Asr1 with Ubp3. These two Ubp3 mutants still associate with Bre5 at levels similar to WT Ubp3, suggesting that Ubp3 mediates the interaction of Asr1 and the Ubp3/Bre5 complex (**Figure 4.2 B**). Because deletion of up to the first 90 amino acids of Ubp3 did not affect the association with Asr1, I attempted to hone in on the exact domain of Asr1 association with Ubp3 by making smaller 20-30 amino acid internal deletions of Ubp3 within the region shown to be required for Asr1 association (residues 91-180 of Ubp3) (**Figure 4.3 A**). However, all internal Ubp3 deletion mutants associate with Asr1 at similar levels to WT, which suggests that Asr1 may not have a small binding motif, but could make several contacts within the 91-180 amino acid region of Ubp3) (**Figure 4.3 B**).

Because deleting the first 180 amino acids of Ubp3 ablated the interaction with Asr1, I wanted to see if this region alone was sufficient for association with Asr1. To test this, I immunoprecipitated either MYC tagged GFP (as a negative control) or the N-terminal 180 amino acids of Ubp3 tagged with GFP and MYC (**Figure 4.4 A**). I found that the first 180 amino acids of Ubp3 are sufficient for binding to both Asr1 as well as to the RNAPII subunit Rpb3, suggesting that this region of Ubp3 coordinates its association with RNAPII.

Ubp3 requires Asr1 to associate with RNAPII

Asr1 and Ubp3 are both known RNAPII interacting proteins (Daulny, Geng et al. 2008, Kvint, Uhler et al. 2008). Because previous studies have shown that Asr1 interacts directly with the C-terminal repeats of Rpb1 (Daulny, Geng et al. 2008) and it is unknown how Ubp3 associates with RNAPII, I tested the requirement of Asr1 for the association of Ubp3 with RNAPII. To do this, I performed a co-immunoprecipitation of Ubp3 and subsequent Western blot of RNAPII to ask 3 questions: 1) Does Ubp3 association with RNAPII occur in the presence of WT Asr1? 2) Does Ubp3 associate with RNAPII in the absence of Asr1? 3) Does a deletion mutant of Ubp3, that cannot bind Asr1, still associate with RNAPII? I found that Ubp3 associates with RNAPII in the

presence of Asr1 by blotting for Rpb3 (a core subunit of RNAPII) and Ser5P Rpb1 (the modified form of Rpb1 that is required for Asr1 association). However, in the absence of Asr1, Ubp3 does not associate with RNAPII (**Figure 4.5**). Additionally, the Ubp3 mutant N180Δ does not associate with RNAPII, despite the presence of Asr1. These observations, together along with the observation that the first 180 amino acids of Ubp3 can associate with RNAPII via association with Asr1 (**Figure 4.5**), suggest that Ubp3/Bre5 interact with RNAPII through Asr1.

4.4 Discussion

In this chapter, I used co-immunoprecipitation analysis of Asr1 and Ubp3 and their respective mutants to gain further insight into their association, and to get a better understanding of the molecular architecture of how Asr1 and Ubp3 interact with other protein partners. I was able to show that the RING and PHD fingers of Asr1 are dispensable for Ubp3 association. The CBD of Asr1 is not sufficient for Ubp3 binding, indicating that the amino-terminus of Asr1 is essential for association with Ubp3. By testing deletion mutants of Ubp3 for association with Asr1 and Bre5, I was able to separate the binding domains of these two proteins on Ubp3 by showing that a deletion mutant of Ubp3 that does not associate with Asr1 still binds Bre5. I was also able to show that Ubp3 requires interaction with Asr1 for it to associate with Rpb1 and Rpb3, two core subunits of RNAPII. This result, taken together with what is known about Asr1, suggests that Ubp3 mediates the interaction between Asr1 and the Ubp3/Bre5 heterodimer. I am unable to definitively conclude whether the interaction between Asr1 and Ubp3 is direct, which would require an *in vitro* binding assay, Far Western analysis, or similar assay, however it is clear that the interaction between Asr1 and Ubp3 is mediated N-terminus of both proteins.

Asr1/Ubp3 interaction impact on association with RNAPII

I was able to establish that Ubp3 associates with Asr1 in a manner that is independent of the activity of the RING and PHD finger of Asr1 and that the CBD of Asr1 is not sufficient for Ubp3 association with Asr1. This result indicates that Ubp3 associates with Asr1 somewhere within the N-terminus of Asr1, independent of the region necessary and sufficient for the association of Asr1 to Rpb1. Also importantly, the RING and PHD finger mutants of Asr1 still bind to Ubp3 and

present a biological tool with which I can use to assay the importance of the RING or PHD finger for a specific cellular process without perturbing the binding capacity of Ubp3 to Asr1.

Mutational analysis of Ubp3 shows that the first 180 amino acids of Ubp3 are both necessary and sufficient for association of Ubp3 to Asr1 as well as to RNAPII. The N180Δ mutant of Ubp3 lies outside of the Bre5 binding domain, and I was able to confirm that this mutant is still capable of binding Bre5, which along with the fact that the DUB catalytic domain lies in the C-terminal end of the protein (Li, Ossareh-Nazari et al. 2007), indicate that the N180 Ubp3 deletion mutant still has the capacity for enzymatic activity. This mutant will allow me to separate the function of Ubp3 DUB activity as a whole from its ability to associate with Asr1 (and subsequently RNAPII). Furthermore, Ubp3 requires the association of Asr1 in order to associate with RNAPII. All of this information together with the fact that Asr1 associates with Rpb1 directly *in vitro* (Daulny, Geng et al. 2008) allow for the conclusion that Asr1 recruits Ubp3 and its cofactor Bre5 to RNAPII through the association of Asr1 and Ubp3. This is an interesting finding given that it is known that Ubp3 is an RNAPII interacting protein, where once bound, can deubiquitylate Rpb1, impacting the ability of stalled RNAPII to clear from chromatin (Kvint, Uhler et al. 2008). Though it may seem contradictory, the interaction of an E3 and DUB is not entirely unique. However, the mediation of Ubp3 association to RNAPII by Asr1 represents a case in which an E3 appears to direct an antagonistic protein to one of its substrates, presumedly for the purpose of a better degree of regulation.

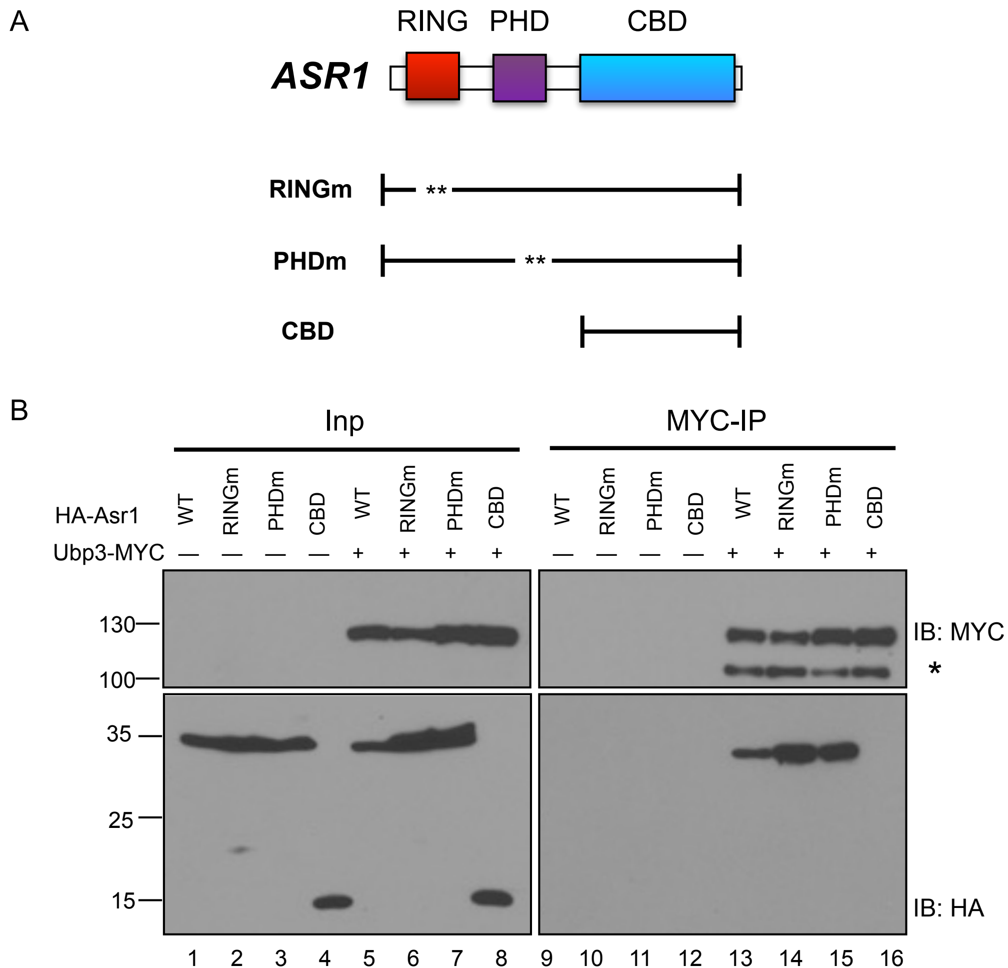
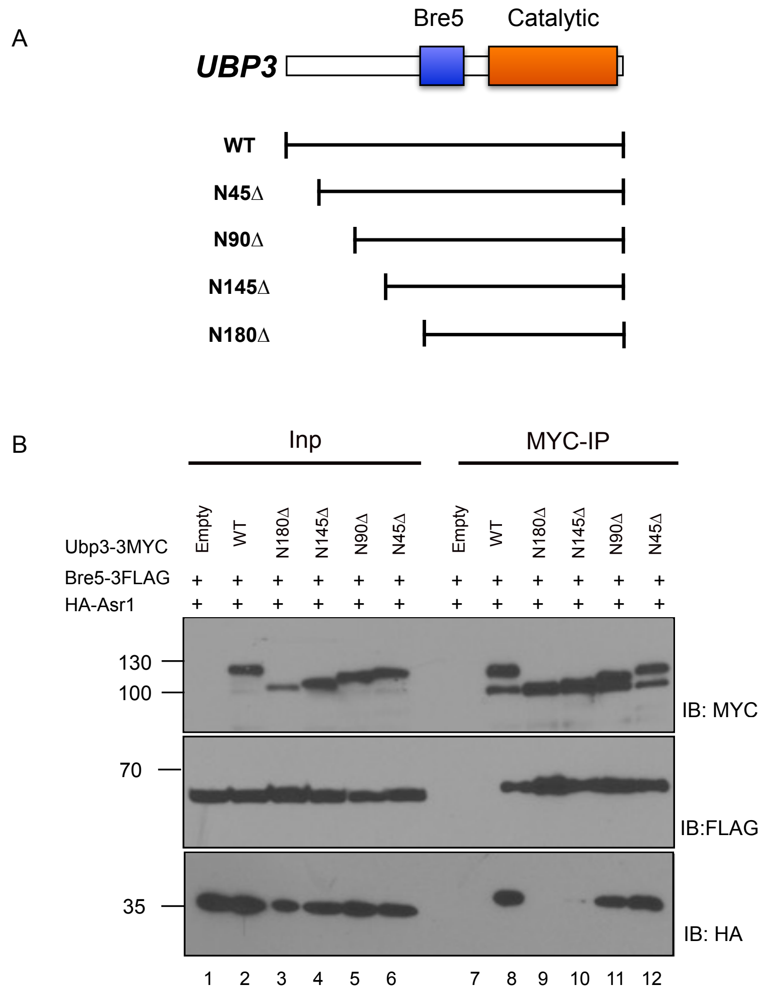


Figure 4.1 The amino-terminus of Asr1 is required for association with Ubp3. (*A*) Cartoon of Asr1, showing the location of the RING and PHD fingers, and the CTD- binding domain (CBD). The RINGm mutation is a simultaneous substitution of cysteine residues 26, 29, 66, and 69 to alanine. The PHDm mutation is a simultaneous substitution of cysteine residues 143, 146, 186, and 189 to alanine. (*B*) Extract was prepared from yeast cells expressing galactose-inducible HA-tagged Asr1 proteins, either alone (WT, YTM9; Asr1^{RINGm}, YTM10; Asr1^{PHDm}, YTM11; CBD, YTM12) or in the presence of a plasmid expressing MYC-tagged Ubp3 (WT, YTM13; Asr1^{RINGm}, YTM14; Asr1^{PHDm},YTM15; CBD, YTM16). Immunoprecipitation (IP) was performed with an anti-MYC antibody (MYC-IP), and products subjected to immunoblotting (IB) with anti–HA and –MYC antibodies. For input (Inp), total cell lysates were extracted with 0.1M NaOH and assayed in parallel. (*) indicates a low molecular weight MYC-reactive species we assume is a degradation product of Ubp3 that forms during the IP.



4.2 **Different elements within Ubp3 mediate interaction with Bre5 versus Asr1.** (*A*) Cartoon of Ubp3, showing the location of the domains required for interaction with Bre5 and for catalysis. Beneath the cartoon is a scaled representation of the location of amino-terminal truncation mutants characterized for interaction with Asr1 in Figure 4.2 B.(*B*) Extract was prepared from yeast cells expressing galactose-inducible HA-tagged Asr1 and FLAG-tagged Bre5, either alone (–, YTM9) or in the presence of a plasmid expressing MYC–tagged Ubp3 proteins (WT, YTM13; ΔN180, YTM17; ΔN145, YTM18; ΔN90, YTM19; ΔN45, YTM20). Immunoprecipitation (IP) was performed with an anti-MYC antibody (MYC-IP), and products subjected to immunoblotting (IB) with anti–HA, –FLAG, and –MYC antibodies. For input (Inp), total cell lysates were extracted with 0.1M NaOH and assayed in parallel.

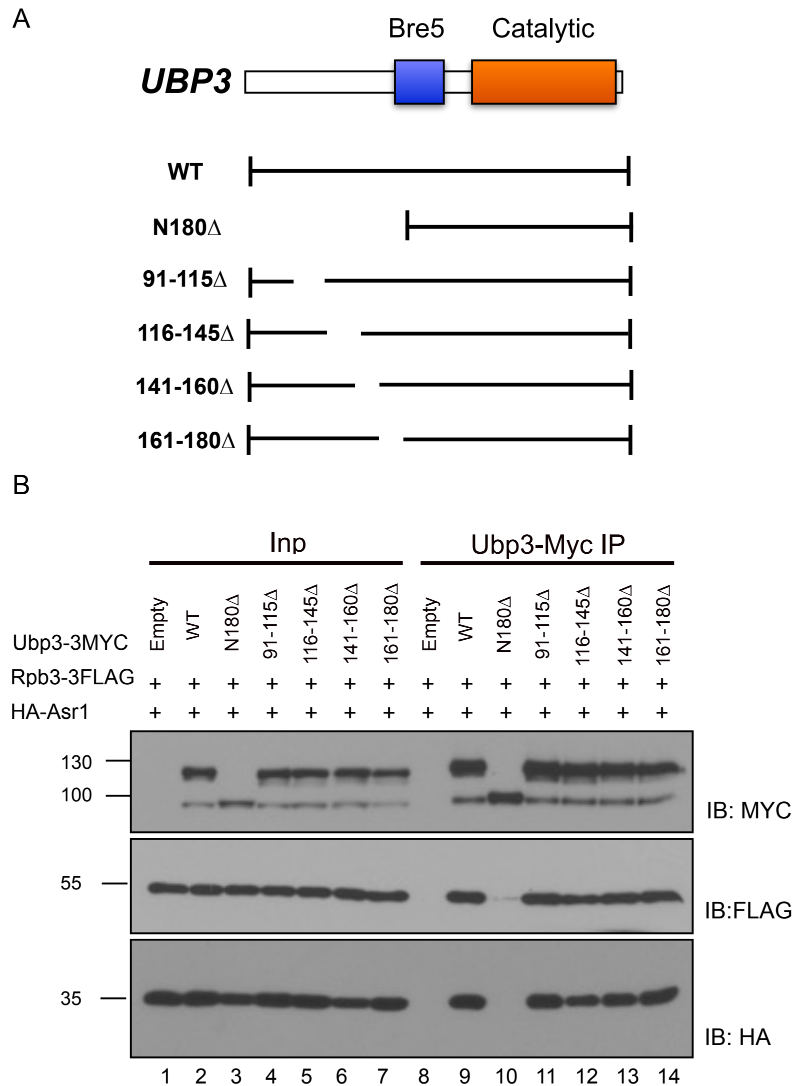


Figure 4.3 Internal deletions of Ubp3 do not affect Asr1 association with Ubp3. (*A*) Cartoon of Ubp3, showing the location of the domains required for interaction with Bre5 and for catalysis. Beneath the cartoon is a scaled representation of the location of amino-terminal truncation mutants characterized for interaction with Asr1 in Figure 4.3 B. (*B*) Extract was prepared from yeast cells expressing galactose-inducible HA-tagged Asr1 and FLAG-tagged Rpb3, either alone (–, YTM22) or in the presence of a plasmid expressing MYC–tagged Ubp3 proteins (WT, YTM24; ΔN180, YTM26; Δ91-115, YTM49; Δ116-145, YTM50; Δ141-160, YTM51; Δ161-180, YTM52). Immunoprecipitation (IP) was performed with an anti-MYC antibody (MYC-IP), and products subjected to immunoblotting (IB) with anti–HA, –FLAG, and –MYC antibodies. For input (Inp), total cell lysates were extracted with 0.1M NaOH and assayed in parallel.

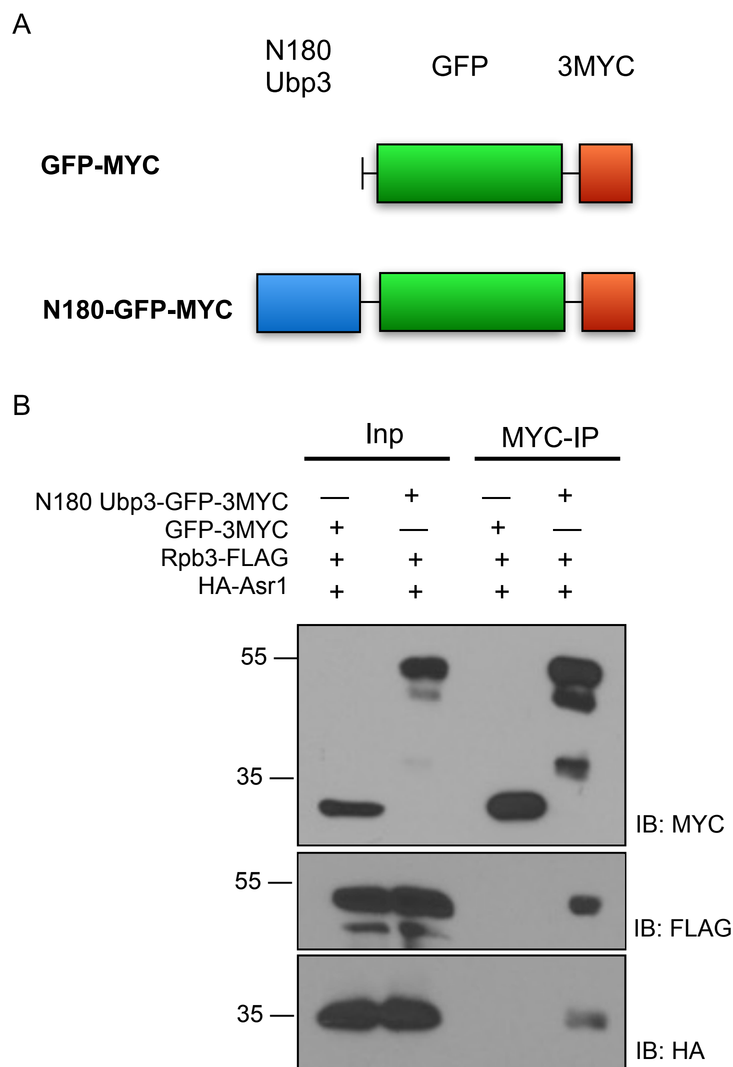


Figure 4.4 The amino-terminus of Ubp3 is sufficient for interaction with Asr1 and RNAPII. (*A*) Cartoon of mutants used to determine sufficiency of N-terminal 180 of Ubp3 for the association with Asr1 and RNAPII. (*B*) Extract was prepared from cells expressing HA-tagged Asr1, FLAG-tagged Rpb3, and either MYC-tagged GFP alone (YTM43), or MYC-tagged GFP fused to the amino-terminal 180 residues of Ubp3 (YTM42). Immunoprecipitation (IP) was performed with an anti-MYC antibody (MYC-IP), and products subjected to immunoblotting (IB) with anti–HA, –FLAG, and –MYC antibodies. For Rpb3–FLAG and Asr1–HA, 2.5% of the input was analyzed; for Ubp3–MYC, 0.1% of the input was analyzed.

