Activator Turnover and Proteolysis In Transcriptional Activation

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Abstract

Regulated gene expression is crucial for maintenance of cellular homeostasis and adaptation to new environments. Therefore, both transcription (the first process in gene expression) and proteolysis (the termination of a protein's existence) are tightly regulated. There is growing evidence that the proteasome, which hydrolyses many proteins in several cellular pathways, regulates transcription. Much of the initial indication that proteasomes are important regulators of transcription was derived from observations that many transcription activators are unstable even during conditions of robust transcription. However, recent attention to the proteasomal regulation of transcription has focused on non-proteolytic functions of the proteasome whereas the importance of proteolysis itself is often dismissed. In my thesis I tested if it was even possible for a proteolytic role to exist in the activation of transcription. If the proteasome is necessary to directly activate transcription several predictions arise. Two of these predictions are (1) transcription activators should dynamically disassociate with their target promoters during transcription, and (2) inhibiting the proteasome inhibits transcription.

To study the dynamics of activators, I focused on a known ubiquitylated activator, Gal4, in which a role for the proteasome but not proteolysis has been proposed. If Gal4 were to stably associate with its target promoter during transcription, then a role for Gal4 proteolysis as part of transcriptional activation could be clearly ruled out. I have improved upon an *in vitro* competition assay to study the association of Gal4 with its target promoter. This technique clearly demonstrates that Gal4 maintains a dynamic association with its targets even under conditions of activation, which is consistent with a potential important role of proteolytic regulation of the activator during transcription.

Similarly, I have also developed a new strain of *Saccharomyces cerevisiae* that has greater sensitivity to proteasome inhibitors such as MG132. This new strain is the product of genetically inactivating two of the three proteolytic subunits of the proteasome. The remaining proteolytic center

is preferentially targeted by proteasome inhibitors resulting in rapid and acute inhibition of yeast proteasomes. This yeast strain will be a valuable tool for many researchers studying ubiquitin and proteolysis. Using this yeast strain to study the induction of gene expression I show that transcriptional induction of certain genes is indeed sensitive to proteasome inhibition, including a gene where there was prior ambiguity regarding the effects of proteasome inhibition. Furthermore, this tool is able to reveal a greater number of genes with significant changes in gene expression under genome-wide transcriptional profiling.

Together my data reveal that previous claims that proteolytic function of the proteasome is dispensable for transcriptional activation were incorrect as my data demonstrate an important role for proteasome-mediated proteolysis in transcription.

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List of Abbreviations

3-AT	3-aminotriazole
4-OHT	4-hydroxytamoxifen
APIS	19S ATPase proteins independent of 20S
cGal4	Competitor Gal4
ChIP	Chromatin immunoprecipitation
CSM	Complete synthetic media
DBD	DNA binding domain
DMSO	Dimethyl sulfoxide
DOC	Deoxycholate
EBD	Estrogen binding domain
EDTA	Ethylenediaminetetra acetic acid
EMSA	Electrophoretic mobility shift assay
Gal4**c**	Phosphorylated Gal4 isoform associated with transcription
gal4D	Mutation in Gal4 that removes the C-terminal 28 amino acids.
gcn4-3T2S	Mutant Gcn4 with thee threonine and two serine changed to alanine
GTF	General transcription factor
HAT	Histone acetyltransferase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hERα	Human estrogen receptor
HRP	Horseradish peroxidase
ORC	Origin recognition complex
ORF	Open reading frame
PEG	Polyethylene glycol
qPCR	Quantitative PCR

RNApolII	RNA polymerase II
SDS	Sodium dodecyl sulfate
TAD	Transcription activation domain
TBP	TATA binding protein
TES	Tris – EDTA – Sodium chloride solution
UAS	Upstream activating sequence
UPS	Ubiquitin proteasome system
YPAD	Yeast peptone adenine dextrose rich media

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Without Bill Tansey, my work would be unfunded, unpublished, poorly planned, and disastrously executed. His help provided the necessary direction to my work, and he has proved to be a mentor in the fullest sense of the term. Thank you, Bill.

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Chapter One: Introduction

Transcription activation and proteolytic destruction are intertwined to properly regulated gene expression in surprising ways. Transcription activators, proteins that bind specific DNA sequences and are necessary to elevate the rate of transcription of specific genes above the basal level (activate transcription), posses a very peculiar quality —the ability of an activator to induce transcription is inversely correlated with its stability (Molinari et al. 1999; Salghetti et al. 2000; Thomas and Tyers 2000; Salghetti et al. 2001). As activators become more potent they are destroyed more rapidly, and the evidence suggests that the activators are destroyed through a specific and highly regulated pathway —the ubiquitin proteasome system (UPS). One intriguing model describing the connection between the UPS and transcription activators is that of transcription activator licensing. In this model transcription activators are ubiquitylated and ultimately destroyed as part of the transcription processes, thereby linking activation and destruction (Lipford and Deshaies 2003; Muratani and Tansey 2003).

The antimony of pairing activation and destruction along with the lack of a mechanism make this model controversial. More significantly for this thesis, the licensing model provides several predictions —two of which are rather simple yet have complicated and contradicting data. Therefore, I aimed to test these predictions in ways that would resolve these controversies and provide improved tools to study the mechanisms of activator licensing.

Chapter two addresses one prediction of the licensing model: if an activator is ubiquitylated and destroyed during the process of transcription then it cannot possibly associate stably with the DNA that it binds. Proteolysis of activators is not the only cause for activator turnover, but if proteolysis of activators does occur then activator turnover is a necessary corollary. Conversely the absence of activator turnover is a clear argument against the licensing model. Although there is a growing body of literature that suggests that transcription factors a highly dynamic (Hager et al. 2009), one study in particular presents a new method to measure activator association with DNA *in*

vivo and concludes from this approach that the model *Saccharomyces cerevisiae* activator Gal4 does indeed lock-onto DNA in an transcription specific context (Nalley et al. 2006). Such clear results are precisely what are needed to test this licensing model. However, several problems lurk beneath the finding of Gal4 stability. Therefore, the turnover of Gal4 during transcription required further examination, which is carried out in the second chapter of this thesis.

A second controversial prediction arising from the activator-licensing model is that inhibition of proteasome-mediated proteolysis should inhibit transcriptional activation. The biggest difficulty for testing the effects of proteasome inhibitors on transcription is that inhibition of the proteasome in *S. cerevisiae* is rather wimpy. Therefore, before testing the effects of inhibiting the proteasome on transcription, I needed to develop an improved strategy to decrease the proteolytic power of the proteasome. This strategy of combining chemical and genetic approaches to inhibit the proteasome is described in the third chapter.

Once I had an effective means to inhibit the proteasome I could turn to address the consequences of proteolysis with transcription. This area of study has its own controversy: two different groups looked at the induction of the same gene using the same methods to inhibit the proteasome and arrived at two opposite results (Lipford et al. 2005; Nalley et al. 2006). I believe one reason such conflicting data arise is that the typical approach used to proteasome-mediated proteolysis pushes the edge of what is sufficient to regulate transcription. Improving the inhibition of the proteasome will also improve the clarity in which the connection between proteolysis and transcription can be studied. In fourth chapter I use this approach to study the activation of three inducible genes: *ARG1, INO1*, and *CHA1*.

Given the success of using this approach at studying individual, strongly induced genes I also investigated the global transcription effects of inhibiting the proteasome. Previous attempts to study the effects of proteasome inhibition have used incomplete inhibition of the proteasome (Fleming et al. 2002; Dembla-Rajpal et al. 2004). In the fifth chapter I demonstrate that my more thorough approach to inhibiting the proteasome results in an increased number of genes that are affected by proteasome

inhibitors. The altered expression of these genes will provide a useful resource for future work into the role of proteasome-mediated proteolysis in transcription.

The sixth and final chapter provides a summary of my work and a discussion on how proteasome mediated proteolysis regulates transcription activators.

The Ubiquitin Proteasome System (UPS)

The ubiquitin proteasome system (UPS) is an ATP-dependent system for the regulated destruction of misfolded, unnecessary, or even harmful proteins from within the cell (Hochstrasser 1996; Hershko and Ciechanover 1998; Schwartz and Ciechanover 1999; Voges *et al.* 1999). The ubiquitylation process begins with the charging of a small 76 amino acid protein, ubiquitin, onto an E1 ubiquitin activating enzyme in one of the two main ATP dependent steps. Ubiquitin is then passed on to a second enzyme, the E2 ubiquitin conjugating enzyme. Then, via an E3 ubiquitin ligating enzyme, the ubiquitin moiety is passed onto the substrate through the C-terminal glycine of ubiquitin and an amino group on a lysine in the substrate (or the N-terminal amino group). Because ubiquitin itself has seven lysines, more ubiquitin molecules can be attached to form chains of ubiquitin on the substrate. It is the conventional wisdom that polyubiquitin chains of four or more ubiquitin peptides attached to the substrate target the protein to the proteasome for destruction.

The proteasome is a large ~2 megadalton complex composed of one to two 19S regulatory subcomplexes and a 20S core (see *Figure 1.1*) (Groll *et al.* 1997; Bochtler *et al.* 1999; Voges *et al.* 1999; Finley 2009; Forster *et al.* 2009; Nickell *et al.* 2009).The 19S regulatory particle itself can be separated into a lid subcomplex and a base subcomplex. The lid of the 19S proteasome is important for recognition and de-ubiquitylation of target substrates. The base of the 19S proteasome is composed of six different ATPase subunits (Rpt1-Rpt6) and two non-ATPase subunits (Rpn1-2). The 19S is able to unwind substrates and translocate the target into the center of the 20S core (Benaroudj and Goldberg 2000; Benaroudj *et al.* 2003; Horwitz *et al.* 2005; Smith *et al.* 2005; Peth *et al.* 2009).

The 20S core is comprised of a series of concentric heteroheptameric rings ordered α_7-β_7-β_7-α_7 to form a cylinder. The three of the seven β subunits provide the proteasome with its proteolytic capability. The β1 subunit, Pre3, has caspase-like proteolytic activity, preferentially cleaving after acidic amino acids of the peptide substrate. The β2 subunit, Pup1, is a trypsin-like protease primarily promoting hydrolysis following basic amino acids. Finally, the β5 subunit, Pre2, has a chymotrypsin-like proteolytic activity with preferential cleavage following large, hydrophobic amino acids (Arendt and Hochstrasser 1997; Heinemeyer *et al.* 1997; Arendt and Hochstrasser 1999; Groll *et al.* 1999; Jager *et al.* 1999). All three proteases depend on a catalytic threonine that is exposed of the N-terminal of the processed peptide and located in the center of the cylinder. Access to the proteases is protected by the α-rings, which form a gate to the interior of the cylinder opened *in vivo* by the 19S base (Smith *et al.* 2005; Smith *et al.* 2007; Rabl *et al*. 2008).

Except for the gene encoding the α3 subunit, *PRE9*, all genes encoding 20S subunits and most 19S subunits are essential for the viability of the yeast. (The α4 subunit Pre6 replaces the absent α3 subunit in Δ*pre9* yeast (Velichutina *et al.* 2004)). However, it is possible to replace the catalytic threonine with an alanine in any one of the proteolytic subunits. This will abolish the associated activity of that protease yet the yeast remains viable. In fact, double mutants without trypsin-like and caspase-like activity or without the chymotrypsin-like and caspase-like activity are viable (Heinemeyer *et al.* 1997; Arendt and Hochstrasser 1999). (There is no specific mention of yeast without chymotrypsin-like and trypsin-like subunits, which may be a nonviable combination of deletions). Of the three subunits, the only the loss of chymotrypsin-like activity stabilized model substrates such as the mating type transcription repressor α2 or ubiquitin-fused β-galactosidase (Arendt and Hochstrasser 1997; Arendt and Hochstrasser 1999). It was also the loss of chymotrypsin-like activity that had the most pronounced affect on yeast growth at elevated temperatures. Together these data have been used to argue that it is the chymotrypsin-like activity that is the primary and

rate-limiting protease for proteasome-mediated proteolysis (Kisselev and Goldberg 2001; Kisselev *et al.* 2006).

The importance of the UPS in the cell is reflected in the numerous cell pathways it regulates such as cell cycle progression (King *et al.* 1996; Zachariae and Nasmyth 1999), DNA repair (Krogan *et al.* 2004; Daulny and Tansey 2009), transcription (Auld and Silver 2006), protein quality control (Goldberg 2003), and organelle distribution (Campbell *et al.* 1994). Although not all ubiquitylation events lead to the proteasome, proteolysis is an important mechanism preventing the toxic accumulation of abundant or misfolded proteins and provides an irreversible step that can provide directionality to any given cell process.

Transcription and Transcription Activators

The process of transcription can be thought of in three phases: initiation, elongation, and termination. Accompanying elongation and termination are several important RNA-processing events. RNA polymerase II (RNApolII) is responsible for the production mRNA and the challenges of regulating transcription center on correct and efficient recruitment of RNApolII and regulating its processivity once transcription initiation has begun. The recruitment of RNApolII itself presents several challenges; not only must RNApolII arrive at the correct genes, it must arrive at the correct start sites, at the correct time. The challenges of recruiting RNApolII are accomplished between the cooperation of *cis* DNA regulatory elements typically located 5' (upstream) of the transcription start site, chromatin structure, and *trans* regulatory proteins (transcription factors).

A certain set of transcription factors is typically associated with RNApolII recruitment and transcription of the initial few nucleotides. These general transcription factors (GTF) include TFIID, which is a protein complex with several proteins necessary to position RNApolII at the transcription start site (*e.g.* TATA-binding protein (TBP)), TFIIB and TFIIA which bridge the binding of DNA and TFIID to the association of RNApolII and TFIIIF, and TFIIE and TFIIH, which assist in the ATP-

dependent opening of DNA to start transcription (Orphanides et al. 1996; Hampsey 1998). In addition to the GTFs, the mediator complex commonly works to coordinate recruitment of RNApolII with the activator (Holstege et al. 1998; Kornberg 2005). The mediator complex has several other functions beyond serving as a bridge between activator and RNApolII. Srb10/Ssn3/Cdk8 regulates the activity of RNA polymerase by phosphorylation of the C-terminal domain (CTD) of the Rpb1 (Kuchin et al. 1995; Liao et al. 1995), and another subunit Nut1 has histone acetyltransferase (HAT) functions (Lorch et al. 2000). Thus, the mediator has been proposed to facilitate the function of activators to recruit RNApolII, dispatch the RNApolII into an elongation phase, and facilitate re-initiation for subsequent rounds of transcription (Svejstrup et al. 1997; Lewis and Reinberg 2003).

In addition to the GTFs and mediator complex, numerous co-activators, such as SAGA, RSC, and SWI/SNF, facilitate RNApolII recruitment and initiation (Guarente 1996; Naar *et al.* 2001; Narlikar *et al.* 2002). These co-activators not only provide additional scaffolding structures to stabilize the association of RNApolII to the promoter, but these co-activators also alter the chromatin architecture, both of the promoter and the transcribed region to facilitate RNApolII binding and later elongation. Co-activators seem to primarily function by modifying or remodeling nucleosomes to make the chromatin more accessible for RNApolII to transcribe the gene while at the same time making the nucleosome structure unfavorable for spurious transcripts to be generated from cryptic promoter-like elements.

Although all these components —GTFs, mediator, co-activators, and nucleosomes —are necessary to have efficient and accurate transcription, the ability to regulate the levels of transcription in response to changing cellular needs typically operates through transcription activators. Most transcription activators have two general modules: a DNA binding domain (DBD) that recognizes specific DNA sequences or upstream activating sequences (UAS) in the promoter and a transcription activation domain (TAD) that is responsible for binding and recruiting co-activators, mediator, and GTFs (Ptashne 1988). For example when the yeast activator Gal4 is induced it is able to bind components of the co-activators SAGA and NuA4, the Mediator complex, and the GTF TFIID

(Reeves and Hahn 2005). Transcription activators are (1) tightly controlled by the cell to adapt to changes in transcriptional demands, (2) flexible to interact with many different molecules of the pre-initiation complex, and (3) specific to bind specific promoters to provide the cell with a regulated means to increase the transcription from particular sets of genes.

There are multiple means to regulate transcription activators: controlling abundance (*e.g.* β-catenin) (Aberle *et al.* 1997; Hart *et al.* 1999; Lagna *et al.* 1999; Latres *et al.* 1999; Winston *et al.* 1999), removal of inhibitory domains (*e.g.* NF-κ-B) (Sears *et al.* 1998; Orian *et al.* 1999; Moorthy *et al.* 2006; Cohen *et al.* 2009; Kravtsova-Ivantsiv *et al.* 2009), localization within the cell (*e.g.* Spt23) (Hoppe *et al.* 2000; Chellappa *et al.* 2001; Rape *et al.* 2001), availability of co-activator targets (*e.g.* LIM homeodomain) (Ostendorff *et al.* 2002; Gungor *et al.* 2007), and stability with co-activators (*e.g.* Gal4) (Lee *et al.* 2005). Interestingly the UPS regulates each of these processes (Lipford and Deshaies 2003; Muratani and Tansey 2003).

The Transcription-Proteasome Nexus

The proteasome has been proposed to regulate several steps throughout the process of transcription (see *Figure 1.2*) (Collins and Tansey 2006). However, not all proposed mechanisms involve the proteolytic ability of the proteasome. Instead much of the debate is not whether there exists a role for the proteasome in transcription but instead it is whether the proteasome requires its proteolytic functions or if it uses non-proteolytic mechanisms to regulate transcription. In addition to proteolysis the proteasome can de-ubiquitylate, unfold, and translocate substrates making the proteasome somewhat of a "reverse chaperone" (Braun *et al.* 1999; Navon and Goldberg 2001; Liu *et al.* 2007).

The issue of the proteasome functioning as a non-proteolytic molecule, even to the extent that a subcomplex of the 19S bases functions independently of the rest of the proteasome is the product of a spontaneous mutational suppressor of a truncated version of Gal4 (Matsumoto *et al.* 1980). This

mutant, *gal4D*, missing much of its activation domain, could not induce *GAL* gene expression. However a pair of spontaneous second site mutations, *sug1* and *sug2*, suppressed the galactose negative phenotype (Swaffield *et al.* 1992). Johnston *et al* building on this observation proposed that these proteins formed a new class of transcriptional co-activators. When these mutations were eventually found to be components of the 19S proteasome (*rpt6* and *rpt4* respectively (Rubin *et al.* 1996)), the relevance of the ability Rpt6 and Rpt4 as coactivators was strongly doubted. The importance of the proteasome in regulating transcription has come from several fronts. First, it has been demonstrated that the ability of the *rpt6* and *rpt4* mutations to suppress specific *gal4* mutations was not simply due to increased abundance of *gal4* (Russell and Johnston 2001). Second, the proteasome has been detected at many genes (Auld *et al.* 2006; Sikder *et al.* 2006)—although conclusions regarding the widespread non-overlap of 19S and 20S subunits may be a consequence of using tags to detect the proteasome (F. Geng, *personal communication*), it seems that the proteasome is indeed found at genes in a transcription dependent manner. Third, there is accumulating evidence that transcription activators are ubiquitylated and unstable (Molinari *et al.* 1999; Salghetti *et al.* 2000; Salghetti *et al.* 2001; Lipford and Deshaies 2003; Muratani and Tansey 2003). Increasing the potency of activators decreases the stability of activators whereas ubiquitylation of an activator allows it to bypass the need for an ubiquitin ligase to induce transcription.

The idea that the proteasome functions non-proteolytically to regulate transcription has merit. First, the proteasome has “reverse chaperone” capabilities of unwinding substrates (Braun *et al.* 1999; Navon and Goldberg 2001; Liu *et al.* 2002). Second, the 19S apart from the 20S proteolytic core, can increase the stability of association between SAGA, Gal4, and the *GAL* UAS (Lee *et al.* 2005). Third, histones, which were the first substrates known to be ubiquitylated, undergo multiple post-translational modifications. In particular, H2B can be mono-ubiquitylated on lysine-123 (Sun and Allis 2002). The mono-ubiquitylation of H2B is necessary for di- and tri-methylation on H3 lysines 4 and 79 in what has been reported to be a 19S but not 20S dependent process (Ezhkova and Tansey 2004; Laribee *et al.* 2007). Finally, another ATPase complex, the Cdc48 complex, has been reported

to destabilize the transcription repressor α2 in an ubiquitylation-dependent process (Wilcox and Laney 2009). Cdc48 does not have proteolytic activities and therefore any mechanism it has on detecting and displacing ubiquitylated α2 is non-proteolytic, providing an example of the type of non-proteolytic remodeling that may be possible by the proteasome.

Given the merit of non-proteolytic mechanisms of the proteasome in regulating transcription it is reasonable to ask if the proteolytic ability of the proteasome is even necessary to directly regulate transcription beyond controlling transcription factor abundance. There are at least two reasons to suspect that the proteolytic role of the proteasome is important to directly regulate transcription. First, there is growing evidence for widespread distribution of the 20S proteolytic core on chromatin coupled with only shaky evidence that 19S subunits are indeed separate from the 20S subunits (Auld and Silver 2006; Sikder *et al.* 2006). Second, and carrying much more weight, is the instability of transcription activators (Molinari *et al.* 1999; Salghetti *et al.* 2000; Salghetti *et al.* 2001; Lipford and Deshaies 2003; Muratani and Tansey 2003). It is not just the ubiquitylation of these activators that is associated with transcription but their instability, which is presumably a product of proteasome-mediated proteolysis. But this then raises the question, "In what capacity is proteasome-mediated proteolysis directly connected to activating transcription?" The model of activator licensing may be relevant.

The Licensing Model of Transcription Activators

The model of transcription activator licensing draws upon analogy with the licensing of the DNA pre-replication complex (pre-RC) (Stillman 1996; Drury and Diffley 2009). In the licensing of the pre-RC, the origin recognition complex (ORC) has bound DNA at specific sequence elements. During early G_1 phase of the cell cycle, Cdc6 binds to ORC and facilitates the binding of MCM proteins. S-Cdk later activates the pre-RC and phosphorylates Cdc6, which is then ubiquitylated and destroyed preventing re-initiation of DNA replication. The analogy between activator licensing and

replication licensing however breaks down in that it is clear that replication must fire once and only once per cell cycle, whereas no such pressing need is placed on transcription.

Nevertheless, the analogy is useful in laying out the three regulatory events and connecting it to activation. In this case, activators facilitate the binding of co-activators, GTFs, and RNApolII. Within the assembled initiation complex are kinases that are not only important for phosphorylation of the RNApolII CTD to facilitate the transition to productive elongation, but also for the phosphorylation of the activator. At least two activators, Gal4 and Gcn4, are known to be phosphorylated by a component of the Mediator complex (Ssn3/Srb10/Cdk8) and by a component of the GTF, TFIIH (Hirst *et al.* 1999; Chi *et al.* 2001; Lipford *et al.* 2005; Muratani *et al.* 2005). Both activators are ubiquitylated in response to these phosphorylation events and both are unstable, particularly during active transcription. Inhibiting the ubiquitylation of Gal4 and Gcn4 has led to loss of transcription in the case of Gcn4 (Lipford *et al.* 2005) and the loss of co-transcriptional processing in the case of Gal4 (Muratani *et al.* 2005). Therefore, it is suspected that Gal4 and Gcn4 and perhaps other activators are phosphorylated to mark that productive transcription is underway. This leads to ubiquitylation and subsequently destruction by the proteasome (see *Figure 1.3*).

The purpose of such a regulatory system may not be immediately obvious (molecular clocks (O'Malley 2009) and removal of “spent” activators (Lipford and Deshaies 2003; Lipford *et al.* 2005) have been proposed), but the model not only accounts for known data but provides clear, testable predictions. Specifically, because I am interested in the connection between the proteasome and transcription, two predictions that I have tested are (1) that transcription activators cannot stably associate with the target promoter if proteolysis is a necessary part in the process of licensing activators, and (2) that inhibiting the proteasome must have some inhibitory effect on genes whose transcription depends on licensed activators if proteolysis is a necessary part in the process of licensing activators. These predictions are interesting because not only are they clear and testable, but there has been controversy and contradictory data that purportedly supports or refutes each of these

predictions. My work then will not only test these two predictions but it will resolve the controversy and provide improved tools to study transcription activator and proteasome function.

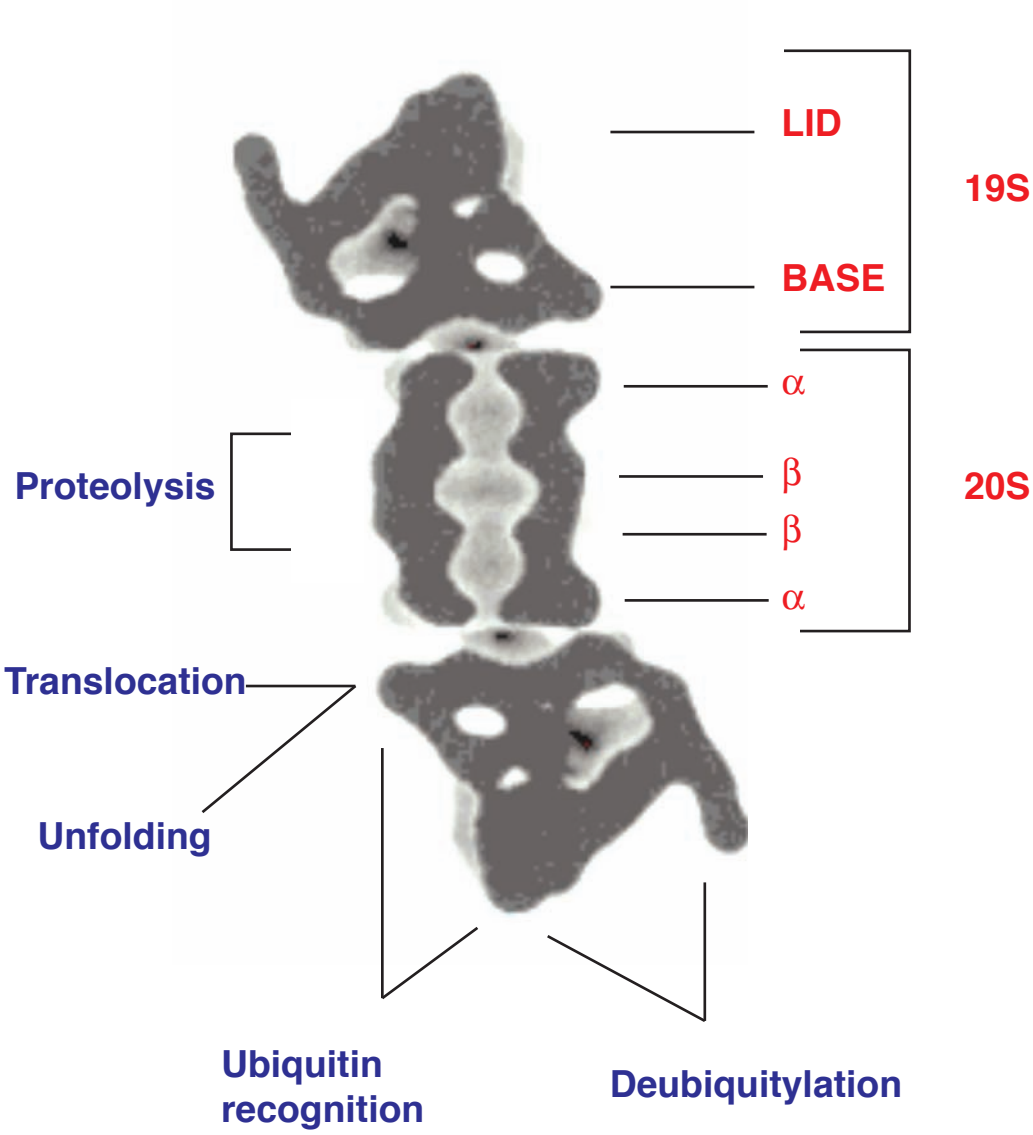

Figure 1.1: Structure and function of the 26S proteasome. The cartoon structure of the proteasome is based on average electron microscopic images of purified proteasome (Nickell *et al*, 2009). The structure (red font) and function (blue font) of the proteasome are listed.

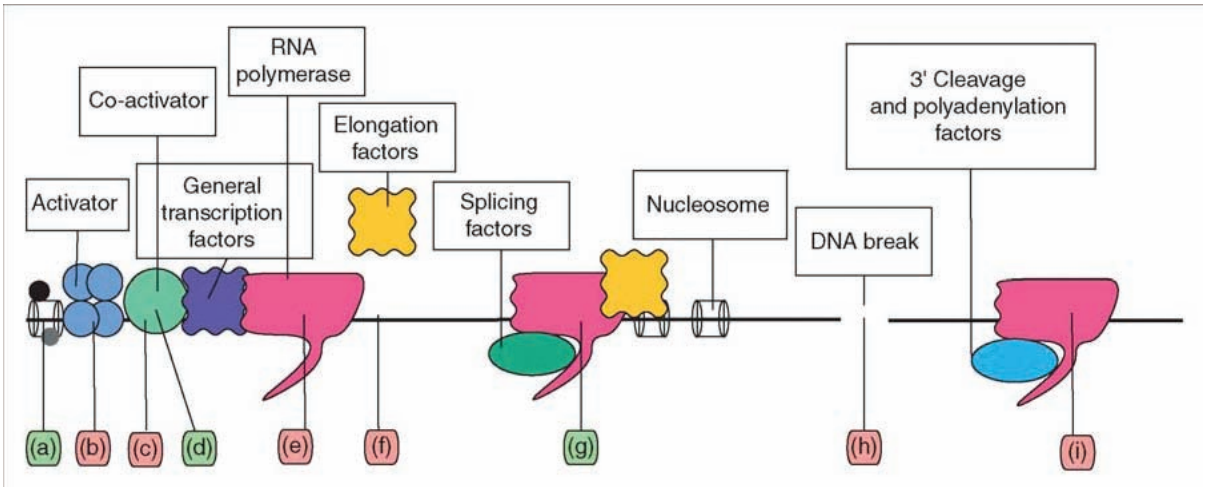

Figure 1.2: The proteasome regulates many aspects of transcription through both proteolytic (red) and non-proteolytic mechanisms (green). (a) Proteasomes interact with chromatin in response to histone H2B ubiquitylation to induce methylation of histone H3. (b) Proteasomes regulate transcription through activator turnover. (c) Proteasomes regulate co-activator and co-repressor exchange and recruitment, possibly through both proteolytic and (d) non-proteoyltic mechanisms. (e) Proteasomes are important for stable recruitment of RNA polymerase to sites of transcription, possibly acting after the first round of transcription. (f) Proteasomes might regulate progression through an elongation checkpoint. (g) 19S base subunits promote efficient transcriptional elongation. (h) Proteasome-mediated proteolytic role in tanscription-coupled repair. (i) Proteasome function may regulate accurate transcription termination.

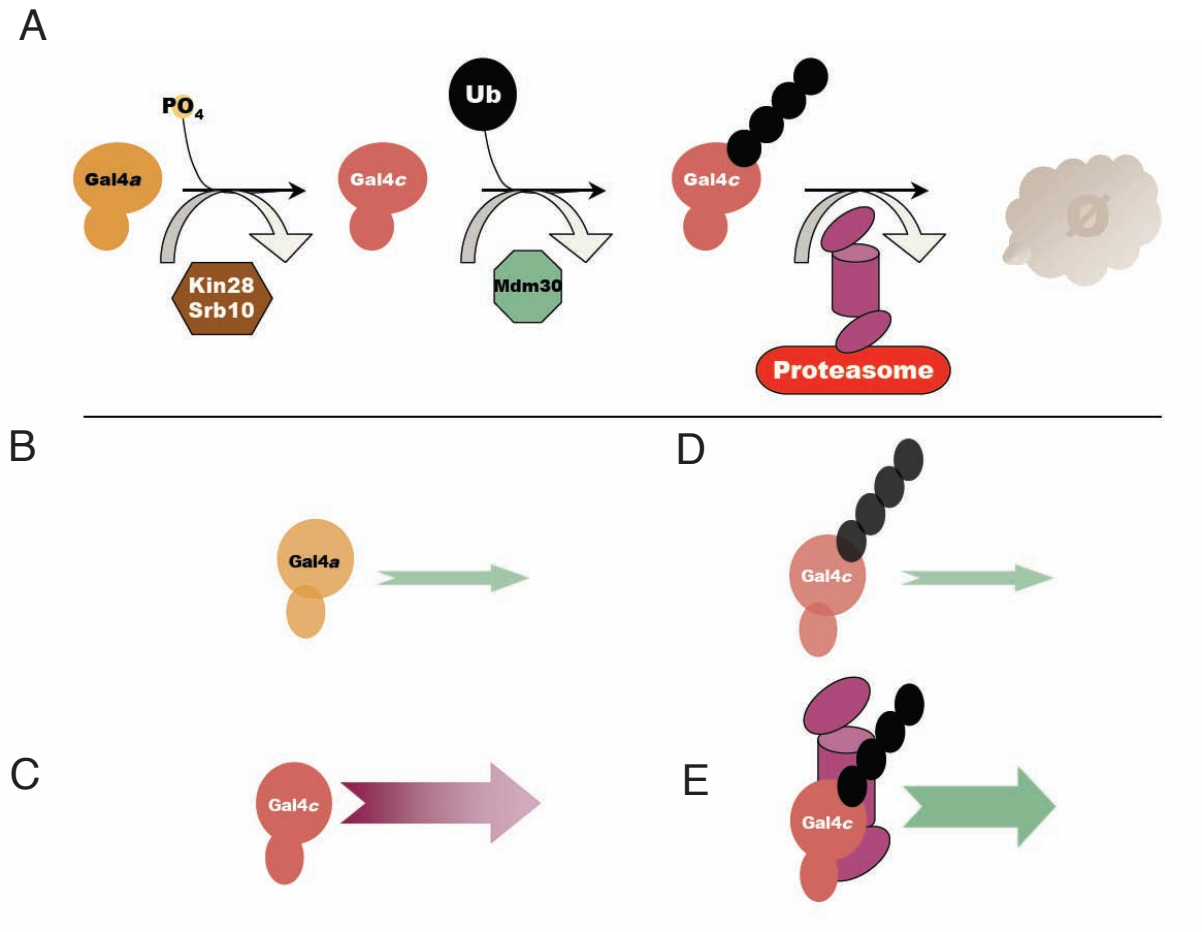

Figure 1.3: **The Proteasome in Activator Licensing with Gal4 as a Model**. (A) Prior to activation, Gal4 is hypophosphorylated. When transcription is induced, Gal4 becomes phosphorylated by the TFIIH kinase Kin28 and the Mediator kinase Srb10. Hyperphosphorylated Gal4, Gal4**c**, is ubiquitylated by Mdm30. Consequently, Gal4 is polyubiquitylated and recognized by the proteasome and destroyed. (B) Overexpression of hypophosphorylated Gal4 can induce low levels of transcription. (C) Preventing ubiquityaltion by the deletion of *MDM30* produces *GAL1* transcripts that are not properly processed and incorporated into polyribosomes. (D) Prevention of proteolysis leads to loss of transcription. (E) With all components in place -phosphorylation, ubiquitylation, and proteolysis –efficient and effective transcription proceeds.