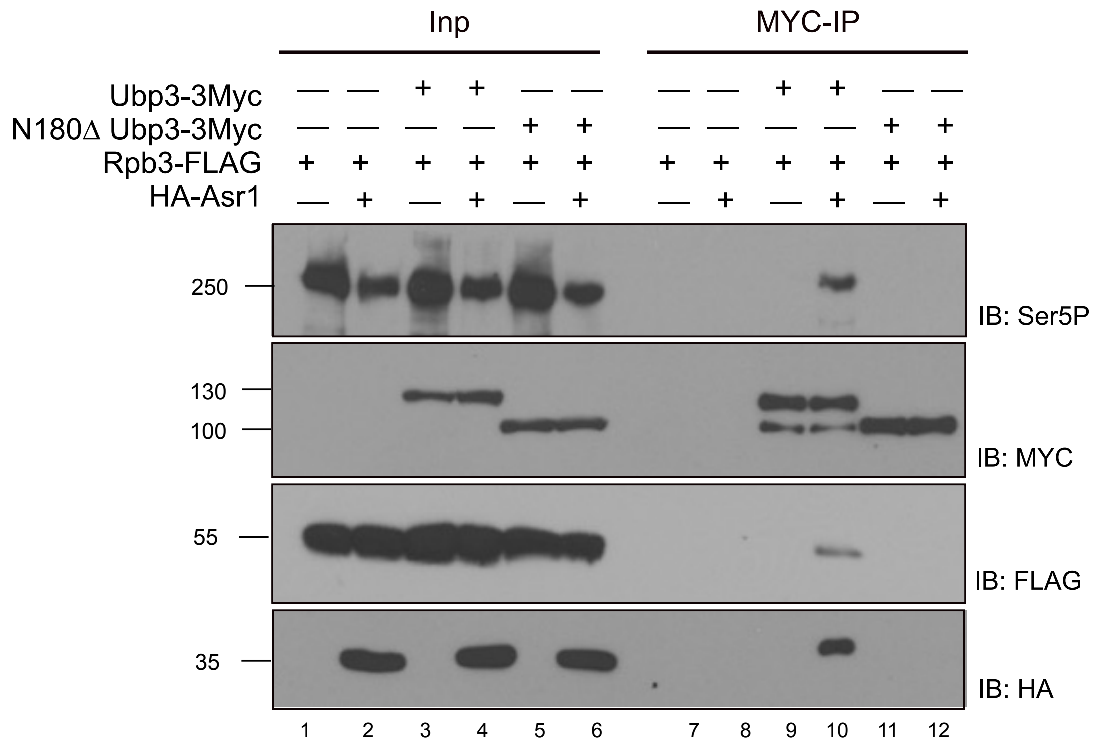


Figure 4.5 Interaction of Ubp3 with RNAPII is mediated by Asr1. Extracts were prepared from yeast cells expressing FLAG-tagged Rpb3, and carrying combinations of (i) an *ASR1* gene deletion (Δ) or expression of wild-type (WT) HA-tagged Asr1, and (ii) WT MYC-tagged Ubp3 (WT) or the ΔN180 MYC-tagged Ubp3 mutant (*lanes 1 and 7*, YTM21; *lanes 2 and 8*, YTM22; *lanes 3 and 9*, YTM23; *lanes 4 and 10*, YTM24; *lanes 5 and 11*, YTM25; *lanes 6 and 12*, YTM26). Immunoprecipitation (IP) was performed with an anti-MYC antibody (MYC-IP), and products subjected to immunoblotting (IB) with anti–pSer5, –FLAG, –HA, and –MYC antibodies. For pSer5, Rpb3–FLAG, and Asr1–HA, 2.5% of the input was analyzed; for Ubp3–MYC, 0.1% of the input was analyzed.

CHAPTER 5

BIOLOGICAL ROLE OF THE ASR1/UBP3 INTERACTION

5.1 Introduction

In the previous chapter, I showed that the E3 Asr1 physically associates with the DUB Ubp3. Ubp3 has been implicated in a multitude of different biological processes, such as: regulation of general autophagy (Kelly and Bedwell 2015), selective forms of autophagy like ribophagy (Kraft, Deplazes et al. 2008, Ossareh-Nazari, Bonizec et al. 2010, Ossareh-Nazari, Nino et al. 2014) and mitophagy (Muller, Kotter et al. 2015), vesicular trafficking (Cohen, Stutz et al. 2003, Ossareh-Nazari, Cohen et al. 2010), regulation of replicative lifespan (Oling, Eisele et al. 2014), regulation of transcriptional elongation during DNA damage (Kvint, Uhler et al. 2008, Mao and Smerdon 2010), and control of telomeric silencing (Moazed and Johnson 1996, Oling, Masoom et al. 2014). Therefore, I will use the insights gained in previous studies and biological tools I have made in order to identify the specific biological role for the Asr1/Ubp3 interaction.

Ubp3 and RPC family in selective autophagy

Autophagy is a process through which the cell engulfs unnecessary or damaged proteins and organelles with a spherical membrane structure called the autophagosome under conditions of stress or starvation (Noda and Inagaki 2015). Ribophagy is a selective form of autophagy where the cell directs the engulfment of ribosomes under nitrogen poor conditions. Ubp3 and Bre5 were identified as factors being required for ribophagy when the process was first being described (Kraft, Deplazes et al. 2008). The model proposed at the time was that an unknown E3 ubiquitylates ribosomal subunits and either the ubiquitin protects these subunits from ribophagy or they need Ubp3 to deubiquitylate them in order to proceed with the process. I originally thought that Asr1 could be the orphan ubiquitin ligase that ubiquitylates ribosomes for ribophagy, however, the Dargemont group just recently discovered that Rkr1 (also known as Ltn1) is the E3 that is involved in this process (although they did not specifically test the requirement of Asr1 in this process) (Ossareh-Nazari, Nino et al. 2014). Additionally, there are RPC family proteins that are involved in selective autophagy in species other than budding yeast. The homolog of Asr1 in the yeast *Pichia pastoris* (*ATG35*) has been shown to regulate the specific autophagic process

called micropexophagy, which is the selective process of autophagic engulfment of peroxisomes when they are no longer needed by the cell, but is not required for general autophagy in this organism (Nazarko, Nazarko et al. 2011). Pexophagy is more specialized in *P. pastoris*, which undergoes both micropexophagy and macropexophagy (with *ATG35* only being involved in the former) whereas most organisms (including budding yeast) only undergo a singular process called pexophagy. Even though S*. cerevisiae* does not undergo micropexophagy, I cannot rule out the involvement of Asr1 in autophagy or pexophagy.

Ubp3 and transcriptional elongation

Transcriptional elongation is a complicated process that utilizes many different mechanisms of regulation including ubiquitylation (Somesh, Reid et al. 2005). In budding yeast, the ubiquitin ligase Rsp5 first ubiquitylates stalled RNAPII when it encounters a transcriptional elongation perturbation such as the drug 6-azauracil or UV damage. The ubiquitin chain is elongated by the Elc1/Cul3 E3 complex, at which time RNAPII is cleared from chromatin and is targeted to the proteasome for destruction (Somesh, Sigurdsson et al. 2007, Harreman, Taschner et al. 2009). Ubp3 has been shown to deubiquitylate this polyubiquitylated form of RNAPII, to a deleterious effect on the cell, preventing the cell from clearing stalled RNAPII from sites of DNA damage (Kvint, Uhler et al. 2008). This finding established a role for Ubp3 in the regulation of RNAPII through the regulation of ubiquitylation under the condition of stalled polymerase, however it remains unclear as to whether Ubp3 deubiquitylates RNAPII under normal conditions.

Ubp3 in telomeric silencing

The first indication that Ubp3 might be involved in telomeric silencing came in 1996 when Ubp3 was identified in a GST purification of Sir4, a member of the Sir complex (Moazed and Johnson 1996). It was discovered in this study that Ubp3 acted as an anti-silencing protein, increasing the amount of telomeric silencing of an ectopic gene reporter upon deletion. Levels of histone modifications that are important for the establishment of heterochromatin (particularly H4K16ac) remained unchanged in *ubp3*Δ cells compared to WT (Kvint, Uhler et al. 2008), and levels of Sir complex proteins remained relatively unchanged at telomeres (Kvint, Uhler et al. 2008, Oling, Masoom et al. 2014), although increased levels of Sir complex proteins were seen at other sites

of heterochromatin such as the mating locus (Oling, Masoom et al. 2014). In all cases, lower levels of RNAPII were observed at heterochromatin in *ubp3Δ* cells (Kvint, Uhler et al. 2008, Oling, Masoom et al. 2014). These observations suggest that Ubp3 may affect telomeric transcription through an as yet unidentified method, possibly through modification of RNAPII.

5.2 Materials and Methods

Spotting assays.

Overnight cultures with appropriate media were grown at 30 °C and diluted the next morning to 0.2 OD_{600}. Cultures were grown to ~0.5 OD_{600} and 5 fold serial dilutions were made. 5 μl of each dilution was pipetted onto CSM agar plates containing either 6-AU (*Sigma*), MMS (*Sigma*), or rapamycin (*Life Technologies*), or the relevant vehicle control, as indicated in the legend to (**Figure 5.1-6)** and grown for 2-3 days at 30 °C prior to being photographed.

His-Ub pulldown assay.

Overnight cultures of yeast strains containing the plasmid pUB221 (containing 6xHis-Ubiquitin-MYC under the control of the *CUP1* promoter) were grown at 30 °C in CSM-Ura media. These cultures were diluted to 0.2 OD_{600} in 200 ml and grown to ~0.6 OD_{600} where 400 μl of 250 mM $CuSO_4$ was added to induce His-Ubiquitin expression. Cultures were grown 2 more hours, pelleted by centrifugation and flash frozen. Cell pellets were resuspended in 2 ml of Buffer A (6M guanidine-HCl, 0.1M Na_2HPO_4/NaH_2PO_4, 10mM imidazole, pH 8.0) and glass beads were added to just below 1 mm from the top of a 2 ml screw cap tube. Cell suspensions were bead beat 8x and lysate was collected by centrifugation. 250μl of 1:1 Ni-NTA beads (*Qiagen*) equilibrated in Buffer A was added and rotated at room temperature for 2 hrs. Beads were washed 3x with Buffer A, 3x with Buffer A/TI (1 volume Buffer A, 3 volumes Buffer TI) and 1x with Buffer TI (25mM Tris-Cl pH 6.8, 20 mM imidazole, pH 6.8). Beads were then resuspended in 200μl 2x Laemmli buffer supplemented with 0.2M imidazole (5% SDS, 20% glycerol, 120mM Tris-Cl pH 6.8, and 5% BME) and run on an SDS-PAGE gel. Immunoblotting was performed using the indicated antibodies together with Supersignal West Pico (*Pierce*) or Supersignal West Femto (*Pierce*).

Telomeric silencing reporter assays.

For *URA3* reporter strains, logarithmic cultures of yeast were serially diluted, divided in two, and spread onto control complete synthetic defined media (CSM) plates, or CSM plates containing 1 g/L 5-fluoroorotic acid (FOA). After growth for 2–3 days at 30 °C, colonies were counted on plates of equal dilution, and relative FOA-resistance calculated by dividing the number of colonies on CSM– FOA plates by the number on the equivalent CSM control plate. For *ADE2* reporter assays, cells were serially diluted, spread onto CSM (or CSM-Leu plates), and grown for 2–3 days at 30 °C. Plates were stored at 4 °C for two days before red and white colonies on each were counted. Percentage colony color was determined by calculating the percentage of red and white colonies for each strain.

5.3 Results

The biological role of Asr1 remains unclear despite its explicit interactions with RNAPII. While deletion of *ASR1* has been implicated to cause sensitivity to ethanol (Betz, Schlenstedt et al. 2004) this claim has been refuted by our lab as well as others (Izawa, Ikeda et al. 2006). Asr1 has more recently been suggested to be involved in Ca^{2+}/calmodulin signaling (Fries, Frank et al. 2011) and in the regulation of the cell cycle (Zou, Yan et al. 2015), however neither of these studies explain the transcriptional role of Asr1. With the new knowledge that Asr1 associates with the Ubp3/Bre5 complex, I was able to use the known biological roles of Ubp3 as a guide to elucidating the role of Asr1 within the cell.

Asr1 and Ubp3 do not interact genetically in several Ubp3 phenotypes

Deletion of *UBP3* has been shown to be sensitive to a variety of different cellular perturbations. Spotting assays to test the viability of *ASR1*/*UBP3* mutants under various conditions were performed to test whether deletion/mutation of *ASR1* would be synthetic or epistatic to *ubp3*∆. Deletion of *UBP3* has been shown to cause sensitivity to 6-azauracil (a drug that causes the reduction of intracellular GTP, which when combined with mutations in transcriptional machinery causes slow growth) (Exinger and Lacroute 1992, Nakanishi, Shimoaraiso et al. 1995) and rapamycin (a macrolide antibiotic that mediates rapid *TOR1* inhibition, which results in the induction of autophagy) (Noda and Ohsumi 1998) as well as lower sensitivity to methyl

methanesulfonate or MMS (a DNA methylating agent) (Pegg 1984). These spotting assays confirm the sensitivity/insensitivity of *ubp3*Δ to these drugs, although deletion of *ASR1* or mutation of the RING finger of Asr1 shows no phenotype change from WT (**Figure 5.1-3**). Additionally, these Asr1 mutations in a *ubp3*Δ background show the same phenotype as *ubp3*Δ, indicating that there is no epistatic or synthetic interaction between *ASR1* and *UBP3* in these contexts. To further confirm the absence of a role for Asr1 ubiquitylation in specific biological roles of Ubp3, a Rpb1 mutant (K1452R/K1458R/K1487R Δ1720-1734; called Δ2KTM Rpb1; (Daulny, Geng et al. 2008)) that has five lysine residues shown to direct ubiquitylation by Asr1 mutated also grows similarly to WT in all three conditions (**Figure 5.1-3**; *compare rows 1 and 8*).

I also performed an assay to specifically test how the ability of Ubp3 to associate with Asr1 (and subsequently RNAPII) affects Ubp3 in these specific biological contexts. To do this, I completed spotting assays comparing WT Ubp3 and a mutant form of Ubp3 that cannot bind Asr1 (both expressed from a plasmid) under similar drug conditions. In all of these assays, expression of mutant Ubp3 performed like the WT/vector control in the 6-AU, rapamycin, and MMS sensitivity assays, indicating that loss of Ubp3 association to Asr1 is not responsible for the phenotype seen in *ubp3*Δ (**Figure 5.4-6**).

Mutation of Asr1 causes a decrease in ubiquitylation of Ser5P Rpb1

As mentioned before, both Asr1 and Ubp3 have been shown to modulate Rpb1 with ubiquitin modifications (Daulny, Geng et al. 2008, Kvint, Uhler et al. 2008). I wanted to confirm these results, as well as test the effect that a double *asr1*/*ubp3* mutant would have on the ubiquitylation of Rpb1. A significant decrease in the ubiquitylation of Ser5P Rpb1 (and no change to total levels of Rpb1) was observed in the case of *asr1*Δ and Asr1^{RINGmut} strains (**Figure 5.7**), which confirmed the earlier finding that disruption of the RING finger of ASR1 decreases Ser5P Rpb1 ubiquitylation but not total Rpb1 ubiquitylation levels (Daulny, Geng et al. 2008). Deletion of *UBP3* has been shown to increase the levels of ubiquitylation of Rpb1 under DNA damage conditions (Kvint, Uhler et al. 2008), however the effect of *ubp3*Δ alone under normal conditions resulted in little to no change in the ubiquitylation status of Rpb1 or Ser5P Rpb1 (**Figure 5.7**; *compare lanes 9 and 10*). There are some notable caveats to this this conclusion however. It is very difficult to

detect these high molecular weight ubiquitylated species, and it may be unreasonable to expect to see even more ubiquitylated Rpb1 in *ubp3Δ* cells. Also, the experiment done by the Svejstrup lab that showed an increase in Rpb1 ubiquitylation was done in the presence of irradiation. Also, instead of purifying ubiquitin and blotting for Rpb1, they purify Rpb1 and blot for ubiquitin. Despite the absence of an effect of *ubp3Δ* alone on the ubiquitylation of Ser5P Rpb1, in my experiment, deletion of *UBP3* appears to antagonize the effect seen by mutation of *ASR1*. Upon *UPB3* deletion in the context of *ASR1* mutants, I observe a reversal in the decrease of the ubiquitylation seen in *ASR1* mutants, suggesting that Ubp3 works antagonistically to Asr1 in ubiquitylation of Rpb1 (**Figure 5.7**; *compare lanes 6 and 8 to 5 and 7*).

Ubp3 and Asr1 affect telomeric silencing of reporter strains

Ubp3 has also been implicated as an anti-silencing factor of telomere proximal genes. Deletion of *UBP3* causes a decrease in the expression of a reporter placed at the right telomere of chromosome V (VR) (Moazed and Johnson 1996). A similar reporter strain in which *URA3* was placed at VR was used to confirm this result in a quantitative silencing assay with a read out of colony resistance to 5-fluoroorotic acid (5-FOA) (Clarke, Samal et al. 2006). This assay takes advantage of the fact that the gene product of *URA3* converts 5-FOA into the toxic compound 5-fluorouracil, which is deleterious to the cell. This allows resistance to 5-FOA to be a proxy for silencing of *URA3* at the telomere. Deletion of *ASR1* causes a mild, but significant, decrease in 5-FOA resistance compared to WT, indicating that Asr1 plays a role in the negative regulation of telomeric silencing (**Figure 5.8 A**). I was also able to confirm that *ubp3Δ* causes an increase of 5-FOA resistance compared to WT, indicating lower transcription of the *URA3* reporter at telomeres in *ubp3Δ* cells (**Figure 5.8 B**). However if *ASR1* and *UBP3* are deleted in the same strain, a close to 4-fold reduction in the number of 5-FOA resistant colonies is observed compared to *ubp3Δ* alone, suggesting that Asr1 and Ubp3 play opposing roles in the regulation of telomeric silencing.