Chapter Two: The Turnover of Gal4 on Chromatin During Activation

Introduction

The Gal4 activator is the central regulator of galactose metabolism in *S. cerevisiae* (Johnston and Carlson 1992). Yeast demonstrate a marked preference for glucose as their primary carbon source to such an extent that yeast repress the synthesis of enzymes required to metabolize other carbohydrates (*e.g.* galactose) when grown in the presence of glucose. If, however, yeast are deprived of glucose and are instead supplied with galactose, transcription of a small set of genes rapidly increases, driven by the Gal4 activator (Ren et al. 2000). In between the active transcription of the induced state (galactose media) and negligible transcription of the repressed state (glucose media), the *GAL* genes have basal levels of transcription in non-inducing media (*e.g.* raffinose or glycerol and lactic acid media) (Johnston and Carlson 1992). Under the repressed and non-induced states, Gal80 inhibits the activity of Gal4. Additionally, during repression, *GAL* genes are tightly controlled to prevent extraneous expression by the repressor Mig1 and the co-repressors Cyc8-Tup1 (Lamphier and Ptashne 1992; Papamichos-Chronakis et al. 2004). However, upon induction with galactose, the inhibitory effects of Gal80 on Gal4 are relieved in a Gal3 dependent manner thereby allowing Gal4 to function as an activator (Bhat and Hopper 1992). (Gal1, which is paralogous to Gal3, can also activate Gal4 in the presence of galactose but the concentration of Gal1 is not typically sufficient to relieve Gal4 repression by Gal80 except in yeast that recently induced *GAL* gene expression (Ptashne 2008)).

Gal4 has three phosphoisoforms that are resolvable by SDS-PAGE western blots (Mylin et al. 1990; Sadowski et al. 1991; Hirst et al. 1999). Under non-inducing conditions only the faster migrating two isoforms (**a** and **b**) can be detected. Gal4**a** and Gal4**b** are unstable; Gal4 levels progressively decrease when treated with cycloheximide. The instability of Gal4**a** and Gal4**b** is presumably regulated by the UPS because deletion *GRR1*, encoding an E3 ligase, stabilizes Gal4 when treated with cycloheximide (Muratani et al. 2005). When yeast are switched to inducing

conditions a third, slower moving, phosphoisoform of Gal4 (**c**) can now be detected. Grr1 no longer regulates Gal4 in the presence of galactose and Gal4**a** and Gal4**b** become stable. However, the activation-associated phosphoisoform of Gal4, Gal4**c**, is unstable and ubiquitylated in an Mdm30-dependent process (Muratani et al. 2005).

Several pieces of evidence provide a compelling case that the ubiquitylation and presumably destruction of Gal4 occurs at its target promoters. First, the unstable portion of Gal4, Gal4**c**, is only detected during conditions that also induce Gal4-dependent transcription (Mylin et al. 1990; Sadowski et al. 1991; Hirst et al. 1999). Second, the kinases that are responsible for Gal4**c** formation have been identified as components of the GTF TFIIH and of the Mediator complex (Hirst et al. 1999). Gal4 is known to interact with the Mediator complex and another GTF, TFIID, at the promoter (Reeves and Hahn 2005), making it plausible that Gal4**c** forms at the promoter. Third, Mdm30, the E3 ligase that mediates Gal4 ubiquitylation in galactose and the instability of Gal4**c,** is detected at the *GAL1* promoter when the yeast are grown in galactose (Muratani et al. 2005). Fourth, Δ*mdm30* yeast are galactose negative and fail to efficiently process *GAL1* mRNA and incorporate those messages into polyribosome complexes for translation (Muratani et al. 2005). Finally, Gal4 is not unusual in this regards; several other activators are unstable in the context of transcription (McNally et al. 2000; Becker et al. 2002; Metivier et al. 2003; Reid et al. 2003; Stavreva et al. 2004; Bosisio et al. 2006; Johnson et al. 2008; Karpova et al. 2008; Hager et al. 2009).

However, using a recently developed competition chromatin immunoprecipitiation (cChIP) technique, Nalley *et al* reported the turnover of Gal4 only occurs when yeast are in non-inducing conditions (Nalley et al. 2006). When transcription occurs, Gal4 is observed to lock onto its target promoter. Elaborating on this model, the Kodadek group proposed that Gal4 is *stabilized* by the monoubiquitylation through a yet unidentified E3 ligase from mammalian nuclear extracts (Ferdous et al. 2007; Archer et al. 2008a; Archer et al. 2008b; Ferdous et al. 2008; Archer and Kodadek 2010). In their model, Gal4 is recognized by a subcomplex of the 19S base (APIS), and dislodged from the promoter. Gal4 is mono-ubiquitylated under inducing conditions, which prevents the recognition of

Gal4 by the APIS complex and consequently Gal4 stably associates with the promoter and activates transcription.

There are many significant problems with the model put forth by the Kodadek group. The primary evidence for the APIS complex comes from the ability to detect certain 19S but not 20S proteins from co-immunoprecipitation with Gal4 or to detect certain 19S but not 20S protein localization to the *GAL1* promoter, (Gonzalez et al. 2002) resulting in an argument from silence for the independent existence of the APIS complex. Second, the evidence of Gal4 ubiquitylation *in vivo* supports that there is substantial *polyubiquitylation* rather than predominantly mono-ubiquitylation of Gal4 (Muratani et al. 2005). Similarly, the E3 responsible for mono-ubiquitylation of Gal4 remains to be identified. Mono-ubiquitylation of Gal4, then, may be an artificial phenomenon that was achieved through the use of mammalian cell extracts. Moreover, this model raises the issue of whether or not Gal4 stably locks onto its target promoter to activate transcription even though the whole cell population of the activation associated Gal4**c** isoform is unstable with cycloheximide treatment.

The competitive ChIP technique used to measure the stability of Gal4 with its promoter is an *in vivo* competition assay between two activators that each bind a common DNA target-sequence. In this case the two activators are two distinct versions of Gal4, endogenous Gal4 and competitor Gal4, which compete for access at the *GAL1* promoter. Endogenous Gal4 is genomically encoded and can be detected at the UAS_{GAL} by ChIP using antibodies directed against the Gal4 TAD. Therefore, although it is necessary that competitor Gal4 share a common DBD with endogenous Gal4, the competitor Gal4 competitor cannot have the same epitope as endogenous Gal4 and therefore it uses a different TAD, namely the VP16 TAD. Association of competitor Gal4 with the *GAL1* promoter is measured by ChIP with antibodies against and N-terminal Myc-epitope tag. Finally, competitor Gal4 is engineered to have an estrogen-binding domain (EBD) from the human estrogen receptor alpha. This EBD is sandwiched between the VP16 TAD and the Gal4 DBD and serves as the trigger sensor for the competition experiment. In the absence of ligand, EBD is bound by Hsp90 and sequestered in the cytoplasm away from the *GAL1* promoter (Fankhauser et al. 1994; Picard 2000). Thus, in the

absence of an appropriate ligand, such as 17-β-estradiol, only endogenous Gal4 is present in the nucleus. Upon addition of an appropriate ligand, Hsp90 releases competitor Gal4, which moves to the nucleus and competition ensues (see *Figure **2.1***). If Gal4 stably binds the UAS_{GAL} then endogenous Gal4 levels should remain constant even after competition is triggered and competitor Gal4 should not be detected at the *GAL1* promoter. Conversely, the loss of endogenous Gal4 and the appearance of competitor Gal4 is evidence of competition and activator turnover.

The Kodadek group argued, based on this assay, that Gal4 is labile under non-inducing conditions but locks onto the *GAL1* promoter upon induction with galactose (Nalley et al. 2006). However, from the data that they presented it was ambiguous whether or not competitor Gal4 was arriving to the *GAL1* promoter, and thus it was not certain that no exchange between endogenous and competitor Gal4 occurred. For this reason, and because Gal4**c** is unstable, I re-examined the stability of Gal4 on the *GAL1* promoter using qPCR rather than ethidium bromide stained gels to quantify the stability of the Gal4 association with its target promoter.

Experimental Procedures

Yeast strains

I list the strains used in the course of this study in table 2.1. The pTK0601 plasmid (Nalley et al. 2006), bearing the competitive Gal4 (***c***Gal4), was transformed using the high efficiency transformation using lithium acetate and PEG, as described by Gietz and Woods (Gietz and Woods 2002). I deleted *PDR5* in the designated strains using gene replacement with *LEU2* by homologous recombination. I synthesized the knockout cassette by PCR using pRS405 as a template, and transformed the cassette again using the lithium acetate and PEG transformation method (Gietz and Woods 2002).

Table 2.1: Yeast Strains Used in This Chapter

Strain	Genotype	Source
BY4741	Mat**a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
BY4741 Δ*gal4*	Mat**a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gal4Δ::KanMX6*	Open Biosystems
GAC101	Mat**a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* + pTK0601 (***c****GAL4, HIS3*)	This study
GAC102	Mat**a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gal4ΔKanMX6* + pTK0601 (***c****GAL4, HIS3*)	This study
BY4742	Matα *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Open Biosystems
BY4742 Δ*gal4*	Matα *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 gal4Δ::KanMX6*	Open Biosystems
BY4742 Δ*pdr5*	Matα *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 pdr5Δ::LEU2*	This study
BY4742 Δ*gal4* Δ*pdr5*	Matα *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 gal4Δ::KanMX6 pdr5Δ::LEU2*	This study
GAC111	Matα *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 pdr5Δ::LEU2* + pTK0601 (***c****GAL4, HIS3*)	This study
GAC112	Matα *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 gal4Δ::KanMX6 pdr5Δ::LEU2* + pTK0601 (***c****GAL4, HIS3*)	This study

Table 2.2: Plasmids Used in This Chapter

Plasmid	Features	Source
pTK0601	Myc - Gal4 DBD - ER EBD -VP16 TAD (*HIS3*)	(Nalley et al. 2006)
pRS405	Yeast integrative vector with *LEU2* marker	(Sikorski and Hieter 1989)

Cycloheximide Treatment

I treated BY4742 yeast grown in CSM 2% galactose with 10 μg /ml cycloheximide (*Sigma*) from a 1000x stock solution in ethanol. I collected samples 15, 30, and 60 minutes after the start of the cycloheximide treatment or ethanol in addition to an untreated control (t_0). I reserved 2ml of each sample to monitor the efficacy of cycloheximide treatment by following the growth of the culture as determined by change in A_{600}. The remaining culture was used for chromatin immunoprecipitation (ChIP) as described below.

Gal4 Competition

Please refer to figure 2.1 for a diagram of the competitive ChIP procedure. For the competitive ChIPs done in non-inducing conditions, yeast were grown in CSM 2% glycerol 2% lactic acid. For the competitive ChIPs in inducing conditions, I induced *GAL* gene transcription by transferring yeast grown in CSM 2% raffinose to CSM 2% galactose. After one hour in galactose, I started the competition assay with 1 μM 17-β-estradiol (*Sigma*) in DMSO or 100 μM 4-hydroxy tamoxifen (4-OHT) (*Sigma*) in ethanol. I collected samples 15, 30, and 60 minutes after the start of competition in addition to an untreated control (t_0) to be processed by ChIP as described below.

Chromatin Immunoprecipitation

I used a ChIP procedure described by Muratani *et al* (Muratani et al. 2005). To 230 ml of yeast culture, I added 6.4 ml of 37% formaldehyde solution (*Fisher*) and cross-linked for 20 minutes at room temperature. I stopped the cross-linking reaction by the addition of 14.5 ml of 2 M glycine for 5 minutes. I then collected the yeast cells by gentle centrifugation (5 minutes at 4000 rpm) and washed the cell pellet twice with ice cold 1x TBS. The washed cell pellet was then flash frozen in liquid nitrogen. I lysed the yeast in ChIP lysis buffer (50 mM HEPES/KOH (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.1% DOC, 0.1% SDS, 5 mM NaF) by bead beating. Cell lysates were sonicated to approximately 500 base pairs fragments. I incubated the sheared chromatin solution with protein A / protein G agarose beads (*Roche*) for 1 hour at 4°C. Then I collected the cleared chromatin solution to be incubated with the appropriate antibody. Endogenous Gal4 was monitored using C-10 anti-Gal4 anitbody (*Santa Cruz*), whereas the competitive Gal4 was followed using the Myc-epitope tag and the AB1 anti-Myc antibody (*Calbiochem*). Following overnight incubation with the antibodies, I added protein A / protein G agarose beads for an hour at 4°C, after which I washed the beads twice with ChIP lysis buffer, once with DOC wash buffer (10 mM Tris-Cl (pH 8.0), 0.25 M LiCl, 0.5% NP-40 alternative, 0.5% DOC, 1 mM EDTA), and twice with 25x Tris-EDTA buffer. I eluted the pulldown chromatin using TES buffer (50 mM Tris-Cl (pH 8.0), 10 mM EDTA, 1% SDS)

incubated at 65°C for 20 minutes. I reversed the cross-linking of the enriched chromatin by incubating overnight at 65°C. Proteins were digested using 2µg proteinase K (*Roche*) at 42°C for two hours, followed with a phenol:chloroform extraction and ethanol precipitation of the DNA.

I quantified the amount of DNA that came down in the immunoprecipitation by qPCR with SYBR green PCR mix (*Applied Biosystems*). Calculating enrichment was done as described by Ezhkova *et al*. The automatically-derived cycle thresholds were obtained from triplicate samples of the immunoprecipitated DNA for both the amplicon of interest (C^{IPA}) and a reference locus (C^{IPR}) as well as corresponding threshold values from chromatin reserved from prior to the immunoprecipitation steps (C^{INA} and C^{INR}) respectively. Fold enrichment was then calculated as $[2^{(IPR-IPA)}] \div [2^{(INR-INA)}]$. I used the *ACT1* ORF (ACT1-Q1 ACT1-Q2 oligonucleotides) as my reference locus to normalize for Gal4 signal binding to the *GAL1* promoter (GAL1-Q1 and GAL1-Q2 oligonucleotides). ChIP signals are expressed as percent of the untreated (t_0) control except for *Figure **2.7***, which expresses the ratio of treated (or competition present) versus mock treated (or no competition present), and for *Figure **2.8***, which is expressed as percent of a competitor Gal4 bound after one hour of treatment with 100 µM hydroxy tamoxifen in a Δ*gal4* (no endogenous Gal4) strain.

Results

Cycloheximide treatment leads to a decrease in Gal4 at the promoter

One approach to monitor the stability of proteins is to shutdown translation with cycloheximide. Muratani *et al* used this technique to demonstrate that Gal4 is unstable, particularly the phosphoisoform associated with transcriptional activity (Muratani et al. 2005). The success of this approach to study the stability of Gal4 in whole cell extracts, motivated me to determine if the stability of Gal4 on chromatin corresponded with the instability of Gal4**c** when treated with cycloheximide. I treated yeast with either cycloheximide to inhibit translation or ethanol as a control and measured the levels of *GAL1* promoter DNA bound by Gal4 at three time points post-treatment

by ChIP (*Figure **2.2***). Whereas ethanol treatment did not significantly change the Gal4 ChIP signal at the *GAL1* promoter, treatment with cycloheximide produced a rapid and substantial loss of Gal4 bound to the *GAL1* promoter. Within fifteen minutes, nearly half the initial Gal4 signal was lost, and by an hour as much as eighty percent of the initial Gal4 no longer associates with the promoter. Thus, the loss of Gal4 on the promoter approximates the global instability of Gal4**c** with cycloheximide treatment but not the stability of isoforms Gal4**a** and Gal4**b** (Muratani et al. 2005). Furthermore, these results are in stark contrast to the report that Gal4 locks onto chromatin during active transcription (Nalley et al. 2006). Therefore, to address the disconnect between stable Gal4 observed by Nalley *et al* and unstable Gal4 that both Muratani *et al* and I observed, I turned to the competitive ChIP assay.

17-β-estradiol is not suitable for Gal4 competition assays

The competitive ChIP assay has the advantage over cycloheximide treatment in that —at least in theory —it is more direct in its ability to focus in on a single DNA binding protein (see *Figure **2.1**)*. To demonstrate that I could obtain similar results as Nalley *et al* (Nalley et al. 2006) I performed their competitive ChIP as described using 17-β-estradiol to induce competition and DMSO treatment for the non-competition control (*Figure **2.3***). Under galactose inducing conditions, the Gal4 ChIP signal does not significantly change from the untreated (t_0) control. The DMSO treated, non-competitive controls, have a slight increase in their Gal4 ChIP signal but overall treatment with DMSO does is not significantly different from the untreated (t_0) control or the competition samples. Therefore, performed this way, there does not appear to be any significant effect by competition, and endogenous Gal4 appears to stably associate with the *GAL1* promoter consistent with observations of the Kodadek group (Nalley et al. 2006).

The induction of *GAL* genes in the presence of 17-β-estradiol is a novel approach that has not been fully characterized. Therefore, I repeated the competition experiments using a strain without competitor Gal4 rather than treating with DMSO as the non-competitive control. Consequently I

treated all samples with 17-β-estradiol (*Figure **2.4***). As in the previous set of experiments, the endogenous Gal4 ChIP signal in the presence of competitor did not significantly deviate from the Gal4 signal prior to competition (t_0). However, when yeast without competitor Gal4 were treated with 17-β-estradiol the Gal4 signal increased significantly (nearly a four-fold increase at one point) rather than remaining flat. This finding indicates that 17-β-estradiol potentially increases the Gal4 signal apart during competition and as such renders the competition experiment invalid. Done properly, competition experiments only change one variable at a time (*i.e.* competitor Gal4 concentration in the nucleus), while keeping the other conditions constant (*i.e.*. the amount of endogenous Gal4). Treatment with 17-β-estradiol, by increasing the Gal4 ChIP signal in the absence of competitor Gal4 violates this basic tenant of carefully designed competition assays.

The odd phenomenon of increasing Gal4 ChIP signals through treatment with 17-β-estradiol is only observed in the context of inducing conditions (*i.e.* galactose media) but not during non-inducing conditions (*i.e.* glycerol-lactic acid media) (*Figure **2.5**)*. When yeast grown in non-inducing conditions are treated with 17-β-estradiol, the Gal4 ChIP signal does not significantly change from initial (t_0) levels of Gal4 when no competitor is present. This is the type of conditions that are necessary to conduct proper competition experiments, and as such it is possible to observe the rapid and dramatic loss of Gal4 from the *GAL1* promoter. Nearly ninety percent of the signal is lost in just the first fifteen minutes of competition. The peculiar effect of 17-β-estradiol on the Gal4 ChIP signal with inducing media but non-inducing media explains why Nalley *et al* observed that Gal4 was stable during inducing whereas it was unstable in non-inducing media (Nalley et al. 2006).

Gal4 Turnover at An Active Locus

The mechanism behind the increased Gal4 ChIP signal as a consequence of treatment with 17-β-estradiol under activating conditions is unknown. If another molecule could bind to the estrogen-binding domain of competitor Gal4 to initiate competition and yet not artificially inflate

Gal4 ChIP signals in the absence of competitor Gal4, then the competition ChIP assay could be salvaged to test if Gal4 stably associates with its promoter. I hypothesized that 4-hydroxy tamoxifen (4-OHT) was possibly such a molecule. Yeasts do have known estrogen binding proteins: *S. cerevisiae* has two old yellow enzymes (Feldman et al. 1982; Burshell et al. 1984) that bind estrogen and the *Candida albicans* homologue of *S. cerevisiae OYE2*, *EBP1* (for estrogen binding protein 1), is an important virulence factor and has been proposed to mediate the metabolic changes necessary for tissue invasion for yeast infections (Skowronski and Feldman 1989; Madani et al. 1994). Significantly, although Oye2, Oye3, and *C. albicans* Ebp1 have a strong affinity for 17-β-estradiol, they do not efficiently bind 4-hydroxy tamoxifen (4-OHT) (Burshell et al. 1984; Madani et al. 1994). Thus, 4-OHT represents a ligand for the human EBD in competitor Gal4 without any known binding proteins in yeast and may therefore be a more suited for a Gal4 competition ChIP assay.

I used 4-OHT to drive the Gal4 competition assay (*Figure **2.6***). Unlike 17-β-estradiol, 4-OHT did not significantly alter Gal4 signals from the initial (t_0) levels of Gal4 in no competitor controls. The increase in Gal4 after fifteen minutes of treatment with 4-OHT in the no competitor samples is not significantly different from the brief momentary increases in Gal4 signal observed at this time point for the DMSO treated control (*Figure **2.3***) or the effect of 17-β-estradiol in non-inducing conditions (*Figure **2.5**)*. Importantly the Gal4 ChIP signal returns to the reference (t_0) levels rather than increasing as much as four fold as is the case for 17-β-estradiol in galactose media *(Figure **2.4***). When competitor Gal4 is present, treatment with 4-OHT leads to a rapid and significant loss of Gal4 at the *GAL1* promoter. Indeed, most of the signal loss occurs by fifteen minutes with nearly two-thirds of the initial (t_0) signal gone. This rapid loss of Gal4 is consistent with the cycloheximide data and corresponds with the result of 17-β-estradiol when the Gal4 signal from yeast with competitor normalized to the Gal4 signal from yeast without the competitor *(Figure **2.7***). Therefore, I conclude that Gal4 does not lock onto chromatin, contrary to what was previously reported (Nalley et al. 2006).

To confirm that the disappearance of Gal4 signal, representing the turnover of endogenous Gal4 from the *GAL1* promoter, was indeed competitively displaced, I also monitored the arrival of competitor Gal4 to the same promoter when 4-OHT drives competition. The association of competitor Gal4 is measured by ChIP using antibodies directed against the N-terminal Myc-epitope tag. ChIP signals from this experiment were expressed as a percentage of Myc-ChIP signal obtained in a Δ*gal4* strain after one hour of competition with 4-OHT representing the maximal binding of competitor that should be observed in the competition experiments (*Figure **2.8***). This experiment demonstrates that the competitor rapidly and efficiently arrives to the *GAL1* promoter even in the presence of endogenous Gal4. The appearance of competitor Gal4 at the promoter is indeed dependent on 4-OHT to start competition, as the Myc-ChIP signal in ethanol treated yeast is not significantly different than strains without competitor. Thus, the elements of a competition experiment are behaving appropriately: endogenous Gal4 signals are relatively unaffected by treatment with 4-OHT alone, and competitor Gal4 arrives at the target promoter at a rate that is not faster than the loss of endogenous Gal4.

Discussion

I have shown that conclusion by the Kodadek group that Gal4, upon activation, stably associates with the *GAL*1 (Nalley *et al.* 2006) is misfounded, based in large part on competitive ChIP data demonstrating the stability of Gal4 with its target promoter. However, these data were based on experiments that used 17-β-estradiol to stimulate competition, which I discovered to have the unintended consequence of increasing the endogenous Gal4 signal when no competitor is present. I have normalized the Gal4 ChIP signal obtained in the presence of competitor Gal4 to that in which no competitor is present. Over the course of a competition assay this leads to a rapid decrease in Gal4 present at the *GAL1* promoter (*Figure **2.7***). This is consistent with competitive ChIP experiments using 4-OHT and ChIP data using cycloheximide treatment. These data, along with the arrival of

competitor Gal4 after treatment with 4-OHT, refute the conclusion that Gal4 stably occupies the *GAL1* promoter. Instead, there is rapid turnover of Gal4 from chromatin, consistent with the expected behavior of a protein non-covalently bound to chromatin and does not preclude a possible role for the proteasome in regulating activator turnover at promoters undergoing active transcription.

One of the unexpected and interesting findings from my work is that 17-β-estradiol increases the Gal4 ChIP signal. The cause is unknown —more Gal4 binding at the promoter, greater accessibility of the Gal4 epitope, or the establishment of a cellular environment that is more amenable to cross-linking are the most likely options. These are each testable, and could result in improved techniques to study Gal4 binding to DNA or even reveal new facets of what has been thought to be a well characterized activator. Also unknown are the proteins through which 17-β-estradiol functions to increase the Gal4 ChIP signal. Based on the previous characterization of *OYE2* and *OYE3* as genes encoding for estrogen binding proteins (Feldman et al. 1982; Burshell et al. 1984), and that yeast with the deletion of either or both genes are viable (Giaever et al. 2002; Odat et al. 2007), these two genes would be best candidates to begin to study how 17-β-estradiol regulates Gal4.

However, as interesting as the surprise effect of 17-β-estradiol may be, I believe that the true significance of this work is clarifying confusion regarding the importance of activator turnover in transcription. Fluorescent microscopy has demonstrated that several activators are dynamic in their movement to and from the areas of active transcription (Hager et al. 2009). However, there are relatively few examples of studies that demonstrate the turnover of transcription factors that compliment and further validate the observations from live cell imaging.

One of the notable examples of ChIP data demonstrating activator turnover is from the work of the Gannon laboratory studying, incidentally, the human estrogen receptor (hERα) (Metivier et al. 2003; Reid et al. 2003). The association of hERα with its target promoters undergoes a cyclical pattern of binding and loss of binding that occurs roughly every forty minutes — substantially longer than the dynamic association of many activators observed using fluorescent labels, but still

demonstrating the point of activator turnover. Furthermore, these studies indicate that the dynamics of hERα and its associated transcription is dependant on the proteasome.

I have not addressed the role of the UPS in the regulation of activator turnover, which is the important future direction of this work. The processes of Gal4 turnover as described by Muratani *et al* (Muratani et al. 2005) proceeds from phosphorylation of Gal4 to form phosphoisoform Gal4**c**, Mdm30 dependent ubiquitylation of Gal4**c**, and presumably proteolysis through the proteasome. Inhibiting these steps in *trans* is not the ideal approach because of significant off-target effects. The kinases required for Gal4**c** formation, Kin28 and Srb10, are important for the initiation of transcription in general. Mdm30 is so named for its regulation of mitochondrial distribution and morphology, which itself is necessary for respiratory competency and regulating galactose metabolism in multiple and potentially complicated processes (Dimmer et al. 2002; Fritz et al. 2003; Neutzner and Youle 2005; Durr et al. 2006; Escobar-Henriques et al. 2006; Cohen et al. 2008). Therefore, if the *cis* elements in Gal4 can be mapped and mutated that regulate Gal4 phosphorylation or ubiquitylation, I would use those forms of Gal4 to study the potential connection of activator turnover and regulation by the UPS. Such *cis* mutations for the phosphorylation of Gcn4 already exist (*gcn4-3T2S*) (Chi et al. 2001; Lipford et al. 2005) and therefore, establishing the competition assay in Gcn4 to study the importance of phosphorylation to activator turnover may prove to be a simpler and more rapid approach than mapping and mutating sites of phosphorylation in Gal4.

cis mutations of proteins that allow ubiquitylation but not proteolytic destruction are rare and poorly understood. Therefore, to study the contribution of the proteasome turnover demands the inhibition of the proteasome. However, the impact of the proteasome on Gal4 mediated transcription has itself been a controversial subject with one group finding that inhibiting the proteasome blocks transcriptional activation (Lipford et al. 2005) and a second group reporting no effect on transcription from proteasome inhibitors (Nalley et al. 2006). I believe that part of the difficulty exists because the level of proteasome inhibition that is achieved through conventional approaches is not adequate to study transcription with sufficient clarity. The development of a better approach to proteasome

inhibition is the purpose of the next chapter in my thesis, and the extension of this new strategy to studying transcription is discussed in chapters four and five.

I have demonstrated that there is indeed turnover of Gal4 on active promoters despite previous reports to the contrary (Nalley *et al.* 2006; Ferdous *et al.* 2007; Archer *et al.* 2008a; Archer *et al.* 2008b; Ferdous *et al.* 2008; Archer and Kodadek 2010) and as such there remains a possible important role for the UPS in regulating activators in the process of transcription.

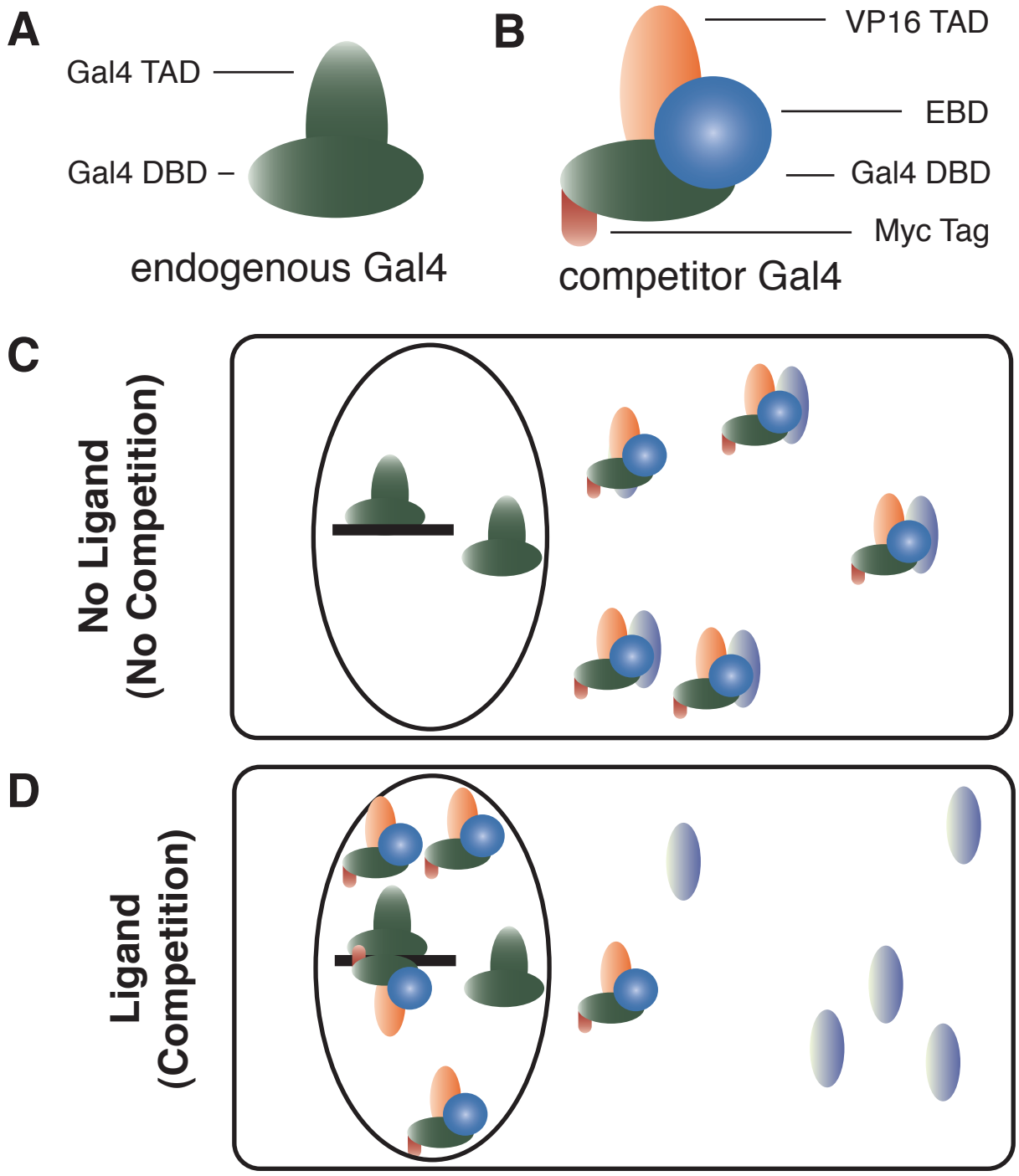

Figure 2.1: Competitive Chromatin Immunoprecipitation. (A) Endogenous Gal4 with the Gal4 DNA binding domain (DBD) and Gal4 transcription activation domain (TAD) shown. (B) Competitor Gal4 with the Gal4 DBD, VP16 TAD, shown with the Myc epitope tag and estrogen binding domain (EBD). (C) Competitor Gal4 is sequestered from the nucleus by Hsp90 and endogenous Gal4 binds to its target UAS. (D) Competition is triggered through the addition of a ligand for the EBD.