The reliability of using *URA3* as a telomeric reporter has come under question as of late. Mutations that cause a perturbation in nucleotide metabolism has led to a multitude of false positives due to the interaction between the *URA3* gene product and 5-FOA (Rossmann, Luo et al. 2011, Yankulov 2011). To address this caveat, I performed a similar assay using a different

reporter strain, with the *ADE2* gene product at the right arm of telomere V (VR) as a reporter gene (Monson, de Bruin et al. 1997). The red/white telomeric silencing assay takes advantage of the adenine biosynthetic pathway in which a mutation of the *ADE2* gene leads to an accumulation of red pigment in the cell. Expression of WT *ADE2* from the telomere in this strain rescues the mutation in the pathway and leads to white cells. WT cells have nearly completely silenced chromatin at this telomere, which results in red colonies. It would be expected that deletion of *UBP3* would result in an increase of red colonies, however, because WT cells are nearly completely silenced, no significant change in colony color from WT (**Figure 5.9**). Deletion of *ASR1* causes a significant increase in white colonies as compared to WT, and the double *asr1*Δ/ *ubp3*Δ cells show a similar phenotype, corroborating the suggestion that Asr1 and Ubp3 play opposing roles in telomeric silencing. These results suggest that the effects seen in the mutant reporter strains are due to the actions Asr1/Ubp3, not a metabolic artifact.

Effect of Asr1/Ubp3 mutants in telomeric silencing reporter strains

To confirm that the silencing phenotypes were due to the deletion of either *ASR1* or *UBP3*, I performed rescue experiments. First, I transformed WT, RING mutant, and PHD mutant Asr1 expressing plasmids into the *ADE2* reporter strain and performed the same red/white colony counting assay. I found that the WT and PHD mutant of Asr1 rescued the defect caused by *ASR1* deletion, however the RING mutant of Asr1 did not (**Figure 5.10**). This suggests that the RING domain, but not the PHD domain, plays an important role in the function of Asr1 in telomeric gene silencing. Because I have established that Ubp3 requires Asr1 to bind RNAPII, I wanted to see how a Ubp3 mutant affects the expression of *ADE2* in these reporter strains, so I tested the WT Ubp3 and the Ubp3 N180Δ mutant the red/white colony assay. Like before, *ubp3*Δ alone caused no significant difference from WT, however when *UBP3* was overexpressed on the plasmid, nearly all colonies were white, suggesting that more *UBP3* causes less telomeric silencing. Expression of the Asr1 binding mutant (N180Δ Ubp3) causes the formation of significantly more red colonies than expression of Ubp3, indicating that Ubp3 requires the association of Asr1 and/ or RNAPII in order to affect telomeric silencing (**Figure 5.11**).

5.4 Discussion

The purpose of this chapter was to use the known biological phenotypes of Ubp3 as a guide to uncover the biological role of Asr1 within the cell. Through the experiments detailed in this section I was able to systematically investigate the specific antagonistic interaction of *ASR1* and *UBP3* in the processes of Rpb1 ubiquitylation and telomeric silencing.

Genetic interaction of *ASR1* and *UBP3*

Spotting assays were utilized as a quick and easy way to use genetics to assess whether *ASR1* and *UBP3* are both involved in a specific process. The spotting assays were performed in a manner to assay if mutation of *ASR1* (through *asr1*Δ or Asr1^{RINGmut}) is involved in this particular process or whether there would be any epistasis/synthetic interaction between *ASR1* and *UBP3.* I also used *UBP3* deletion mutants that disrupt the interaction of Asr1 and Ubp3, to assess how the Asr1/Ubp3 interaction (and in turn the association of Ubp3 with RNAPII) were important for a particular function. The fact that there was no observable genetic interaction between *ASR1* and *UBP3* in 6-AU, MMS, and rapamycin sensitivity conditions, but there is one in other assays (Rpb1 ubiquitylation and telomeric silencing) suggests that Asr1/Ubp3 interaction is specific and highly regulated.

It is particularly curious that expression of the two N-terminal deletion mutants of Ubp3, which are devoid of association with RNAPII, are able to rescue sensitivity to 6-AU (**Figure 5.4**) seeing as growth on 6-AU is normally used as proxy to identify mutants to impair transcriptional elongation (Tansey 2006). This would lead me to believe that the role of Ubp3 in transcriptional elongation is not based in its association with RNAPII, or more likely, the sensitivity of *ubp3*Δ cells to 6-AU is due to an off target effect of the drug.

Asr1 and Ubp3 in the ubiquitylation of Rpb1

In this chapter, I observed that Asr1 and Ubp3 have opposing functions in the ubiquitylation of Ser5P Rpb1. In both the *asr1*Δ and Asr1^{RINGmut} strains, a decrease in the amount ubiquitylated Ser5P Rpb1 species was observed, and when *UBP3* was deleted in this context, a slight increase in ubiquitylated Ser5P Rpb1 species was observed. This result can only be obtained if there is a

second ubiquitin ligase that is targeting Rpb1. There are several reasons why this has to be correct. One reason is that when the ability of Asr1 to ubiquitylate Rpb1 is removed, there is still ubiquitylated Rpb1 observed (Daulny, Geng et al. 2008) (**Figure 5.7**). Second, a double mutant of *asr1*Δ/*ubp3*Δ or Asr1^{RINGmut}/*ubp3*Δ causes an increase in the ubiquitylated Rpb1 pool compared to the single *ASR1* mutant, indicating this increase is due to ubiquitylation by the alternative ubiquitin ligase. The two obvious options for alternative Rbp1 ubiquitin ligases are Rsp5 and the Elc1/Cul3 complex, both of which are known Rpb1 targeting ubiquitin ligases (Harreman, Taschner et al. 2009). These E3s are only known to target RNAPII under conditions of polymerase stalling, such as in DNA damage and other cases of elongated transcriptional arrest that require the clearing of RNAPII from chromatin (Somesh, Reid et al. 2005). Ubp3 was predicted to oppose the clearing of RNAPII from chromatin in these specific cases through deubiquitylation of RNAPII (Kvint, Uhler et al. 2008). Although, there is no evidence that Rsp5 or Elc1/Cul3 target RNAPII for ubiquitylation at telomeres, they are the only E3s (besides Asr1) known to directly target RNAPII subunits. Another option would be that there is an additional ubiquitin ligase that has yet to be identified that targets Rpb1. The presence of multiple E3s for a single substrate is very common, and given that there are over 80 E3s in yeast (Singh, Gonzalez et al. 2012), it is plausible that there is another as yet identified E3 that targets Rpb1.

Asr1 and Ubp3 role in telomeric silencing

I was able to determine that Asr1 plays a role in the silencing of at least two ectopic telomeric reporter genes by observing that upon deletion of *ASR1,* expression of the reporter genes increased compared to WT. This result supports my initial conclusion from the RNA-seq experiment that Asr1 regulates transcription of telomeric genes. I also used the telomeric reporter strains to assay the genetic interaction of *ASR1* and *UPB3* in this context. I observed that deletion of *UBP3* alone causes a dramatic amount of 5-FOA resistant colonies in the *URA3* reporter assay, confirming the results of previous reports (Moazed and Johnson 1996, Oling, Masoom et al. 2014). *ASR1* deletion in the same context partially reverses the phenotype seen by deletion of *UBP3*, exhibiting a strong genetic interaction between *ASR1* and *UBP3* in this strain. In the *ADE2* reporter assay, the vast majority of colonies are red in the WT, so deletion of *UBP3* did not result in more red colonies (which would indicate more silencing of the *ADE2* reporter), however

deletion of *ASR1* in this context drastically reduces the amount of silencing compared to just *UPB3* deletion. These observations establish that *ASR1* and *UBP3* strongly interact genetically in the process of telomeric silencing.

Asr1 also requires the activity of its RING domain in order to regulate an ectopic telomeric reporter, which suggests that ubiquitylation by Asr1 plays an important role in the regulation of telomeric silencing. This result corroborates with the observation in Chapter 3 that the Asr1^{RINGmut} causes an induction of endogenous telomeric genes. It was somewhat surprising to observe that the PHD finger of Asr1 is dispensable for its role in the regulation of telomeric silencing. This result would suggest that the PHD finger of Asr1 is not involved with directing Asr1 to subtelomeric regions through an interaction with chromatin.

Overexpression of Ubp3 in the *ADE2* telomeric reporter strain causes a dramatic decrease in silencing of the ectopic reporter, further confirming the role of Ubp3 as an anti-silencing factor. The Ubp3 N180Δ mutant (which cannot associate with Asr1 or RNAPII) does not reverse this silencing defect, and is nearly identical to deletion of *UPB3* in how it affects telomeric silencing. This observation suggests that Ubp3 requires the association of Asr1 (or RNAPII) in order to have an anti-silencing affect on the expression of telomere proximal genes.

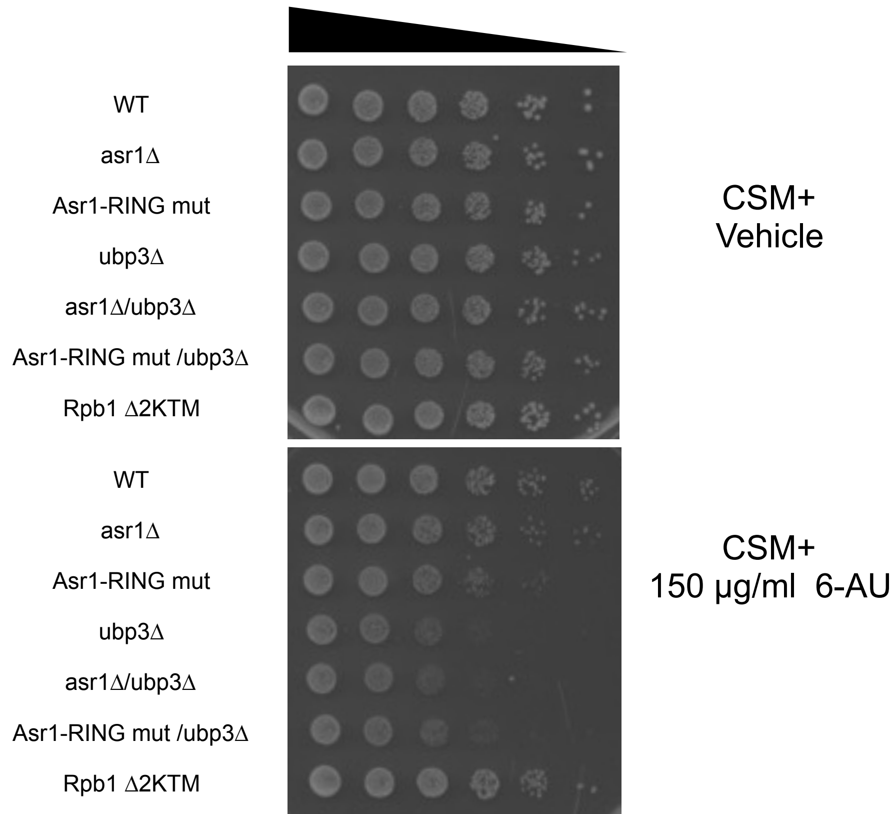


Figure 5.1 Mutation of *ASR1* does not affect growth in the presence of 6-azauracil (6-AU). Growth assays were performed by spotting liquid cultures in 5-fold serial dilutions on a control plate, and a plate that contained 150 µg/ml 6-AU in indicated strains (WT, BY4741; asr1Δ, YTM5; ASR1-RING mut, YTM27; ubp3Δ, ΔUbp3; asr1Δ/ubp3Δ, YTM6; Asr1-RING mut/ubp3Δ, YTM28; Rpb1 Δ2KTM, YTM29). Plates were grown 2-3 days.

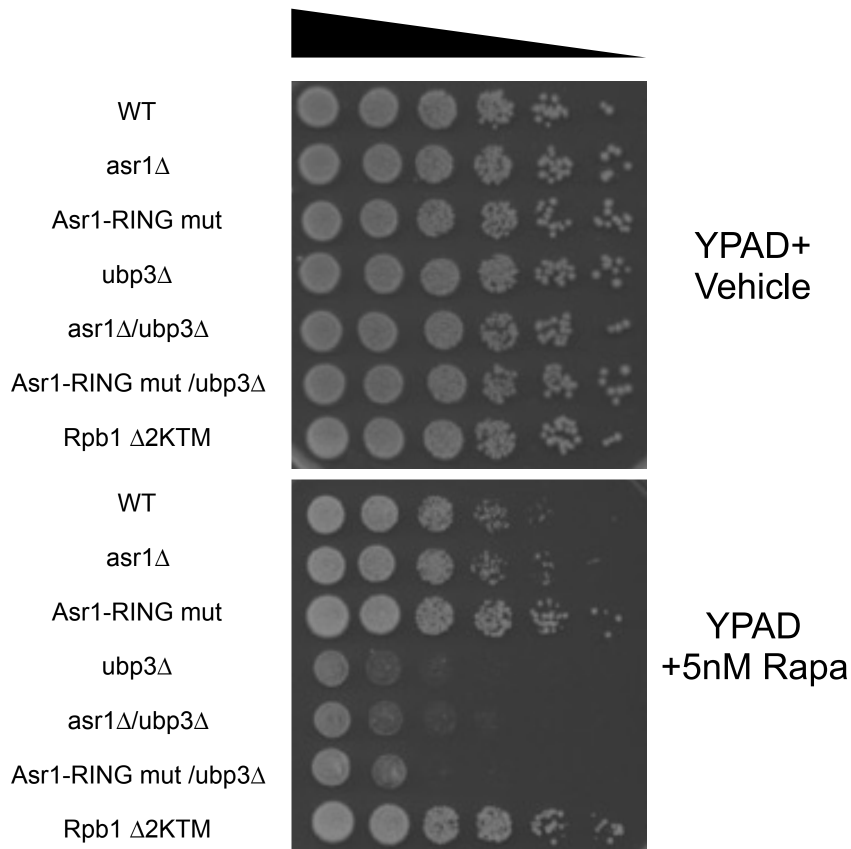


Figure 5.2 Mutation of *ASR1* does not affect growth in the presence of rapamycin. Growth assays were performed by spotting liquid cultures in 5-fold serial dilutions on a control plate, and a plate that contained 5 nM Rapamycin in indicated strains (WT, BY4741; asr1Δ, YTM5; ASR1-RING mut, YTM27; ubp3Δ, ΔUbp3; asr1Δ/ubp3Δ, YTM6; Asr1-RING mut/ubp3Δ, YTM28; Rpb1 Δ2KTM, YTM29). Plates were grown 2-3 days.

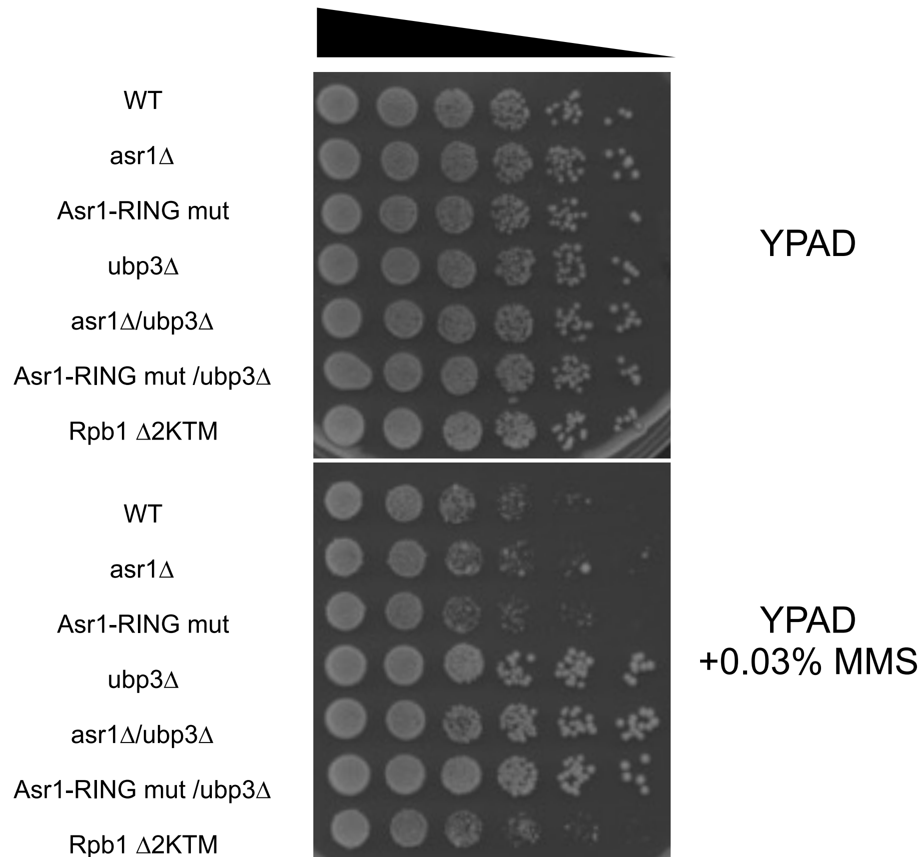


Figure 5.3 Mutation of *ASR1* does not affect growth in the presence of methyl methanesulfonate (MMS). Growth assays were performed by spotting liquid cultures in 5-fold serial dilutions on a control plate, and a plate that contained 0.03% MMS in indicated strains (WT, BY4741; asr1Δ, YTM5; ASR1-RING mut, YTM27; ubp3Δ, ΔUbp3; asr1Δ/ubp3Δ, YTM6; Asr1-RING mut/ubp3Δ, YTM28; Rpb1 Δ2KTM, YTM29). Plates were grown 2-3 days.

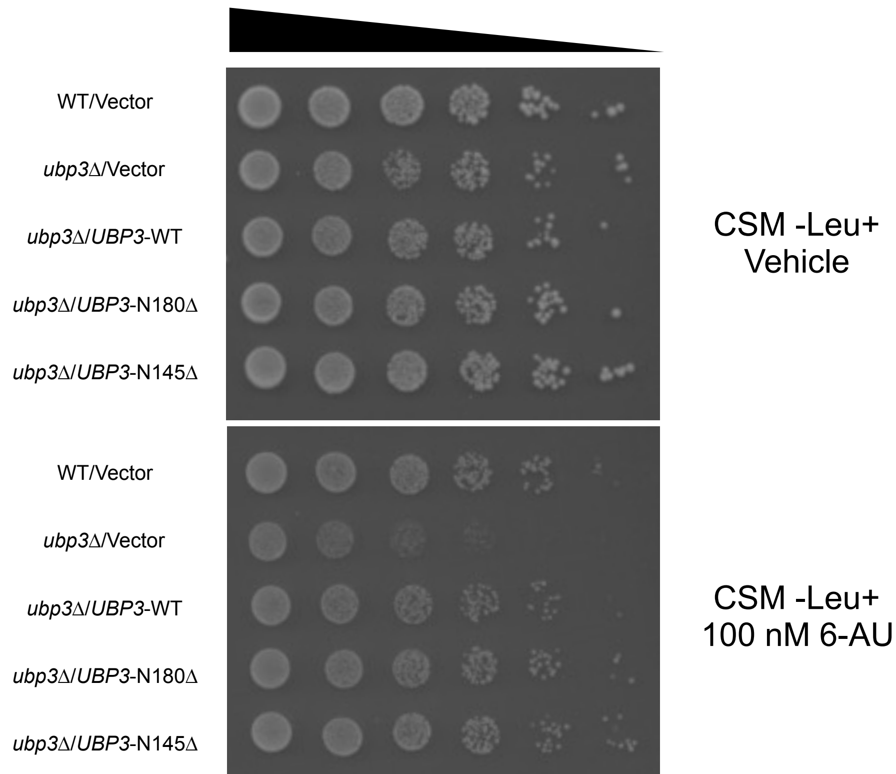


Figure 5.4 Ubp3 deletion mutants exhibit WT behavior when exposed to 6-azauracil (6-AU). Growth assays were performed by spotting liquid cultures in 5-fold serial dilutions on a control plate, and a plate that contained 100 µg/ml 6-AU in indicated strains (*UBP3*/Vector, YTM44; *ubp3*Δ/Vector, YTM45; *ubp3*Δ:*UBP3-WT*, YTM46; Δ*ubp3*:*UBP3*-Δ*N180*, YTM47; Δ*ubp3*:*UBP3*-Δ*N145*, YTM48). Plates were grown 2-3 days. Ubp3 constructs were expressed from pRS415 GPD.

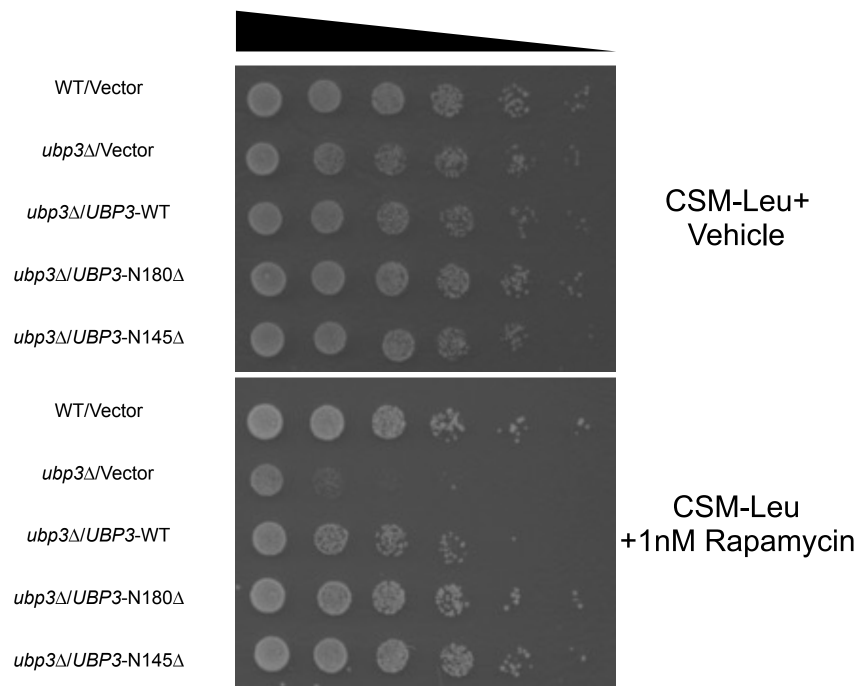


Figure 5.5 Ubp3 deletion mutants exhibit WT behavior when exposed to rapamycin. Growth assays were performed by spotting liquid cultures in 5-fold serial dilutions on a control plate, and a plate that contained 1nM Rapamycin in indicated strains (*UBP3*/Vector, YTM44; *ubp3*Δ/Vector, YTM45; *ubp3*Δ:*UBP3-WT*, YTM46; Δ*ubp3*:*UBP3-*Δ*N180*, YTM47; Δ*ubp3*:*UBP3-* Δ*N145*, YTM48).. Plates were grown 2-3 days. Ubp3 constructs were expressed from pRS415 GPD.

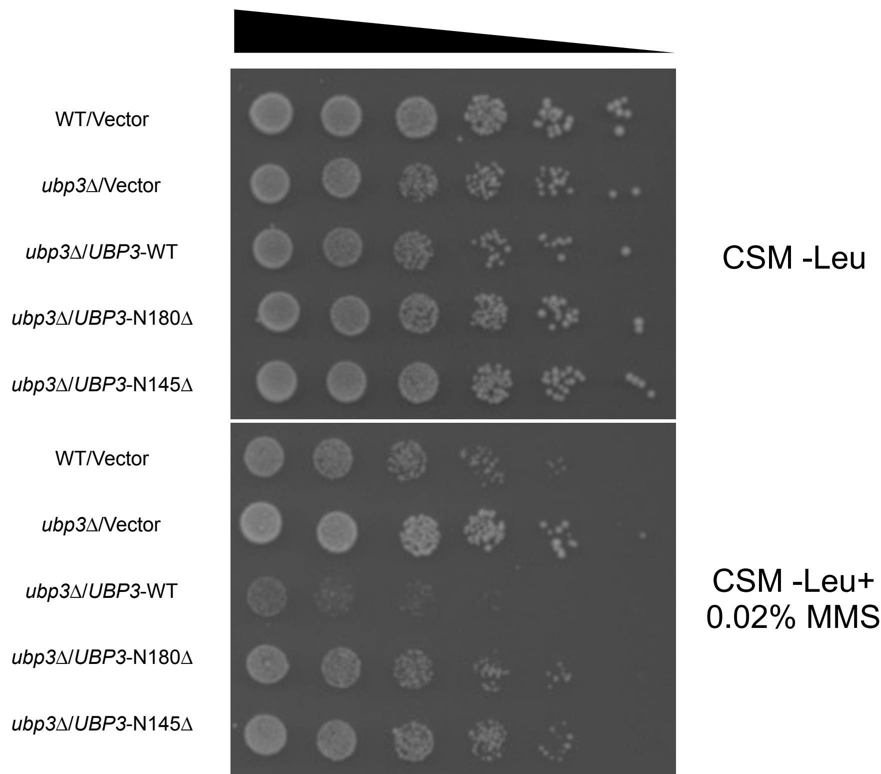


Figure 5.6 Ubp3 deletion mutants exhibit WT behavior when exposed to methyl methanesulfonate (MMS). Growth assays were performed by spotting liquid cultures in 5-fold serial dilutions on a control plate, and a plate that contained 0.02% MMS in indicated strains (*UBP3*/Vector, YTM44; *ubp3Δ*/Vector, YTM45; *ubp3Δ*:*UBP3-WT*, YTM46; Δ*ubp3*:*UBP3-ΔN180*, YTM47; Δ*ubp3*:*UBP3- ΔN145*, YTM48). Plates were grown 2-3 days. Ubp3 constructs were expressed from pRS415 GPD.

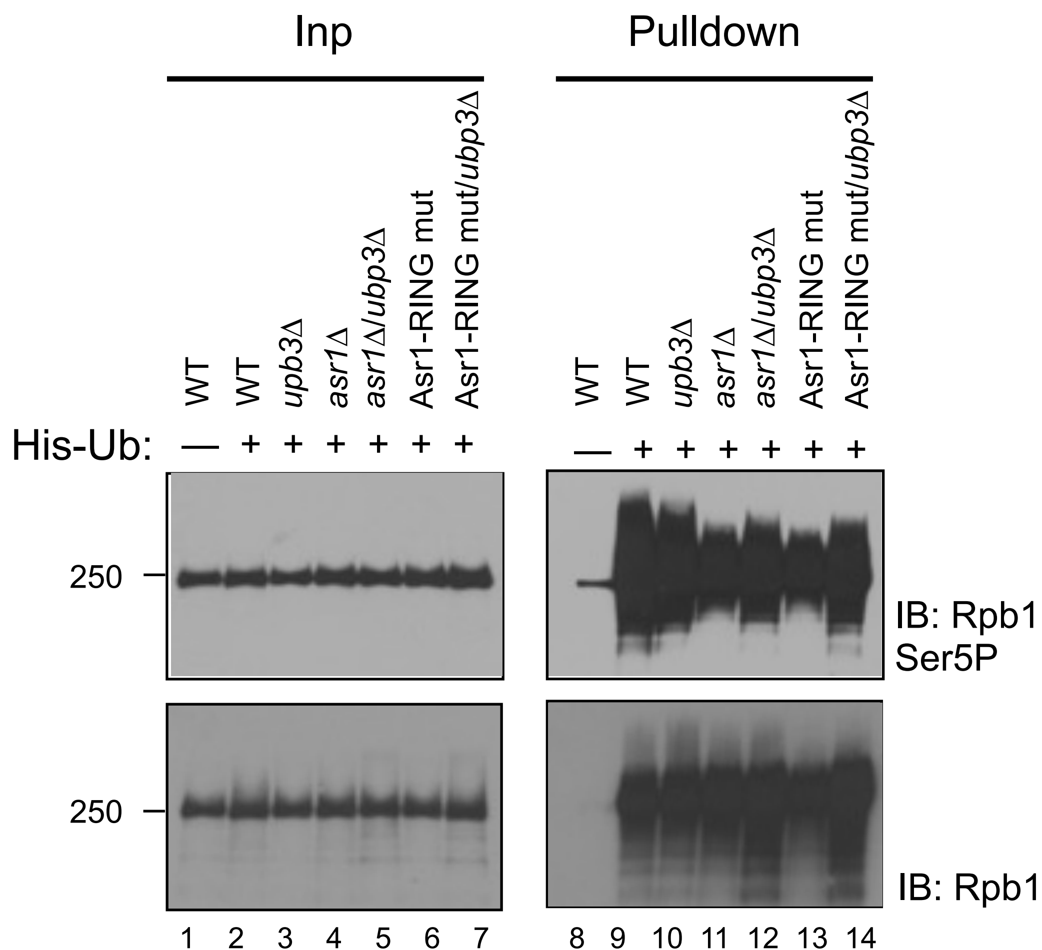


Figure 5.7 Asr1 and Ubp3 oppositely impact ubiquitylation of Ser5P Rpb1. Denaturing extracts were prepared from a WT yeast strain (*lane 1,* YTM31), or yeast expressing polyhistidine-tagged ubiquitin (His–Ub) and carrying the indicated genotype (*WT*, YTM32; *ubp3*Δ, YTM35; *asr1*Δ, YTM33; *asr1*Δ*/ubp3*Δ, YTM36; *asr1*^{RINGm}, YTM34; *asr1*^{RINGm}Δ*ubp3,* YTM37). Ubiquitylated proteins were recovered on Ni-NTA resin. Levels of protein in the Ni-NTA-bound material, and a sample of the input, were detected by immunoblotting with a pSer5-specific antibody (pSer5 IB) and total Rpb1 CTD specific antibody (Rpb1 IB).

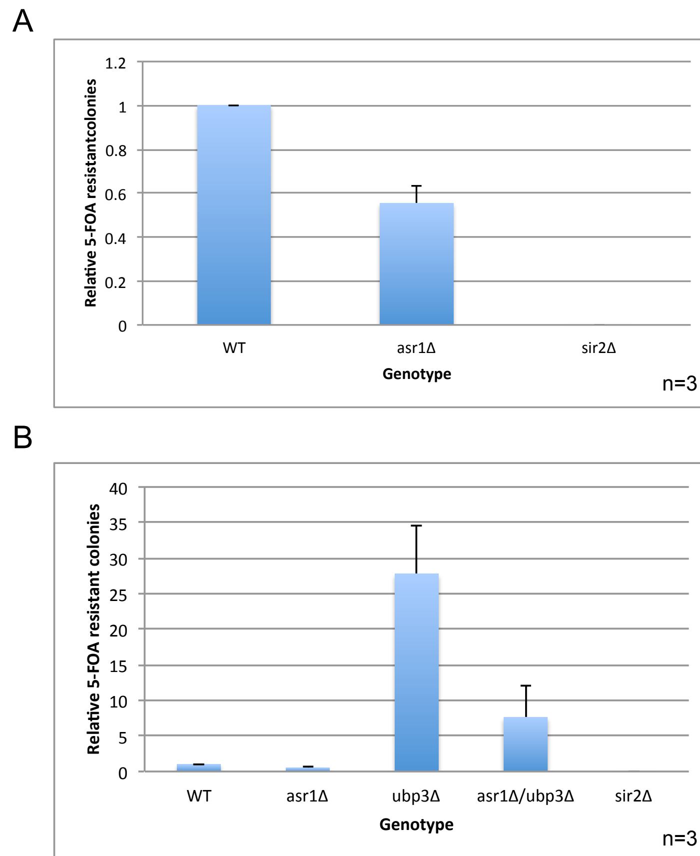


Figure 5.8 Deletion of *ASR1* causes a decrease in 5-fluoroorotic acid (5-FOA) resistant colonies in *URA3* telomeric reporter strain. (*A*) Asr1 is required for full silencing of a telomere-proximal *URA3* reporter. Equal amounts of yeast cells of the indicated genotype (*WT*, LPY4819; *asr1*Δ, LPY4819 ΔAsr1; *sir2*Δ, LPY4977) were plated on media with or without 5-fluoroorotic acid (FOA) and colonies counted. The relative number of FOA-resistant colonies were then calculated. Error bars represent s.e.m. (*n=3*). (*B*) *asr1*Δ partially rescues silencing defect seen by *ubp3*Δ. Assay performed as in (A) but with additional strains (*WT*, LPY4819; *asr1*Δ, LPY4819 ΔAsr1; *ubp3*Δ, LPY4819 ΔUbp3; *asr1*Δ/*ubp3*Δ, LPY4819 ΔAsr1ΔUbp3; *sir2*Δ, LPY4977).

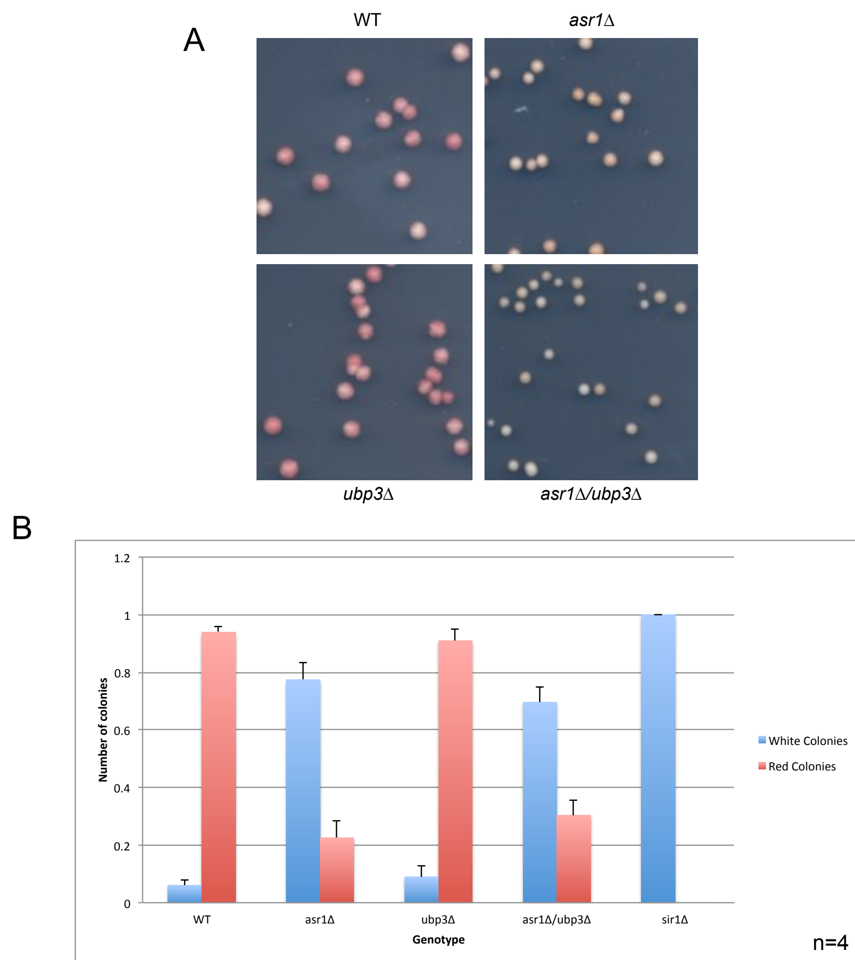


Figure 5.9 Deletion of *ASR1* causes an increase in transcription of *ADE2* telomeric reporter. (*A*) Photograph of representative colonies used to calculate colony color ratios for *ADE2* telomeric reporter strain. (*B*) Loss of *ASR1* is epistatic to loss of *UBP3*. Yeast cells of the indicated genotype (*WT*, YPH499UTAT; *asr1*Δ, UTAT TM1; *ubp3*Δ, UTAT TM2; *asr1Δ/ubp3*Δ*,* UTAT TM3; *sir2*Δ, UTAT TM12) were plated to single colony density on non-selective media and the ratio of white and red colonies for each plate calculated.

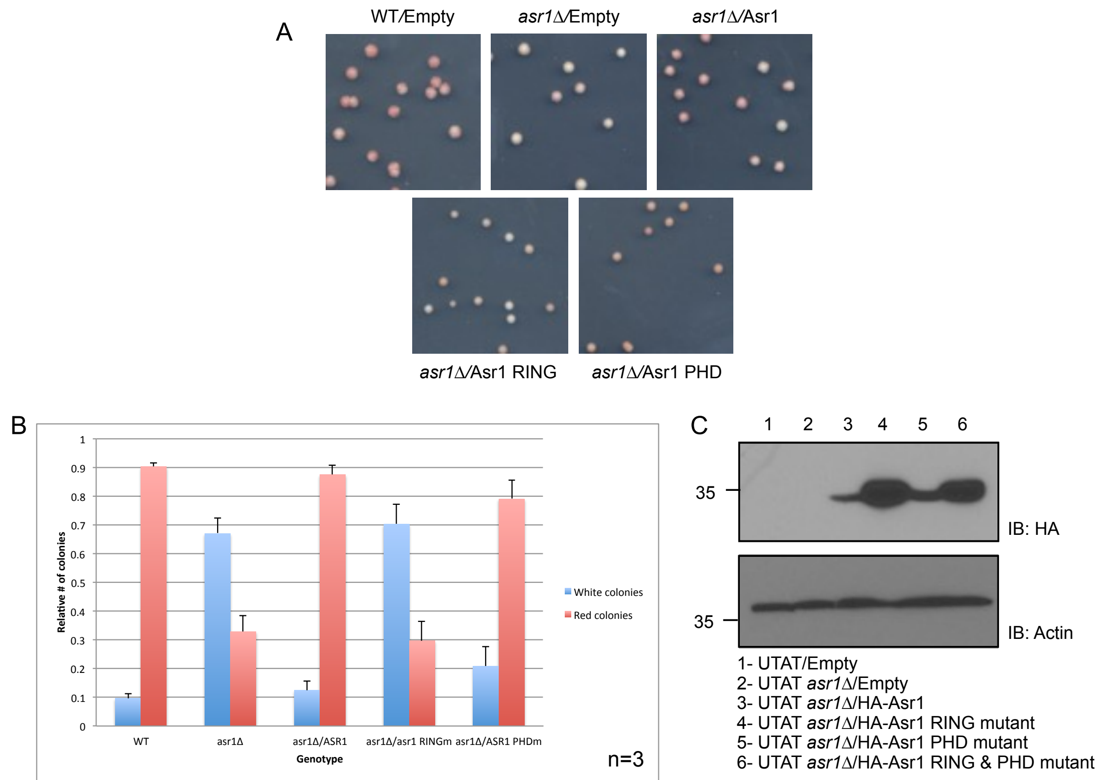


Figure 5.10 The RING finger of Asr1 is required in the regulation of an ectopic *ADE2* telomeric reporter. (A) Photograph of representative colonies used to calculate colony color ratios for *ADE2* telomeric reporter strain. (*B*) Yeast cells (*WT*, UTAT TM4; *asr1*Δ, UTAT TM5; *asr1Δ/ASR1*, UTAT TM6; *asrΔ/asr1*^{RINGm}, UTAT TM7; *asr1Δ/ASR1*^{PHDm}, UTAT TM8) were plated to single colony density on non-selective media and the ratio of white and red colonies for each plate calculated. Quantification of *ADE2* telomeric reporter assay. Error bars represent s.e.m. (*n=3*). (C) Steady-state levels of HA-tagged Asr1 proteins assayed in Figure 5.10 A and B. Immunoblot (IB) was performed using an anti-HA antibody. An antibody against actin was used as a loading control. Note that the double RING & PHD mutant of Asr1 was not included in functional assays.