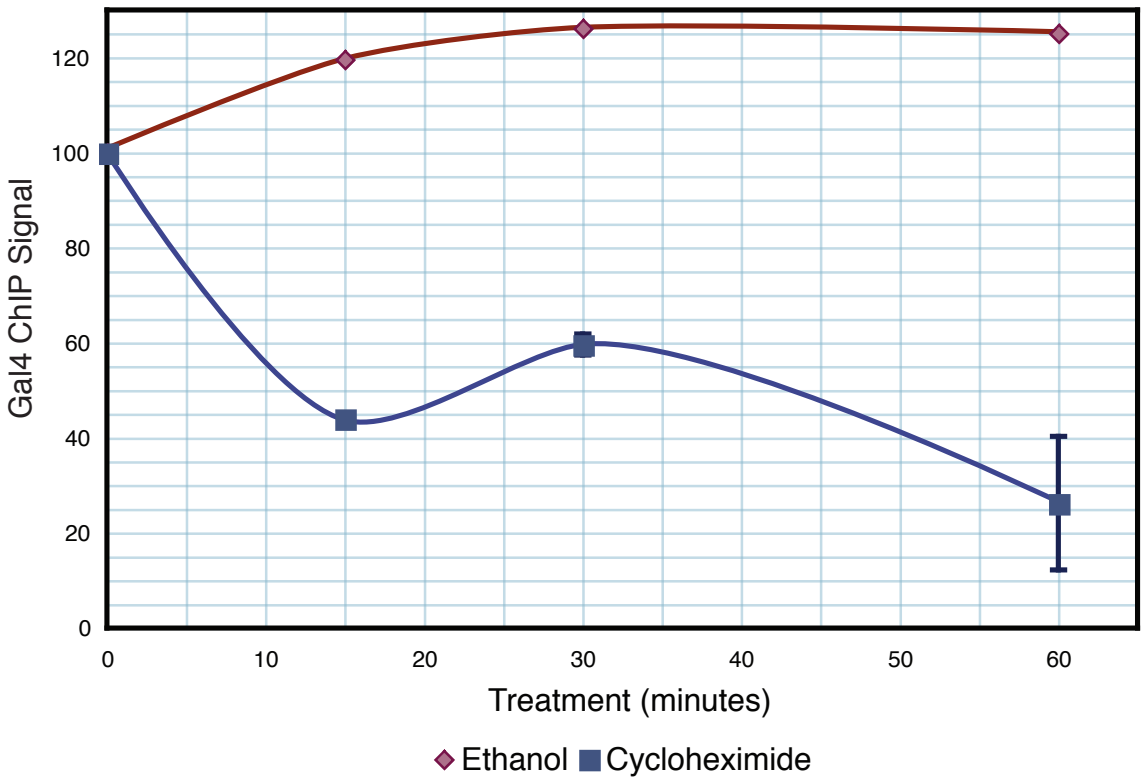

Figure 2.2: Cycloheximide treatment leads to a decrease in Gal4 at the promoter. Gal4 ChIP signal at the *GAL1* promoter expressed as a percentage of non-treated signal as a function of cycloheximide treatment (blue-squares) or ethanol mock treatment (red-diamonds). n = 2; SEM; p = 0.004.

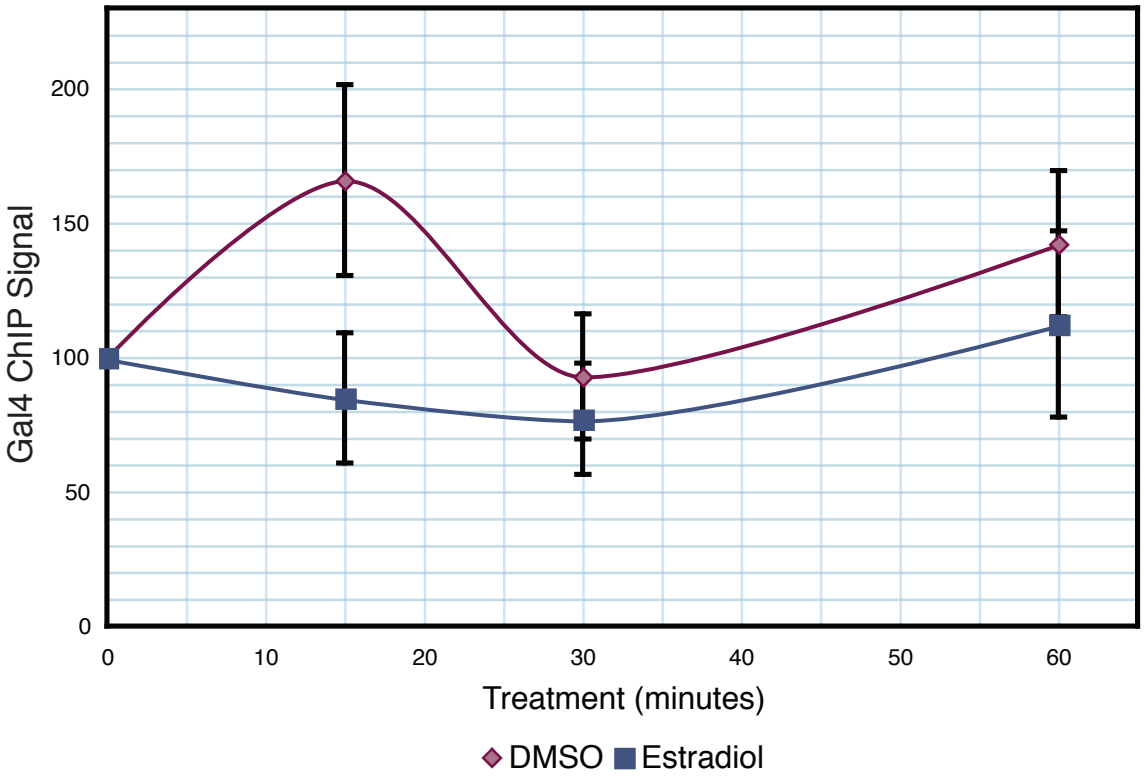

Figure 2.3: Competitive ChIP Shows Gal4 Stability. Gal4 ChIP signal at the GAL1 promoter as a percentage of signal at t_0 of competition as a function of competition time in minutes. BY4741 with competitor Gal4 had competition induced through 17-beta-estradiol (Estradiol; blue-squares) or went through a mock competition with DMSO as a vehicle control (DMSO; red-diamonds). n = 4; SEM; p = 0.06.

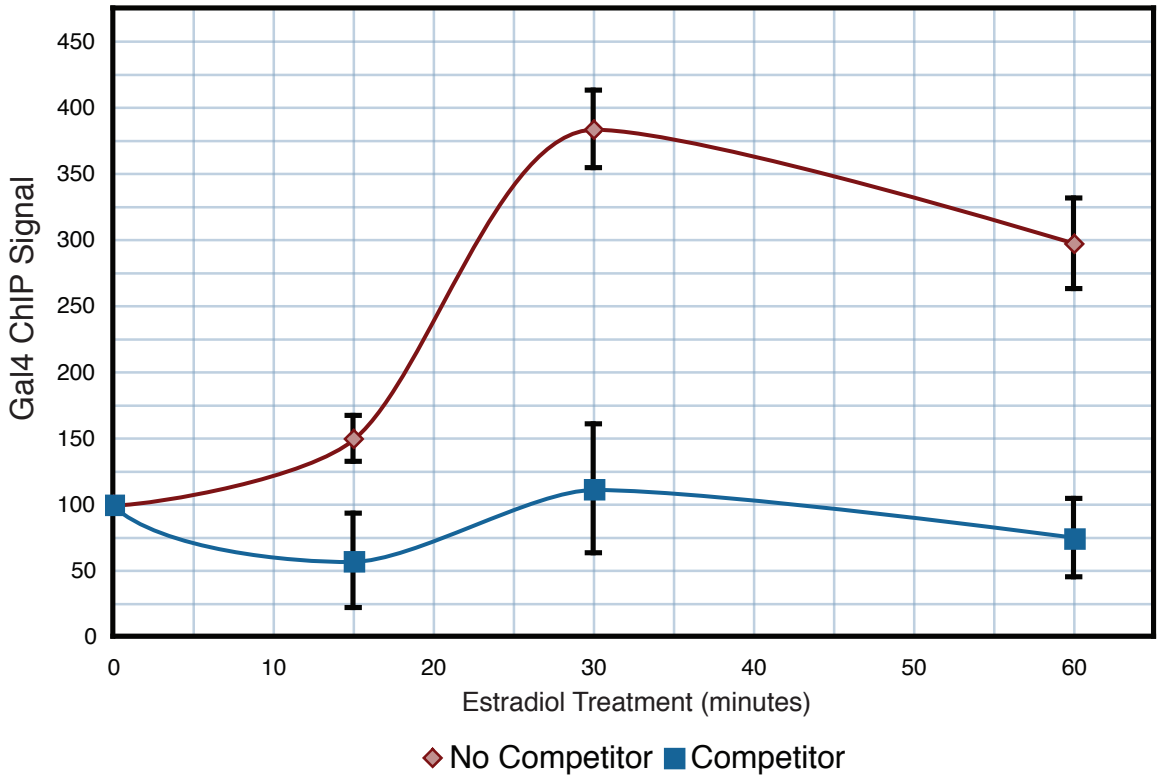

Figure 2.4: Competitive ChIP under Inducing Conditions is Affected by Estradiol (No Competitor Control). Gal4 ChIP signal at the *GAL1* promoter as a percentage of signal at t_0 of competition as a function of competition time in minutes. BY4741 without competitor Gal4 (No Competitor; red-diamonds) compared with BY4741 with competitor Gal4 (Competitor; blue squares). n = 3; SEM; p = 0.004.

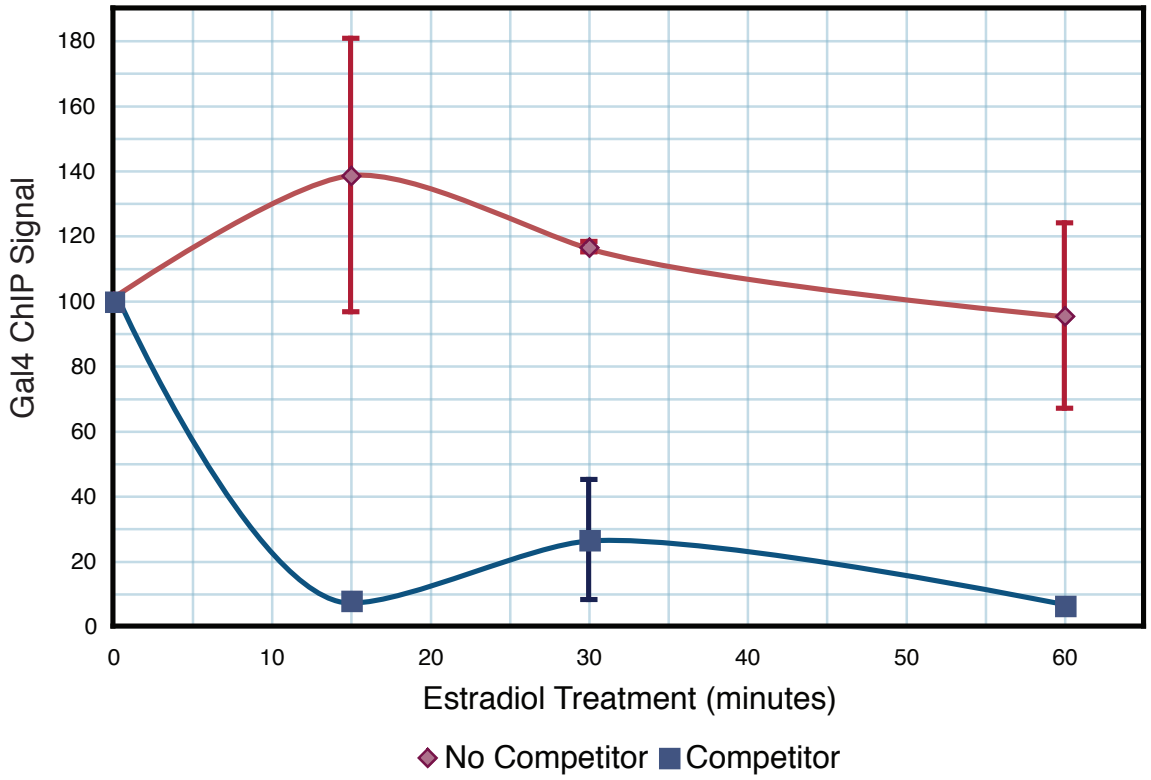

Figure 2.5: There is no estradiol induced Gal4 ChIP artifact in non-inducing conditions. Gal4 ChIP signal at the *GAL1* promoter as a percentage of signal at t_0 of competition as a function of 17-beta-estradiol treatment in minutes. BY4741 without competitor Gal4 (No Competitor; red-diamonds) compared to BY 4741 with competitor Gal4 (Competitor; blue-squares). n =2; SEM; p = 0.007.

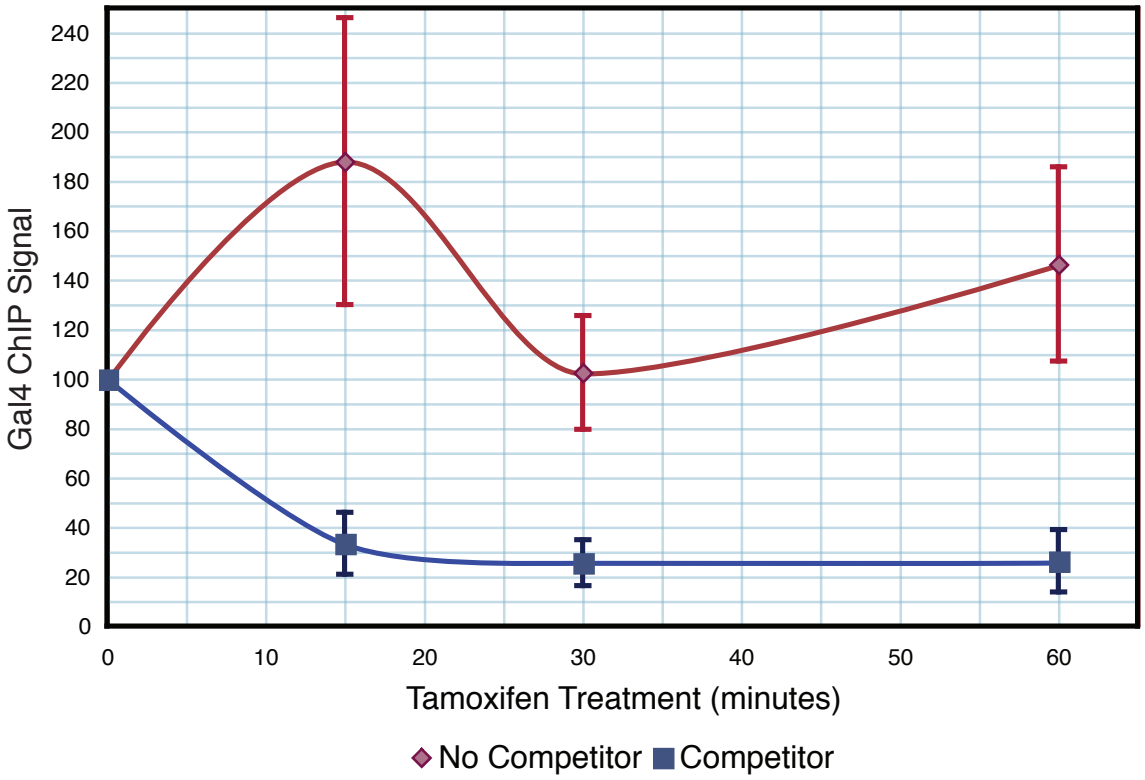

Figure 2.6: Competitve ChIP under inducing conditions using 4-hydroxy tamoxifen. Gal4 ChIP signal at the *GAL1* promoter as a percentage of signal at t_0 of competition as a function of competition time in minutes. BY4741 without competitor Gal4 (No Competitor; red-diamonds) compared with BY4741 with competitor Gal4 (Competitor; blue-squares). n = 3; SEM; p = 0.002.

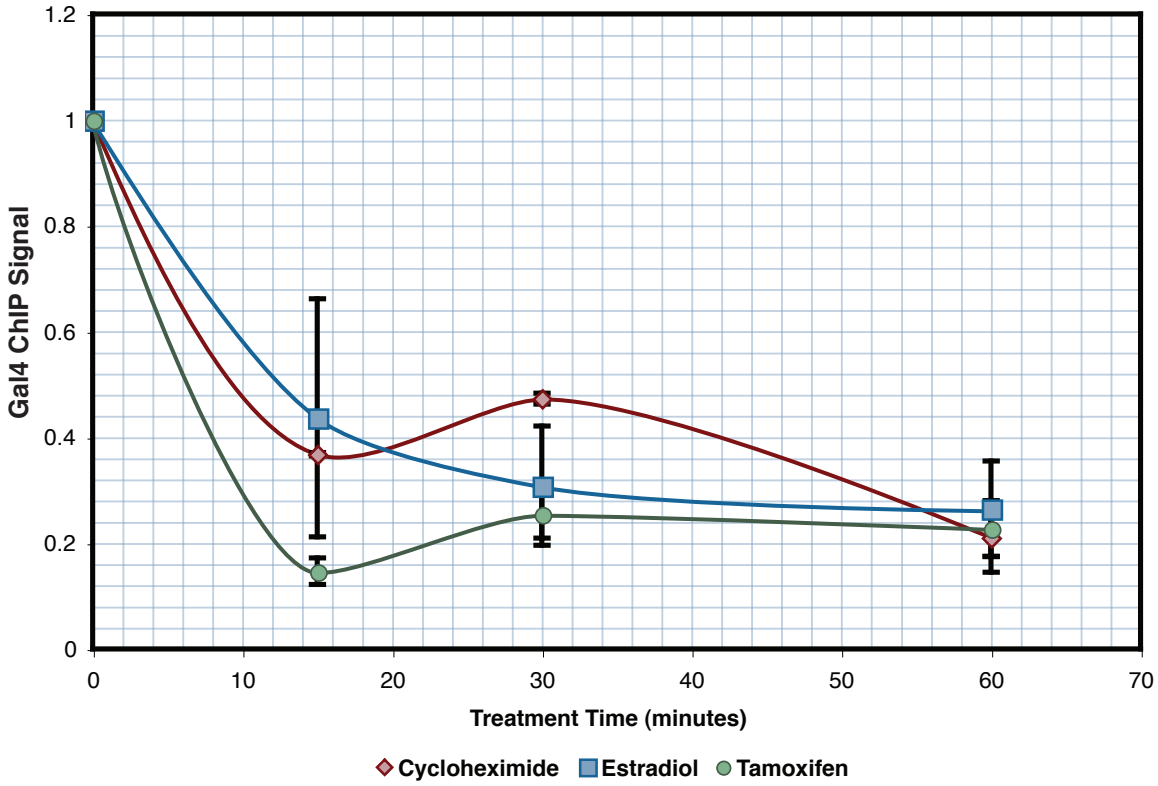

Figure 2.7: Normalization of Gal4 ChIP data. Gal4 ChIP signal at the *GAL1* promoter as a function of time in minutes. Cycloheximide treatment as a percentage of ethanol control (red-diamonds; n = 2). Competitive ChIP with 17-beta-estradiol as the competition trigger with the signal of endogenous Gal4 in the presence of competitor Gal4 as a percentage of endogenous Gal4 signal without competitor Gal4 (blue-squares; n = 3). Competitive ChIP with 4-hydroxy tamoxifen as the competition trigger with the signal of endogenous Gal4 in the presence of competitor Gal4 as a percentage of endogenous Gal4 signal without competitor Gal4 (green-circles; n = 3). SEM. ANOVA: p = 0.69.

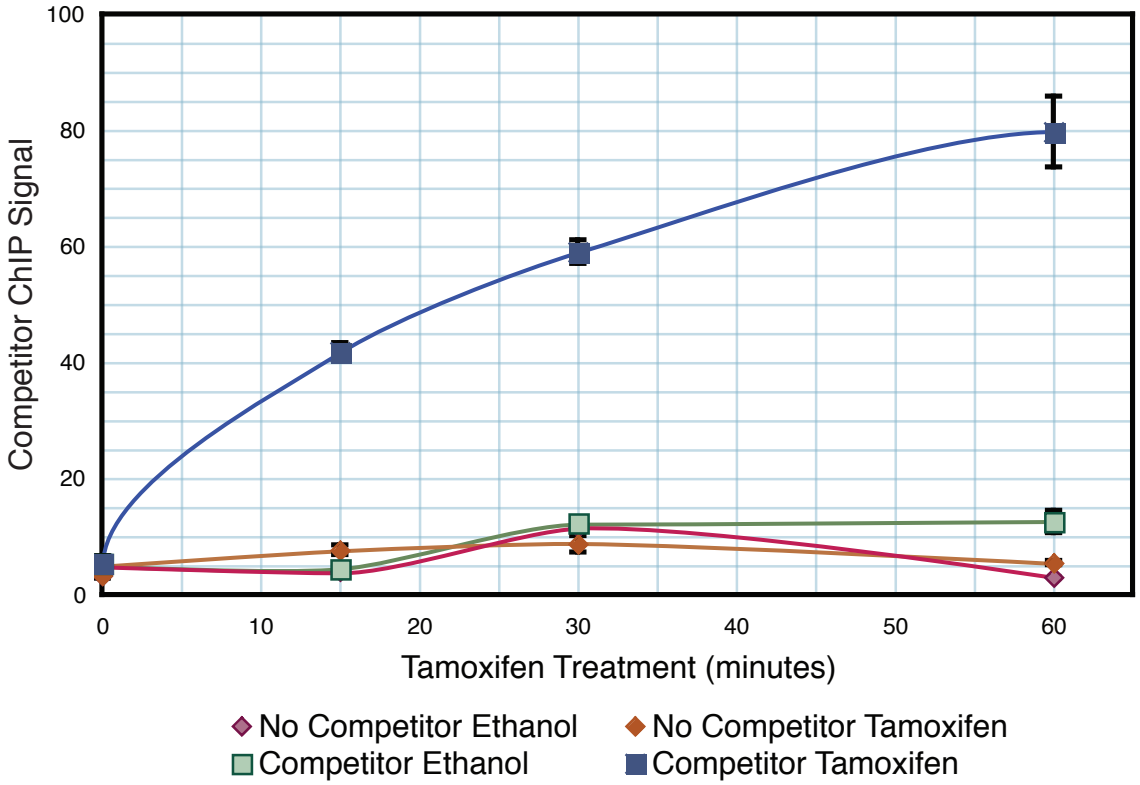

Figure 2.8: Competive ChIP under inducing conditions using 4-hydroxy tamoxifen. Myc ChIP signal (competitor Gal4) at the *GAL1* promoter as a percent of competitor binding after one hour in a delta *gal4* strain as a function of competition time in minutes. BY4741 without competitor Gal4 (No competitor; diamonds) compared with BY4741 with competitor Gal4 (Competitor; squares). n = 2; SEM; p = 0.04.

Chapter Three: Increased Sensitivity to Proteasome Inhibition in *S. cerevisiae*

Introduction

In this chapter I will discuss my approach to studying the proteasome by combining chemical inhibitors with genetic inactivation of the proteolytic subunits. Small molecule inhibitors of the proteasome are powerful tools to study the importance and mechanisms of the ubiquitin proteasome system (UPS) in many diverse cell pathways. These drugs are important, not only as tools for research but also as naturally synthesized compounds and as useful pharmacological agents. Bortezomib (Velcade/PS-341) has become a valuable proteasome inhibitor for the treatment of multiple myeloma (Richardson *et al*. 2005). In addition to bortezomib, at least four other proteasome inhibitors (NPI-0052, carfilzomib/PR-171, CEP-18770, and MLN9708) are in phase I or phase II clinical trials for the treatment of various cancers.

However, the typical approach of inhibiting the proteasome in *S. cerevisiae* has produced several instances in which the physiological effects are much smaller than would be expected given the importance of the proteasome in numerous cell processes such as cycle progression (King *et al*. 1996; Zachariae and Nasmyth 1999), DNA repair (Krogan *et al*. 2004; Daulny and Tansey 2009), transcription (Auld and Silver 2006; Collins and Tansey 2006), protein quality control (Goldberg 2003; Kostova and Wolf 2003), and organelle distribution (Campbell *et al*. 1994). For example, proteomic profiling of ubiquitylated proteins routinely uses proteasome inhibitors to stabilize ubiquitylated population of proteins. However, these proteome wide data sets of ubiquitylated proteins routinely fail to detect cyclins (Mayor *et al.* 2007), which are classically unstable proteins regulated by the UPS. This is consistent with the notion that inhibition of the proteasome through small molecule inhibitors is often incomplete.

A second example in which the physiological response to proteasome inhibition is much smaller than one would expect *a priori* that inhibition of the proteasome would essentially halt growth. However, treatment with MG132 or bortezomib has little impact on cellular proliferation

(Lee and Goldberg 1996; Fleming *et al.* 2002). In contrast to the resistance of yeast to proteasome inhibition, inactivation of the only E1 activating enzyme, Uba1, rapidly terminates yeast growth (Ghaboosi and Deshaies 2007).

If I am going to test the prediction that inhibiting the proteasome will inhibit activation of transcription then it will be useful to have a yeast strain in which the effects of proteasome inhibitors is unambiguous.

My strategy to improve the responsiveness of *S. cerevisiae* to proteasome inhibition was to target all three of the proteasome proteolytic subunits: the β1 subunit Pre3 with its associated caspase-like activity, the β2 subunit Pup1 with its associated trypsin-like activity, and the β5 subunit Pre2 with chymotrypsin-like associated activity (Arendt and Hochstrasser 1997; Heinemeyer et al. 1997; Jager et al. 1999). Proteasome inhibitors have a marked bias towards a single proteolytic subunit, most commonly the chymotryptic subunit Pre2 (Lee and Goldberg 1996; Bogyo et al. 1998; Elofsson et al. 1999; Kisselev and Goldberg 2001; Kisselev et al. 2006; Groll et al. 2009). The bias of proteasome inhibitors towards the chymotryptic activity of the proteasome has not been typically considered a problem because the chymotryptic activity has been presumed to be the primary and rate-limiting step in proteasome mediated proteolysis (Kisselev et al. 2006). Although the chymotryptic activity is commonly considered to be the most significant proteolytic activity, the other two proteases presumably make important contributions in the cell. Analysis of the mammalian proteasome revealed a differential requirement for each proteasome subunit depending on the substrate (Kisselev et al. 2006). It would be very surprising if the same were not true for the yeast proteasome too, considering the similarity of the yeast and mammalian proteasomes. Therefore I have combined the chemical inhibition of the proteasome with genetic inactivation of the two non-chymotryptic proteasome proteases.

Genetically inactivating the proteasome proteases is straightforward —each protease depends on a single catalytic threonine. Although the genes encoding each proteasome protease are all essential (as are most other proteasome genes (Giaever et al. 2002)) the loss of any one catalytic site

is not lethal. In fact yeast without the *PUP1* and *PRE3* catalytic threonines are also viable making it possible to have the proteasome operate entirely on through chymotryptic activity, the activity towards which most proteasome inhibitors are directed against. Therefore, treatment with an inhibitor, such as MG132, should acutely shutdown the remaining proteolytic activity of the proteasome.

One other necessary step to generate yeast that are hyper-sensitive to proteasome inhibition is to ensure adequate concentration of MG132 in the cell. The effective intracellular concentration of proteasome inhibitors is not very high in wild type yeast for three main reasons: ineffective permeability of the inhibitors against the yeast cell wall (Lee and Goldberg 1996; Liu et al. 2007), efflux of the inhibitors under the control of pleiotropic drug resistance genes (Fleming et al. 2002), and up regulation of the proteasome subunits through the transcription activator Rpn4 in response to proteasome inhibition (Xie and Varshavsky 2001; Fleming et al. 2002; Ju et al. 2004; Wang et al. 2008; Wang et al. 2010). Increasing the effective concentration by deleting a single pleiotropic drug response gene, *PDR5*, is sufficient to sensitize the yeast to many proteasome inhibitors and permits future study of the transcription of the proteasome genes themselves, which if transcription activation typically depends on proteolysis represents an interesting counter example of a transcription activator that is activated by inhibiting proteolysis.

Experimental Procedures

Yeast Strains

Strains used in this study are listed in table 3.1. The strains MHY1177 and MHY178 (Arendt and Hochstrasser 1999), were a gift from Mark Hochstrasser. I deleted *PDR5* from these strains using gene replacement with *KanMX6* by homologous recombination using sequences flanking the *PDR5* ORF. I synthesized the knockout cassette by PCR using pYM1 (Knop et al. 1999) as a template, and transformed the cassette as described by Gietz and Woods (Gietz and Woods 2002). Correct targeting was validated by PCR. The resulting strains are GAC201 (*PUP1PRE3pdr5*) and GAC202

(*pup1pre3pdr5*). For α-arrest experiments, GAC201 and GAC202 were converted to the **a** mating type by expressing the *HO* endonuclease from a *URA3* selectable vector (Ycp50-*HO* (Krishnamoorthy et al. 2006); gift from Shelly Berger) followed by counter-selection with 5-FOA (*US Biological*). I verified mating type by testing growth of *bar1* yeast (RC634 (Chan and Otte 1982); gift from Brehon Laurent) in the presence of patches of potentially switched yeast and by growth sensitivity to α-factor (*Zymo Research*). I confirmed that the *MAT***a** and *MATa* yeast had similar growth rates and flow cytometric profiles following nocodazole block and release.

Table 3.1: Yeast Strains Used

Strain	**Genotype**	**Source**
BY4742 Δ*pdr5*	Matα *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0 pdr5*Δ*::KanMX6*	This study
MHY1177	Matα *his3-11 leu2-3,112 ura3-52 lys2-801 trp1-1 pre3*Δ*::HIS3 pup1*Δ*::leu2::HIS3* [pRS317-*PUP1*] [YCplac22-*PRE3*] gal$^-$	(Arendt and Hochstrasser 1999)
MHY1178	Matα *his3-11 leu2-3,112 ura3-52 lys2-801 trp1-1 pre3*Δ*::HIS3 pup1*Δ*::leu2::HIS3* [pRS317-*pup1T30A*] [YCplac22-*pre3T20A*] gal$^-$	(Arendt and Hochstrasser 1999)
GAC201	Matα *his3-11 leu2-3,112 ura3-52 lys2-801 trp1-1 pre3*Δ*2::HIS3 pup1::*Δ*leu2::HIS3* [pRS317-*PUP1*] [YCplac22-*PRE3*] gal^{--}	This study
GAC202	Matα *his3-11 leu2-3,112 ura3-52 lys2-801 trp1-1 pre3*Δ*::HIS3 pup1*Δ*::leu2::HIS3* [pRS317-*pup1T30A*] [YCplac22-*pre3T20A*] gal$^-$ ρ^0	This study
GAC201**a**	Mat**a** *his3-11 leu2-3,112 ura3-52 lys2-801 trp1-1 pre3*Δ*::HIS3 pup1*Δ*::leu2::HIS3* [pRS317-*PUP1*] [YCplac22-*PRE3*] gal$^-$	This study
GAC202**a**	Mat**a** *his3-11 leu2-3,112 ura3-52 lys2-801 trp1-1 pre3*Δ*::HIS3 pup1*Δ*::leu2::HIS3* [pRS317-*pup1T30A*] [YCplac22-*pre3T20A*] gal$^-$ ρ^0	This study
RC634	Mat**a** *rme1 ade2-1 ura1 his6 met1 can1 cyh2 sst1-3*	(Chan and Otte 1982)
RJD3269	Mat**a** *can1-100 leu2-3,112 his3-11,15 trp1-1 ura3-1 ade2-1 uba1*Δ*::KanMX6* [pRS313-*uba1-204-His*]	(Ghaboosi and Deshaies 2007)

Table 3.2 Plasmids Used

Plasmid	**Features**	**Source**
pYM1	*KanMX6* tag	(Knop et al. 1999)
Ycp50-*HO*	*HO* mating type switching	(Krishnamoorthy et al. 2006)

Proliferation assay

I grew *S. cerevisiae* cultures at 30°C from an $A_{600\ nm}$ 0.2 in 10 ml YPAD with either 50 μM MG132 (*American Peptide*) or an equivalent volume DMSO (*Sigma*). At the indicated time points following treatment, I measure the $A_{600\ nm}$ from 1 ml of each sample.

For the MG132 titration experiment, 10 ml YPAD cultures were started at an $A_{600\ nm}$ 0.5 with the indicated concentrations of MG132. Following twenty-four hours of treatment I measured the $A_{600\ nm}$ from 1 ml of each sample. IC_{50} was approximated by first calculating the percent inhibition of the maximum possible inhibition (assuming no change in absorbance is that maximum) and then calculating the best fit curve (least squares) of the equation $I = (Max \cdot MC) \div (IC_{50} + MC)$ to the data where *I* is the percent inhibition for a given concentration of MG132 (*MC*) and *Max* is maximum inhibition.

Cell cycle assays

GAC201**a** and GAC202**a** were arrested in G1 using 30 μM α-factor in 10 ml YPAD cultures for two hours at 25°C. Samples were then treated with an additional 15 μM α-factor and 50 μM MG132 (or equivalent volume of DMSO) for another hour at 25°C. I collected 1ml of culture for the "time zero" (t_0) sample. I released the remaining culture from arrest by washing twice with YPAD before growing as a 9 ml YPAD culture with 50 μM MG132 or equivalent volume DMSO at 30° C. I collected 1 ml of culture at each of the indicated time points.

I arrested GAC201 and GAC202 in G2/M by treating 10 ml YPAD cultures with 150 μg nocodazole (Sigma) for 90 minutes at 30°C. I then treated the cultures with an additional 75 μg nocodazole and 50 μM MG132 (or equivalent volume DMSO) for an hour at 30°C. I collected 1ml of culture for the "time zero" (t_0) sample. I released the remaining culture from arrest by washing twice with YPAD before growing as a 9 ml YPAD culture with 50 μM MG132 or equivalent volume DMSO at 30° C. I collected 1 ml of culture at each of the indicated time points.