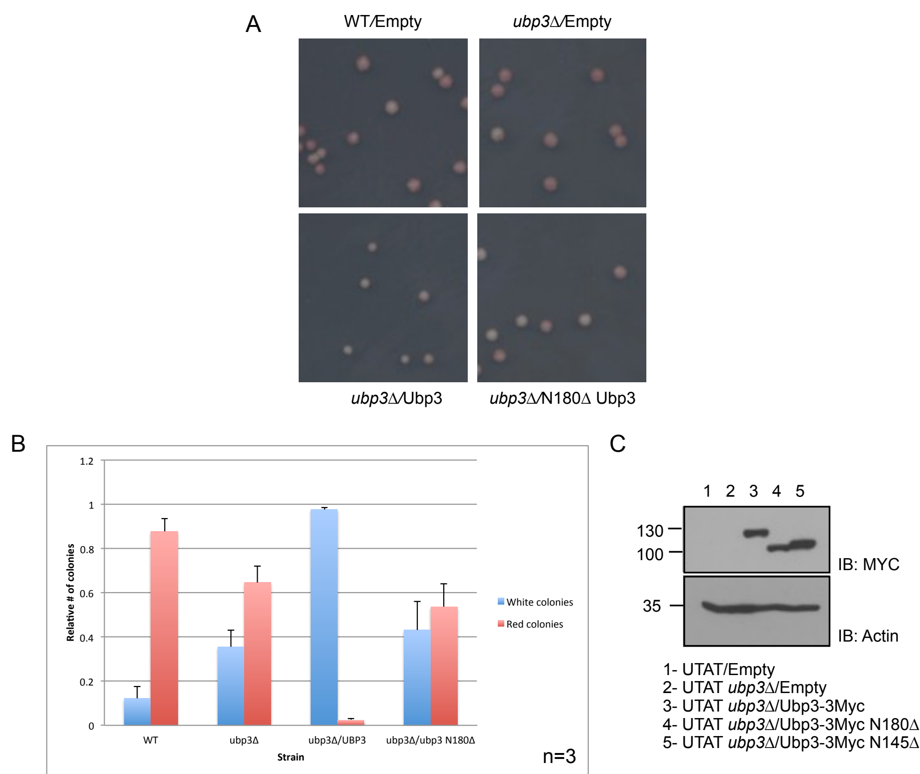


Figure 5.11 Expression of WT Ubp3, but not mutant Ubp3, causes an increase in expression of *ADE2* in telomeric reporter strain. (*A*) Photograph of representative colonies used to calculate colony color ratios for *ADE2* telomeric reporter strain. (*B*) The amino-terminus of Ubp3 is required for its pro-silencing function. Yeast cells of the indicated genotype (*WT*, UTAT TM4; *ubp3Δ*, UTAT TM9; *ubp3Δ/UBP3,* UTAT TM10; *Δubp3/ubp3 ΔN180*, UTAT TM11) were plated to single colony density on non-selective media and the ratio of white and red colonies for each plate calculated. Error bars represent s.e.m. (*n=3*). (*C*) Steady-state levels of MYC-tagged Ubp3 proteins assayed in Figure 5.11 A and B. Immunoblot (IB) was performed using an anti-MYC antibody. An antibody against actin was used as a loading control.

CHAPTER 6

ROLE OF ASR1 IN SUBTELOMERIC GENE SILENCING

6.1 Introduction

In the previous chapter, I showed that the biological end result of the interaction between Asr1 and Ubp3 is specific to two processes: the ubiquitylation of RNAPII and the regulation of subtelomeric transcription. I hypothesize that Asr1 and Ubp3 regulate transcription through direct modification of ubiquitylation of RNAPII. Through the experiments described in this chapter, I aimed to gain a clearer understanding as to the mechanism and scope of the role Asr1 and Ubp3 play in the regulation of transcription of telomere proximal genes. To accomplish this this, I used qRT-PCR to assay how affecting ubiquitylation of Rpb1 by Asr1 affects the transcription of subtelomeric genes identified in the RNA-seq analysis, as well as several subtelomeric genes that have been described to be induced upon disruption of the Sir complex. I assessed whether Asr1 directly associates with the subtelomeric regions in which mutation of Asr1 causes induction of transcription. I also addressed whether the induction telomere proximal genes caused by disruption of ubiquitylation of Rpb1 by Asr1 is caused by or commensurate with a change in the acetylation status of H4K16, similar to that seen by disruption of the Sir complex.

Ubiquitin and the proteasome in subtelomeric silencing

Signaling by the ubiquitin proteasome system has previously been shown to play an integral role in the regulation of telomeric silencing. As mentioned earlier, H2B ubiquitylation is essential for histone crosstalk to signal H3K4 and H3K79 di- and trimethylation by Set1 (Sun and Allis 2002) and Dot1 (Ng, Xu et al. 2002). These chromatin marks are thought to act as a barrier between euchromatic and heterochromatic regions, preventing the spread of the Sir complex into euchromatin (Venkatasubrahmanyam, Hwang et al. 2007). Proper silencing of telomeric regions also requires the proteasome, as mutation of two subunits of the attached ATPase portion (Rpt4 and Rpt6) result in an increase in transcription in areas of heterochromatin such as subtelomeric regions and the mating locus (Ezhkova and Tansey 2004). Further understanding about how Asr1 and Ubp3 affect the regulation of telomere proximal genes will further develop the connection between regulation of subtelomeric transcription by the UPS.

6.2 Materials and Methods

RNA isolation.

Cells were grown from a single colony in YPAD overnight at 30 °C. The next day, 25 ml cultures were diluted to 0.2 OD_{600} and grown to log phase. RNA was collected using the Hot Acid Phenol method (Collart and Oliviero 2001). The RNA was treated with DNaseI (*NEB*) to remove contaminating DNA and reverse transcription was carried out to create a cDNA library using the following reaction: 1x PCR buffer (*Applied Biosystems*), 5mM $MgCl_2$, 0.5mM dNTPs (*Roche*), 20U RNase inhibitor (Applied Biosystems), 2.5μM random hexamers (*Applied Biosystems*), 50U Multiscribe Reverse Transcriptase (*Applied Biosystems* #4311235) and 1 μg RNA. The 20 μl reaction was diluted 1:10 and 5 μl was used for each 10 μl PCR reaction using KAPA SYBR FAST qPCR Master Mix 2X Universal and quantified on a Eppendorf Realplex2 Mastercycler in triplicate. The expression of specific genes were calculated relative to the housekeeping gene *ACT1* by $2^{\wedge(ACT1\text{-}GOI)}$ where the value of *ACT1* is the average CT value of *ACT1_F* and *ACT1_R* and GOI is the average CT value using primers specific to the gene of interest. All expression values were then normalized to the WT value of 1.

Chromatin immunoprecipitation (ChIP)

Cultures were grown overnight at 30 °C in YPAD. In the morning, 100 ml cultures were diluted to 0.2 OD_{600} and grown to log phase (~0.8-1 OD_{600}) and treated with a final concentration of freshly made 1% paraformaldehyde (PFA) for 15 minutes at room temperature to crosslink. Cross-linking was quenched with a final concentration of 0.25M glycine at room temperature. Pellets were washed 2x with ice cold 1x PBS, flash frozen, and stored at -80°C for later use. The next day, cell pellets were resuspended in 800 μl chromatin FA buffer (50 mM Hepes, 150mM NaCl, 10mM EDTA, 1% Triton, 0.5% SDS, 0.1% DOC), and glass beads were added to 1 mm below the top of screw cap tube and bead beat 6x for 40 seconds with 1 min on ice in between. Lysate was collected by centrifugation and cleared with successive 15 minute spins at 16.1 RCF. Samples were sonicated to shear chromatin to a mean size of 250 bp. 25 μl of chromatin was diluted with 1 ml FA buffer (50mM Hepes, 150mM NaCl, 10mM EDTA, 1% Triton, 0.1% SDS, 0.1% DOC) and incubated overnight at 4 °C with 5 μl anti H4K16ac antibody (*Millipore*, #07-329). Protein A beads were prepped by washing 35 μl 1:1 slurry 2x with FA buffer, then incubated with 1 mL FA buffer,

75 μg/μl salmon sperm DNA and 10 ng/mL BSA for 30 minutes, then added to chromatin/antibody mixture for 2 hours. Immune complexes were washed 1x with FA buffer, 1x with 0.5M NaCl FA buffer (50 mM Hepes, 500mM NaCl, 10mM EDTA, 1% Triton, 0.1% SDS, 0.1% DOC), 1x with LiCl-NP40 buffer (250mM LiCl, 0.5% NP40, 0.5% DOC, 1mM EDTA, 10 mM Tris-Cl pH 8), and 2x with TE (10mM Tris-Cl pH 8, 1mM EDTA). Supernatant was removed, and 200 μl elution buffer (1% SDS, 200mM NaCl, 100mM $NaHCO_3$) and 5 μl 20 mg/mL proteinase K was added and incubated at 65 °C overnight to both digest proteins and reverse cross-linking. Supernatant was removed and DNA was recovered by phenol-chloroform extraction and ethanol precipitation. DNA pellet was resuspended in 200 μl TE and 5 μl was used for each 10 μl PCR reaction using KAPA SYBR FAST qPCR Master Mix 2X Universal and quantified on a Eppendorf Realplex2 Mastercycler in triplicate.

DAM-ID profiling

For each protein to be assessed, two strains are needed: one strain has the DNA adenine methylase (DAM) integrated just before the stop codon of the protein of interest, and the control strain has DAM integrated just after to start codon, to express untagged DAM from the endogenous promoter. Cells were grown from a single colony in YPAD overnight. The next day 25 ml cultures were diluted to 0.2 OD_{600} and grown to log phase. Clean yeast genomic DNA prep was adapted from (van Steensel and Henikoff 2000). Add 200 μl DAM-ID DNA extraction buffer (25mM Tris pH8, 25mM EDTA pH 8, 100mM NaCl, and 0.4% SDS), 200μl phenol:chloroform:isoamyl alcohol (25:24:1) (*Sigma*), and 200 μl glass beads and bead beat 3 times 1 minute with 1 minute of rest on ice. 200 μl of TE (10mM Tris pH 8.0, 1mM EDTA) was added and spun 10 minutes at high speed to collect the supernatant. 1 μl of 10 mg/ml RNase A (*Clontech*) was added and incubated at 37 °C for 15 minutes. 5 μl of 20 mg/ml Proteinase K (*Roche*) was added and incubated at 65 °C for 2 hours. DNA was extracted with 200 μl of phenol:chloroform:isoamyl alcohol 25:24:1 and ethanol precipitated (1ml EtOH, 12 μl 3M NaOAc) overnight. Samples were spun at high speed, washed one time with 70% ethanol and let dry. The DNA pellet was resuspended in 50 μl water then diluted to 100 ng/μl. 100 ng genomic DNA was digested with DpnII at 37 °C overnight (1x DpnII buffer, 10U DpnII, 100 ng DNA) in a 50 μl reaction with and without DpnII. DpnII was heat inactivated for 20 minutes at 65°C. 5 μl was used

for each 10 µl PCR reaction using KAPA SYBR FAST qPCR Master Mix 2X Universal and quantified on a Eppendorf Realplex2 Mastercycler in triplicate. To calculate percent DNA methylation, the signal in the digest sample (with DpnII) was divided by the signal in the mock sample.

$$\text{Percent DNA Methylation} = \frac{2^{\wedge(\text{DpnII control CT value} - \text{DpnII tagged CT value})}}{2^{\wedge(\text{no enzyme control CT value} - \text{no enzyme tagged CT value})}}$$

6.3 Results

Asr1 and Ubp3 affect transcription at native telomeres

Because of the effect *ASR1* and *UBP3* mutants had on the expression of telomeric genes in the telomeric reporter strains, as well as the large percentage of telomeric genes found to be induced in the RNA-seq analysis described in Chapter 3, I used qRT-PCR to see how different *ASR1* and *UBP3* mutants affected native telomeric gene transcription. First, I looked at the top three telomeric genes induced in the RNA-seq: *PHO12*, *PHO84*, and *PHO89* (**Table 3.3**). I found that there was very little change in the expression of all three genes in *asr1*Δ cells. When I tested the expression of these three telomeric genes in Asr1^{RINGmut}, I observed between a 15-40 fold induction, similar to the level of induction seen in *sir*2Δ cells. What was interesting is that when *UBP3* is deleted in the context of Asr1^{RINGmut}, there is a complete loss of induction of telomeric genes that are seen upon mutation of *ASR1* (**Figure 6.1**). These findings suggest that Asr1 and Ubp3 play antagonistic roles in the regulation of telomeric gene silencing in budding yeast.

I also wanted to see if this trend held for other subtelomeric genes shown to be affected by disruption of the Sir complex. *YFR057W* is a subtelomeric gene located in a heterochromatic region on the right arm of telomere VI. This gene is a hallmark for the study of silencing at telomeres, often used as an indicator for a telomeric silencing factor (Vega-Palas, Venditti et al. 1997, Tham and Zakian 2002). In WT cells, *YFR057W* is silenced to a level that is undetectable by RNA-seq (not shown). Upon perturbation of the Sir complex, expression of this gene can be seen to increase ~10-40 fold (Yang, Miller et al. 2008, Koch and Pillus 2009, Wang, Li et al.

2013). Deletion of *ASR1* caused a mild increase in the expression of *YFR057W*, just under 3 fold above the levels of WT, with no detectable difference in the expression of *ALD6*, an internal gene located in an euchromatic region on the right arm of chromosome XVI (**Figure 6.2**). The impact on the expression of *YFR057W* was much more pronounced in the Asr1^{RINGmut}, causing an increase in expression of over 17 fold above WT levels. Deletion of *UBP3* alone caused little effect on the expression of *YFR057W*, however, deletion of *UBP3* in the context of either *asr1*Δ or Asr1^{RINGmut} reversed any induction seen by these mutants. These results were consistent when quantifying two other published genes located in silenced subtelomeric regions: *YNR077C* (located on the right arm of chromosome XIV) and *YCL074W* (located on the left arm of chromosome III) (Ezhkova and Tansey 2004, Leung, Cajigas et al. 2011). These further establish an antagonistic role for Ubp3 and Asr1 in the regulation of transcription at telomeres.

Effect of Rpb1 ∆2KTM mutant on transcription of telomeric genes

The downstream effect of *asr1*Δ and Asr1^{RINGmut} is a decrease in ubiquitylation of Rpb1, which is presumed to cause an increase in transcription of these previously silenced subtelomeric genes. To more directly test my model, I used the Rpb1 mutant previously described in Chapter 5, in which the sites of Asr1 directed ubiquitylation on Rbp1 were mutated (Daulny, Geng et al. 2008). The steady state levels of the ∆2KTM Rpb1 mutant are indistinguishable from WT Rpb1 in total Rpb1 and Ser5P levels, suggesting that this mutant does not affect the stability of the protein (**Figure 6.3**).

I first tested expression of the top three subtelomeric genes identified in the RNA-seq analysis: *PHO12*, *PHO84*, and *PHO89*. In the case of all three genes, the ∆2KTM Rpb1 mutant showed induction compared to WT, however the induction was lower than was observed in the Asr1^{RINGmut} strain (**Figure 6.4**). A similar result was observed when measuring the expression of *YFR057W*, *YCL074W*, and *YNR077C* (**Figure 6.5**). Therefore, mutation of the ability of Asr1 to ubiquitylate Rpb1 *in trans* (through mutation of the RING finger of Asr1) and *in cis* (through the ∆2KTM Rpb1 mutant) is suggestive of a direct role for Asr1 in the modification of RNAPII via ubiquitylation to regulate subtelomeric transcription. However, the levels of induction are lower than that of the Asr1^{RINGmut}. This result suggests that the induction of telomere proximal genes caused by

mutation of the RING activity of Asr1 is caused by more than a lack of ubiquitylation of Rpb1 by Asr1. There is either another protein that regulates Rpb1, either through ubiquitylation or another method, or Asr1 targets some other positive regulator of subtelomeric gene transcription that would not be affected by the mutation of Rpb1.

Induction of telomeric genes by mutation of Asr1 ubiquitylation of Rpb1 is not caused by hyper-H4K16ac

The method by which the Sir complex regulates silenced chromatin is relatively well known, the end result of which is deacetylation of H4K16 by the NAD+ dependent histone deacetylase (HDAC) Sir2. This chromatin mark causes a tightening of the chromatin, preventing the access of transcriptional machinery in that area (Suka, Luo et al. 2002). This method of transcriptional silencing is not promoter or transcript specific per se, but rather targets a specific location on the chromosome to form heterochromatin (Kueng, Oppikofer et al. 2013). To assess whether the induction of telomeric genes in the Asr1 and Rpb1 mutants is associated with a general perturbation of Sir regulation, which would cause an increase in H4K16 acetylation in these areas, I performed a ChIP for H4K16ac and measured the enrichment of this chromatin mark at the telomeric genes *YFR057W*, *YNR077C*, and *YCL074W*. Upon deletion of *SIR2*, there is approximately a 15-25 fold enrichment of H4K16ac compared to the levels of WT. However, in *asr1*Δ, Asr1^{RINGmut} and Rpb1 Δ2KTM mutants, all of which cause at least a slight induction of expression level of these genes, H4K16ac remains relatively unchanged (**Figure 6.6**). This result suggests that the induction of subtelomeric gene caused by *ASR1* and *RPB1* mutants is not associated with some direct or indirect perturbation of the Sir complex but rather an independent process by which the cell can regulate telomeric transcription.

Asr1 associates with telomeric genes

Although I have strong evidence that shows mutations in *ASR1* causes immense induction of the transcription of many subtelomeric genes, and that Asr1 associates with a modified form of RNAPII seen commensurate with the initiation of transcription, I have not definitively shown that Asr1 acts on RNAPII at the level of chromatin. One way to show this would be to ChIP Asr1 at telomeres, however to get a robust signal for Asr1 gene enrichment, overexpression of Asr1 was

needed (Daulny, Geng et al. 2008). There are several limitations of ChIP that may make it difficult to use as an assay to detect Asr1 association with subtelomeric genes. ChIP utilizes formaldehyde to crosslink proteins and nucleic acid together for immunoprecipitation. The cross-linking occurs through the formation of methylene bridges between lysine and arginine residues and nucleic acids (Lu, Ye et al. 2010), and the majority of these protein-DNA contacts occur within histones. Proteins that don't make direct contact with DNA or make transient interactions with DNA are less likely to form these bridges, and therefore they are often difficult to immunoprecipitate on chromatin (Aughey and Southall 2015). Therefore an alternative method was utilized to show association of particular proteins at chromatin that does not rely on formaldehyde cross-linking. In this procedure, called DNA adenine methyltransferase identification (DAM-ID) profiling, Asr1 and Asr1^{RINGmut} are fused to the *Escherichia coli* DNA adenine methyltransferase. This enzyme methylates GATC DNA sequences it encounters, therefore, any GATC sequence that comes into contact with Asr1-DAM proteins become methylated. That DNA can then be cleaved by a methylation-sensitive restriction enzyme to detect levels of association of the protein with DNA. This method has previously been used to show the interaction of Sir proteins with chromatin (Venkatasubrahmanyam, Hwang et al. 2007, Leung, Cajigas et al. 2011).

I selected two telomeric genes from our RNA-seq list (*PHO84* and *PHO89*), as well as a euchromatic gene that is not induced upon mutation of the RING finger of Asr1 (*ALD6*) as a control. Both the Asr1-DAM and Asr1^{RINGmut}-DAM proteins are seen to have enriched methylation across *PHO84* and *PHO89*, indicating that they make contact with DNA at these regions, while there is no enrichment in either strain in *ALD6* (**Figure 6.7**). Additionally, both Asr1-DAM and Asr1^{RINGmut}-DAM appear to be enriched specifically within the gene body compared to outside of the gene (**Figure 6.8-9**). These results show that Asr1 preferentially associates with these genes and provides more evidence that Asr1 plays an active role at chromatin in the regulation of subtelomeric genes.

Induction of non-telomeric genes

Through the analyses in this chapter, I have established a role for Asr1/Ubp3 as playing an antagonistic role in the regulation of transcription of subtelomeric genes. However, as seen in the RNA-seq, only ~33% of the induced genes in the Asr1^{RINGmut} strain were telomere proximal, which means ~67% of the induced genes are located along the arm of the chromosome, in presumably euchromatic DNA. How or if Asr1 directly regulates these genes is unclear. Therefore I used qRT-PCR to measure the expression of three candidate genes from this pool of non-telomeric genes: *SPL2*, *PRM7*, and *YBR056C-B*. In the case of *PRM7* and *YBR056C-B*, mutation of *ASR1* has a moderate effect on the expression of the gene, but mutation of *UBP3* had no real impact when combined with these mutations. These two genes were also only marginally responsive *sir2*Δ. However, in the case of *SPL2*, the Asr1^{RINGmut} causes a 17 fold increase in induction compared to WT. Deletion of *UBP3* in this context reverses this induction, an effect that is similar to what is seen in the subtelomeric genes that are responsive to the mutation of the RING finger of Asr1. Unexpectedly, *SPL2* is induced by *sir2*Δ, to a level of ~30 fold (**Figure 6.10**). This was an unanticipated result, as the Sir complex has generally been thought to only affect the transcription of traditionally heterochromatin regions. *SPL2* could potentially be representative of a new class of Sir responsive genes. A similar trend is seen in the Δ2KTM *rpb1* mutant. Expression of *PRM7* and *YBR056C-B* are near WT levels in the Δ2KTM mutant, however expression of *SPL2* is induced ~3.5 fold (**Figure 6.11**). These observations together support the idea that Asr1/Ubp3 regulate the transcription of Sir responsive genes through modification of Rpb1 by ubiquitylation.

6.4 Discussion

In this chapter I explored the transcriptional effect that mutation of the RING finger of Asr1 has on cells. Because of the lack of a phenotype seen by deletion of *ASR1* in previous whole transcriptome approaches, I focused my experiments on a point mutant of *ASR1* that mutated two cysteine residues within the RING finger in order to eliminate RING activity. Through this analysis I was able to observe an induction of a subset of genes located near telomeres caused by loss of ubiquitylation of Rpb1 by Asr1.

Antagonistic role of Asr1 and Ubp3 in subtelomeric silencing

Because Asr1 and Ubp3 have opposing effects on the ubiquitylation of Rpb1, with Asr1 promoting its ubiquitylation and Ubp3 promoting its deubiquitylation, it would seem fit that the transcriptional effect of these proteins would be antagonistic as well. Similar to what was observed in the His-Ubiquitin assay and the telomeric reporter assay, I was able to show that Asr1 and Ubp3 play opposing roles in the regulation of native telomeric genes. This antagonistic relationship appears to only be true for specific genes, as observed in **Figure 6.10**, deletion of *UBP3* in the context of the mutation of *ASR1* does not reverse the phenotype of *ASR1* mutation alone in the genes *PRM7* and *YBR056C-B*. One explanation for this could be the regulation of silent genes by Asr1 and Ubp3 only occurs on the same panel of genes as the Sir complex, as deletion of *SIR2* only marginally affects the transcription of *PRM7* and *YBR056C-B*.

Effect of Asr1^{RINGmut} vs *asr1*Δ on subtelomeric silencing

One of the surprising results that was observed is that the Asr1^{RINGmut} strain confers a larger phenotypic effect on the induction of telomeric genes than the *asr1*Δ mutant does. Why is this the case? Steady state protein levels of Asr1^{RINGmut} remain higher than WT Asr1, with a half life of over 3 hours compared to half life of under 0.5 hours respectively (Daulny, Geng et al. 2008). This is almost certainly because of self regulation through auto-ubiquitylation. However, WT Asr1 and Asr1^{RINGmut} associate at near identical levels to telomeric genes (**Figure 6.7**), so mutation of the RING finger of Asr1 does not appear to change the amount of Asr1 directly associating with genes. One very important distinction between the Asr1^{RINGmut} and the *asr1*Δ mutant is the ability of RING mutant Asr1 to still associate with Ubp3, thereby directing the association of Ubp3 to RNAPII. Because deletion of *UBP3* in addition to mutation of the RING finger of Asr1 reverses the induction of telomeric genes seen in the Asr1^{RINGmut} strain alone, I can conclude that Ubp3 must be acting on a non-Asr1 substrate as well in its role as an anti-silencing protein.

Model of antagonistic role of Asr1 and Ubp3 in the regulation of telomeric silencing

Through the work in this thesis, I was able to construct a working model for how Asr1 and Ubp3 work antagonistically to regulate the transcription of telomeric genes (**Figure 6.12**). Prior to

initiation, RNAPII is hypophosphorylated on serine 5 of its CTD. Because Asr1 requires the hyperphosphorylation of Ser5P on the CTD of Rpb1 to associate with RNAPII, Asr1 remains unbound prior to initiation of RNAPII with the promoter (**Figure 6.12 A**). Upon initiation of RNAPII to a subtelomeric gene, RNAPII becomes hyperphosphorylated on serine 5. This modification allows the association of the Asr1 and subsequently Ubp3 (**Figure 6.12 B**). Once bound, Asr1 ubiquitylates RNAPII, which ejects the heterodimer Rpb4/7 from the RNAPII complex and blocks transcription (**Figure 6.12 C**). Ubp3 can rapidly deubiquitylate RNAPII to reform the 12 subunit complex, which can then initiate transcription at a different gene and transcribe there (**Figure 6.12 D**). This model provides the cell with a more versatile method of gene silencing that can be implemented or rapidly reversed through ubiquitylation of RNAPII, without the transcription and translation of new proteins.

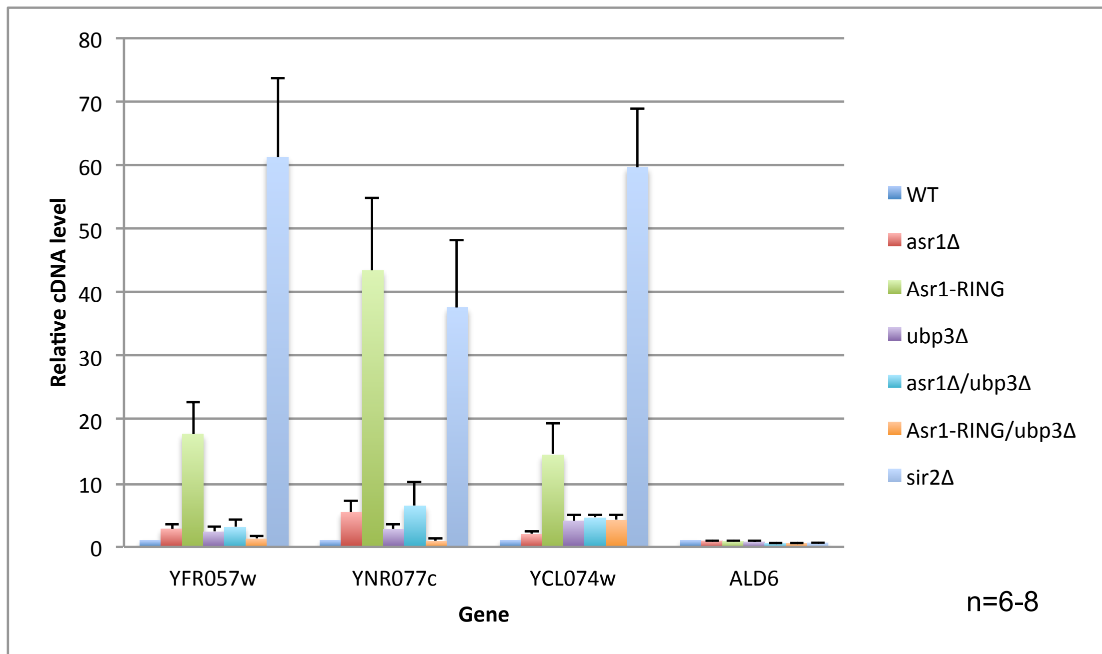


Figure 6.1 Deletion of *UBP3* reverses the impact of the Asr1^{RINGmut} on silencing of native telomere-proximal genes identified in Asr1^{RINGmut} RNA-seq. Total RNA was extracted from yeast cells of the indicated genotype and RT–QPCR used to measure transcript levels from the indicated loci. *ACT1* was used as control locus. The expression level of each gene in wild-type (*WT*) congenic cells was set to one. Error bars represent s.e.m. (*n=8*).

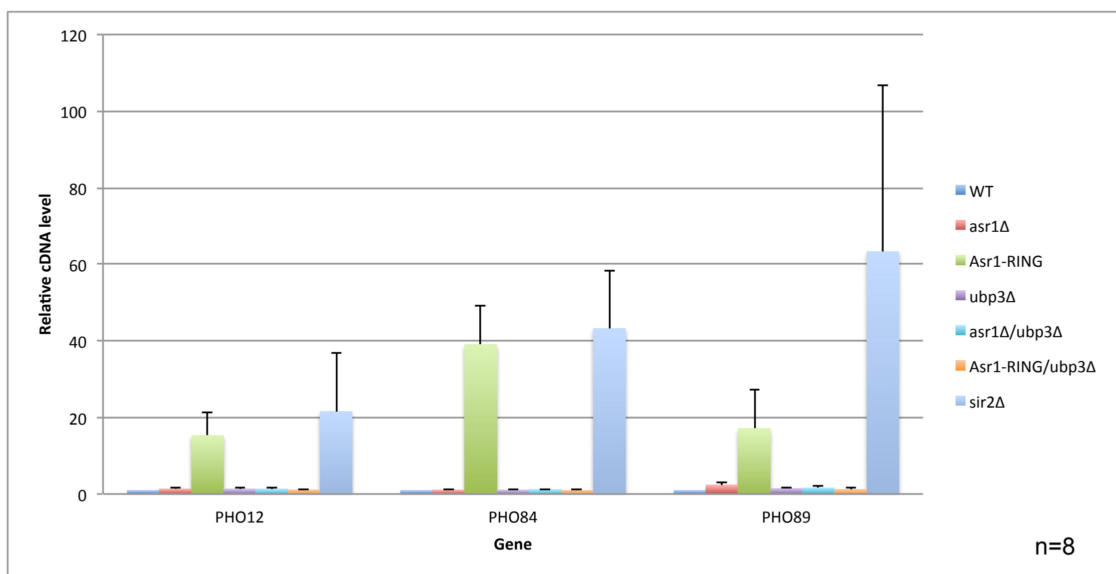


Figure 6.2 Deletion of *UBP3* reverses the impact of the Asr1^{RINGmut} on silencing of native Sir responsive telomere-proximal genes. Total RNA was extracted from yeast cells of the indicated genotype and RT–QPCR used to measure transcript levels from the indicated loci. *ACT1* was used as control locus. The expression level of each gene in wild-type (*WT*) congenic cells was set to one. Error bars represent s.e.m. (*n=6–8*).

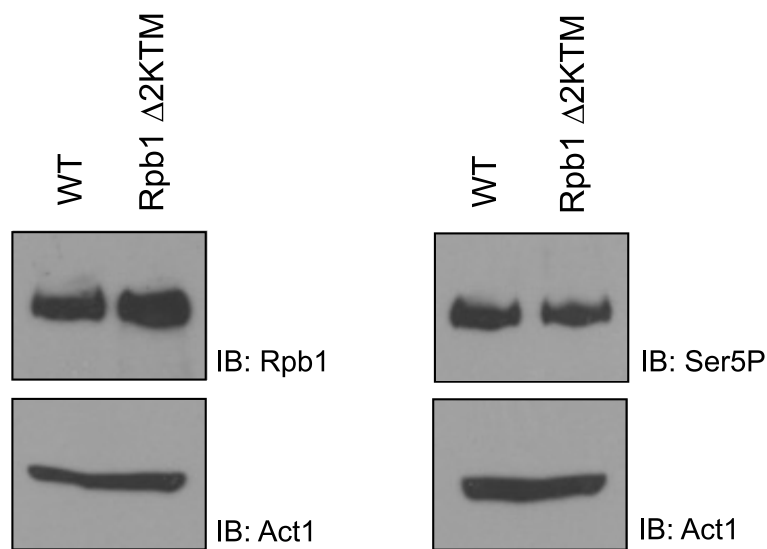


Figure 6.3 Steady state levels of WT Rpb1 and Δ2KTM Rpb1 are equal. Lysates were collected by rapid alkali treatment lysis. Immunoblot (IB) was performed using an anti-Rpb1 antibody, anti-Ser5P Rpb1 antibody, and anti-Actin antibody.

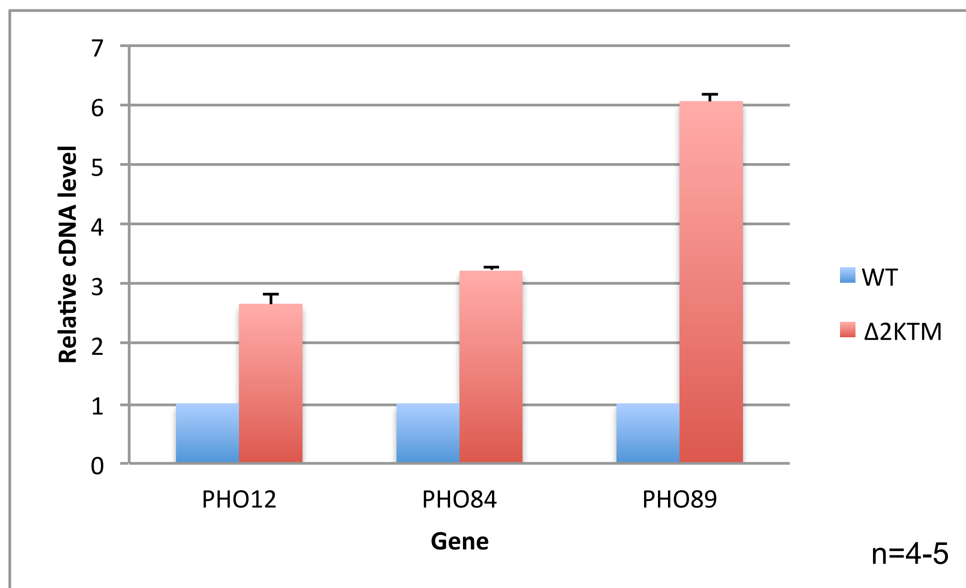


Figure 6.4 Asr1-dependent ubiquitylation sites on Rpb1 are required for silencing of native telomere-proximal genes. Total RNA was extracted from yeast cells of the indicated genotype and RT–QPCR used to measure transcript levels from the indicated loci. *ACT1* was used as control locus. The expression level of each gene in wild-type (*WT*) congenic cells was set to one. Error bars s.e.m. (*n=4–5*).

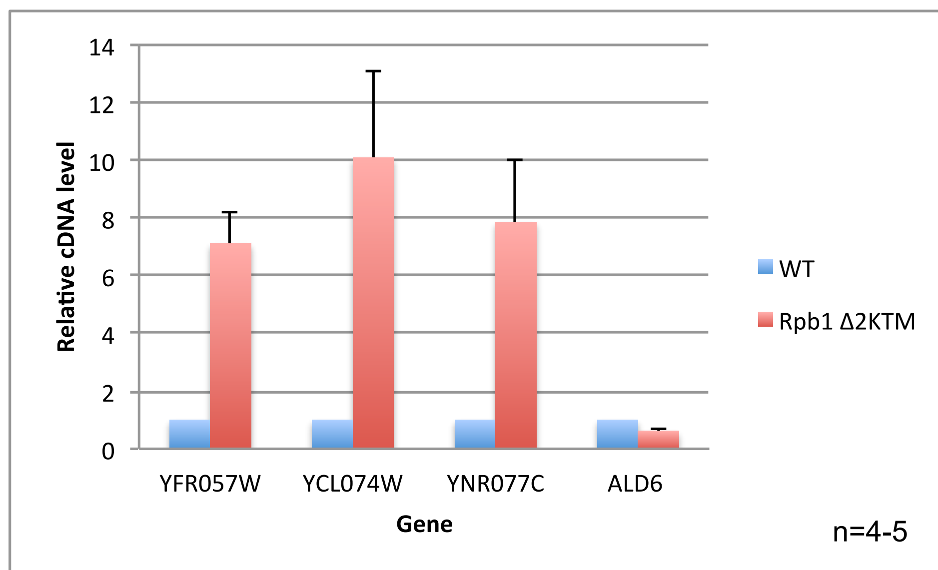


Figure 6.5 Asr1-dependent ubiquitylation sites on Rpb1 are required for silencing of native telomere-proximal genes found in RNA-seq. Total RNA was extracted from yeast cells of the indicated genotype and RT–QPCR used to measure transcript levels from the indicated loci. *ACT1* was used as control locus. The expression level of each gene in wild-type (*WT*) congenic cells was set to one. Error bars s.e.m. (*n=4–5*).