From each of the arrested and released samples and from asynchronously growing yeast populations, I resuspend the cells in 70% ethanol and fixed at 4°C overnight. These cells were then washed with water, treated for 12 hours at 37°C with 1 μg RNase, DNase free (*Roche*), sonicated, treated for 2 hours at 42°C with 500 μg proteinase K (Roche), and stored in a 50 mM Tris-HCl (pH 7.5) solution. 1 x 10^6 cells were diluted in 1 ml of SYBR gold solution (*Invitrogen*). I counted cells and DNA content using a LSR II cell analyzer (*BD Biosciences*).

Ubiquitylation assay

I grew 15 ml YPAD cultures treated with 50 μM MG132 for the indicated time. From these cultures I extracted proteins by boiling in EZ buffer (60 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 2% 2-mercaptoethanol). I used the Bio-Rad protein assay to determine protein concentration to run 50 μg protein on 8% polyacrylamide gels. These gels were transferred onto a 0.45 μM nitrocellulose membrane (*Whatman*). 1:1,000 anti-ubiquitin antibody (MAB1510, *Millipore*) and 1:15,000 anti-mouse-HRP (*GE Health Care*). I checked that equivalent amounts of protein were loaded using Ponceau S staining of the membrane.

Results

Combined genetic and chemical inhibition of the proteasome prevents cell proliferation

Although proteasome inhibitors are useful tools to study the mechanism and biology of the proteasome, I have been concerned that the physiological response of *S. cerevisiae* to MG132 is too subtle. For example, I grew BY4742 yeast with a deletion of the pleiotropic drug response gene *PDR5* in the presence or absence of 50 μM MG132, the typical concentration of proteasome inhibitor used in *S. cerevisiae* (*Figure **3.1***). Under these conditions the proliferation of yeast does not halt. Treatment with MG132 did slow the initial growth for the first eight hours of treatment after which, although there is nearly a two hour delay between when DMSO treated yeast reach a given $A_{600\ nm}$

and when MG132 treated yeast reach that optical density, the shape of the growth curves a very similar indicating similar rates of proliferation after eight hours of MG132 treatment. Most significantly, this treatment did not halt growth. Similar results have been observed in MG132 treated yeast in increased drug uptake through *ISE1* deletion (Lee and Goldberg 1998) or in bortezomib treated Δ*pdr5* yeast (Fleming *et al.* 2002). However, inactivation of the first step of the UPS, the E1 activating enzyme *uba1-204*, results in rapid and dramatic growth arrest (Ghaboosi and Deshaies 2007), supporting the conclusion that MG132 alone is insufficient to fully inhibit the proteasome.

Therefore, I developed a new strategy combining chemical inhibition of the chymotryptic subunit, Pre2, with genetic inactivation of the other two proteolytic proteasome subunits, Pup1 and Pre3, for the purpose of improving proteasome inhibition. The new strain with *pup1-T30A*, *pre3-T20A* mutations and deletion of *PDR5* (*pup1pre3pdr5*) proliferated slower than its related *PUP1PRE3pdr5* strain (*Figure **3.2,*** compare the blue shaded squares to the green shaded circles), consistent with the previous report that inactivating Pup1 and Pre3 by replacing those catalytic threonines to alanines (Arendt and Hochstrasser 1999). (This effect may be due to this strain being a petite mutant as discovered by genome-wide analysis of transcription, see *Chapter Five*). Furthermore, in the context of the *pup1-T30A* and *pre3-T20A* mutations, the effect of MG132 inhibiting proliferation was striking as there was comparatively little increase in $A_{600\ nm}$ over the sixteen hours of treatment (*Figure **3.2***, orange solid circles). These findings support two conclusions. First, the standard use of the proteasome inhibitor MG132 does not fully inhibit the proteasome. Second, although the chymotryptic activity is considered the most important proteasome protease and as such is the best characterized and most commonly targeted of the proteasome subunits, the other to proteolytic enzymes make important contributions to the cell —at least as measured by proliferative growth.

I tested the dose sensitivity of the *pup1pre3pdr5* to MG132 to determine if the growth defects observed at 50 μM concentrations of MG132 represented an appropriate level of inhibitor to use (*Figure **3.3***). Titration of MG132 from 10 μM to 100 μM concentrations did not significantly change

proliferation of *PUP1PRE3pdr5* yeast over the course of one day of treatment. A similar result in a Δ*ise1* strain was previously reported with concentrations extending to 200 μM (Lee and Goldberg 1998). However, in the *pup1pre3pdr5*, the change in $A_{600\ nm}$ after twenty-four hours of treatment was significant. With as little as 10 μM MG132 there was a marked decrease in cell proliferation that rapidly decreased to an average of two cell divisions in a twenty-four hour period. The titration of MG132 over this scale permits for the calculation of an approximate IC_{50} for MG12 in the *pup1pre3pdr5* strain as rough 24 μM. This indicates 50 μM MG132 is a reasonable level of proteasome inhibitor to target the *pup1pre3pdr5* proteasome.

Inhibition of the proteasome impairs progression through the cell cycle

If treatment of yeast with MG132 in the genetic context of *pup1-T30A* and *pre3-T20A* mutations results in substantial decrease in grow rates of *S. cerevisiae*, then a reasonable question to address is what stage or stages of the cell cycle were inhibited by MG132. Some of the classic examples of protein ubiquitylation and destruction involve the G1 and mitotic cyclins (King et al. 1996; Zachariae and Nasmyth 1999), which would suggest multiple stages of the cell cycle responsible for the growth arrest for *pup1pre3pdr5* cells treated with MG132. Likewise, inactivating the E1 ubiquitin activating enzyme *uba1-204* arrests cells throughout the cell cycle (Ghaboosi and Deshaies 2007). To test that cell cycle arrest occurred at multiple steps in the cell cycle I labeled the DNA of yeast using SYBR gold and counted the DNA content per cell using flow cytometry.

I studied the effects of proteasome inhibition on asynchronous populations after one hour of treatment with proteasome inhibition (***Figure 3.4***). Comparing cytometric profiles (***Figure 3.4 A***) and counts of cell with 1n (G_1), 2n (G_2/M), or intermediate (S) levels of DNA (***Figure 3.4 B***), I do not observe any dramatic changes. The loss of Pup1 and Pre3 in *pup1pre3pdr5* may lead to a decrease in the population of cells in S phase regardless of treatment with MG132. Additionally, there may be an increase in the 1n DNA content cell population of *pup1pre3pdr5* yeast treated with DMSO compared to *PUP1PRE3pdr5* yeast, and a corresponding decrease in 2n DNA content. But with this effect not

reproduced in MG132 treated yeast, these findings may not be significant. In general analysis of the asynchronous population does not reveal any particular phase of the cell cycle in which the proliferative arrest occurs.

Next, I synchronized the yeast populations by arresting in G2/M phase using nocodazole (*Figure **3.5***). Measuring the changes in DNA content after release from nocodazole can reveal the ability of yeast to exit past this stage of the cell cycle. Once again, in the *PUP1PRE3pdr5* yeast, treatment with MG132 has negligible effect on cell cycle progression. In *pup1pre3pdr5* yeast, loss of the Pup1 function or Pre3 function or both leads to slower progression out of 2n DNA content and into 1n DNA content. Treatment with MG132 seems to result in either slightly slower G2/M exist in *pup1pre3pdr5* than treatment with DMSO or a relative flat progression through the cell cycle.

I also synchronized the yeast populations in G_1 with α-factor mating pheromone (*Figure **3.6***). When released from α-factor induced arrest, treatment of *PUP1PRE3pdr5* yeast with MG132 produced a slight lag in rate at which cells progressed from G_1 into later stages of the cell cycle. In contrast, loss of Pup1 and Pre3 function in DMSO treated *pup1pre3pdr5* yeast had a higher initial level of cells with 1n DNA content, but as the shape of the curve for progressive loss of 1n DNA content is similar to DMSO treated *PUP1PRE3pdr5* (*Figure **3.6 B***) the rate of cell cycle progression from 1n to 2n DNA content is not dependent on Pup1 and Pre3 activity. Inhibition of the chymotryptic site with MG132 and loss of Pup1 and Pre3 function combine to pause the cells mostly in G_1 but there is a fraction of cells that arrested in G_2 instead (23.8 ± 0.7%; 95% CI).

These data are consistent with the notion that impairment of the UPS in general, and proteolysis in particular, prevents the efficient progression through the cell cycle. Furthermore, the progression of through the various stages seem to highlight not only the synergistic effects of inhibiting the chymotryptic activity in *pup1pre3pdr5* yeast but also the different relative effects of MG132 and loss of Pup1 and Pre3 activity. Cells released from α-arrest were not greatly inhibited by loss of Pup1 and Pre3 function alone but were affected by MG132 treatment in *PUP1PRE3pdr5* yeast

(*Figure* ***3.6***). In contrast, treatment of MG132 alone had negligible impact on the release from nocodazole-induced block but *pup1pre3pdr5* exited nocodazole arrest more slowly (*Figure* ***3.5***).

Inhibition of the proteasome accumulates ubiquitylated proteins

Presumably, inhibiting the proteasome by targeting all three of its proteolytic activities delays proliferation and progression through the cell cycle by stabilizing unstable protein targets. It should, therefore, be possible to detect the accumulation of high molecular weight ubiquitylated proteins when the proteasome is inhibited at all three of its proteolytic sites, to an extent greater than that observed by treating with MG132 alone or by inactivating *PUP1* and *PRE3* genetically. Compared to untreated *PUP1PRE3pdr5* yeast, the loss of the tryptic and caspase-like activity increases the detection of ubiquitin conjugated proteins by western blot using anti-ubiquitin antibodies (*Figure* ***3.7*** compare lanes 1 and 6). Treating yeast with MG132 increases the accumulation of ubiquitylated proteins in *PUP1PRE3pdr5* yeast, which is often used demonstrate the efficacy of MG132 treatment in yeast. However MG132 treatment increases the accumulation of ubiquitylated proteins and to a greater extent in *pup1pre3pdr5* strains than in *PUP1PRE3pdr5* (*Figure* ***3.7***). Therefore, in addition to the chymotryptic activity, at least one of either the caspase-like or tryptic activities of the proteasome contributes to the normal processing of the ubiquitin conjugated proteins.

Discussion

I have developed a new strain of yeast with increased sensitivity to proteasome inhibitors by combining chemical inhibition of the chymotryptic site with genetic inactivation of the tryptic and caspase-like subunits to simultaneously target all three of the proteasome proteases. This approach elicits more dramatic effects than treatment with MG132 alone, and establishes that previous work with proteasome inhibitors need to be interpreted carefully because the use of proteasome inhibitors does not necessarily mean that the proteolytic ability of the proteasome is fully shutdown. Failure to

detect significant changes after treatment with a proteasome inhibitor should not be interpreted as proof that there is not a proteolytic role for that observed process. I have shown that there is in fact significant proteolytic ability within at least one, if not both, of the Pup1 and Pre3 subunits. Presumably it is this remaining proteolytic activity that prevents MG132 treatment alone from arresting proliferation. Indeed, the loss of Pup1 and Pre3 activity has a significant impact on yeast proliferation, cell cycle progression, and accumulation of ubiquitylated species. Because I have studied the loss of both subunits at once, the relative contribution of each subunit remains an important area for future investigation.

For my purposes I see this strain as valuable tool for studying the intersection of the proteasome and transcription. It is not a useful strain to study *GAL* inducible genes, because this strain is galactose negative and is not rescued by expression of wild-type *GAL2*, a common basis for galactose negative laboratory strains (Winston et al. 1995). However, with many other inducible genes that are potentially regulated by the UPS (Lipford and Deshaies 2003; Muratani and Tansey 2003; Lipford et al. 2005), this strain is a valuable resource to study transcription.

This new strain of yeast establishes a valuable resource for the yeast community. I have only begun to highlight the impact of the proteasome on proliferation, cell cycle progression, and the stability of ubiquitylated proteins. The later observation suggests that this strain might be particularly amenable to proteomic analysis of ubiquitylated proteins. The absence of classically unstable proteins, such as cyclins, from ubiquitylated protein data sets is a striking example of the incompleteness of these data sets.

The combination of a demonstrable growth defect and ability to use lower concentrations of proteasome inhibitor make this strain amenable to using plates to study the effects of proteasome inhibitors such as MG132, YU101, epoximycin, and carfilzomib (*personal communication*, Tara Gomez and Raymond Deshaies). One application of these plates is to screen other genes or chemical compounds that enhance or suppress the ability to grow in the presence of proteasome inhibitors. The

use of other proteasome inhibitors —YU101, epoximycin, and carfilzomib —demonstrates that this strain also has increased sensitivity to other chymotryptic-biased inhibitors.

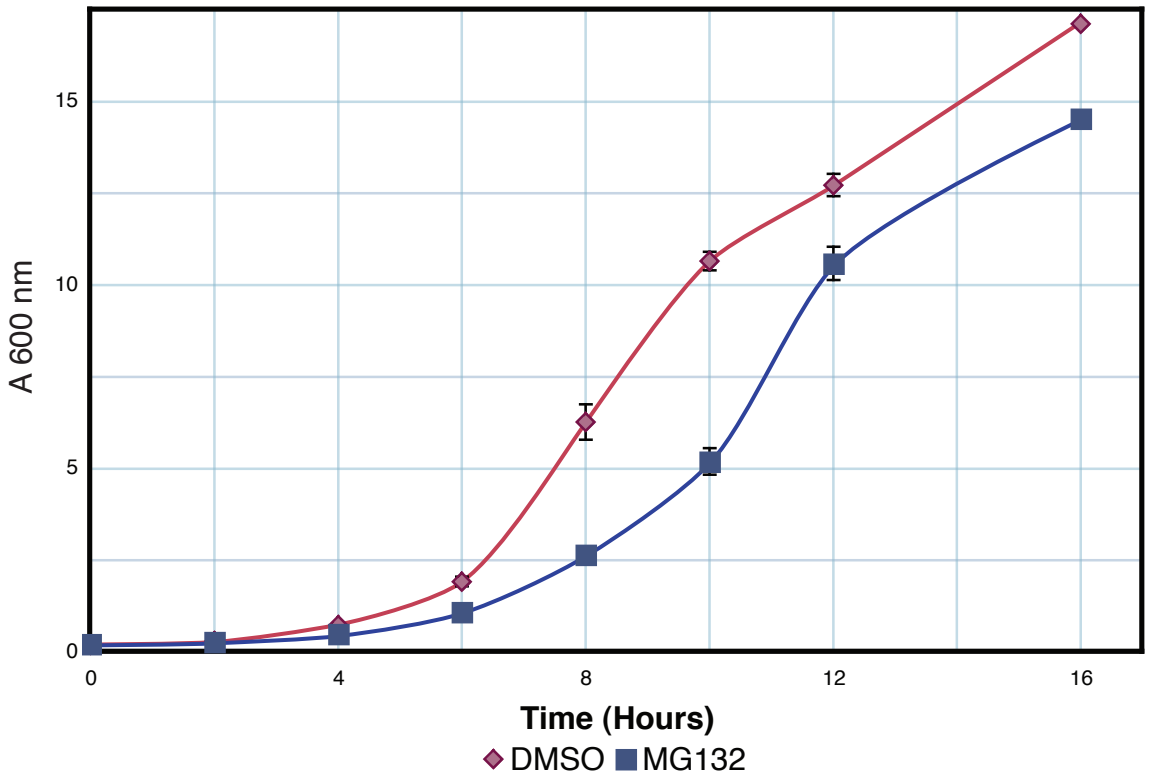

Figure 3.1: MG132 Does Not Halt The Proliferation Of *Saccharomyces cerevisiae*. BY474 Δ*pdr5* yeast treated with either DMSO (Red Diamonds) or 50 µM MG132 (Blue Squares). Absorbance at 600 nm was measured for a sample from each culture at the indicated times post treatment. n = 3; SEM; p = 0.0004.

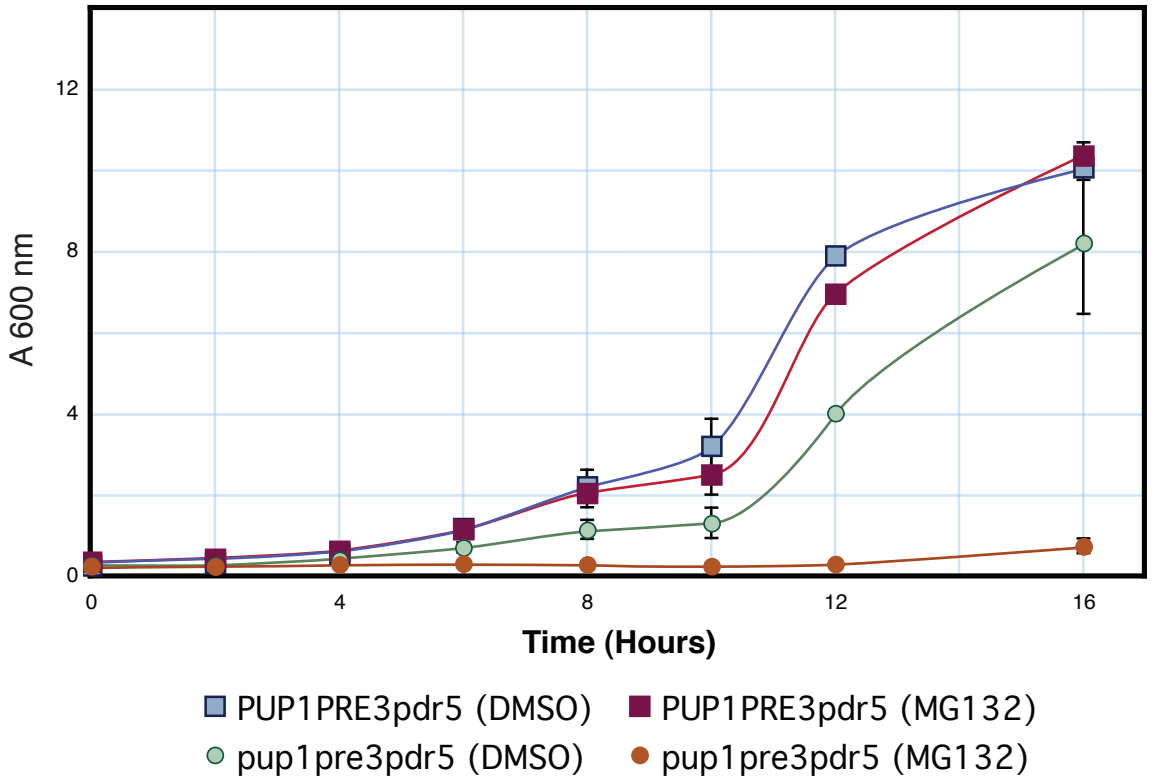

Figure 3.2: MG132 and the Proliferation Of *Saccharomyces cerevisiae*. BY474 Δ*pdr5* yeast treated with either DMSO (Red Diamonds) or 50 µM MG132 (Blue Squares). Absorbance at 600 nm was measured for a sample from each culture at the indicated times post treatment. n = 3; SEM. ANOVA: $p = 4 \times 10^{-8}$.

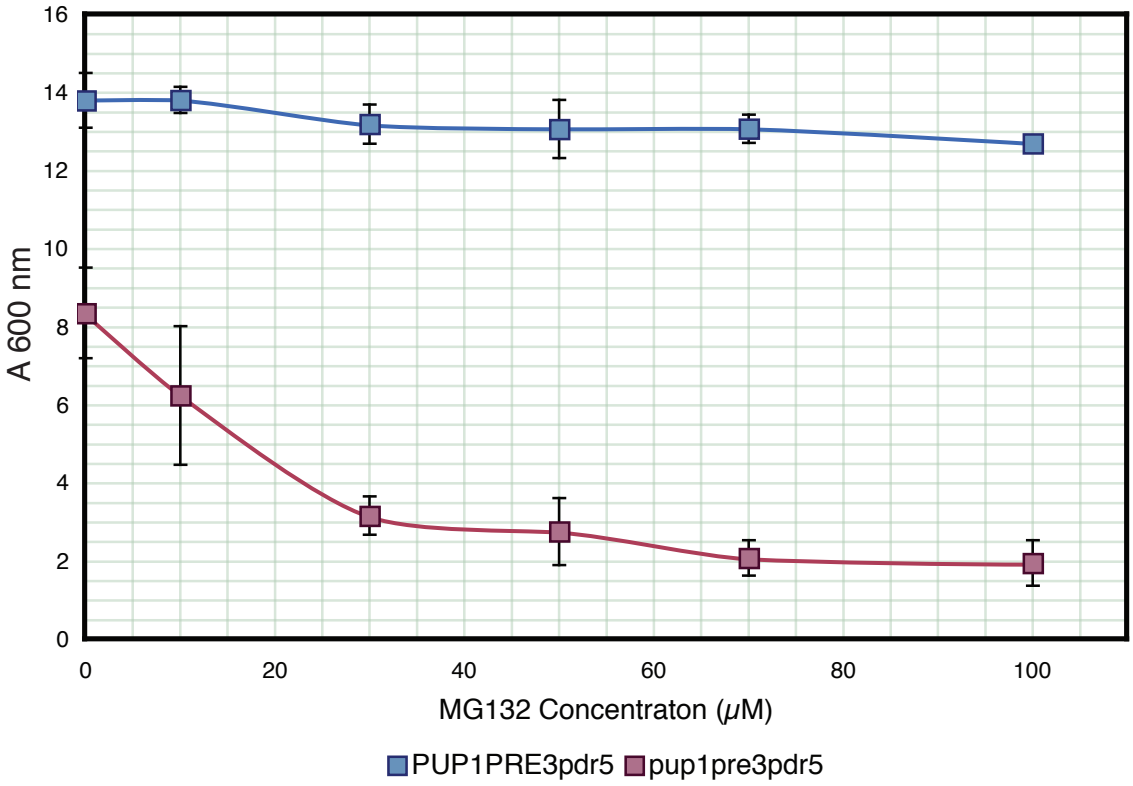

Figure 3.3: Titration of MG132. *PUP1PRE3pdr5* (Blue) and *pup1pre3pdr5* (Red) yeast treated with the indicated concentrations of MG132. Absorbance at 600 nm was measured for a sample from each culture one hour post-treatment. n = 3; SEM; p = 2 x 10^{-7}.

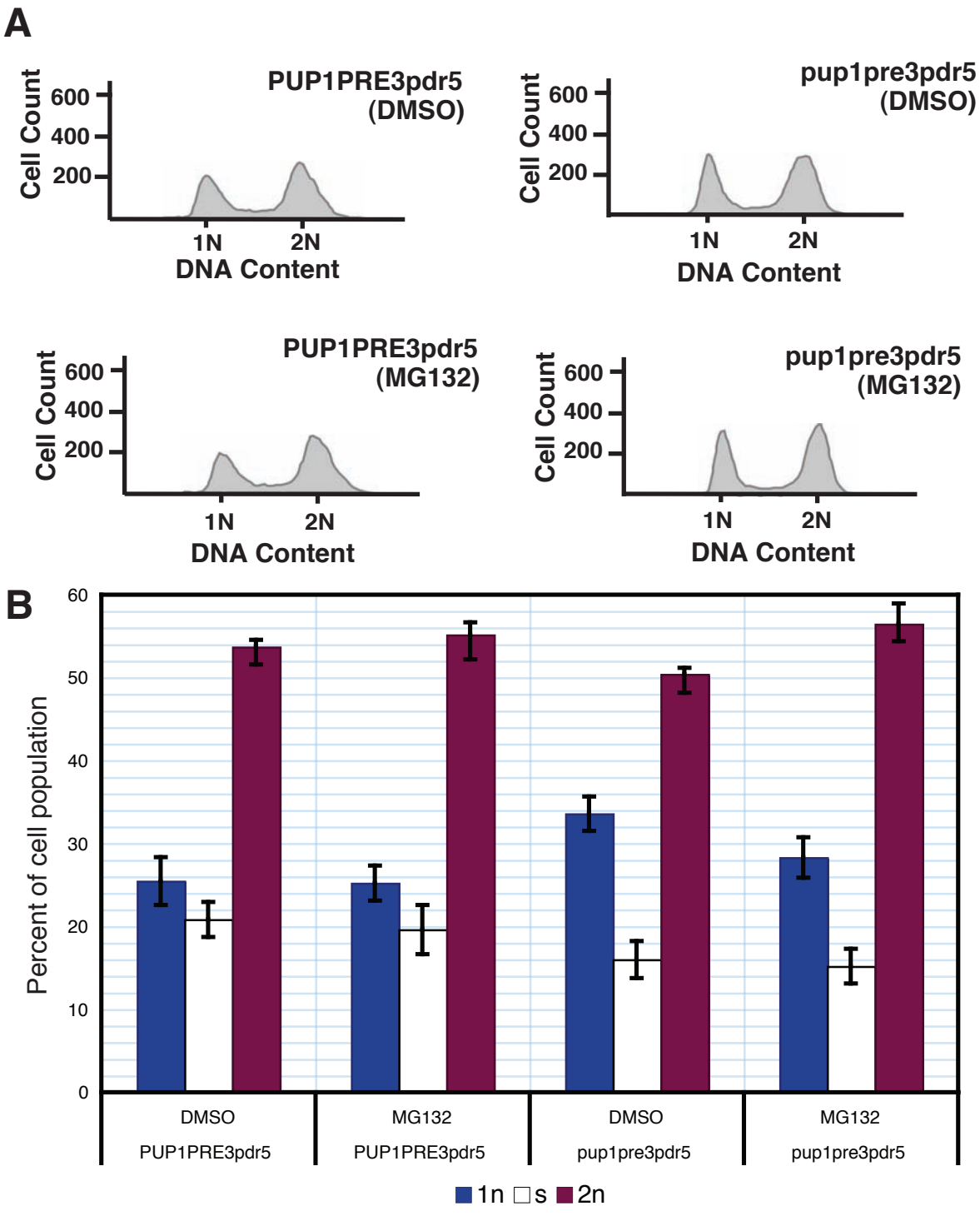

Figure 3.4: Cell cycle distribution of asynchronous populations. (A) Representative of four flow cytometric profiles counting the number of cells with a given DNA content. (B) Quantification of the percentage of cells within each cell cycle phase; n = 4; SEM. ANOVA: p = 1.

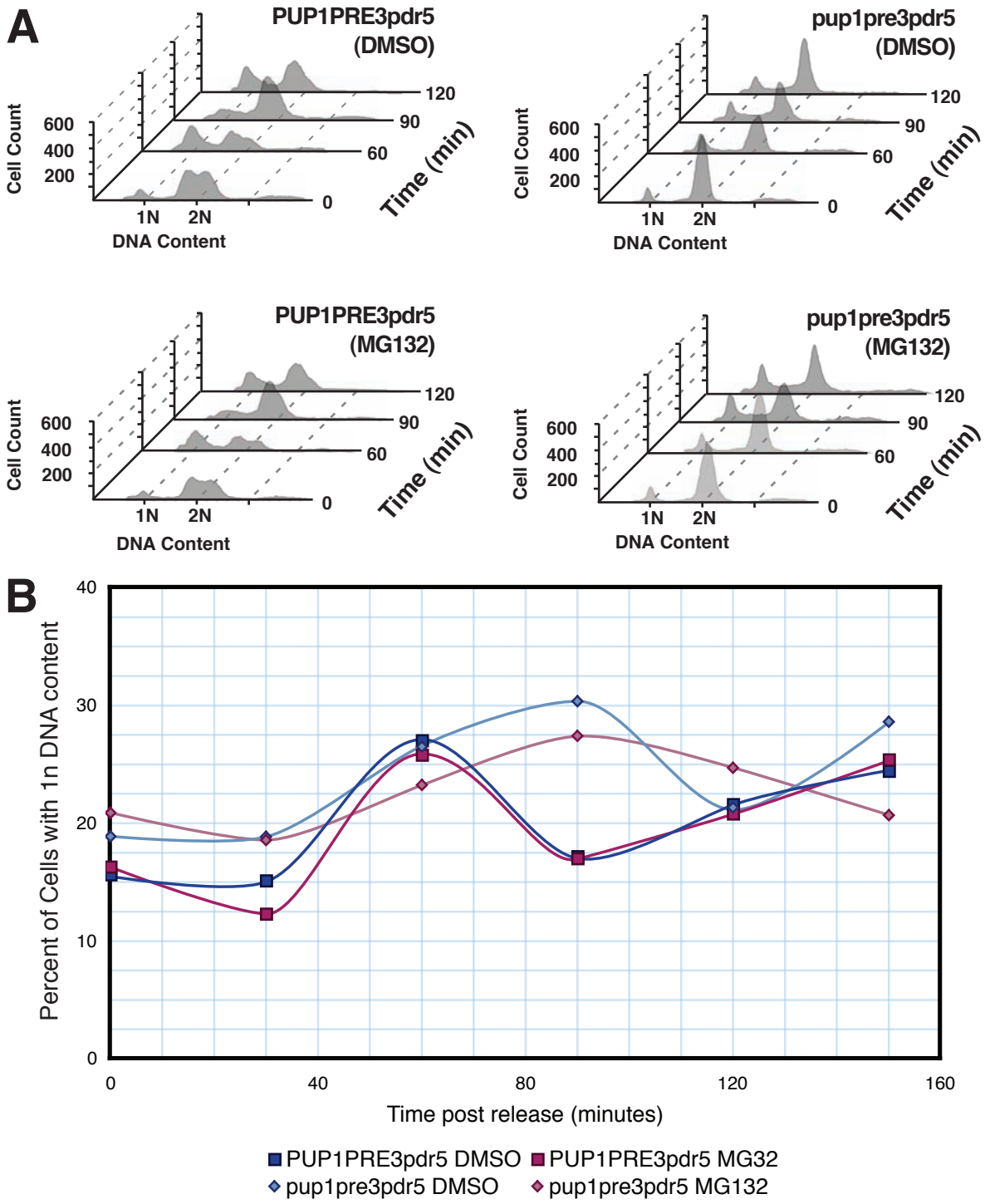

Figure 3.5: Inhibition of the proteasome delays exit from nocodazole induced arrest. (A) Flow cytometric profiles counting the number of cells with a given DNA content as a function of time (minutes) post-release from nocodazole induced arrest. (B) Quantification of the percentage of cells with 1n DNA content as a function of time. ANOVA: $p = 0.1$

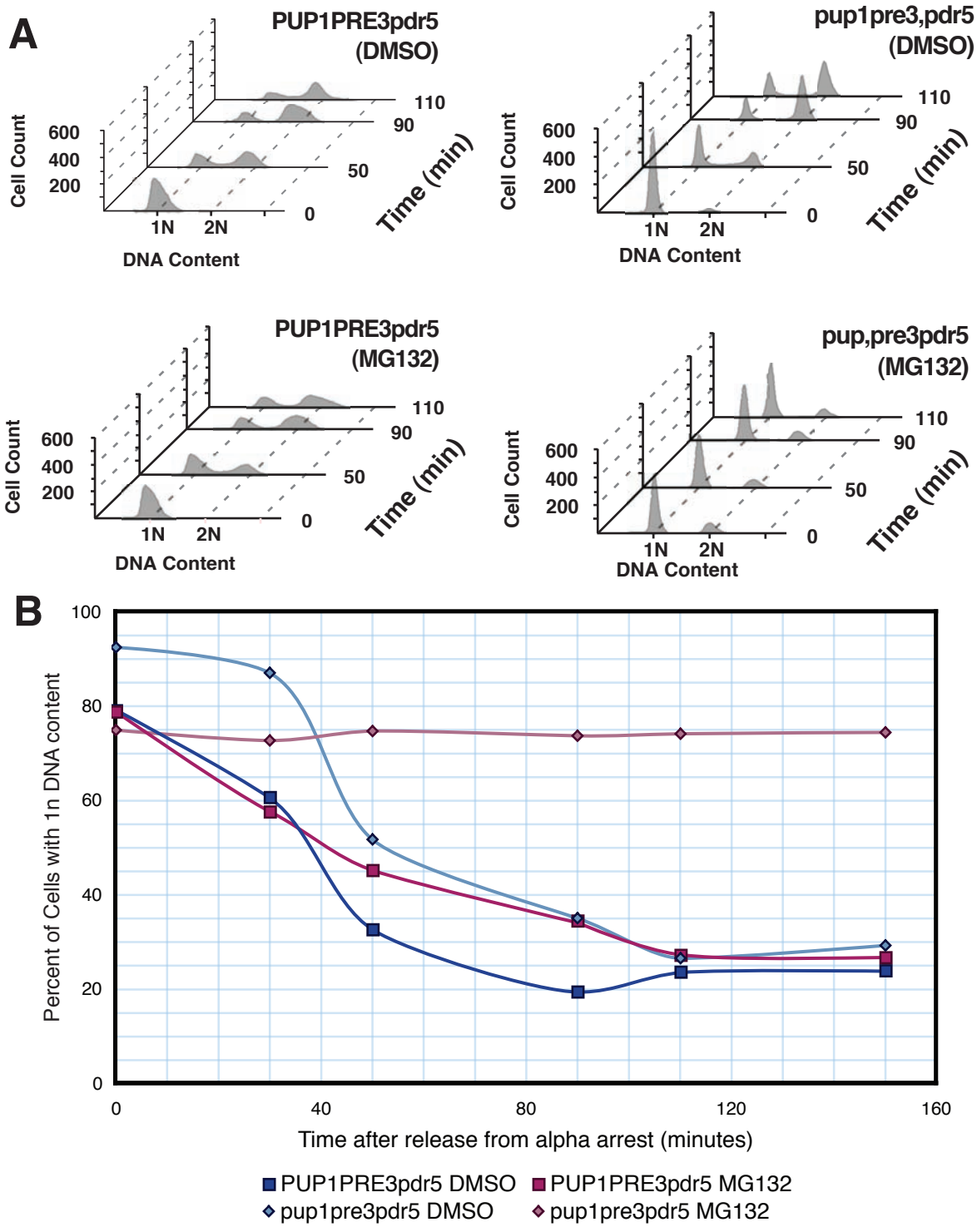

Figure 3.6: Inhibition of the proteasome delays exit from alpha-factor induced arrest. (A) Flow cytometric profiles counting the number of cells with a given DNA content as a function of time (minutes) post-release from alpha factor induced arrest. (B) Quantification of the percentage of cells with 1n DNA content as a function of time. ANOVA: $p = 0.002$.

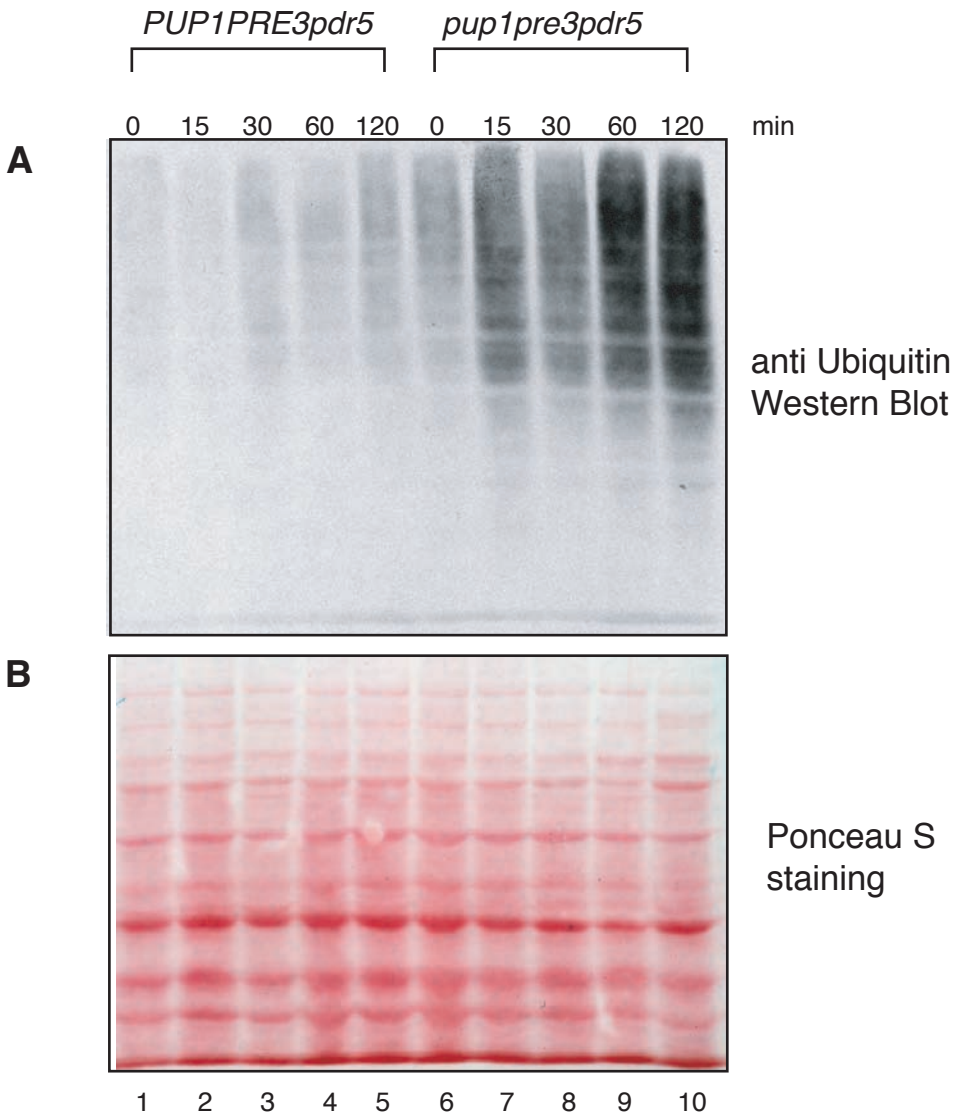

Figure 3.7: Inhibition of the proteasome leads to accumulation of ubiquitylated proteins. (A) anti-ubiquitin western blot of *PUP1PRE3pdr5* and *pup1pre3pdr5* treated with 50 μM MG132 for the indicated period of time (minutes). (B) Corresponding Ponceau S stain to demonstrate equivalent loading and transfer of proteins to the membrane. Lane numbers appear at the bottom.

Chapter Four: Inhibition of the Proteasome Inhibits Activation of Certain Genes

Introduction

In this chapter I will describe the effects of proteasome inhibition on the ability to activate transcription from four different model loci. If proteolytic turnover of activators is important for transcriptional activation then inhibiting the proteasome should have substantial impact on the ability to drive transcription. Although many groups examined the impact of proteasome inhibition on transcription (Kawazoe et al. 1998; Wallace and Cidlowski 2001; Deroo et al. 2002; Fleming et al. 2002; Mitsiades et al. 2002; Dembla-Rajpal et al. 2004; Dennis et al. 2005; Yew et al. 2005; Kinyamu and Archer 2007; Lassot et al. 2007; Tirard et al. 2007; Kinyamu et al. 2008; Middledorp et al. 2009), the resulting data is often contradictory resulting in continued debate regarding the importance of proteolysis on transcription. Gal4 represents a microcosm of this debate; two separate groups have arrived at diametrically opposed conclusions regarding the importance of proteasome-mediated proteolysis on the activation of transcription (Lipford et al. 2005; Nalley et al. 2006). Both groups studied the activation of the same gene, *GAL1*, and used the same proteasome inhibitor, MG132. Whereas Lipford *et al* found that *GAL* induction was lost when yeast were treated with MG132 (Lipford et al. 2005), Nalley *et al* reported that inhibition of the proteasome had negligible effect on transcription of *GAL* genes (Nalley et al. 2006). I have since examined this question myself to determine which result I have the most confidence in to build my model of how the proteasome regulates transcription activators.

In light of my work developing a strain with increased sensitivity to proteasome inhibition (see *Chapter Three*), the inability to detect an impact on transcription after treatment with a proteasome inhibitor, such as MG132, is not compelling evidence that the proteasome is dispensable for transcription. Increasing the overall sensitivity of yeast to proteasome inhibitors might also increase the degree to which transcription is affected by proteasome inhibitors. Therefore, I investigated the impact of proteasome inhibition in the context of this strain. Both MHY1177 and MHY1178, which were the parental strains to my *PUP1PRE3pdr5* (GAC201) and *pup1pre3pdr5*

(GAC202) are galactose negative and not made galactose positive by expression of wild-type *GAL2*, a common cause for the galactose negative phenotype in laboratory strains of *S. cerevisiae* (Winston et al. 1995). Although these strains were unsuitable to further analyze Gal4 dependent transcription in the presence of increased inhibition of the proteasome, many other active genes were available to study. I chose *ARG1* (regulated by the activator Gcn4 (Swanson et al. 2003; Yoon et al. 2004; Govind et al. 2005; Kim et al. 2005; Qiu et al. 2005)) and *INO1* (regulated by the heterodimeric activator Ino2/Ino4 (Lopes and Henr 1991; Lai and McGraw 1994; Nikoloff and Henry 1994; Graves and Henry 2000; Shirra *et al.* 2005; Esposito *et al.* 2010)) as genes whose induction was previously reported as sensitive to MG132 (Lipford et al. 2005) but had the same pallor of doubt cast over it as for the finding that *GAL1* is sensitive to proteasome inhibition. I also selected *CHA1* for my analysis, a gene that had not previously been analyzed for its dependence on proteasome and represents a different class of activated genes in that its regulation depends critically on the movement of a positioned nucleosome at the TATA box (Petersen et al. 1988; Bornaes et al. 1993; Holmberg and Schjerling 1996; Moreira and Holmberg 1998; Zawadzki et al. 2009).

Experimental Procedures

Yeast Strains

Strains used in this study are listed in Table 4.1. I deleted the *PDR5* gene from BY4742 using gene replacement with *KanMX6* by homologous recombination using sequences flanking the *PDR5* ORF. I synthesized the knockout cassette by PCR using pYM1 as a template (Knop et al. 1999), and transformed the cassette as described by Gietz and Woods (Gietz and Woods 2002). Correct targeting was validated by PCR. Similarly I deleted *PDR5* in W303-1**a** using gene replacement with a *TRP1* cassette using pRS404 as template for PCR (Sikorski and Hieter 1989). The preparation of GAC201 (*PUP1PRE3pdr5*) and GAC202 (*pup1pre3pdr5*) is described in the methods section of Chapter Three.

I induced *GAL* gene expression by addition of 20% (w/v) aqueous solution of galactose to yeast grown in CSM 2% raffinose for a final concentration of 2% galactose. Unless otherwise indicated, samples were collected one hour post galactose induction. I inhibited the proteasome by treating with 50 μM MG132 for half an hour prior to induction to be consistent with the methods previously used to study the affect of MG132 on transcription (Lipford *et al.* 2005; Nalley *et al.* 2006).

I induced *ARG1* expression by transferring yeast grown in YPAD to CSM lacking histidine (*Formedium*) and supplemented with 100 mM 3-aminotriazole (3-AT) (*Sigma*). Non-induced *ARG1* controls were transferred from YPAD to CSM and mock treated with a volume of water equivalent to 3-AT used to induce *ARG1*. I induced *INO1* by rinsing yeast grown in YPAD with water and then transferring the yeast to CSM lacking inositol *(Formedium*). Non-induced *INO1* controls were similarly rinsed and then transferred into CSM. I induced *CHA1* with the addition of 1 mg/ml L-serine (*Formedium*). *ARG1*, *INO1*, and *CHA1* induction proceeded for 90 minutes before collecting RNA for expression analysis or formaldehyde cross-linking for ChIP analysis. I inhibited the proteasome by treating with 50 μM MG132 at the time of induction.

Table 4.1: Yeast Strains Used

Strain	**Genotype**	**Source**
BY4741 Δ*pdr5*	Mat**a** *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 pdr5::KanMX6*	This study
W303-1**a** Δ*pdr5*	Mat**a** *leu2-3,112 trp1-1, can1-100, ura3-1, ade2-1, his3-11,15, ypb1-1 Δpdr5::TRP1*	This study
GAC201	Matα *his3-11 leu2-3,112 ura3-52 lys2-801 trp1-1 pre3Δ2::HIS3 pup1::Δleu2::HIS3* [pRS317-*PUP1*] [YCplac22-*PRE3*] gal$^-$	This study
GAC202	Matα *his3-11 leu2-3,112 ura3-52 lys2-801 trp1-1 pre3Δ2::HIS3 pup1::Δleu2::HIS3* [pRS317-*pup1T30A*] [Ycplac22-*PRE3*] gal$^-$ ρ^0	This study

Table 4.2: Plasmids Used

Plasmid	**Features**	**Source**
pYM1	*KanMX6* selectable marker	(Knop et al. 1999)
PRS404	*TRP1* selectable marker	(Sikorski and Hieter 1989)

RNA extraction and analysis

I collected RNA from 15 ml of yeast at mid-log phase growth ($A_{600\ nm}$ = 0.6 – 1.0) using a Hot Phenol RNA extraction method (Collart and Oliviero 1993). Cells were lysed in RNA extraction buffer (50 mM sodium acetate (pH 4.8), 0.5% SDS, and 10 mM EDTA) and an equal volume of acid (pH 4.3) phenol (*Sigma*) by incubating at 65 °C for one hour. Samples were chilled on ice for five minutes, centrifuged for five minutes, and the aqueous phase was collected for purification through another extraction with acidic (pH 4.3) phenol and an extraction with 25:24:1 phenol:chlorofom:isoamyl alcohol (*Sigma*). Samples were precipitated in ethanol for less than 20 minutes at –20°C. RNA was resuspend in nuclease free water. Contaminating DNA was removed with *DNase I* (*invitrogen*). 1 µg of RNA was used for first strand cDNA synthesis with *SuperScript II* reverse transcriptase (*invitrogen*).

I quantified cDNA by qPCR with SYBR fast PCR mix (*Kapa Biosystems*). Expression was calculated relative *ACT1* by $2^{ACT1\text{-}GOI}$ where *ACT1* is the signal from the *ACT1* ORF (ACT1q1 and ACT1q2 oligonucleotides) and GOI is the gene of interest. *GAL1* gene signals in induction time course experiments were normalized setting the maximum signal to 1000 arbitrary units to minimize the noise of experiment-to-experiment variation in *GAL1* induction. (1000 was selected because the median expression of *GAL1* was 951 fold above *ACT1* expression).

Chromatin Immunoprecipitation

For ChIP I fixed 50 ml of yeast culture with 1.5 ml paraformaldehyde solution (37% (w/v) paraformaldehyde (*Sigma*) in 1x phosphate buffer solution (PBS) and 0.2% 10N KOH) for 30 minutes. Cross-linking was stopped with 3 ml 2.5 M glycine for 5 minutes at room temperature before washing the cells with PBS. Cells were lysed in 800 µl lysis buffer (50 mM HEPES (pH 7.5), 15 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% SDS, 0.1% DOC) by bead beating. Cell lysates were sonicated to approximately 500 base pairs median fragments. Chromatin was incubated with 1:1000 dilution of anti-Rpb3 antibody (1Y26, *Neoclone*) overnight at 4 °C followed with an hour

incubation at 4 °C with a 1:100 dilution of rabbit anti mouse IgG antibody (*invitrogen*) before pulldown with protein A sepharose beads (*GE Healthcare*). Immunoprecipitated chromatin bound to protein A sepharose beads was washed in IP buffer (50 mM HEPES (pH 7.5), 15 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% DOC), high salt buffer (50 mM HEPES (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% DOC), lithium-DOC buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.25 M LiCl, 5% NP-40 alternative, 5% DOC), and twice with 10x Tris-EDTA (TE) buffer (10 mM Tris-HCl (pH 8.0), 100 mM EDTA) before elution for 20 minutes in elution buffer (100 mM $NaHCO_3$, 1% SDS). Eluted chromatin and input chromatin were reverse-cross-linked overnight at 65 °C. Samples were treated with 1.5 mg proteinase K (*Roche*). DNA was purified with a phenol:chloroform extraction and precipitated in ethanol and stored in TE buffer.

I quantified the amount of DNA that came down in the immunoprecipitation by qPCR with SYBR fast PCR mix (*Kapa Biosystems*). I calculated enrichment as described by Ezhkova *et al* (Ezhkova and Tansey 2004). Automatically-derived cycle thresholds were obtained from triplicate IP samples for both the amplicon of interest (C^{IPA}) and a reference locus (C^{IPR}) as well as corresponding threshold values from input chromatin (C^{INA} and C^{INR} respectively). Fold enrichment is then calculated as $[2^{(IPR-IPA)}] \div [2^{(INR-INA)}]$. I used V(L) intergenic sequence as my reference to normalize for Rpb3 binding.

Results

GAL *gene induction is impaired by proteasome inhibition*

Given my interest in the connection between the proteasome and Gal4 function and in light of the controversy regarding the effect of proteasome inhibition on *GAL1* induction (Lipford et al. 2005; Nalley et al. 2006) I have analyzed the expression of *GAL1* genes in the presence or absence of the proteasome inhibitor MG132. Following the same protocol as both Lipford *et al* and Nalley *et al* of inhibiting the proteasome with 50 μM MG132 half an hour prior to induction with galactose, I

observed greater than five-fold inhibition of *GAL1* induction compared to DMSO control samples after an hour of induction (*Figure **4.1***). The effects of proteasome inhibition on transcription are detectable as early as 15 minutes post-induction (students t-test p = 0.07; n =5) and continue to increase over the course of the hour. This result demonstrates a significant dependence on proteasome-mediated proteolysis in the activation of *GAL1* contrary to the report of Nalley *et al*.

To alleviate concerns that MG132 may affect *GAL*1 expression in a strain specific context I also tested the response of *GAL1* expression to MG132 in the yeast strain W303-1**a** Δ*pdr5*, whereas previously I worked with BY4741 Δ*pdr5 (Figure **4.2***). Once again I detected approximately five-fold decrease in transcription in both strains, ruling out differences between these two strains.

I decided to determine if the effects of MG132 inhibition were limited to just *GAL1* or extended to other strongly induced Gal4 regulated genes. Therefore, I measured the transcription at *GAL2*, *GAL7*, and *GAL10* in addition to *GAL1 (Figure **4.3**)*. All genes, despite having different levels of expression relative to each other, demonstrated a typically five-fold decrease in activation when treated with MG132. Therefore, I am convinced of the importance of the proteolytic ability of the proteasome to strongly induce Gal4 dependent transcription sharp contrast to the report of Nalley *et al* (Nalley et al. 2006).

Proteasome Inhibition of Transcription Activation Is Sensitive to the Activity of Pup1 or Pre3 or Both Subunits

The parental strains to make *PUP1PRE3pdr5* and *pup1pre3pdr5* were galactose negative. Therefore, to use *PUP1PRE3pdr5* and *pup1pre3pdr5* to study gene activation I looked at the transcriptional effects of proteasome inhibition on the activation several other genes. *ARG1* transcription is induced, along with several other genes, by amino acid starvation through the transcription activator Gcn4 (Swanson et al. 2003; Yoon et al. 2004; Govind et al. 2005; Kim et al. 2005; Qiu et al. 2005). This activator, like Gal4 is both well characterized and unstable during conditions associated with its function (Meimoun et al. 2000; Chi et al. 2001; Shemer et al. 2002;

Lipford et al. 2005). Like Gal4, Gcn4 is phosphorylated two kinases associated with the transcription initiation complex, Kin28 and Pho85. Gcn4 is ubiquitylated by the essential E3 ligase Cdc4 and inactivation of the temperature-sensitive version of Cdc4 by shifting to the non-permissive temperature results in the loss of transcription in response to amino acid starvation (Lipford et al. 2005). Furthermore, inhibition of the proteasome also prevented induction of Gcn4 target genes. However, based on the debate over *GAL1* dependence on the proteasome this later finding has been doubted (Kodadek 2010). I hypothesized that this was the type of situation that would benefit from increased sensitivity to proteasome inhibition. I used the histidine analogue 3-AT to induce *ARG1* transcription in both *PUP1PRE3pdr5* and *pup1pre3pdr5* strains (***Figure 4.4***). In the essentially wild-type proteasome context of *PUP1PRE3pdr5* I find a non-statistically significant decrease in *ARG1* activation when yeast were treated with MG132 (t-test $p = 0.111$; $n = 5$). When I examined *ARG1* activation in *pup1pre3pdr5* yeast treated with MG132 not only was there a significant inhibition of transcriptional activation but the levels of *ARG1* expressed were now comparable to the basal level of expression consistent with failure to induce the *ARG1* gene. I conclude the *ARG1* activation is dependent on the proteasome activity. Whereas in my *PUP1PRE3pdr5* yeast I could find a significant loss of *ARG1* expression, the combined activity of MG132 and loss of Pup1 and Pre3 yielded unambiguous results consistent with the potential of this strain to more clearly study the connection between proteasome-mediated proteolysis and transcription.

To begin to determine where the loss of proteasome function was regulating transcription I looked at the recruitment of RNApolII to the early ORF of *ARG1*. If the proteasome was necessary for the signaling pathway upstream of Gcn4 or for the initiation of transcription then the loss of proteasome function should lead to the loss of RNApolII recruitment. Conversely, defects in elongation should still permit for significant detection of RNApolII at the early ORF. I performed the ChIP with antibodies against the Rpb3 subunit of RNApolII and I observed recruitment of RNApolII to the *ARG1* locus in *PUP1PRE3pdr5* yeast only when yeast were treated with 3-AT (***Figure 4.5***). MG132 did not affect this recruitment, consistent with the inability to detect a significant loss if

ARG1 transcription. In *pup1pre3pdr5* yeast, I detected lower Rpb3 ChIP signals with in 3-AT induction conditions than what I observed in the *PUP1PRE3pdr5* strain. This is in contrast to the level of transcripts observed in these strains. Nevertheless, treatment with MG132 further decreases the level of RNApolII at *ARG1* to levels that are now similar to RNApolII when the *ARG1* transcription is off. These data are consistent with impairment in transcription initiation as a result of impairing the proteolytic function of the proteasome.

I looked at a different locus, *INO1*, to begin to gauge how general common sensitivity of transcription activation to proteasome inhibition in *S. cerevisiae*. The *INO1* gene is activated through the heterodimeric activator Ino2 and Ino4 in response to depletion of inositol (Lopes and Henr 1991; Lai and McGraw 1994; Nikoloff and Henry 1994; Graves and Henry 2000; Shirra *et al.* 2005; Esposito *et al.* 2010). In *PUP1PRE3pdr5* yeast, treatment with MG132 does not inhibit activation and, if anything, elevates the level of *INO1* expression (*Figure **4.6***). In contrast, the loss of at least on of the activities of Pup1 and Pre3 hinders the activation of *INO1*. Furthermore, consistent with synergistic effect of MG132 treatment with the loss of Pup1 and Pre3 proteolytic activities, *INO1* transcription becomes even lower, suggesting a critical dependence on proteasome function for the activation of this gene. The levels of RNApolII recruitment to *INO1* display a pattern similar to the *ARG1* locus (*Figure **4.7***), which belies the difference in the effect of the Pup1 and Pre3 subunits alone on transcription. In the case of *INO1* the transcription levels correspond very accurately with RNApolII levels.

Not all inducible genes are dependent upon proteasome-mediated proteolysis. *CHA1* is induced by multiple signals, such as stress to the cell wall integrity triggered by treatment with Congo red (Garcia et al. 2004) or with elevated temperatures (A. Leung, *Personal Communication*). Alternatively, excess serine levels in the media induce *CHA1* expression (Petersen et al. 1988; Bornaes et al. 1993; Holmberg and Schjerling 1996), which is the method I selected to use to induce *CHA1* because it provides a rapid and robust response with little secondary impact on the cell as compared to heat shock. Induction of *CHA1* with serine was unaffected by addition of MG132 in both

the *PUP1PRE3pdr5* and *pup1pre3pdr5* strains (*Figure* ***4.8***), providing an example of a gene not dependent on proteasome-mediate proteolysis for activation. The recruitment of RNApolII at this locus is affected by the triple inhibition of loss of Pup1 and Pre3 function with the addition of MG132 *(Figure* ***4.9***). However, the level of Rpb3 detected at *CHA1* remains substantially higher than when no serine is added to the cell, and this residual presence of RNApolII may explain why I observe *CHA1* transcription even when I inhibit the proteasome in *pup1pre3pdr5* yeast.

Discussion

I have examined the effects of proteasome inhibition on four model inducible genes. Using MG132 to inhibit the proteasome, *GAL* induction is significantly reduced. However, the variability between different laboratories and the knowledge that MG132 incompletely inhibits the proteasome leaves uncertainty regarding the degree to which proteasome mediated proteolysis is necessary for *GAL* gene induction. For example, in my analysis, MG132 inhibits *GAL* gene induction by approximately eighty percent. The nature of the remaining twenty percent of transcription is also of interest. Does this represent a proteasome independent set of transcripts or does this remaining transcription reflect the incompleteness of inhibition achieved by biasing the inhibition of the proteasome to the chymotryptic activity? I believe that the data from *ARG1* and *INO1* in the *pup1pre3pdr5* support that residual *GAL* transcription arises from incompletely inhibiting the proteasome rather than reflecting transcription that is independent of proteasome mediated proteolysis because the more thorough inhibition of the proteasome in *pup1pre3pdr5* significantly reduces the level of transcripts produced to near basal amounts. Inactivating Pup1, Pre3, and deleting *PDR5* in a strain that is galactose positive will allow this hypothesis to be tested.

The differences in effect of loosing Pup1 and Pre3 proteolytic activity between *ARG1* and *INO1* transcription lead to an interesting problem. It makes sense that, as has been described *in vitro* (Kisselev et al. 2006), that the *rate* at which proteolysis for a given substrate should be differentially

dependent on each of the proteolytic functions. For example loss of the caspase-like activity would have a significant effect on proteins with many acidic residues but not on those with relatively few acidic amino acids. But although the *rate* of proteolysis may depend on the substrate's composition, proteolysis should eventually occur. Furthermore the non-proteolytic processes of the proteasome — de-ubiquitylation, unfolding, and translocation —should inhibit the function of a substrate. If there is a limited potential to increase the proteasome recruitment to an actively transcribed gene compared to a much greater potential to increase the recruitment of the critical proteasomal substrate, say an activator, then slowing down the rate of proteolysis would lead to occupied proteasomes that cannot function. Proteasomes without caspase-like or tryptic activities will be jammed with to-be-digested substrates differently than proteasomes with inhibited chymotryptic activity.

The relationship between the rate of proteolysis and substrate composition emphasizes the need to identify the important proteasome substrates in transcription. Inhibition of the proteasome leads to loss of RNApolII concentration to the very 5' of the ORF, consistent with a defect in recruitment. The instability and ubiquitylation of transcription activators make these proteins primary candidates. The characterization of the UPS regulation of Gcn4 provides valuable tools in testing the contributions of activator proteolysis. In addition to inhibiting proteolysis acutely in the *pup1pre3pdr5* strain and inhibiting ubiquitylation using Cdc4 temperature sensitive mutants, the phosphorylation of Gcn4 can be blocked using *cis* mutations to three threonines and two serines that are necessary for Gcn4 phosphorylation (*gcn4-3T2S*). Importantly, although *gcn4-3T2S* cannot be ubiquitylated by Cdc4, induction of *ARG1* still occurs even with thermal inactivation of Cdc4 temperature sensitive mutants. If the report that *gcn4-3T2S* can also activate *ARG1* when treated with MG132 extends to the *pup1pre3pdr5* strain then it will be useful to study the changes in the initiation complex that are recruited to Gc4n regulated promoters, such as *ARG1*. Furthermore, the ability of *gcn4-3T2S* to activate transcription in the context of proteasome inhibition is suggestive that the basic amino acid sensing pathway remains intact and that the defect is indeed with transcription.

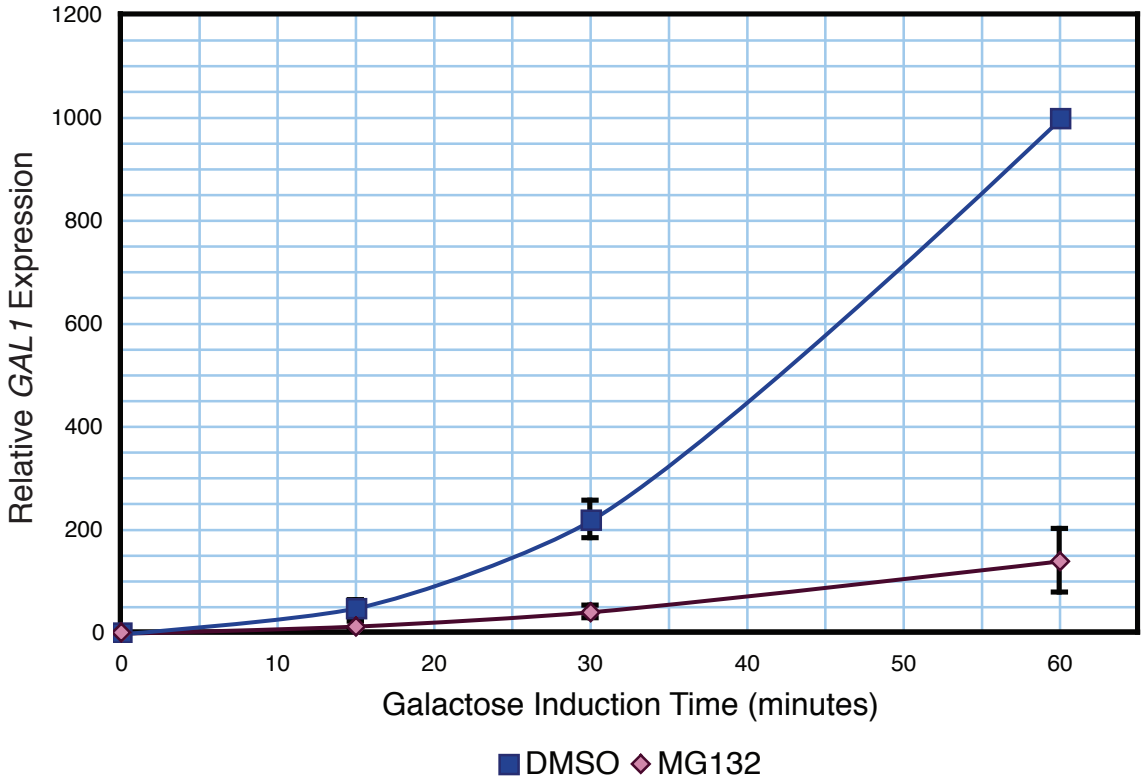

Figure 4.1: MG132 Treatment Inhibits *GAL1* Induction. *GAL1* expression relative to *ACT1* and normalized to *GAL1* expression after one hour of galactose induction as a function of galactose induction time (minutes). Treatment 50 μM MG132 (red diamonds) or mock treatment with DMSO (blue squares). n = 5; SEM.

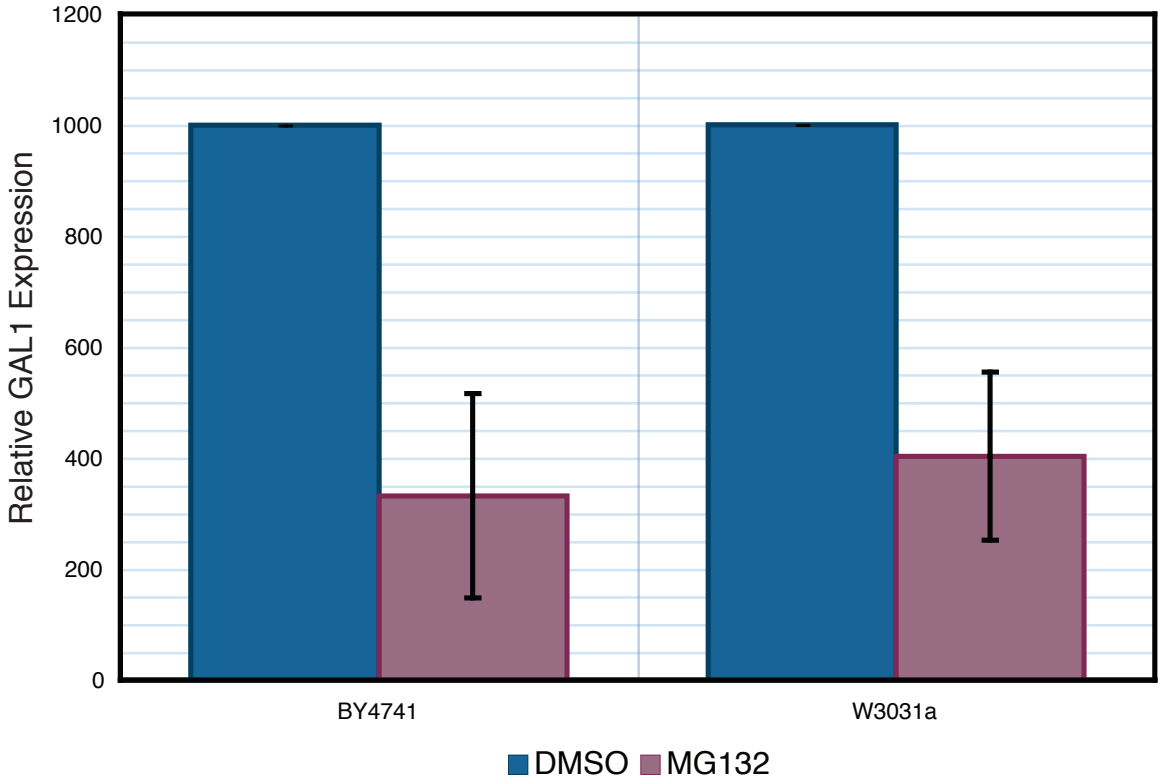

Figure 4.2: MG132 Inhibits *GAL1* Expression in Different Genetic Backgrounds. *GAL1* expression one hour post induction relative to *ACT1* and normalized to *GAL1* treated with DMSO. DMSO (Blue) and 50 μM MG132 (Red) in both BY4741 Δ*pdr5* and W3031**a** Δ*pdr5*. n = 5; SEM.