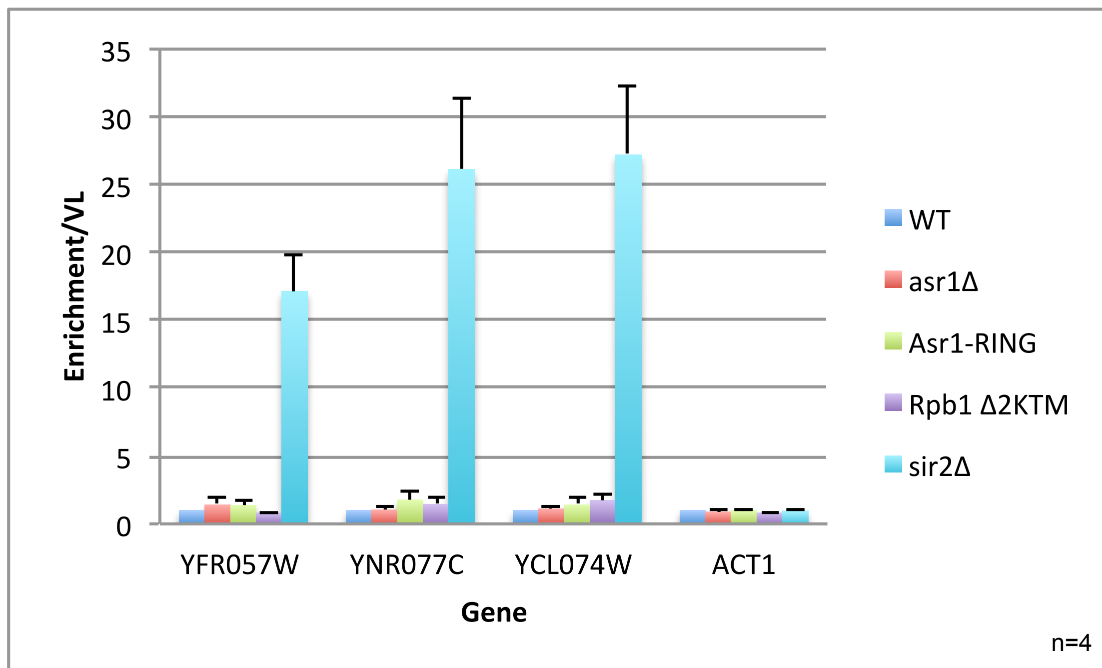


Figure 6.6 Loss of silencing in Asr1 mutant cells is not accompanied by an increase in H4K16 acetylation. ChIP was performed on chromatin isolated from the indicated strains (*WT*, BY4741; Δ*asr1*, YTM5; *asr1*^{RINGm}, YTM27; *Rpb1–Δ2KTM*, YTM29; Δ*sir2*, ΔSir2), using an anti-H4K16 acetylation (H4K16Ac)-specific antibody. Co-precipitating DNAs were quantified by QPCR using primers that recognize the 5' portion of each, and normalized to the signal from a primer set that amplifies an intergenic portion on the left arm of chromosome V (VL). Error bars s.e.m. (n=*4*).

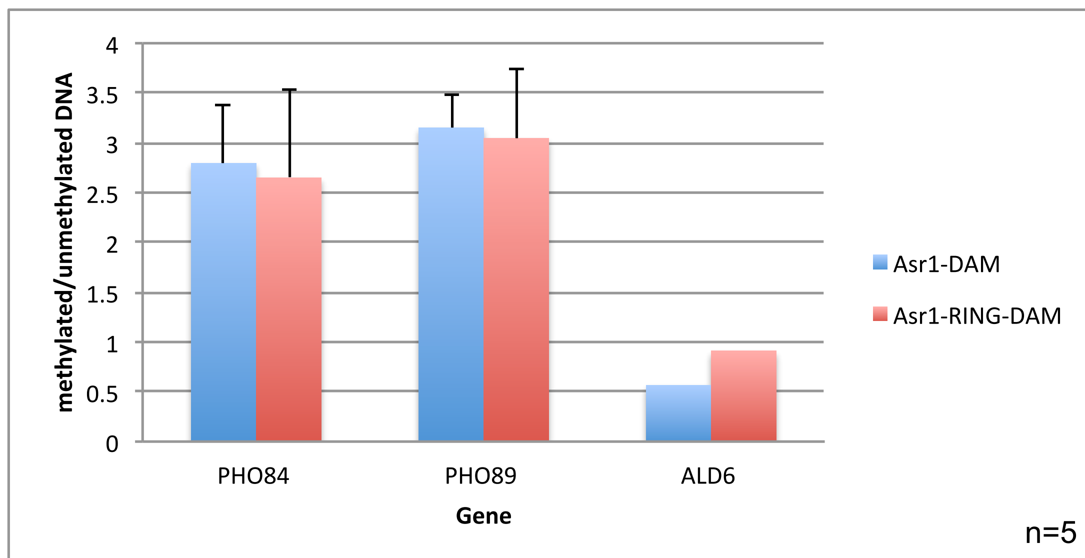


Figure 6.7 Asr1 associates with subtelomeric chromatin. DNA was isolated from congenic strains expressing either an Asr1–DAM (YTM38) or Asr1^{RINGm}–DAM (YTM39) fusion, or unfused DAM expressed from the *ASR1* promoter (YTM40). DNA was then either untreated, or cleaved with the methylation-sensitive DpnII restriction enzyme. QPCR was performed using primers flanking a DpnII site within each gene to determine the extent of cutting by DpnII. Signals for each fusion strain were normalized to that from the strain expressing unfused DAM protein, and data plotted as the ratio of methylated/non-methylated DNA. Error bars s.e.m. (*n=5*).

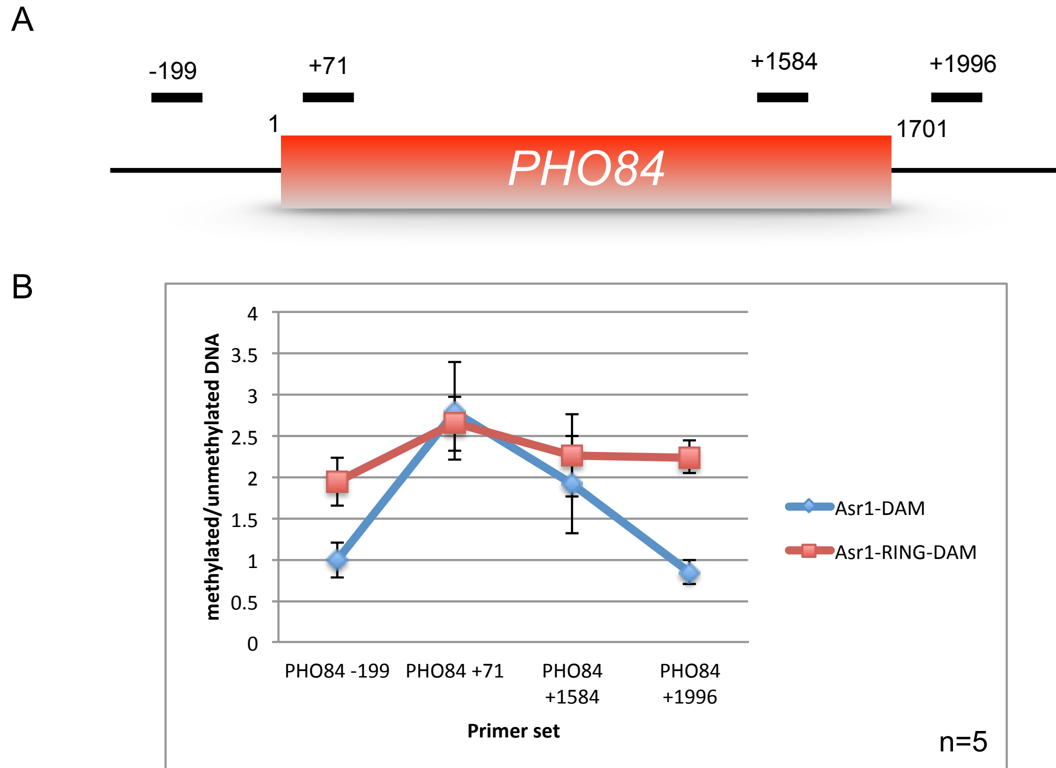


Figure 6.8 Asr1 associates with *PHO84* gene body. (*A*) Schematic of the primer sets used for DAM-ID with the gene *PHO84*. (*B*) DNA was isolated from congenic strains expressing either an Asr1–DAM (YTM38) or Asr1^{RINGm}–DAM (YTM39) fusion, or unfused DAM expressed from the *ASR1* promoter (YTM40). DNA was then either untreated, or cleaved with the methylation-sensitive DpnII restriction enzyme. QPCR was performed using primers flanking a DpnII site within each gene to determine the extent of cutting by DpnII. Signals for each fusion strain were normalized to that from the strain expressing unfused DAM protein, and data plotted as the ratio of methylated/non-methylated DNA. Error bars s.e.m. (*n=5*).

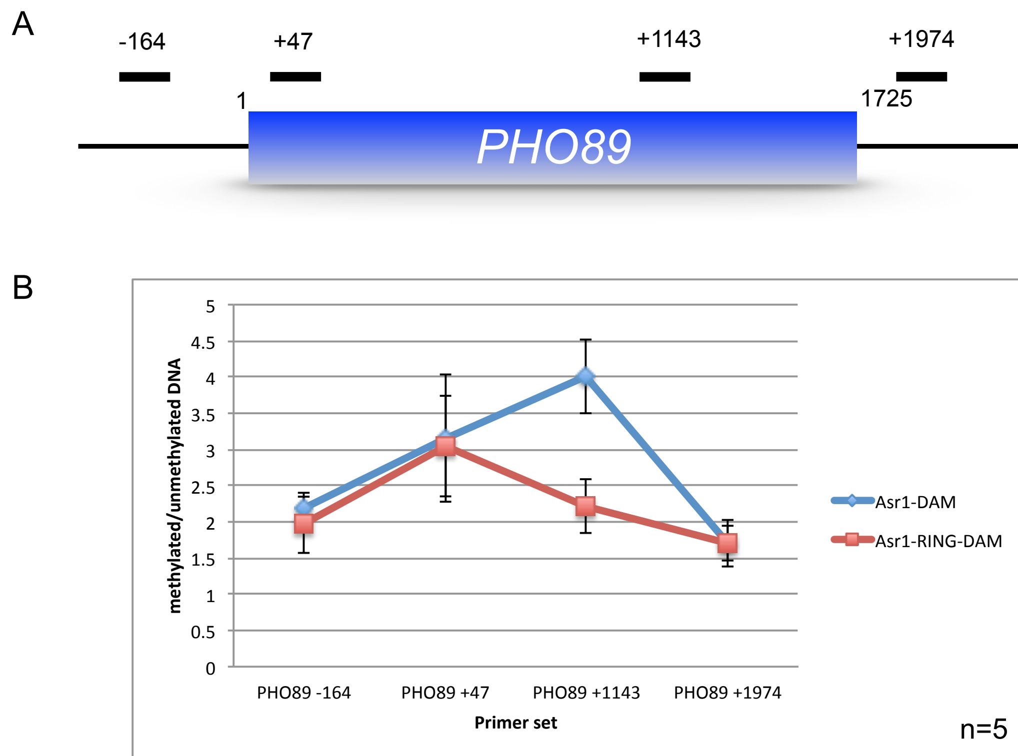


Figure 6.9 Asr1 associates with *PHO89* gene body. (*A*) Schematic of the primer sets used for DAM-ID with the gene *PHO89*. (*B*) DNA was isolated from congenic strains expressing either an Asr1–DAM (YTM38) or Asr1^{RINGm}–DAM (YTM39) fusion, or unfused DAM expressed from the *ASR1* promoter (YTM40). DNA was then either untreated, or cleaved with the methylation-sensitive DpnII restriction enzyme. QPCR was performed using primers flanking a DpnII site within each gene to determine the extent of cutting by DpnII. Signals for each fusion strain were normalized to that from the strain expressing unfused DAM protein, and data plotted as the ratio of methylated/non-methylated DNA. Error bars s.e.m. (*n=5*).

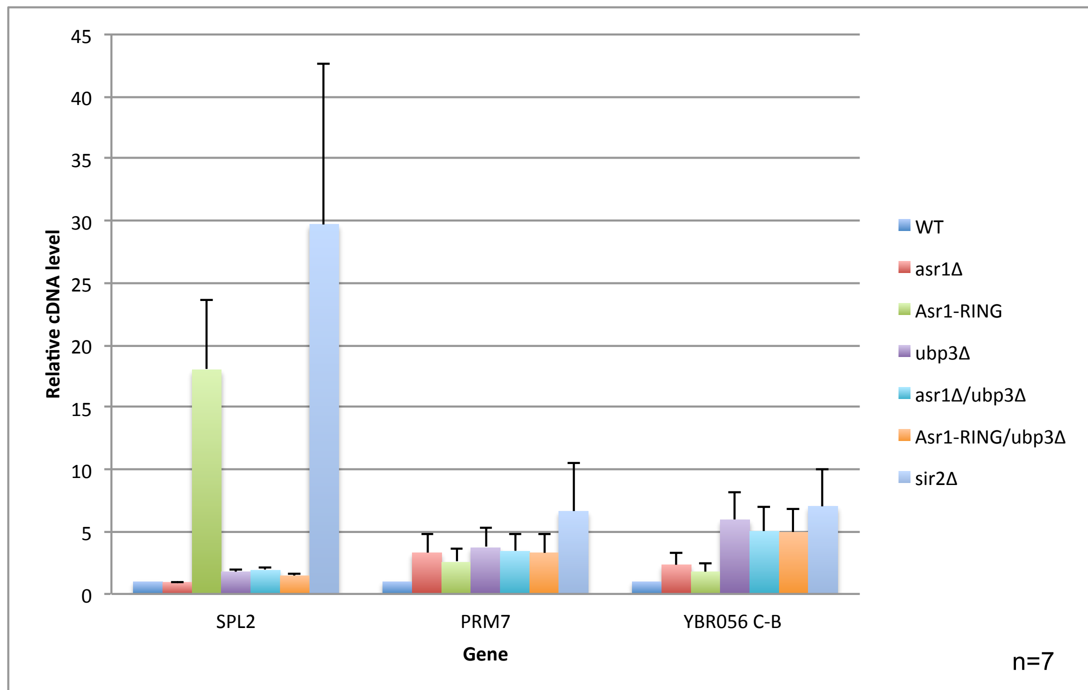


Figure 6.10 Asr1 and Ubp3 control expression of a euchromatic gene also regulated by Sir2. Total RNA was extracted from yeast cells of the indicated genotype (*WT*, BY4741; Δ*asr1*, YTM5; *asr1*^{RINGm}, YTM27; Δ*ubp3*, ΔUbp3; Δ*asr1*Δ*ubp3*, YTM6; *asr1*^{RINGm}Δ*ubp3,* YTM28; Δ*sir2*; ΔSir2) and RT–QPCR used to measure transcript levels from the indicated loci. *ACT1* was used as control locus. The expression level of each gene in wild-type (*WT*) congenic cells was set to one. Error bars represent s.e.m. (*n=7*).

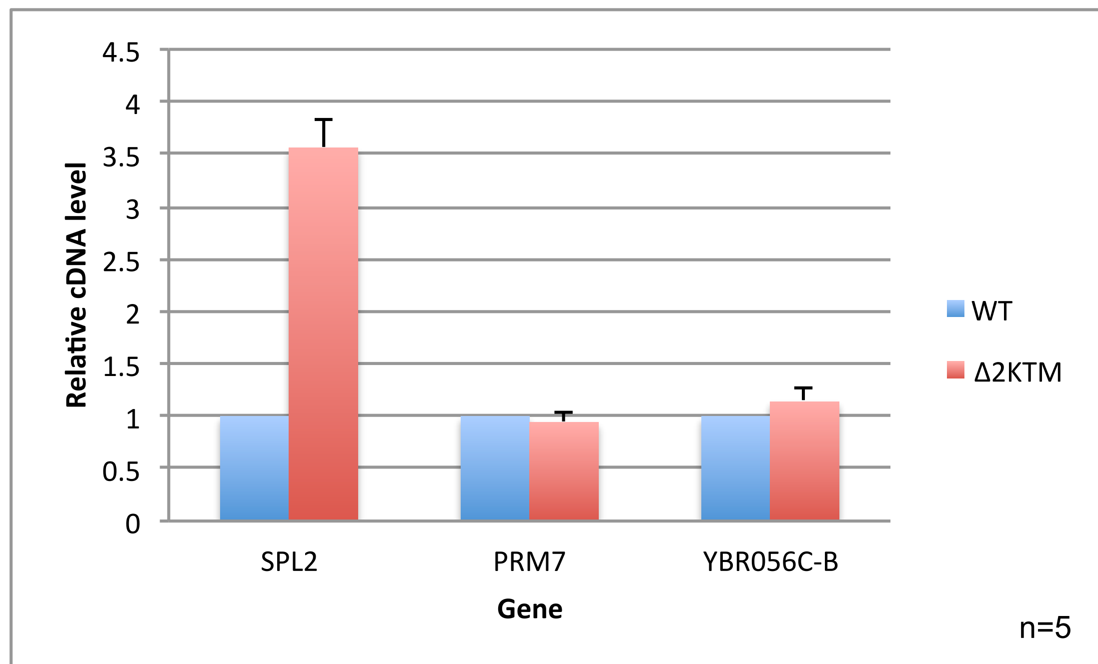


Figure 6.11 Asr1-dependent ubiquitylation sites on Rpb1 are required for control of expression of a euchromatic gene also regulated by Sir2. Total RNA was extracted from yeast cells of the indicated genotype and RT–QPCR used to measure transcript levels from the indicated loci. *ACT1* was used as control locus. The expression level of each gene in wild-type (*WT*) congenic cells was set to one. Error bars s.e.m. (*n=5*).

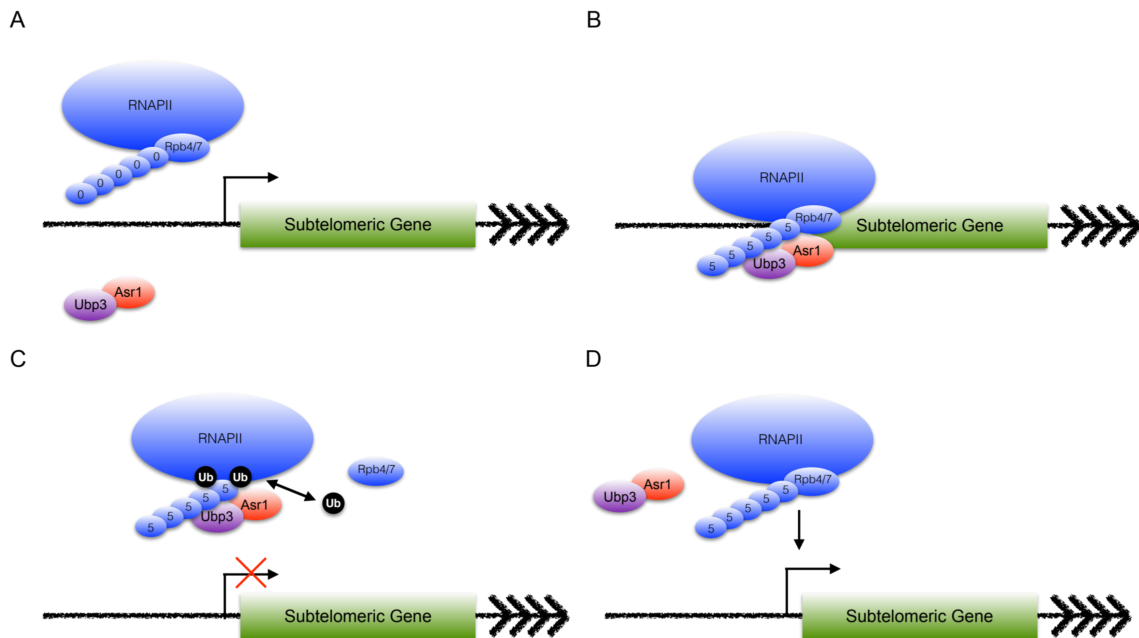


Figure 6.12 Model of the antagonistic roles of Asr1 and Ubp3. (*A*) Prior to transcriptional initiation, RNAPII is hypophosphorylated on it's CTD tail. (*B*) Once initiated, RNAPII becomes hyperphosphorylated on serine 5 of the CTD repeats, which attracts the association of Asr1/ Ubp3. (*C*) Upon binding, Asr1/Ubp3 can control the ubiquitylation status of RNAPII. If ubiquitylated, Rpb4/7 become dissociated from the holoenzyme, and the transcriptional activity of RNAPII is impeded. (*D*) Because the ubiquitylation is reversible, Ubp3 can deubiquitylate RNAPII, which can reassociate with Rpb4/7 and reengage with chromatin to initiate transcription.