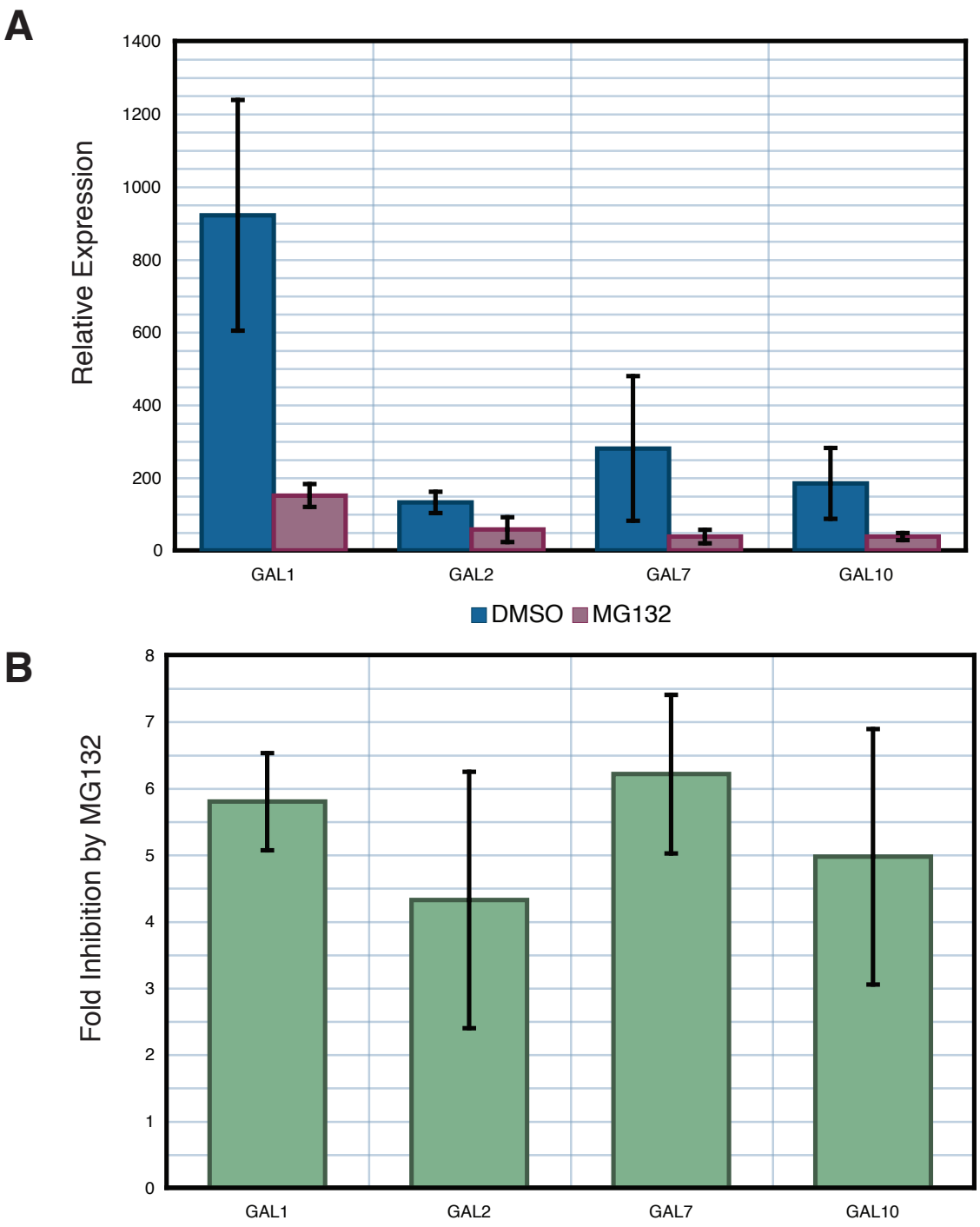

Figure 4.3 MG132 Inhibits the Expression of Multiple *GAL* Genes. (A) Expression of *GAL1*, *GAL2*, *GAL7*, and *GAL10* relative to *ACT1* after one hour of induction. DMSO treatment (Blue) and 50 μM MG132 (Red). (B) Ratio of expression in DMSO to MG132 treatment from above. n = 5; SEM.

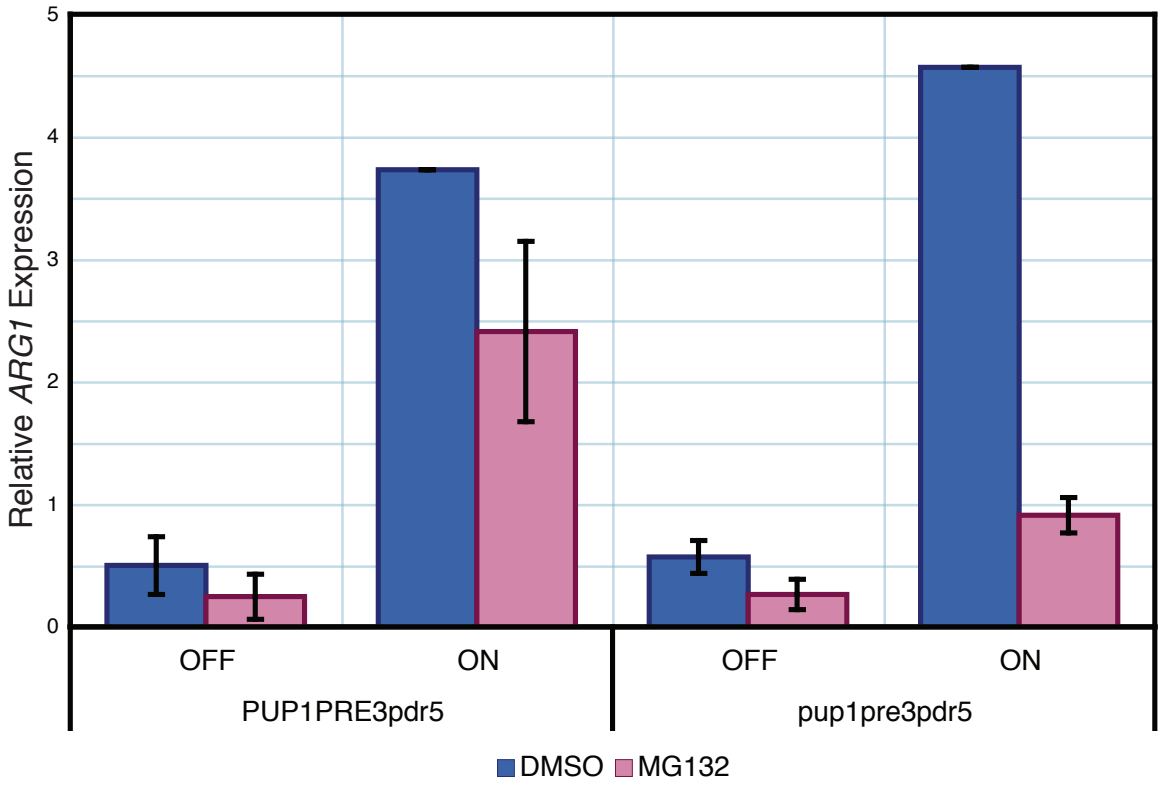

Figure 4.4 Proteasome Inhibition Leads to Loss of *ARG1* Induction. *ARG1* expression ninety minutes post induction with 3-AT relative to *ACT1*. DMSO treatment (Blue) and 50 µM MG132 (Red). n = 5; SEM. *PUP1PRE3pdr5* DMSO ON compared to *PUP1PREpdr5* MG132 ON, p = 0.15. *PUP1PRE3pdr5* MG132 ON compared to *PUP1PRE3pdr5* DMSO OFF, p = 0.039. *pup1pre3pdr5* DMSO ON compared to *pup1pre3pdr5* MG132 ON, p = 1×10^{-5}. *pup1pre3pdr5* MG132 ON compared to *pup1pre3pdr5* DMSO OFF, p = 0.12.

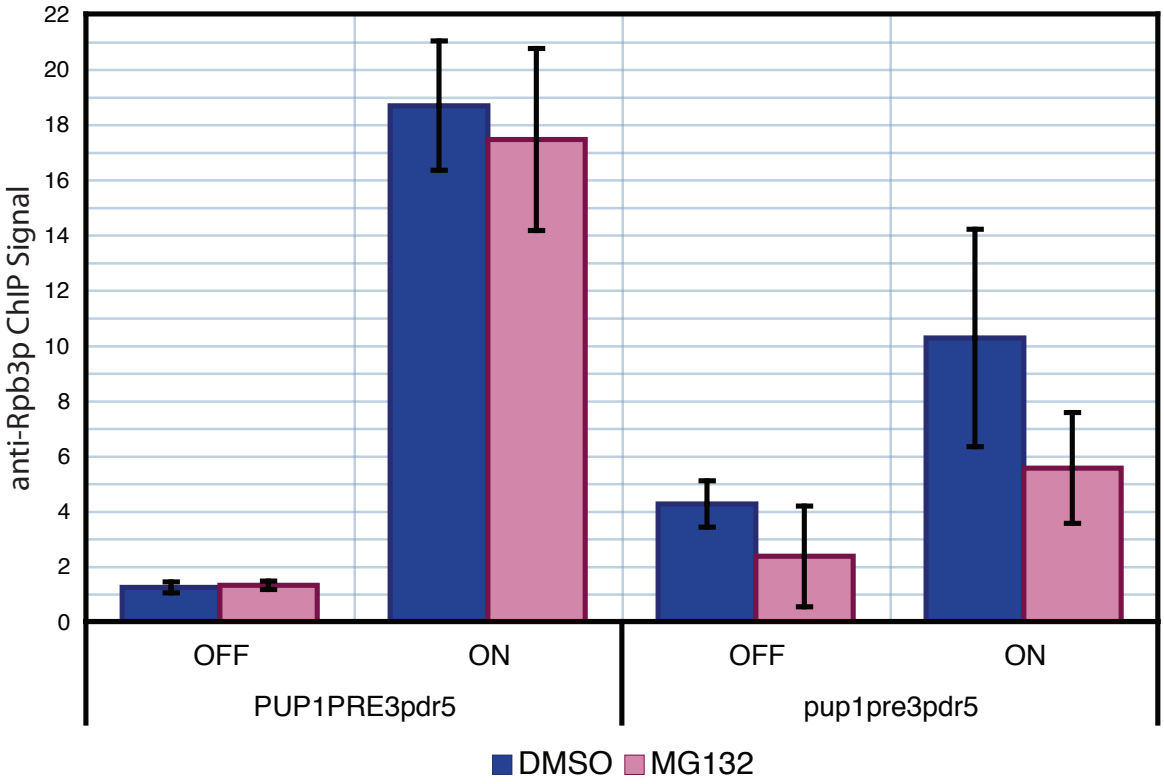

Figure 4.5 Proteasome Inhibition Leads to Loss of RNApolII at *ARG1*. ChIP signal from anti-Rpb3p ChIP at the *ARG1* 5′ ORF relative to V(L) intergenic sequence for when *ARG1* transcription is OFF (water) or ON (3-AT). n = 5; SEM. *PUP1PRE3pdr5* DMSO ON compared to *PUP1PREpdr5* MG132 ON, p = 0.78. *PUP1PRE3pdr5* MG132 ON compared to *PUP1PRE3pdr5* DMSO OFF, p = 0.0016. *pup1pre3pdr5* DMSO ON compared to *pup1pre3pdr5* MG132 ON, p = 0.29. *pup1pre3pdr5* MG132 ON compared to *pup1pre3pdr5* DMSO OFF, p = 0.59.

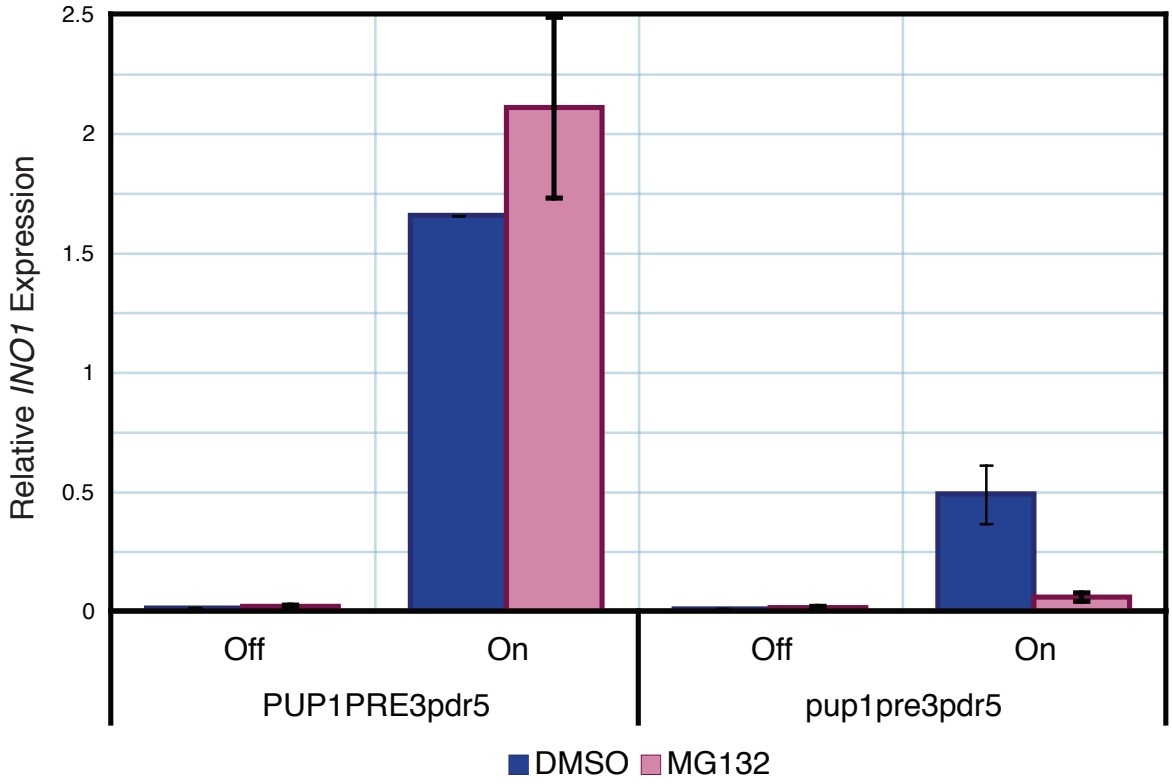

Figure 4.6 Proteasome Inhibition Leads to Loss of *INO1* Induction. *INO1* expression ninety minutes post-induction by inositol starvation relative to *ACT1*. n = 4; SEM. *PUP1PRE3pdr5* DMSO ON compared to *PUP1PREpdr5* MG132 ON, p = 32. *PUP1PRE3pdr5* MG132 ON compared to *PUP1PRE3pdr5* DMSO OFF, p = 0.001. *pup1pre3pdr5* DMSO ON compared to *pup1pre3pdr5* MG132 ON, p = 0.013. *pup1pre3pdr5* MG132 ON compared to *pup1pre3pdr5* DMSO OFF, p = 0.038.

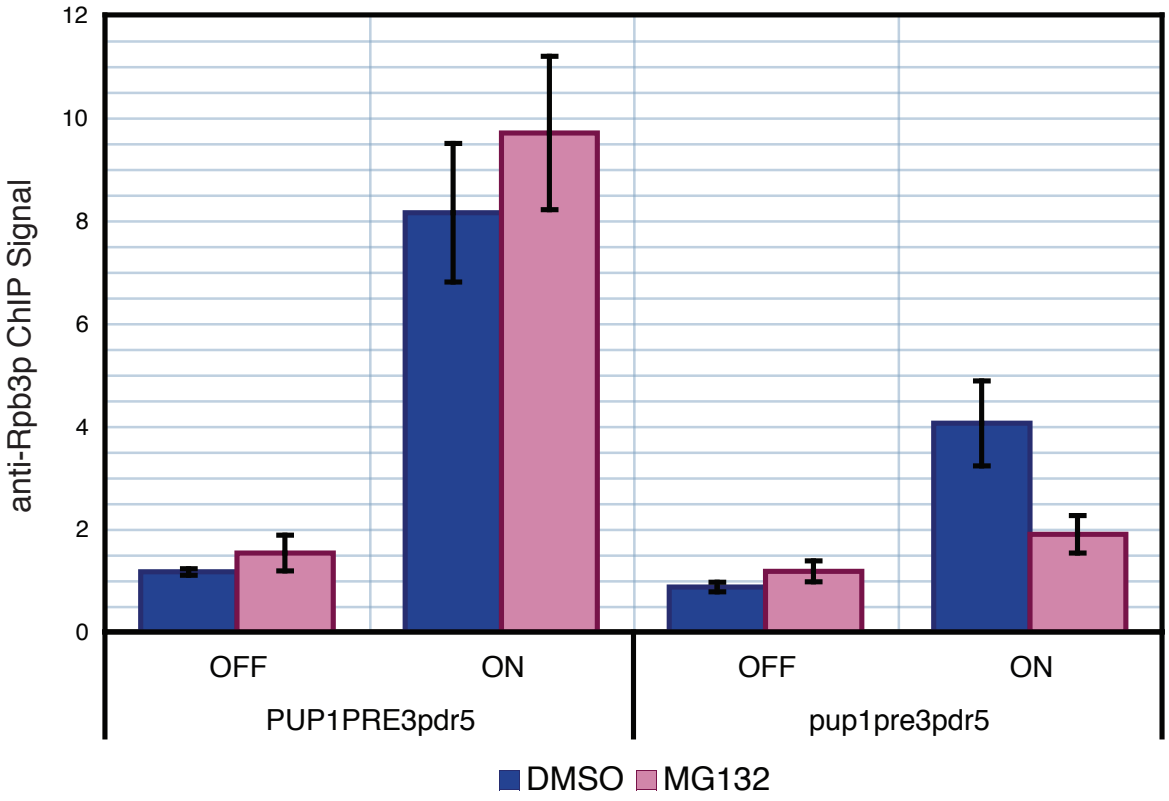

Figure 4.7 Proteasome Inhibition Leads to Loss of RNApolII at *INO1*. ChIP signal from anti-Rpb3p ChIP at the *INO1 5′ ORF* relative to V(L) intergenic sequence for when *INO1* transcription is OFF (CSM) or ON (-inositol). n = 4; SEM. *PUP1PRE3pdr5* DMSO ON compared to *PUP1PREpdr5* MG132 ON, p = 0.46. *PUP1PRE3pdr5* MG132 ON compared to *PUP1PRE3pdr5* DMSO OFF, p = 0.001. *pup1pre3pdr5* DMSO ON compared to *pup1pre3pdr5* MG132 ON, p = 0.03. *pup1pre3pdr5* MG132 ON compared to *pup1pre3pdr5* DMSO OFF, p = 0.11.

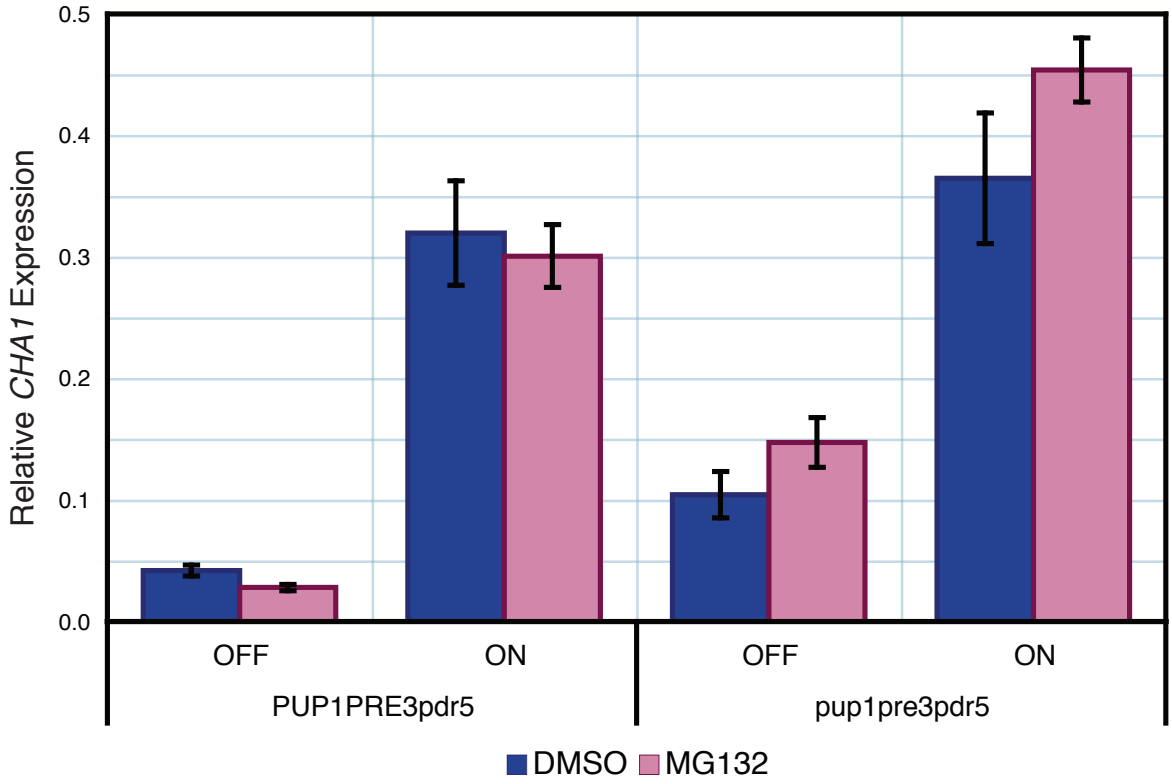

Figure 4.8 Proteasome Inhibition Does Not Affect *CHA1* Induction. *CHA1* expression ninety minutes post-induction by serine addition relative to *ACT1*. n = 2; SEM. *PUP1PRE3pdr5* DMSO ON compared to *PUP1PREpdr5* MG132 ON, p = 0.71. *PUP1PRE3pdr5* MG132 ON compared to *PUP1PRE3pdr5* DMSO OFF, p = 6 x 10^{-10}. *pup1pre3pdr5* DMSO ON compared to *pup1pre3pdr5* MG132 ON, p = 0.15. *pup1pre3pdr5* MG132 ON compared to *pup1pre3pdr5* DMSO OFF, p = 2 x 10^{-9}.

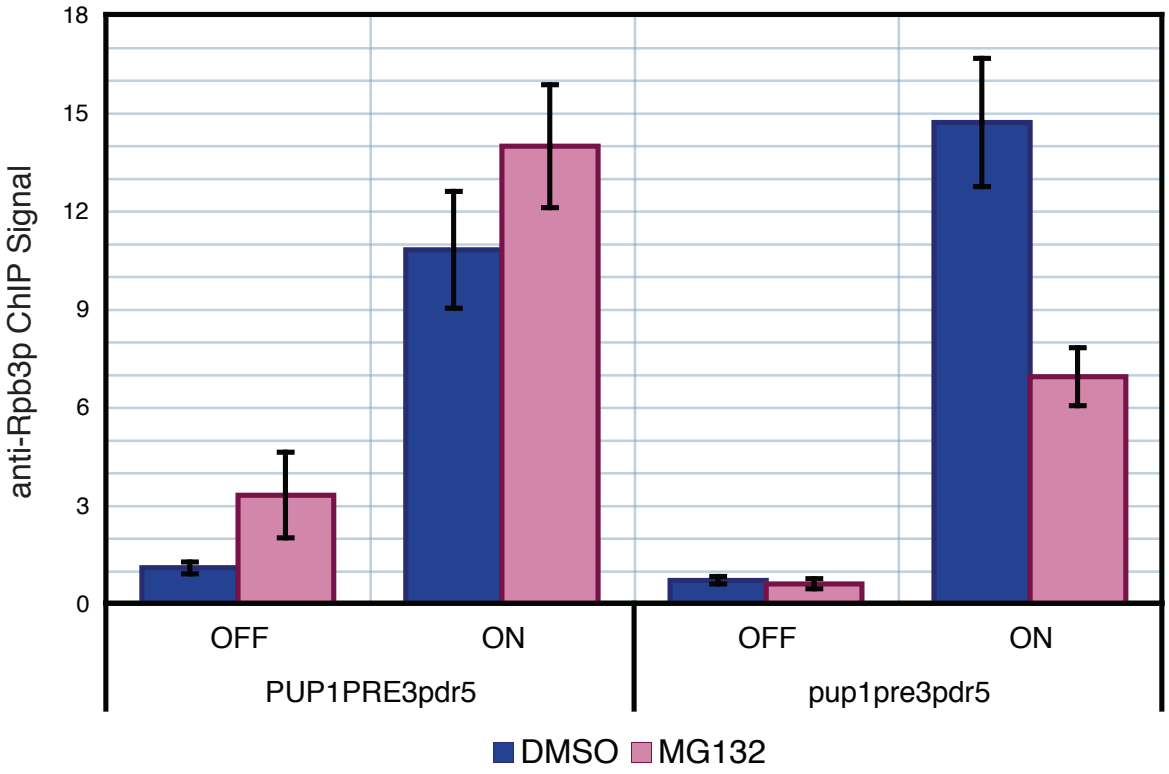

Figure 4.9 Proteasome Inhibition and Rpb3 Recruitment with *CHA1* Induction. ChIP signal from anti-Rpb3p ChIP at the *CHA1* 5′ ORF relative to V(L) intergenic sequence for when *CHA1* transcription is OFF (water) or ON (serine). n = 2; SEM. *PUP1PRE3pdr5* DMSO ON compared to *PUP1PREpdr5* MG132 ON, p = 0.29. *PUP1PRE3pdr5* MG132 ON compared to *PUP1PRE3pdr5* DMSO OFF, p = 0.0030. *pup1pre3pdr5* DMSO ON compared to *pup1pre3pdr5* MG132 ON, p = 0.0069. *pup1pre3pdr5* MG132 ON compared to *pup1pre3pdr5* DMSO OFF, p = 3.1×10^{-5}.

Chapter Five: Proteasome Inhibition and Global Transcription

Introduction

In this chapter I will discuss the effects on global transcription of treating yeast with the proteasome inhibitor MG132. In contrast to similar studies in the past (Fleming *et al.* 2002; Dembla-Rajpal *et al*. 2004) I am doing this in the context of inactive *PUP1* and *PRE3*, which as I have demonstrated increases sensitivity to proteasome both at the physiological level of proliferation (*Chapter Three*) and the level of transcriptional activation (*Chapter Four*).

A surprisingly small number of genes were identified as targets of the proteasome inhibitor PS341 (bortezomib/Velcade) in a time-series study of transcription (Fleming *et al.* 2002). This study is noteworthy as both one of the first genome-wide characterization of the proteasome and its inhibitors and in its contribution for understanding the proteasome in *S. cerevisiae*. It was from this study that the drug efflux-pump *PDR5* was convincingly demonstrated to inhibit the potency of proteasome inhibitors *in vivo*. Furthermore, Fleming *et al* identified Rpn4 as an important regulator of the transcriptional response to PS341. Rpn4 is an important activator that induces the expression of most proteasome subunits and ubiquitylation in response to decrease proteasome activity (Fleming *et al.* 2002; Dembla-Rajpal *et al.* 2004). Even after four hours of treatment, the majority of the genes with expression altered after treatment with PS341 depend on functional Rpn4.

Another set of analysis, by the Rymond group, found substantially more genes with expression that was elevated in the presence of proteasome inhibitor, in this case MG132 (Dembla-Rajpal *et al.* 2004). Included in their set of genes that were induced by proteasome inhibition were proteasome subunits, genes likely involved in responding to general cellular stressors such as temperature, and mitochondrial function.

One other notable genome wide analysis of the proteasome examined the association of the proteasome to genes rather than the effects of proteasome inhibition on transcription (Auld *et al.* 2006). The Silver laboratory performed these ChIP-on-chip (microarray analysis of DNA from a chromatin immunoprecipitation) experiments using epitope tagged versions of Pre1 (20S proteasome

β-subunit), Rpt1 (19S proteasome base), and Rpn11 (19S proteasome lid) to determine the distribution and overlap of these subunits. Tagging proteasome subunits alters the stability of the proteasome (F. Geng, *personal communication*), and consequently to what extent a proteasome subunit associates with chromatin independently of the other subunits remains in doubt. However, this study did suggest that the majority of proteasome association with chromatin was with highly transcribed genes, consistent with the possible role of the proteasome as a regulator and even facilitator of transcription.

I examined the data from these experiments to better understand how the proteasome regulates transcription. In particular I was looking for classes of genes that were both repressed by proteasome inhibition and bound by the proteasome subunit Pre1 (the 20S component) to reveal common regulatory patterns. Instead, I found that there was relatively little overlap between the two expression data sets or with either of the expression sets and Pre1 ChIP signals (*Figure **5.1**)*. Indeed the consistency among the expression data was particularly poor for repressed genes. The poor level of agreement among the data sets might reflect differences in the activity of the drugs used, strain differences, and laboratory-to-laboratory variation such as what I found with *GAL1* transcriptional inhibition by MG132 (*Chapter Four*). Alternatively, the data might be due to failure to completely inhibit the proteasomal activity. I showed that the combined chemical and genetic approaches to target all three proteolytic sites of the proteasome increases my ability to detect a role of proteasome-mediated proteolysis in transcriptional activation for certain genes (*Chapter Four*). With the strain that I developed and characterized that have increased sensitivity to proteasome inhibition, I can study the genome wide transcription effects of inhibiting proteasome-mediated proteolysis without concern that the inhibition of the proteasome may be incomplete. Furthermore, studying the genome wide effects of proteasome inhibition can give new insights on the importance of the Pup1 and Pre3 proteasome subunits in the cell.

Based on the time series data from Millenium Pharmaceuticals (Fleming *et al.* 2002) the peak of transcriptional response to MG132 was at one hour of treatment. Therefore, I selected that time of

treatment for my analysis. Neither of the two expression studies used commercially available arrays, therefore I chose to use a Nimblegen array because it provided comprehensive coverage of the *S. cerevisiae* genome with, at the time of my experiments, the largest and most recently updated arrays commercially available. Furthermore, the microarray facility at Vanderbilt University Medical Center, where this set of experiments was conducted, had previously used this platform with success (V. Amman, *personal communication*).

Experimental procedures

Yeast strains

I list the strains used in the course of this study in Table 5.1. The preparation of the strains GAC201 (*PUP1PRE3pdr5*) and GAC202 (*pup1pre3pdr5*) was described in the methods section of Chapter Three.

Yeast were grown in YPAD cultures at an initial $A_{600\ nm}$ of 0.5. One part of the sample was left untreated while the remaining sample was treated for one hour with 50 μM MG132. (Samples treated for two and four hours with MG132 were also collected for later analysis).

Table 5.1: Yeast Strains Used

Strain	Genotype	Source
GAC201	Matα *his3-11 leu2-3,112 ura3-52 lys2-801 trp1-1 pre3Δ::HIS3 pup1Δ::leu2::HIS3* [pRS317-*PUP1*] [YCplac22-*PRE3*] gal$^-$	This Study
GAC201	Matα *his3-11 leu2-3,112 ura3-52 lys2-801 trp1-1 pre3Δ::HIS3 pup1Δ::leu2:HIS3* [pRS317-*pup1-T30A*] [YCplac22-*pre3-T20A*] gal$^-$ ρ^0	This Study

RNA extraction and purification

I collected RNA from 15 ml of yeast using a Hot Phenol RNA extraction method. Cells were lysed in RNA extraction buffer (50 mM sodium acetate (pH 4.8), 0.5% SDS, and 10 mM EDTA) and an equal volume of acid (pH 4.3) phenol (*Sigma*) by incubating at 65 °C for one hour. Samples were chilled on ice for five minutes, centrifuged for five minutes, and the aqueous phases was collected for

purification through another extraction with acidic (pH 4.3) phoenol followed with a phenol:chloroform:isoamyl alcohol extraction. Samples were precipitated in ethanol for 20 minutes at –20°C. RNA was resuspend in nuclease free water. Contaminating DNA was removed with *DNaseI* (*Roche*). RNA was further purified by passing the RNA solution through RNeasy coloumns (*Qiagen*) and delivered to the Vanderbilt University Medical Center Functional Genomics Shared Resource Center (FGSRC) for labeling and hybridizaiton.

Labeling and Hybridization

All RNA Preps submitted to the FGSRC were run on an Agilent 2100 Bioanalyzer to assess RNA integrity. Those samples meeting minimum requirements of RNA integrity number of 7.0 and greater were used to generate cDNA targets for hybridization to Nimblegen arrays. 10ug of total RNA from each sample was reverse transcribed at 42 °C using 400 units Superscript II (*Invitrogen*) in the presence of 6 μg anchored oligo dT and an Amino-allyl tagged dUTP (*Sigma*). Final concentrations of dNTP's in the reaction were 200 μM dA,dG,dC, 51 μM dT, and 149 μM AAdUTP (*Sigma*). cDNA targets generated were incubated with NaOH to hydrolyze any remaining total RNA, then neutralized with HCl, and cleaned over Qiaquick PCR Purification columns (*Qiagen*) following manufacturer's protocol with the exception of the following: Two washes were completed with 80% ETOH instead of PE buffer and the elution was completed with nuclease-free H2O instead of EB buffer. The targets were dried to completion and then coupled to either ester-linked Cy3 dyes (*GE*) in 0.1 M Sodium Bicarbonate, pH. 9.0 for 120 minutes in a 20 μl volume. After quenching of unbound dye with 4 M Hydroxylamine (*Aldrich*) Cy3 targets were cleaned up over Qiaquick columns (*Qiagen*). The targets were quantitated and then a total of 125 ng of each target was dried to completion. Each target was resuspended in one of Nimblegen's Sample Tracking Controls, mixed with Hyb cocktail, heat denatured and then loaded on the 12-plex arrays, following standard Nimblegen protocols. The targets were hybridized 16 hours at 42 °C on a Maui Hyb Station (*BioMicro Systems, Inc*., 4 chamber model) mixing program B per Nimblegen protocol. Following post hybridization washes, as per

standard Nimblegen protocol, all arrays were scanned on an AXON 4000B scanner and gene expression levels were determined using the associated feature extraction software (V. Amman, *personal communication*).

Data Analysis

Support for the data analysis of the microarrays for feature extraction and the determination of significant difference between *PUP1PRE3pdr5* and *pup1pre3pdr5* using log-odds ratios was provided by D. Vaka and J. Huang. Significance for comparisons between untreated and MG132 treated samples was based on p-values < 0.05 from student's t-test and FDR values < 0.05 when indicated. Cluster analysis using a Self Organizing Map algorithm and heatmap generation was done using MeV_4_5 software (Saeed *et al.* 2003). Identification of promoter motifs was done using CERES software (Morris *et al.* 2010). Data from the Fleming *et al* microarray experiments was retrieved using yMGV (Lelandais *et al.* 2004). Data from Dembla-Rajpal *et al* and Auld *et al* experiments was obtained in the supplemental data (Dembla-Rajpal *et al.* 2004; Auld *et al.* 2006).