CHAPTER 7

CONCLUSIONS AND PERSPECTIVES

7.1 Conclusions and perspectives

Despite the defined molecular effect of Asr1 on RNAPII, and despite its conservation throughout eukaryotic life, deletion of the *ASR1* gene results in no overt phenotype in budding yeast. Therefore, I aimed to uncover the biological function of Asr1 through a multi-pronged approach through the identification of physical interactors of Asr1 and the global transcriptomic effects of Asr1. Through my studies, I was able to determine that Asr1 associates with the deubiquitylase Ubp3 in order to regulate telomeric transcription through the ubiquitylation of RNAPII.

Association of DUBs and E3s

It may seem illogical that two enzymes with opposing functions would associate with each other, but there are many examples in the literature of ubiquitin ligases and DUBs interacting with each other. One such example is the association of the E3 BRCA1/BARD1 with the DUB BAP1 in human cells (Jensen, Proctor et al. 1998). BRCA1 and BARD1 are both RING finger containing proteins that heterodimerize to form a tumor suppressor (Shakya, Szabolcs et al. 2008). The BRCA1/BARD1 E3 activity is further activated by autoubiquitylation of polyubiquitin chains (Mallery, Vandenberg et al. 2002), however the E3 activity of BRCA1/BARD1 is inhibited by BAP1 (Nishikawa, Wu et al. 2009). The exact method of this inhibition is unclear, however it appears to be a combination of the removal of the activating polyubiquitin chains by BAP1 and an interference with the association of BRCA1/BARD1 by the binding of BAP1 to each subunit of the heterodimer (Nishikawa, Wu et al. 2009).

Another example of an E3 and DUB associating *in vivo* is the interaction between the DUB HAUSP and the E3 Mdm2 in humans. Mdm2 plays an important role in the maintenance of cellular homeostasis, as it targets the tumor suppressor p53, ubiquitylating it to keep levels low under normal cellular conditions (Marine, Francoz et al. 2006). HAUSP has been shown to bind and deubiquitylate both p53 directly (Li, Chen et al. 2002) as well as Mdm2 (Cummins, Rago et al. 2004). Furthermore, Mdm2 has been shown to direct the binding of HAUSP to p53 by forming

a trimeric complex (Brooks, Li et al. 2007). This creates a unique situation in which HAUSP is involved in the direct stabilization and indirect destabilization of p53 through the association with Mdm2.

The human homolog of Ubp3, USP10, has also been shown to associate with an E3, Huwe1 (Li, Martinez et al. 2015). In this study, it was shown that Huwe1 and USP10 work antagonistically to control the steady state levels of TATA-binding protein (TBP) in a cell type specific manner to control gene expression as these cells differentiate. This is an interesting example of how an E3 and DUB work antagonistically to control the transcription of a large panel of genes within the cell.

Lastly, there is the association of the DUB USP28 and the F-box protein Fbw7α, which is part of the SCF ubiquitin ligase that targets the oncogene Myc. Fbw7α and Fbw7γ are both members of different multisubunit E3 complexes that can control the protein stability of Myc, however Fbw7α is localized to the nucleus whereas Fbw7γ is located to the nucleolus. In this case, USP28 is able to bind Fbw7α, but does not bind Fbw7γ. Through its association with Fbw7α (Popov, Wanzel et al. 2007), USP28 can form a ternary complex with Myc, reversing the ubiquitylation by Fbw7α, thereby stabilizing the protein specifically in the nucleus, but not the nucleolus. So in this example, an E3 is able to direct a DUB to a specific substrate in order to specifically control the ubiquitylation state in a certain compartment of the cell.

These are four unique examples of how E3s and DUBs associate in nature, which could provide an indication as to the purpose of the association of the E3/DUB pair Ubp3 and Asr1. It seems unlikely that Ubp3 associates with Asr1 for the purpose of stabilization as is the case with BAP1 and BRCA1/BARD1 and HAUSP and Mdm2 because the half life of Asr1 in WT cells is already less that 30 minutes (Daulny, Geng et al. 2008) and deletion of *UBP3* does not appear to affect steady state levels of Asr1 (**Figure 4.1 B**, *lane 1*). After completion of these studies, I prefer a model similar to how Fbw7α directs USP28 to Myc to regulate stability or how Huwe1 and USP10 antagonistically control the stability of TBP. In the case of Asr1 and Ubp3, I have established that Asr1 directs Ubp3 to it known substrate, Rpb1. Similar to how the E3 Huwe1 and and the DUB USP10 control ubiquitylation of TBP, my the qRT-PCR and ubiquitylation data suggest that when

both Asr1 and Ubp3 are bound to RNAPII, they control the ubiquitylation status of Rpb1 to regulate the transcriptional activity of RNAPII. Upon monoubiquitylation of Rpb1 by Asr1, Rpb4 and Rpb7 dissociate from the rest of the RNAPII complex and the transcriptional activity is perturbed, which can be reversed through the deubiquitylation by Ubp3.

How does Asr1 specifically recognize subtelomeric genes?

As described in Chapter 6, Asr1 is able to physically associate with at least two telomeric genes independent of its RING activity. It is known that Asr1 binds to RNAPII through its C-terminal binding domain (CBD), however it is still an open question as to how Asr1 is able to specifically recognize and affect the activity subtelomeric genes. It is tempting to suggest that Asr1 recognizes a specific chromatin modification through its PHD finger, as PHD fingers have been identified in many different proteins for this explicit purpose (Taverna, Li et al. 2007). There are a few observations that suggest that this is not the case. First, the PHD finger of Asr1 (and many RPC proteins) is missing a tryptophan or similar aromatic residue just before the third grouping of histidine and cysteine zinc chelating residues (**Figure 1.9**). In other PHD finger proteins, this tryptophan forms an “aromatic cage” that is essential in the binding of H3 methylated tails (Sanchez and Zhou 2011), so it seems unlikely that Asr1 would bind methylated H3 through its PHD finger. However, a recent study showed the ability of the Asr1 human homolog PHRF1 to bind H3K36me3 via its PHD finger (Chang, Chu et al. 2015), so more investigation may be needed. Another observation, probably more convincing, is that the PHD domain of Asr1 is dispensable for the regulation of telomeric silencing as measured by the red/white colony assay (**Figure 5.10**). If the PHD finger were responsible for the association of Asr1 with subtelomeric regions, it would be logical for this domain to be essential for the regulation of telomere proximal genes.

If the PHD finger of Asr1 is not responsible for its specificity to subtelomeric genes, then what is? One possibility is that Asr1 is recruited to subtelomeric genes through an association with Sir complex proteins. Ubp3 is a known Sir4 interacting protein, so it is possible that Ubp3 directs Asr1 to subtelomeric genes through association with the Sir complex (Moazed and Johnson 1996). Sir3 and Sir4, as well as the Sir4 interacting protein Sif2 were identified as possible Asr1

interacting proteins in the TAP/Mass spectrometry experiment, however they were not included in **Table 3.1** because they fell below the specified spectral count threshold that was established. More evidence for the hypothesis that the Sir complex directs Asr1 and Ubp3 to subtelomeric genes comes from the gene expression data in Chapter 6. The Sir complex is not known to be a transcriptional regulator for euchromatic genes, however, when *SIR2* is deleted from cells, expression of the euchromatic gene *SPL2* is induced, but not the euchromatic genes *PRM7* or *YBR056C-B* (**Figure 6.10**). Similarly, mutation of the ability of Asr1 to ubiquitylate Rpb1 (either through the RING finger mutation or the ∆2KTM Rpb1 mutant) results in a similar induction of *SPL2* expression (**Figure 6.10-11**), suggesting that Asr1 and the Sir complex affect a similar panel of genes, whether they are located in euchromatic or heterochromatic regions. If this is the case, it is possible that the Asr1/Ubp3 complex associate with genes through the association of the Sir complex. It is also possible that Asr1 coordinates its association with RNAPII initiated with subtelomeric genes entirely via its CBD through a unique combination of modifications to the CTD of Rpb1. This is an area of study for Asr1 that requires more investigation.

Regulation of transcription through dissociation of Rpb4/7 from RNAPII

In this study, I show evidence that Asr1 directly targets RNAPII for ubiquitylation in a manner that is dependent upon Ser5P, the end result being the dissociation of the Rpb4/7 heterodimer from the rest of the RNAPII complex, preventing transcription of telomeric genes. This model does not initially appear to be completely consistent with everything that has been published about Rpb4/7 in budding yeast. Rpb4/7 have been shown to be required for the initiation of transcription *in vitro*, a step antecedent to phosphorylation of Ser5 of the CTD of Rpb1 (Edwards, Kane et al. 1991). However, association of Rpb4/7 with the rest of the RNAPII complex has been shown to be dispensable for the elongation of transcription in budding yeast, and it has been suggested that as little as 20% of RNAPII complex are Rpb4/7 bound during stationary phase in budding yeast (Choder 2004). Therefore, one could argue that the only step of transcription that requires Rpb4/7 occurs temporally prior to the association of Asr1 with RNAPII (through Ser5P). There is however considerable evidence in favor of the model presented in Chapter 6 for the regulation of telomeric transcription through ubiquitylation by Asr1. First, the Rpb4/7 heterodimer has been shown to be required for transcription under stressful conditions, and the majority of telomeric genes have

been shown to be stress related (Burhans, Ramachandran et al. 2006). I would also argue that polymerase that lacks the Rpb4/7 heterodimer complex or its homologs has been shown to lack optimal transcriptional processivity, suggesting that although Rpb4/7 may not be essential for transcription, they are essential for an optimally transcribing RNAPII complex (Runner, Podolny et al. 2008, Hirtreiter, Grohmann et al. 2010). It is also possible that the dissociation of Rpb4/7 from the rest of RNAPII is not singularly responsible for the loss of transcriptional activity seen in Asr1 associated RNAPII alone, but could be a combination of a high degree of ubiquitylation of Rpb1 and ejection of Rpb4/7 from the RNAPII complex.

Relationship of Asr1/Ubp3 with the Sir complex

The method by which the Sir complex regulates subtelomeric transcription has been researched extensively throughout the years, however the exact role of Ubp3 in the regulation of telomere proximal genes has remained unknown. The Sir complex forms areas of heterochromatin based on location, in a non-gene specific manner (Sherman and Pillus 1997) and heterochromatin formed by the Sir complex is heritable and results in an on/off mode of silencing of genes that is not easily reversible (Kitada, Kuryan et al. 2012, Dodson and Rine 2015). The regulation of telomeric silencing by Asr1/Ubp3 does not appear to permanently alter the chromatin architecture around these genes, as the levels of H4K16ac of *ASR1* mutants remain unchanged from WT, but rather they regulate individual polymerase complexes, thereby representing a more subtle method of regulation. This subtle regulation could also explain why the levels of gene induction caused by the disruption of Asr1 ubiquitylation of Rpb1 are not generally as strong as that seen by disruption of the Sir complex alone.

What does the function of Asr1 suggest about the function of other RPC family proteins?

Very little has been published about the RPC family of proteins, particularly in reference to their association with RNAPII. The function of Asr1 in budding yeast could give insight into the function of RPC family proteins as a whole. As of yet, current literature lacks any publications about the fission yeast *Schizosaccharomyces pombe* RPC member Pbr1 (Polymerase Binding RING/PHD finger). *S. pombe* is an interesting model organism because it contains some cellular features not present in *S. cerevisiae* that make it more relatable to higher eukaryotes, such as the presence of

RNAi machinery and a wider array of non-coding RNAs (Russell and Nurse 1986, Yanagida 2002, Martienssen, Zaratiegui et al. 2005). There are many similarities between budding yeast and fission yeast, such as the presence of silenced chromatin at telomeres and the fact that the Rpb4/7 heterodimer is dissociable (Choder 2004), suggests that Pbr1 might have a similar function in fission yeast. Preliminary data that I have collected suggests that Pbr1 associates with Ser5P Rpb1, indicating it could have a regulatory affect on transcription similar to Asr1. Additionally, deletion of *PBR1* causes an induction of a subset of ncRNA, but it is unclear as to whether the RING activity of Pbr1 is required for this regulation. There is a direct homolog for the *S. cerevisiae* DUB Ubp3 and its cofactor Bre5 in *S. pombe*, called Ubp3 and Nxt3 respectively. In future studies, it would be interesting to see if Pbr1 associates with these proteins as well. Telomeric silencing is not as well elucidated in *S. pombe*, but given my current studies, I would predict that Pbr1 is involved in this process.

The human RPC family member PHRF1 has recently been suggested to function as a DNA repair protein, promoting non-homologous end joining (NHEJ) in the case of double strand DNA breaks (Chang, Chu et al. 2015). In this paper, the researchers show that full length PHRF1 binds both Ku70 and Ku80, which in the case of NHEJ, forms a heterodimer to bind DNA ends and recruit other important proteins for DNA repair (Lieber 2010). In budding yeast, the homologs Yku70 and Yku80 form a heterodimer that not only binds double strand DNA breaks, but binds telomere ends, and has been predicted to loop back into subtelomeric regions to aid in the establishment of heterochromatic DNA (Kueng, Oppikofer et al. 2013). Additionally, recruitment of DNA repair proteins has been implicated in the formation of heterochromatin in budding yeast (Kirkland, Peterson et al. 2015). If Ku70 and Ku80 function similarly in humans, these results suggest that PHRF1 may associate with the Ku70/80 heterodimer in the context of silencing of telomeres in human cells, however further investigation will be needed.

7.2 Future Studies

Association of Asr1 with telomeres

In these studies, I have established that Asr1 can directly associate with at least two subtelomeric genes, however it remains unclear how Asr1 is able to specifically target these regions. One

interesting hypothesis to test would be whether Asr1 recognizes a unique combination of modified residues of the CTD code. From experiments done in the Tansey laboratory, it has been established that Asr1 requires phosphorylation of serine 5, whereas phosphorylation of serine 2 of the CTD of Rbp1 is dispensable for Asr1 association (Daulny, Geng et al. 2008). The first experiment I would perform would be to assess whether Asr1 requires any of the other residues of the C-terminal repeats in order to associate with RNAPII. This could be accomplished through a similar assay as was performed in Daulny *et al.* 2008 in Figure 2C, in which a Far Western was performed, using Asr1 as a probe, and each residue of the CTD was sequentially mutated to assay whether or not theses residues are essential for Asr1 association. Another option to determine whether Asr1 recognizes a specific modification of the CTD would be to perform a modified peptide array containing different modified CTD synthetic peptides and use WT Asr1 as a probe, similar to those done with modified histone peptides (Nady, Min et al. 2008). If Asr1 does specifically recognize a unique combination of modified CTD, I could then test whether this modification is required for the recruitment of Asr1 to subtelomeric genes.

An additional possibility is that Asr1 and Ubp3 are recruited to subtelomeric regions by a protein that specifically localizes to these regions. One hypothesis is that the Sir complex recruits Asr1 and Ubp3 to subtelomeric genes, which is supported by the fact that Ubp3 has been shown to associate with Sir4 (Moazed and Johnson 1996). To test this, I would first delete *SIR4* and assess the association of Ubp3 and Asr1 to telomeric regions using DAM-ID profiling. If Asr1 and/or Ubp3 association with telomeric genes is negatively affected by deletion of the *SIR4*, this would suggest that the Sir complex plays a role in the recruitment of Asr1 and Ubp3 to subtelomeric genes.

It would also be interesting to know what genes Asr1 associates with globally. The standard method to do this would be through a ChIP-seq experiment. These experiments require large amounts of material to complete, are difficult to perform with proteins that are expressed at low levels, and have been proven to be problematic for proteins that make transient interactions with chromatin (Aughey and Southall 2015). ChIP-seq would most likely be difficult for Asr1 for these reasons, especially considering its low level of association to gene loci it is known to interact with as measured by ChIP/qPCR (Daulny, Geng et al. 2008). Another option would be to use genome

wide DAM-ID, an assay that has been verified in drosophila, but could be adapted for use in *S. cerevisiae* (Greil, Moorman et al. 2006). I have already used DAM-ID profiling to assess the association of Asr1 to individual genes, therefore I am confident it would be a useful assay to analyze the genome wide chromatin association of Asr1.

RPC proteins in transcriptional silencing

I have established the role of Asr1 in the regulation of subtelomeric gene silencing, however, it is still unclear as to whether other RPC family proteins play a role in this process in their respective organisms. The study of heterochromatin has been established in the model organism *Schizosaccharomyces pombe*, so investigating this process would be a logical next step to determine if the function of Asr1 is conserved in other species. Like *S. cerevisiae*, there are *S. pombe* strains that have integrated telomeric reporter genes (Tong, Keller et al. 2012). Therefore, I would first delete *PBR1* (the fission yeast RPC family member) from the telomeric reporter strain to see if Pbr1 affects the transcription of an ectopic telomeric reporter gene. At the same time, I would construct a *PBR1* deletion and a *PBR1* RING mutant strain and use qRT-PCR to measure how these mutations affect the steady state levels of known silenced subtelomeric genes. There is no published evidence that Ubp3 acts as an anti-silencing factor in *S. pombe*, however it would be interesting to assess whether the function of this protein is conserved across yeast species as well.

As mentioned earlier, the human RPC family member, PHRF1, has not been extensively studied until recently, and these analyses have not explored the role of PHRF1 in RNAPII regulation. PHRF1 is predicted to associate with Rpb1, however the first step in its study would be to confirm the association of PHRF1 with Ser5P Rpb1, as this has not yet been published. Like in yeast, there are human telomere reporter cell lines as well (Baur, Zou et al. 2001). In this cell line, stable cells of PHRF1 mutants (RING mutant and CBD mutant) could be made to assess whether PHRF1 is involved in the regulation of telomeric transcription in human cells. One major difference between human and yeast transcriptional machinery of note is that the human homolog of Rpb4/7 is essential for transcription, and does not form a dissociable heterodimer as is seen in budding and fission yeast (Choder 2004). PHRF1 could still potentially regulate RNAPII

transcription through direct ubiquitylation without the dissociation of Rpb4/7. One possibility is that PHRF1 ubiquitylates initiated RNAPII, and this ubiquitylation prevents the association of transcription elongation factors to RNAPII, thereby blocking the progression of transcription. Alternatively, PHRF1 could oligoubiquitylate RNAPII in a manner that ubiquitin physically halts the progression of transcription through the gene. Since PHRF1 has been identified as a tumor suppressor, I would anticipate it to be a high interest protein to investigate in the future (Ettahar, Ferrigno et al. 2013, Prunier, Zhang et al. 2015).

7.3 Summary of thesis

In summary, through my work, I have uncovered a new method of gene silencing in the budding yeast *S. cerevisiae*. Through non-proteolytic ubiquitylation, Asr1 is able to modify at least two subunits of RNAPII, which disengages the enzyme from chromatin, preventing transcription at subtelomeric regions. The DUB Ubp3 is able to bind with Asr1, and through Asr1 it can associate with RNAPII, and reverse the gene silencing action caused by Asr1. This silencing occurs independently of deacetylation by the silent information regulator (Sir) complex, as mutants that interfere with the ability of Asr1 to ubiquitylate Rpb1 cause an induction of telomeric transcripts, but do not affect the acetylation state of H4K16 residues in the area. The evidence presented in this dissertation reveal the importance of non-proteolytic ubiquitylation in the control of transcription, but also sheds light on a poorly understood family of eukaryotic ubiquitin-ligases.

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