Results

Meta-analysis of previous genome wide studies of the proteasome

I examined the published data sets from three genome-wide analysis of the proteasome in *S. cerevisiae*. Millenium Pharmaceuticals characterized the small molecule proteasome inhibitor that they developed, PS341 (bortezomib/Velcade), in a times series set of experiments (Fleming *et al.* 2002). I compared the genes described as significant after one hour of treatment to those genes discovered by the Rymond group (Dembla-Rajpal *et al.* 2004), which looked at MG132 on using a macroarray of dot blots using P^{33} labeled nucleotide probes. The degree of overlap between these two data sets was rather poor (*Figure* ***5.1***). For example, of the 1026 genes Dembla-Rajpa *et al* determined to be significantly induced upon MG132 only 65 (6%) were common to the genes

identified by Fleming *et al* as significantly induced with PS341. In comparison 13 of the 1026 genes *al* in the Dembla-Rajpa *et al* up-regulated set were also in the repressed genes reported by Fleming *et*.

I also compared the effects of proteasome inhibition on gene expression to genome localization of the 20S proteasome subunit Pre1 by the Silver laboratory (Auld *et al.* 2006). I did not expect to find a significant number of genes that were induced with proteasome inhibitor to have high concentrations of proteasome associated at the locus. Consistent with that expectation, none of the genes reported to be induced by Fleming *et al* were reported to have strong Pre1 ChIP signals associated with their locus, and only 1% of the over expressed genes in the Dembla-Rajpal data set were represented in the anti-Pre1 ChIP enriched genes. There were only marginally more repressed genes in common with the Pre1 binding, which was not strong support for direct regulation of those genes by the proteasome.

The disparity between these data and my findings that the use of chemical inhibition of the proteasome can be improved by combining small molecule inhibitors with genetic inactivation of non-targeted proteolytic subunits was sufficient motivation to study the genome wide transcriptional effects of proteasome inhibition in strains without active Pup1 and Pre3.

Transcriptional consequences of inactive pup1 and pre3

Before examining the effects of acute treatment with proteasome inhibitor in yeast without active Pup1 and Pre3, I wanted to determine what the transcriptional consequence of these inactivating mutations on their own had. Therefore, I compared gene expression of untreated *PUP1PRE3pdr5* to untreated *pup1pre3pdr5* yeast. This analysis revealed relatively few genes that were significantly affected (*Figure **5.2**)*. Many of the genes with altered expression in *pup1pre3pdr5* yeast were mitochondrial encoded. The level of transcripts detected was towards the lower limits of signal consistent with a loss of mitochondrial transcription and perhaps genome. Furthermore, many of the other genes that were detected as having significantly lower expression in the *pup1pre3pdr5* strain than the *PUP1PRE3pdr5* strain were nuclear genes encoding for mitochondrial functioning

proteins such as ATP-synthase or components of the electron transport chain (ETC) (*Table **5.2**)*. Other genes with decreased expression in *pup1pre3pdr5* yeast include several hexose transport proteins. Thus, the overall picture that arises from this analysis is that *pup1pre3pdr5* are respiratory deficient. Of the few genes are expressed at significantly higher levels in *pup1pre3pdr5* yeast (*Table **5.3***), *PRE3* is notable, representing a proteasomal subunit, which, because the loss of proteasome function induces the expression of proteasome subunits, may reflect that a degree Rpn4 accumulation and activation. Beyond the changes in these few genes, untreated *pup1pre3pdr5* is nearly indistinguishable, transcriptionally, from *PUP1PRE3pdr5*.

Table 5.2: Genes with significantly lower gene expression in *pup1pre3pdr5* compared to *PUP1PRE3pdr5*. (ETC: Electron Transport Chain; mtRNA: mitochondria RNA).

GENE		Fold Change	p	Function	Complex
Q0130	OLI1	689.00310	0.00000		ATP synthase
Q0105	COB	89.85021	0.00133		ETC
Q0050	AI1	84.95832	0.00175	mtRNA processing	
Q0110	BI2	40.11398	0.00000	mtRNA processing	
Q0065	AI4	38.60458	0.00037	mtRNAprocessing	
Q0045	COX1	29.09804	0.00011		ETC
Q0075	AI5_Beta	27.69429	0.00069		
Q0070	AI5_Alpha	18.60562	0.00105	mtRNA processing	
Q0160	SCE1	15.66131	0.00053	mtRNA processing	
Q0055	AI2	14.56365	0.00157	mtRNA processing	
YDL181W	INH1	13.60847	0.00087		ATP synthase
Q0080	ATP8	10.49211	0.00357		ATP synthase
Q0085	ATP6	8.12190	0.00039		ATP synthase
YHR092C	HXT4	7.98329	0.00022	Hexose transport	
Q0275	COX3	7.13139	0.00008		ETC
YKL163W	PIR3	5.51305	0.00070	Cell Wall Stability	
YDR342C	HXT7	4.57266	0.00013	Hexose transport	
YDR343C	HXT6	4.45961	0.00003	Hexose transport	
YOR065W	CYT1	4.45574	0.00048		ETC
YHR001W-A	QCR10	3.69016	0.00127		ETC
YOL058W	ARG1	3.60404	0.00099	Amino acid biosynthesis	
YFL014W	HSP12	3.04083	0.00113	Cell Stress	
YGR183C	QCR9	2.60904	0.00013		ETC
YEL024W	RIP1	2.38070	0.00036		ETC

Table 5.3: Genes with significantly higher gene expression in *pup1pre3pdr5* compared to *PUP1PRE3pdr5*.

GENE		Fold Change	p	Function	Complex
YKL071W	YKL071W	11.33949	0.00016		
YJL001W	PRE3	3.46515	0.00218		Proteasome
YBR085W	AAC3	3.36576	0.00071	Mitochondrial ATP transport	
YNR034W	SOL1	2.59116	0.00114		
YEL071W	DLD3	2.52412	0.00031		

Expression Differences Upon Treatment with MG132

To test the effects of proteasome inhibitor on transcription, I looked at the effects of MG132 in the *PUP1PRE3pdr5* yeast. Using an FDR threshold of 0.05, no genes were significantly reported as being changed. With a less stringent threshold of significance (p value < 0.05), only 72 genes of the 5777 assayed were reported as significantly changed. Even with this generous threshold, the fold change for these genes is relatively modest, as apparent by the similarity of the heatmap of the statistically significant changes in expression (*Figure **5.3***). The leftmost three columns represent the Log_2 expression of untreated *PUP1PRE3pdr5* yeast. The expression of these three roughly matches those of the right set of three values of expression after one hour of MG132 treatment. These data reflect the general resistance of this *PUP1PRE3pdr5* strain to MG132, which may be due in part to the expression of the proteasome subunits on a plasmid. Because of the lack of substantial changes in the expression profile, I did not invest significant time further investigating the data I obtained for *PUP1PRE3pdr5*. Instead I turned my attention to focus on the transcription changes of *pup1pre3pdr5* yeast in response to MG132 treatment.

The combination of chemical inhibition with MG132 and genetic inactivation of the catalytic sites of Pup1 and Pre3 results in an increase in the number of genes that change in response to proteasome inhibition as compared to *PUP1PRE3pdr5* (*Figure **5.5**)*. Using the same threshold for significance, 671 genes change in response to MG132, which is nearly ten times the number detected in *PUP1PRE3pdr5*. Between *pup1pre3pdr5* and *PUP1PRE3pdr5* there is limited overlap in genes

with significant changes in expression, which may reflect a *bona fide* difference in genes regulated by the proteasome or represent a high false positive rate tolerated to detect any significant level of change in *PUP1PRE3pdr5* yeast. Importantly, there are no cases in which a gene was induced in *pup1pre3pdr5* and repressed *PUP1PRE3pdr5*, or *vice versa*. Using a more stringent cutoff of an FDR threshold less than 0.05, 59 genes still are determined to be significantly altered in expression in response to MG132 treatment (*Figure **5.4***).

In contrast to *PUPPRE3pdr5*, in which the heatmap of untreated samples was nearly identical with samples treated with MG132 (*Figure **5.3***), the left-side (untreated) of the *pup1pre3pdr5* heatmap does not look like the right-side (treated). In fact, there are rather striking examples of strongly induced and strongly repressed genes (*Figure **5.4***). However, unlike comparing the difference between untreated *pup1pre3pdr5* and untreated *PUP1PRE3pdr5*, no clear pathways are obvious in this set.

To be able to make sense of the large number of genes changing in *pup1pre3pdr5* I search for transcription factor binding motifs that were either enriched in genes that were induced by MG132 treatment or genes that were repressed by MG132. I used this approach for several reasons. First, common transcription factor motifs in the promoter represent a potential set of genes with a common regulator, which would be useful as comparison to Gcn4 regulation of *ARG1* and Ino2/Ino4 regulation of *INO1* in order to better define what steps in transcriptional activation are changed in a proteasome-dependent manner. Second, genes that share common transcription factor motifs often belong in the same cellular pathway, which provides a biological meaning to the changes in gene expression that are occurring. Finally, this approach was simplified by the availability of a searchable database of yeast promoters with their corresponding binding motifs, based on both sequence prediction and empirical evidence from ChIP experiments (Morris *et al.* 2010).

The $\log_2$ fold change in the significant genes that I found in this experiment took on a nearly normal distribution (*Figure **5.6 A***): very few genes had substantial changes in excess of four-fold more (yellow) or less (blue) than untreated yeast, whereas the majority of genes found to be

significantly different had one and half to two fold changes. Moreover, with slightly more than half the significant genes induced in response to MG132, it becomes straightforward to test if transcription factor motifs predicted by the CERES algorithm to be enriched in induced genes actually are enriched. If a transcription factor motif is incorrectly predicted by the CERES algorithm to be enriched for induced genes, then plotting log fold changes of the genes that have a given motif reveals whether such genes are randomly assorted with up (yellow) or down (blue) regulated genes or if there is indeed a particular bias for up-regulation (yellow). Rpn4 (*Figure **5.6 B**)* an activator that is known to induce transcription of proteasome encoding genes in response to proteasome inhibition provides an example for this type of analysis. CERES identified this as a motif commonly occurring in genes induced by MG132, as is demonstrated by plotting the log fold change for genes with Rpn4 motifs. Similarly, Fhl1, which regulates many ribosomal subunits, which Fleming *et al* observed to be repressed in response to proteasome inhibition is detected in my analysis as significantly enriched in the repressed gene category (*Figure **5.6 C**)*.

Other transcription factors with enrichment in induced genes are Hsf1 (*Figure **5.7 A***), Msn2 (*Figure **5.7 B***), Msn4 (*Figure **5.8 C***). These are genes commonly associated with response to cell stress such as heat-shock, osmotic stress, or accumulation of unfolded proteins as would occur with severe inhibition of the proteasome. Not observed in previous genome-wide studies, there is significant enrichment of Gcn4 *(Figure **5.8 A***), Rap1 (*Figure **5.8 B***), and Sfp1 (*Figure **5.8 C***). These represent amino-acid biosynthesis genes (Gcn4) and ribosomal genes (Sfp1) that indicate that concomitant with an increase in chaperones and proteins in the UPS to handle the accumulation of unfolded and damaged proteins there is a decrease in translation to prevent further accumulation of protein levels.

Compared to previous analysis of transcription response to proteasome inhibition, my analysis tended to pick out different genes (*Figure **5.9***). Part of this is expected because the physiological effects of proteasome inhibition are different in the *pup1pre3pdr5* than typical wild type strains. However, the degree of overlap with the other two data sets surprisingly low, particularly

with Dembla-Rajpal with not only lower agreement as a percentage of the genes they identified but also in terms of disagreement as to the direction in which genes responded to proteasome inhibitor.

I also compared the genes that I found significant to the genes that Auld *et al* identified as being bound by Pre1 (*Figure* ***5.10***) because having found more genes that were repressed by proteasome inhibition, I speculated that I would find more genes that were differentially regulated in response to MG132 that were bound by Pre1. To a modest extent this is true (25 repressed genes with significant Pre1 binding compared to 15 genes in the Debmla-Rajpal and Fleming datasets combined). However, the majority of Pre1 bound sites did not significantly change.

Discussion

One of the most striking features of studying global gene expression changes in response to proteasome inhibition is the relatively small number of genes that are affected. The number of genes that I identified as significantly changed represents little more than ten percent of the entire genome. Considering the importance of the proteasome to the cell, as evidenced by the growth defects of *pup1pre3pdr5* yeast treated with MG132, there is a robustness in transcription to proteasome inhibition that is not just the result of incompletely targeting the three proteolytic sites. I believe that this robustness occurs for two reasons. First, the patterns of gene expression observed with treatment of the cells with proteasome inhibitor – increase in proteasome subunits and chaperones and corresponding transcriptional decrease in protein synthesis – provide a buffer on the impact of loss of proteasome function. Second, much of the evidence that leads to the hypothesis of a direct role of the proteasome in transcription is based on studies of inducible genes. My study, and the previous gene expression profiles, used rich media that did not induce many genes. Consequently the genes that tend to provide evidence for a role of the proteasome in transcription are off or at basal levels throughout these experiments. One notable exception is Gcn4 and its transcriptional targets. *ARG1* expression was modestly (1.4 fold) decreased by proteasome inhibition. This is unexpected when its expression

should be towards basal levels. Nevertheless, observing many Gcn4 targets as repressed by proteasome inhibition is encouraging for use of this activator in studying the connection between proteolysis and transcriptional activation. In contrast to Gcn4, Msn2 dependent transcripts are induced in the context of proteasome inhibition. This is interesting because Gcn4 and Msn2 are regulated by similar pathways such as Srb10 (Chi *et al.* 2001). Thus, the separation of response between Gcn4 and Msn2 is interesting and studying the two in parallel could prove insightful for how the UPS stimulates activators such as Gcn4 but inhibits Msn2.

Related to the small number of genes that were observed to be regulated by the proteasome, there is relatively little overlap between Pre1 binding and MG132 regulated gene expression. This is an important concern, because if the proteasome-mediated proteolysis is directly involved in regulating transcription it should not only be detected with actively transcribed genes but also be associated with those genes that significantly change in response to MG132. The differences in genes observed in studying Pre1 binding and proteasome inhibition may be due to strain differences, consequences of using tagged proteasome subunits for ChIP, or once again the inability to look at inducible genes in rich YPAD media.

One of the unanticipated findings is the loss of mitochondrial gene expression in *pup1pre3pdr5* even without treatment with MG132. It will be interesting to determine if this reflects a loss of transcription or a more severe loss of mitochondrial DNA. Generating another *pup1pre3pdr5* strain will be important to test if the mitochondrial defect is a consequence of prolonged loss of Pup1 and Pre3 function or if it represents a unique event in the history of this particularly strain. On-the-other hand, the *pup1pre3pdr5* strain had a very similar transcription profile to *PUP1PRE3pdr5* yeast, suggesting that other significant changes are not already occurring that would complicate studying transcription in *pup1pre3pdr5*.

The one significant result from this work is that it highlights the contribution of removing Pup1 and Pre3 to improving the ability to detect transcriptional defects as a consequence of acute proteasome inhibition with MG132. There was significantly more numerous and larger changes in

response to MG132 in the *pup1pre3pdr5* strain than with the *PUP1PRE3pdr5* yeast, validating it as an important tool to study transcription and proteasome-mediated proteolysis.

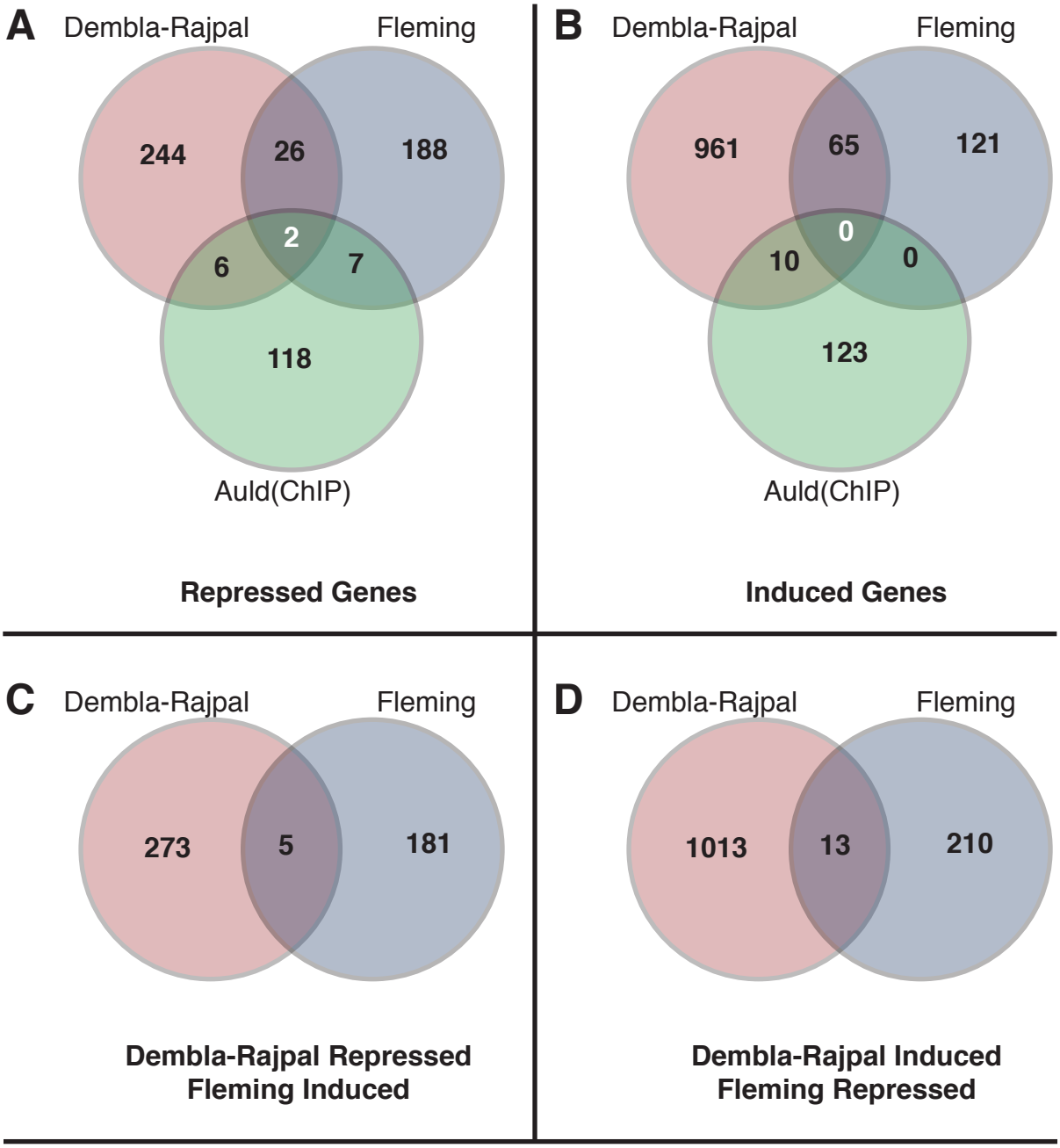

Figure 5.1: Meta-analysis of previous genome wide studies of the proteasome. (A) Venn diagram of genes repressed by MG13 (Red; (Dembla-Rajpal *et al,* 2004) and PS341 (Blue; Fleming *et al*, 2002) compared to genes bound by Pre1 (Green; Auld *et al,* 2006). (B) Corresponding diagram of genes induced by MG132 (Red; Dembla-Rajpal *et al*, 2004) and PS341 (Blue; Flemng *et al*, 2002) compared to genes bound by Pre1 (Green; Auld *et al*, 2006). (C) Disharmony between the two expression data sets. Genes observed as repressed by Dembla-Rajpal *et al* (Red) and induced by Fleming *et al* (Blue). (D) Inverse, corresponding data of genes that Debla-Rajpal *et al* observed as induced whereas where observed by Fleming *et al* as repressed.

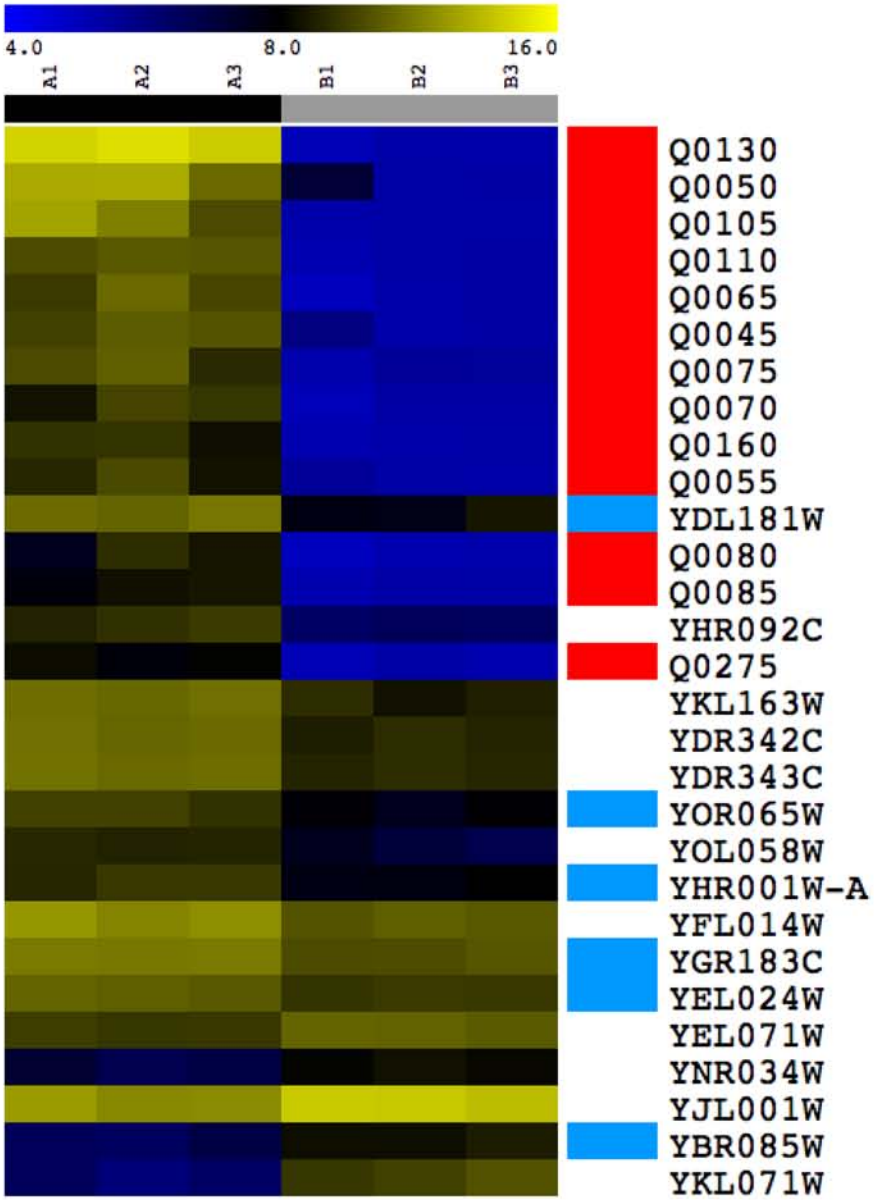

Figure 5.2: Expression differences between *PUP1PRE3pdr5* and *pup1pre3pdr5* without MG132 treatment. Heatmap of genes with significantly different (log-odds) between *PUP1PRE3pdr5* yeast (Samples A1, A2, A3 – black x-cluster) and *pup1pre3pdr5* yeast (Samples B1, B2, B3 – grey x-cluster). Genes encoded in the mitochondrial genome are indicated in red. Genes necessary for mitochondrial-mediated respiration are indicated in sky-blue.

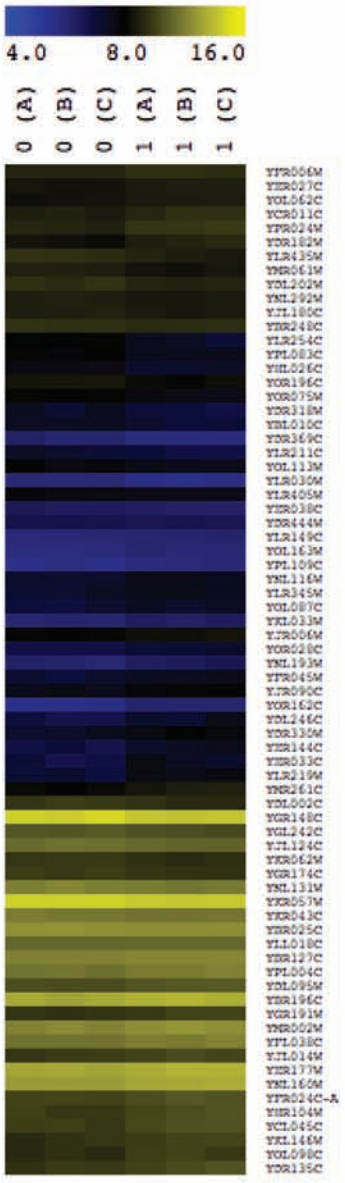

Figure 5.3: Expression differences in *PUP1PRE3pdr5* between untreated and one hour treatment with MG132. Heatmap of genes with significantly different ($p < 0.05$) between untreated *PUP1PRE3pdr5* yeast (Samples 0 (A-C)) and *PUP1PRE3pdr5* treated with MG132 for one hour (Samples 1 (A-C)).

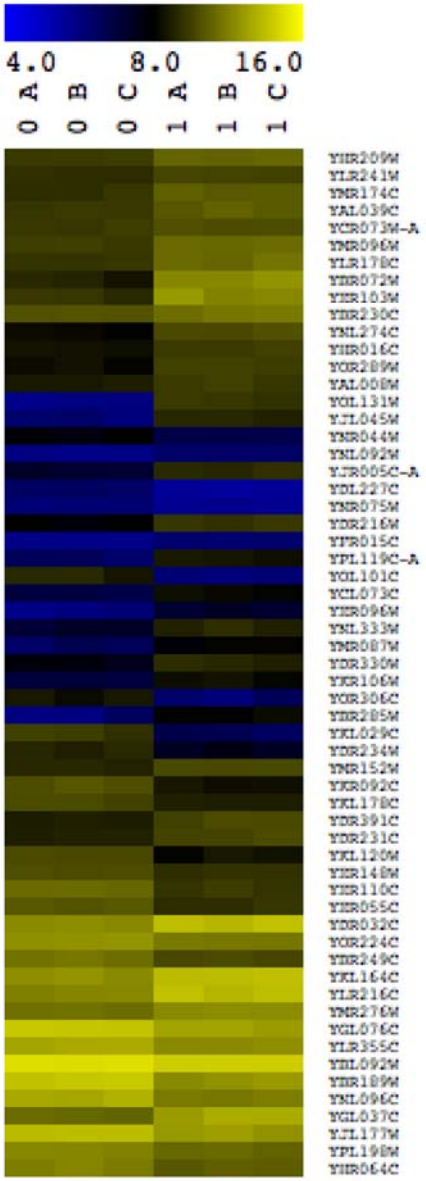

Figure 5.4: Expression differences in *pup1pre3pdr5* between untreated and one hour treatment with MG132. Heatmap of genes with significantly different expression (FDR < 0.05) between untreated *pup1pre3pdr5* yeast (Samples 0 A-C) and *PUP1PRE3pdr5* treated with MG132 for one hour.

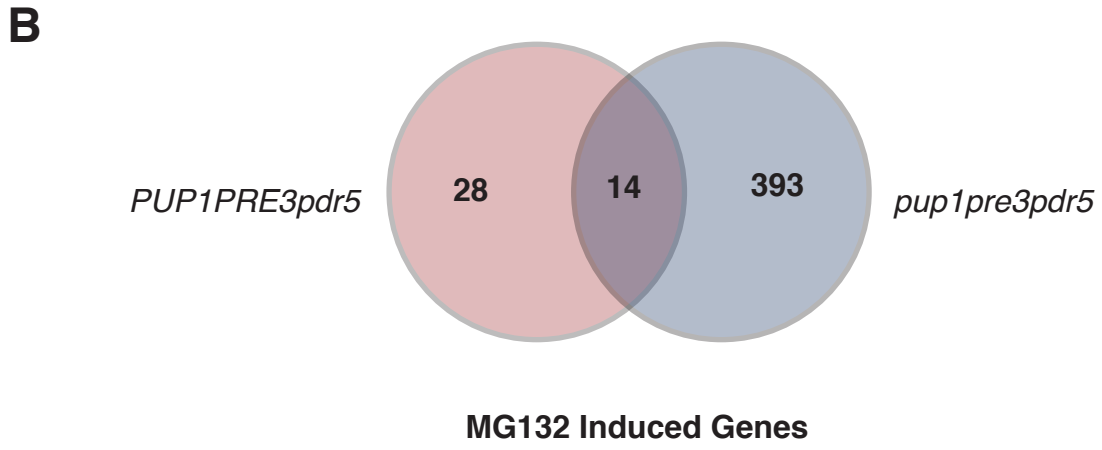

Figure 5.5: Comparison of significantly differentially expressed genes in response to MG132 treatment – *PUP1PRE3pdr5* compared to *pup1pre3pdr5*. (A) Venn diagram of genes repressed by MG132 in *PUP1PRE3pdr5* (Red) and *pup1pre3pdr5* (Blue). (Significance is $p < 0.05$). (B) Venn diagram of genes induced by MG132 in *PUP1PRE3pdr5* (Red) and *pup1pre3pdr5* (Blue). (Significance is $p < 0.05$).

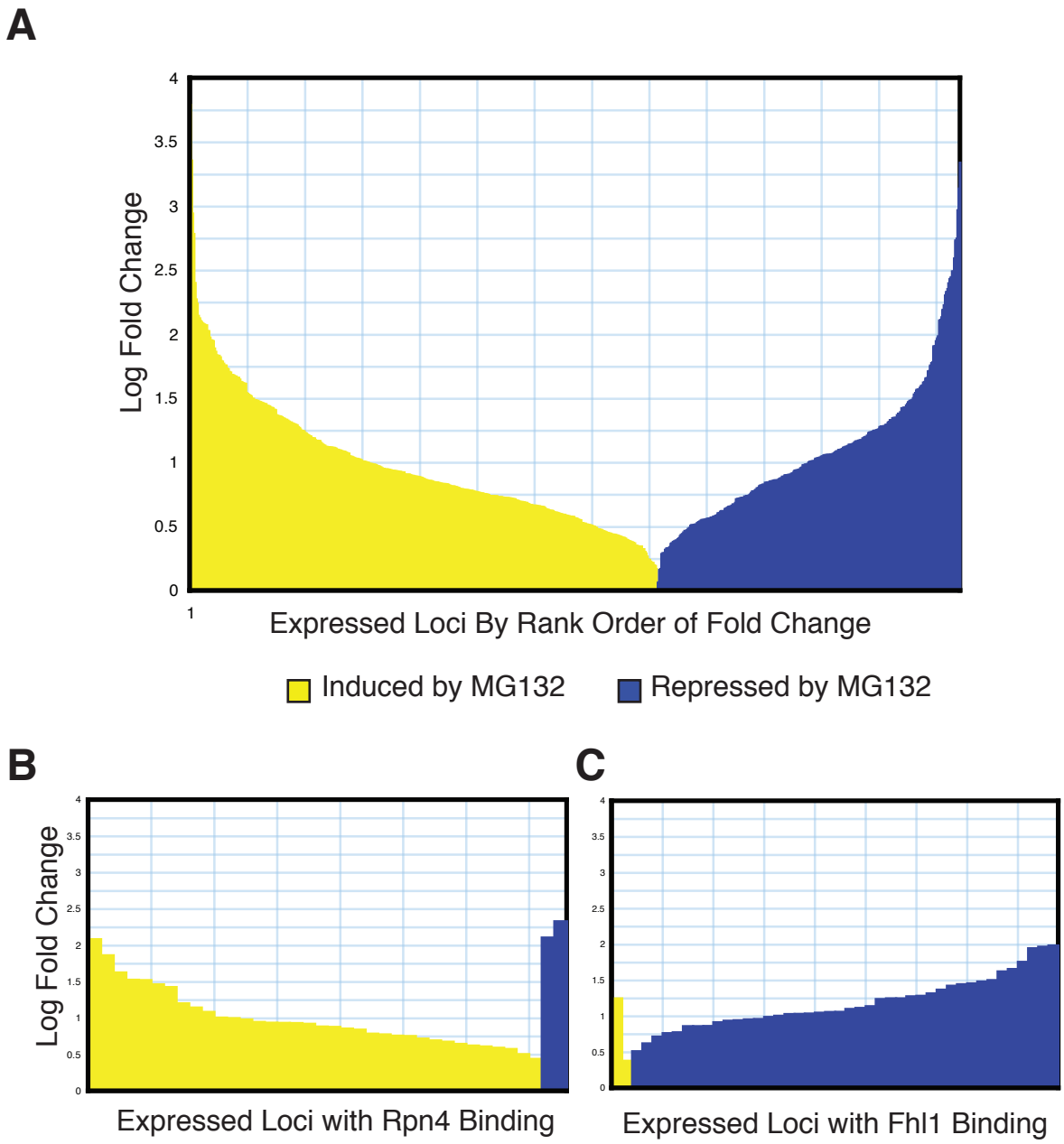

Figure 5.6: Identification of common promoter motifs in genes in which expression is responsive to MG132 treatment. (A) Distribution of Log2 fold change from 0 to 1 hours of treatment. Induction of expression after one hour of treatment with MG132 in yellow. Repression of expression in response to MG132 treatment in Blue. (B) Example of an activator, Rpn4, with binding motifs enriched in genes with increased expression in response to MG132. (C) Example of an activator, Fhl1, with binding motifs enriched in genes with increased expression in response to MG132.

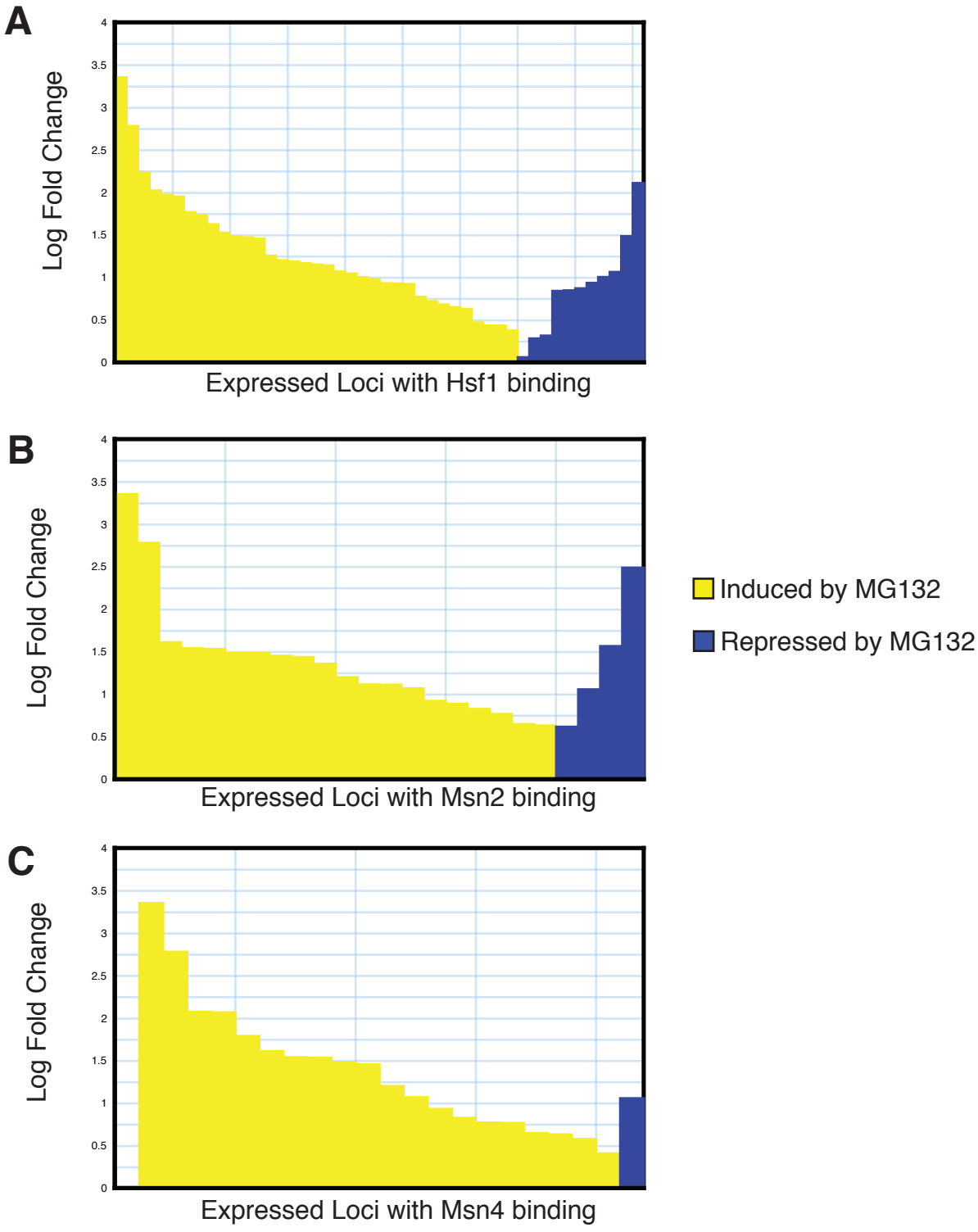

Figure 5.7: Common promoter motifs in genes in which expression is induced by MG132 treatment. (A) Log fold change in expression of genes with Hsf1 binding motifs in their promoters. (B) Log fold change in expression of genes with Msn2 binding motifs in their promoters. (C) Log fold change in expression of genes with Msn4 binding motifs in their promoters.

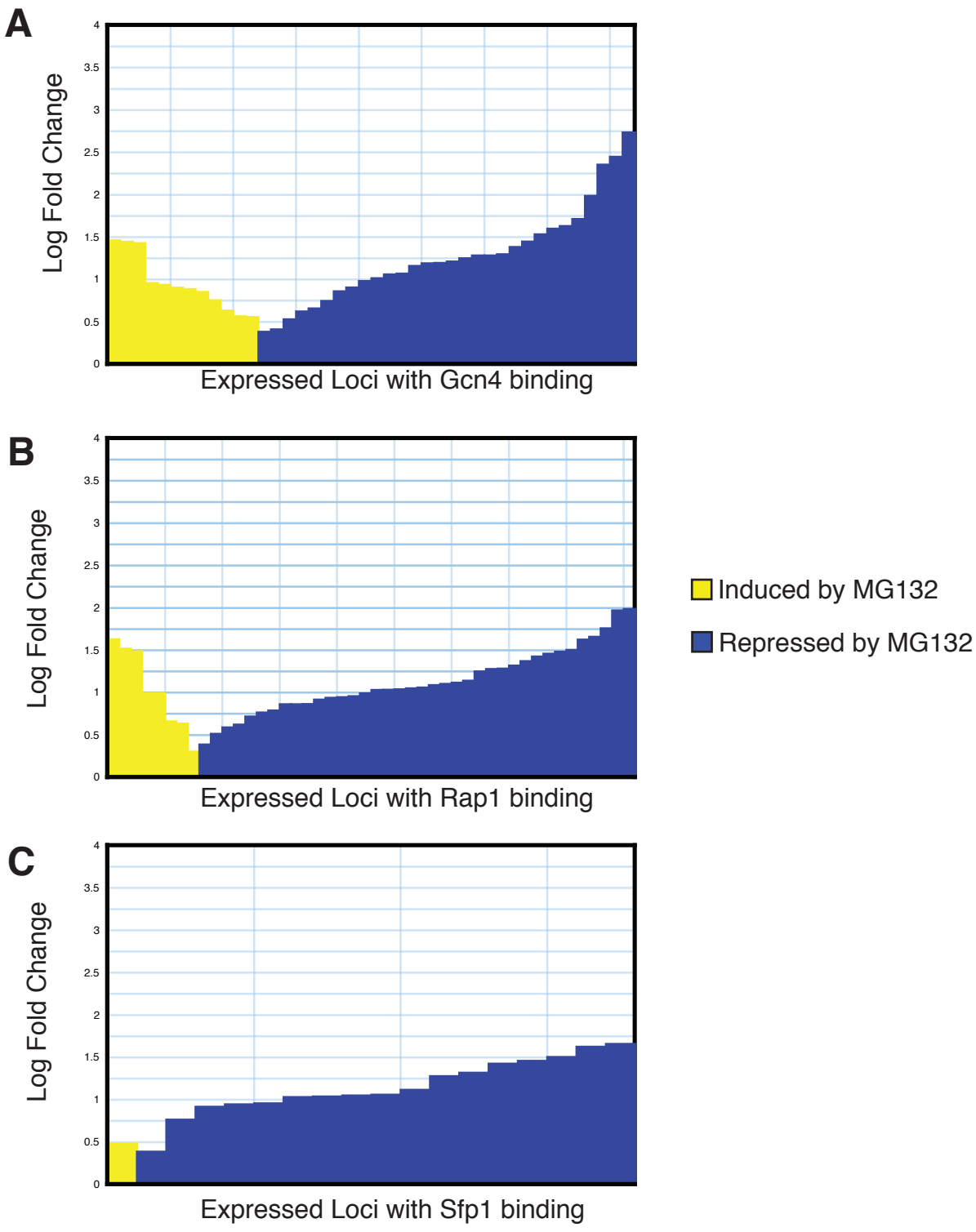

Figure 5.8: Common promoter motifs in genes in which expression is repressed by MG132 treatment. (A) Log fold change in expression of genes with Gcn4 binding motifs in their promoters. (B) Log fold change in expression of genes with Rap1 binding motifs in their promoters. (C) Log fold change in expression of genes with Sfp1 binding motifs in their promoters.

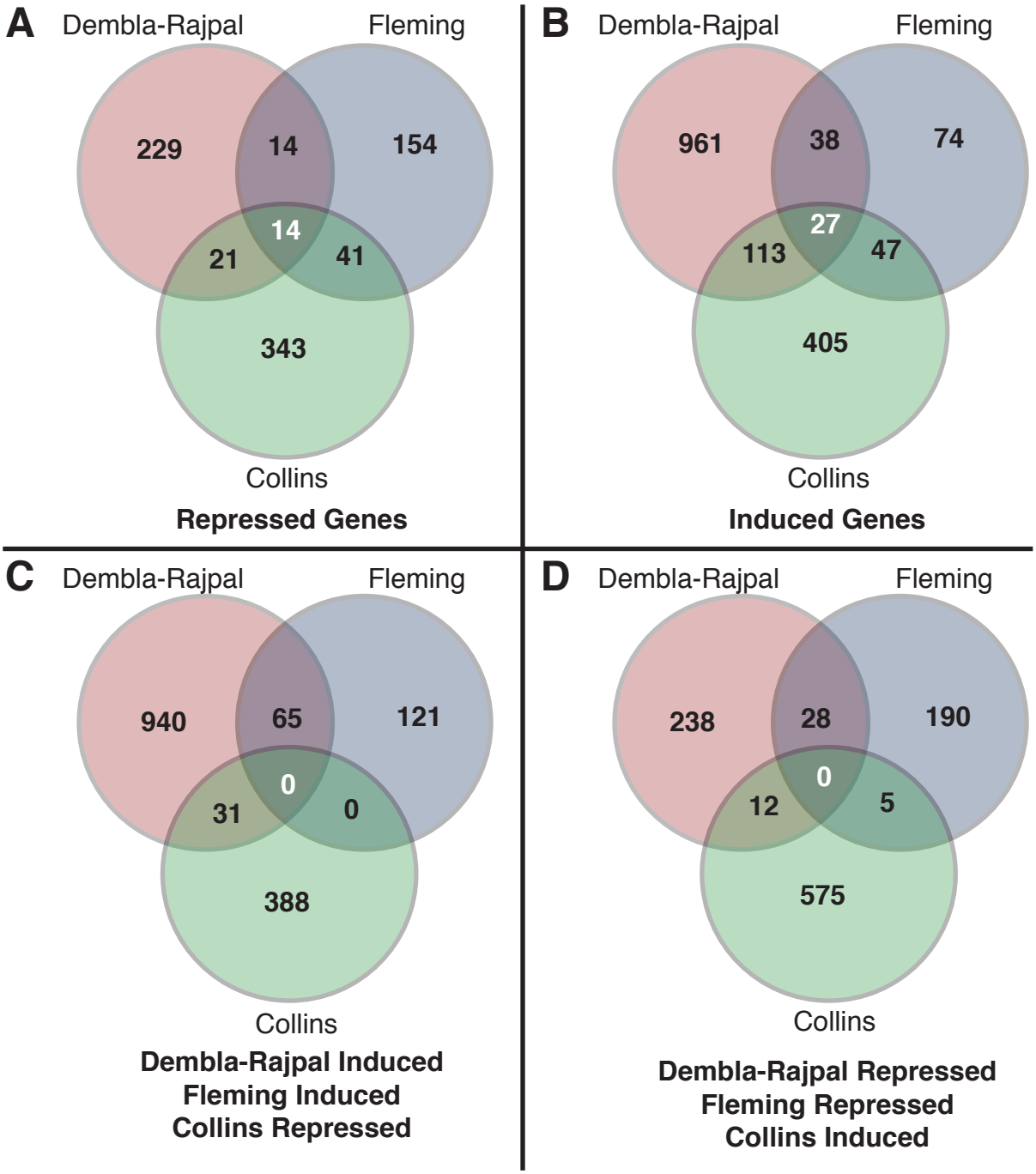

Figure 5.9: Comparison of my significant genes with previous genome wide studies of proteasome inhibition. (A) Overlap of genes repressed in response to MG132 treatment (Red; Dembla-Rajpal *et al*, 2004), (Green; Current work), or to PS341 (Blue; Fleming *et al* 2002). (B) Overlap of genes induced in response to MG132 treatment (Red; Dembla-Rajpal *et al*, 2004), (Green; Current work), or to PS341 (Blue; Fleming *et al* 2002). (C) Disharmony between the two expression data sets. Genes observed as induced by Dembla-Rajpal *et al* (Red) and Fleming *et al* (Blue) compared to those that were repressed in my data set (Green). (D) Disharmony between the two expression data sets. Genes observed as repressed by Dembla-Rajpal *et al* (Red) and Fleming *et al* (Blue) compared to those that were induced in my data set (Green).

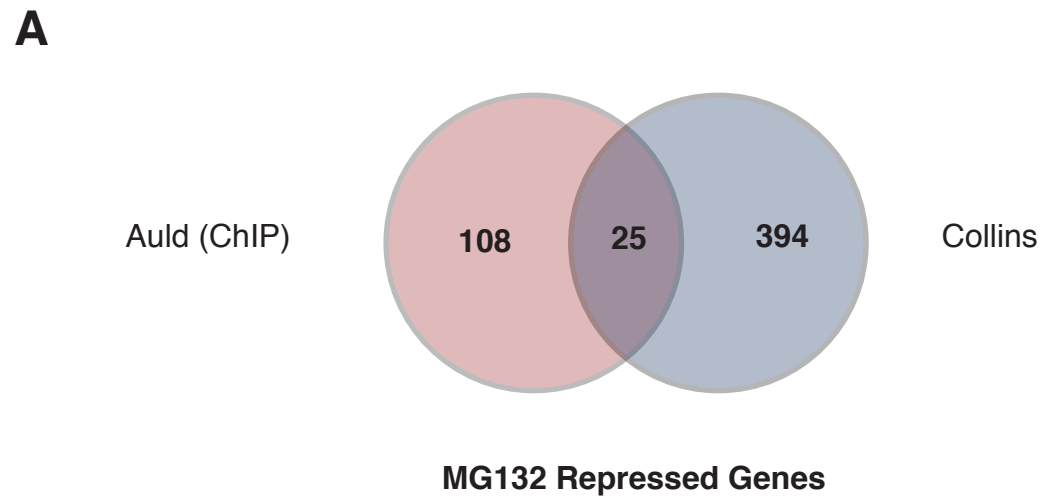

Figure 5.10: Comparison of significantly differentially expressed genes in response to MG132 treatment to localization of Pre1 across the genome (A) Venn diagram of genes bound by Pre1 (Red) and repressed by MG132 (Blue). (B) Venn diagram of genes genes bound by Pre1 (Red) and induced by MG132 (Blue).

Chapter Six: Protein Turnover and Transcriptional Activation

My research focused on addressing if the proteasome regulates transcriptional activators through licensing in which activators are ubiquitylated and destroyed as part of transcription. From this model of activator function, two testable models arise. 1) Activators should not stably associate with their target promoters when transcription occurs. 2) Inhibition of proteolysis should inhibit the ability to activate transcription. To test the first prediction, that activators do not lock onto promoters during transcription, I used the classical activator Gal4 to demonstrate that this activator is labile and undergoes turnover on the promoter during conditions of active transcription (*Chapter Two*). I have also used Gal4 to demonstrate sensitivity of transcriptional activation to proteasome inhibition (*Chapter Four*). Developing a new strain of yeast that has increased sensitivity to proteasome inhibition (*Chapter Three*), I also demonstrated that two other inducible genes, *ARG1* and *INO1*, failed to activate when treated with MG132 (*Chapter Four*). However, not all genes are regulated by the proteasome in this fashion. Activation of *CHA1* is not affected by shutdown of the proteasomal proteases (*Chapter Four*). I also extended my work with the new yeast strain, which improves inhibition by combining chemical inhibition of MG132 with the genetic abolition of the critical catalytic sites of Pup1 and Pre3, to characterizing the whole genome transcriptional defects of loosing proteasome function. The improved method of proteolytic inhibition enables the detection of many more genes under the control of the proteasome than is possible by simple treatment of MG132 without inactivating Pup1 and Pre3 (*Chapter Five*).

Activator turnover may occur by non-proteasome-mediated means through other chaperones or by basic association-dissociation kinetics. Furthermore, proteasome inhibition may not regulate transcription through transcription activator but by stabilizing repressors or impairing the signaling pathways for induction. However, given the known role of ubiquitylation and ubiquitin ligases in regulating several model activators such as Gal4 and Gcn4 and the importance of proteasome-mediated proteolysis for hundreds of genes in the yeast cell, the potential for the proteasome to

regulate transcription at the level of the activator is not only a plausible model, but one that seems to best account for the known data.

Many improved techniques and basic characterization of the impact of the proteasome on transcription should provide for the ability to advance the understanding of the mechanisms of how the proteasome regulates transcriptional activation. The corrected system for studying transcription activator turnover, the improved inhibition of the proteasome, and a list of genes that are regulated by the proteasome are tools that my work provides that can be combined with existing and new mutations in model activators (*e.g.* Gal4 and Gcn4) and the promoters of the genes that they target (*e.g. GAL1* and *ARG1*) and emerging data regarding the positioning of proteasomes across the genome and in response to changes in transcription to advance this field.

The immediate steps that I would take would be to use the activator Gcn4 and its mutant variant *gcn4-3T2S,* which by virtue of its inability to be phosphorylated also bypasses the requirement for ubiquitylation and functional proteolysis to drive transcription (Lipford and Deshaies 2003). The changes in the recruitment of the initiation complex should be investigated in detail as presumably proteasome inhibition decreases the levels of RNApolII that can be recruited to Gcn4 regulated promoters. The *gcn4-3T2S* mutant may also be a tool to determine if ubiquitylation and proteolysis regulate activator turnover.

I have shown that the typical approach of treating yeast with MG132, even with an impaired drug efflux system from deleting *PDR5,* does not achieve a very strong phenotype. Cell proliferation in this context continues to occur, and it has been documented that several known ubiquitylated proteins, such as cyclins, are notably absent from proteomic profiling of ubiquitylated proteins even when MG132 or other proteasome inhibitors are used to stabilize ubiquitylated substrates. I have demonstrated that a much more robust inhibition of the proteasome can be accomplished by not only targeting the chymotryptic proteasome subunit with MG132 but by also removing the catalytic centers of the tryptic and caspase-like subunits. This approach results in significant impairment of the cell proliferation and increased accumulation of ubiquitylated substrates. The combined chemical and

genetic inhibition of the proteasome should thus provide a valuable technique for other researchers studying the biology and mechanisms of the UPS.

My work also suggests that more research needs to be directed to the contributions of the tryptic and caspase-like subunits of the proteasome to various cell processes. I am working on a next generation version of yeast that not only is amenable to studying galactose induction but also is able to separate the relative contributions of Pup1 and Pre3. This work may revise our current model of the chymotryptic subunit being *the* rate limiting subunit for *all* processes regulated by the proteasome. Such information would not only alter our understanding regarding the function of the proteasome but might also redesign approaches to inhibiting the proteasome in human disease.

My work has examined two predictions of the proteasome to regulate transcription. The natural consequence of destroying activators during transcription is that there should be rapid turnover of the activator. The results of Nalley *et al* for Gal4 using competitive ChIP to measure this turnover provided compelling evidence that a potent and unstable activator none-the-less locked onto the promoter during transcription (Nalley et al. 2006). However, when using this technique I noticed that 17-β-estradiol in the absence of competitor Gal4 increased the level of endogenous Gal4 detected with ChIP. This fact alone explained the apparent stability of Gal4. An alternative means of inducing competition using 4-hydroxy tamoxifen did not induce the same artifact. Using this method demonstrated that Gal4 does not lock onto the promoter during active transcription but remains dynamic.

My work studying the effects of proteasome inhibitors accomplished two things. First, my work led to the development of a new strain that has increased sensitivity to proteasome inhibition. Not only did this technical improvement lead to an improved means to study the proteasome in transcription but it also provides a useful resource to study other pathways regulated by the UPS. Moreover, this strain demonstrates the importance of Pup1 and Pre3 to the biology of *S. cerevisiae*. Inactivation of these two proteasome subunits results in decreased proliferation, accumulation of ubiquitylated protein, and even impacts the activation of certain genes. These subunits have typically

been considered secondary to the chymotryptic activity of Pre2, but my data suggest that further investigation needs to be directed towards the tryptic and caspase-activities alongside the chymotryptic subunit.

Finally I have shown that, while not a universal mechanism for regulating transcription, inhibition of the proteasome can inhibit the induction of certain genes. These data reshape our understanding of the proteasome in transcription. Previous means of inhibiting the proteasome by chemical means using MG132 alone have had difficulty revealing the role of proteolysis in transcription. However, inhibiting multiple subunits by combining chemical and genetic approaches demonstrates an important role of proteolysis in regulating transcriptional activation. Given the current evidence, an important regulatory role of the proteasome through proteolysis is plausible.

What then would be the role of proteasome-mediated proteolysis in transcription? As central regulators of gene expression, transcription activators are likely targets of proteolysis. Targeting activators would generate a cycle of activator recruitment, transcription, and activator destruction followed by renewed activator recruitment and so on. Thus, when this cycle of activation is arrested (at renewed activator recruitment), transcription rapidly stops. I propose that a key function of proteasome-mediated proteolysis is to maintain such transcriptional programs in a responsive state to fluctuations in the signaling environment.

Proteasome-mediated proteolysis may also provide dynamic control to the process of moving from an assembled initiation complex to effective transcription elongation. The assembled initiation complex —including not just RNApolII and its general transcription factors, but also the mediator complex, several co-activators, and critically the activator itself —must be re-arranged to permit efficient transition to the elongation phase of transcription. There has been speculation that in the process of transcriptional activation there is a special pioneer round of transcription (Jiang and Gralla 1993; Yudkovsky *et al.* 2000; Ansari and Hampsey 2005; Arndt and Winston 2005; Malik *et al.* 2007; Singh and Hampsey 2007; Tran and Gralla 2008; Kaderi *et al.* 2009). In this round of transcription, the transition from initiation to elongation phases occurs without fully recruiting

components of the elongation or the co-transcriptional RNA processing machinery. Instead this initial round of transcription remodels the chromatin along the gene to facilitate greater efficiency in subsequent rounds of transcription, an effect that has been termed "transcriptional memory" (Ng *et al.* 2003; Xiao *et al.* 2005; Kaderi *et al.* 2009). I propose that instead of there simply being a pioneer round and then suddenly efficient transcription, there is instead a gradual transition from inefficient transcription to effective transcription that is accelerated by the ubiquitylation and subsequent proteolysis of activators.

In my model of how proteolysis activators transcription (see *Figure* ***6.1***) there is a clear progression wherein the activator is first phosphorylated (*Figure* ***6.1-B***), then ubiquitylated (*Figure* ***6.1-C***), and ultimately destroyed (*Figure* ***6.1-D***). Initial, or pioneer, rounds of transcription are inefficient, with slow rates of transcription and failure to recruit elongation factors and co-transcriptional RNA processing enzymes (*Figure* ***6.1-A***). Phosphorylation of the activator alters the association of the transcriptional initiation complex so that RNApolII can transition more quickly from being recruited to the promoter into the elongation phase of RNA synthesis. However, the phosphorylation of potent activators, such as Gal4 and Gcn4, might be too effective, causing premature escape of RNApolII before the phosphorylation of RNApolII at its CTD and before the recruitment of elongation and co-transcriptional processing complexes (*Figure* ***6.1-B***). Ubiquitylation, therefore, functions analogously to a resistor in an electrical circuit in that it stabilizes the progression of RNApolII from an initiated to elongating state (*Figure* ***6.1-C***). How precisely this is achieved is not yet clear. One possibility is that ubiquitylation serves as a scaffold to stabilize the initiation complex by increasing the interaction surface. Alternatively, instead of being recognized by the initiation complex, ubiquitin because of its bulk in an already massive initiation complex could act as a wedge to misalign the initiation complex into an arrangement that delays the escape of RNApolII from the promoter. In addition to its role as a "resistor," ubiquitylation of the activator also serves to recruit the elongation factors (Hobeika *et al.* 2007) and the proteasome, which is required for efficient histone methylation (Ezhkova and Tansey 2004; Laribee *et al.* 2007) and transcriptional

elongation (Ferdous *et al.* 2001; Lassot *et al.* 2007). So far the evidence points to non-proteolytic roles for the proteasome in the processes of histone methylation and RNApolII elongation. The proteolytic role of the proteasome, therefore, is to modulate the resistance of activator ubiquitylation (*Figure **6.1-D***) and to bail out stalled RNApolII (Svejstrup 2003; Somesh *et al.* 2005). An activator with multiple conjugated ubiquitin proteins conjugated not only is a more powerful "resistor" but now becomes a target for proteolysis resulting in the dual benefit reducing the resistance and increasing the dynamics of the activator to be more responsive to changes in inducing signals.

In my model, ubiquitylation and proteolysis is one means to transition to efficient transcription. Cycles of ubiquitylation and proteolysis speed the change from inefficient and ineffective pioneer rounds of transcription to a state of "transcription memory" —be it delocalization of the chromatin to the nuclear periphery (Brickner and Walter 2004; Brickner 2009; Brickner 2010), the formation of chromatin loops (Ansari and Hampsey 2005), opening the chromatin by nucleosome remodeling (Kundu *et al.* 2007), or nucleosome modifications such as methylation of histone H3 on lysine 36 (Xiao *et al.* 2007; Youdell *et al.* 2008). Once this transition to a "transcriptional memory" state has been achieved, RNApolII is more effectively phosphorylated, elongation factors and co-transcriptional RNA processing complexes are more effectively recruited, and the cycles of ubiquitylation and proteolysis of the activator are no longer necessary to increase efficient transcription (Li *et al.* 2010). Instead ubiquitylation and subsequent proteolysis of the activators maintains the sensitivity of the promoter complex to changes in the activating signals (*Figure **6.1-E***).

There are several predictions that naturally arise from this model. First, this need not be a universal model for the function of transcriptional activators at all genes. Genes that are transcribed at low levels will not exhibit the same hallmarks of activator ubiquitylation and proteolysis that will occur at genes with high levels of induced transcription. Similarly, weak activators may be phosphorylated without pushing the rate of polymerase escape beyond the rate of phosphorylation of RNApolII and recruitment of elongation factors and co-transcriptional RNA processing complexes. Furthermore, activators do not have to be the sole target of ubiquitylation. The same pattern of

phosphorylation, ubiquitylation, and degradation of the human estrogen receptor co-activator SRC-3 has been documented (Wu *et al*. 2007). Finally, prolonged activation should not be dependent on ubiquitylation and proteolysis, but only the initial build up to efficient transcription will be dependent on ubiquitylation and proteolysis.

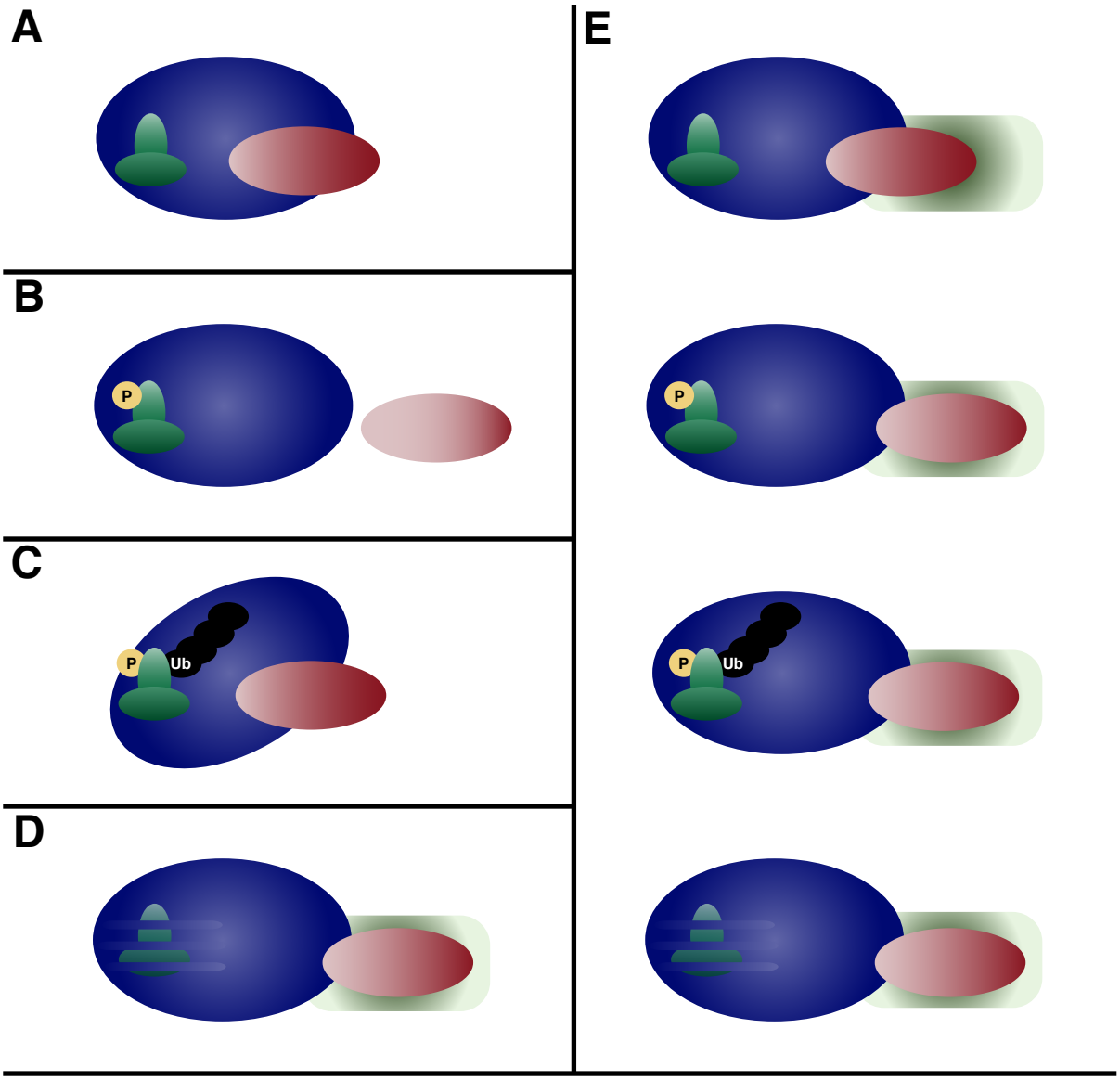

Figure 6.1 Model of How Ubiquitylation and Proteolysis of a Transcription Activator Might Regulate Transcription. (A) Activator (green) recruits RNApolII (red) and the initiation complex. In this "pioneer" round of transcription RNApolII is inefficient. (B) Phosphorylation of the activator increases the rate at which RNApolII can escape. For a potent activator this process is too efficient. (C) Ubiquitylation of the activator alters the conformation of the initiation complex providing "resistance" against the excessive rate of escape induced by phosphorylation. (D) This buys time for the phosphorylation of RNApolII and recruitment of elongation and co-transcriptional RNA processing complexes (green halo around RNApolII). The "resistance" imposed by ubiquitylation is relieved by proteolytic turnover of the activator. (E) In a state of "transcriptional memory", RNApolII is more efficiently phosphorylated along with more rapid recruitment of the elongation and co-transcriptional RNA processing complexes. Thus, while phosphorylation may enhance transcription, the cycle of ubiquitylation and proteolysis is not as necessary as in the early rounds of transcription.

Appendix: Publications Produced in the Course of This Thesis

Collins, G.A. and Tansey W.P. The proteasome: a utility tool for transcription? *Current Opinions in Genetics and Development* (2006) **16**:197-202

A review I wrote with Dr. Tansey summarizing the recent advances in connecting the proteasome to transcription. In this review we discussed the potential of both the proteolytic and non-proteolytic roles of the proteasome throughout transcription.

Leung, A. Geng, F. Daulny, A. Collins, G. Guzzardo, P. Tansey, W.P. Transcriptional control and the ubiquitin-proteasome system. *Ernst Schering Foundation Symposium Proceedings* (2008) 75-97.

A review, that Dr. Tansey and the yeast group of the Tansey laboratory wrote on how the UPS regulates multiple steps of the transcription process.

Collins, G.A. Lipford, J.R., Deshaies, R.J. Tansey, W.P. Gal4 turnover and transcription activation. *Nature* (2009) **461**:E7

A brief communications arising to *Nature*, which on the basis of my work (see *Chapter **2**)*, demonstrates that an artifact exists in the competitive ChIP signals for Gal4 when 17-β-estradiol is used to trigger competition. This artifact leads to the mistaken conclusion that Gal4 "locks" onto promoters during activation. I demonstrated that 4-hydroxytamoxifen is a more suitable trigger for studying Gal4 competition because it does not lead to the same artificial increase in Gal4 binding as was observed with 17-βestradiol. Making this adjustment to the competition ChIP results in finding that Gal4 is labile and does not lock in on the promoter, which is a result that is in better agreement with previous literature describing the behavior of Gal4 and other activators.

Collins, G.A., Gomez, T.A., Deshaies, R.J. Tansey, W.P. Combined chemical and genetic approach to inhibit proteolysis by the proteasome. *Yeast* Submitted

A paper describing the need for a more sensitive strain of yeast to proteasome inhibitors such as MG132 and demonstrating that the combination of chemically targeting the chymotryptic subunit with genetic inactivation of the tryptic and caspase-like subunits of the proteasome results in such a strain (see *Chapter Three*). This strain is characterized for defects in proliferation, cell cycle progression, stability of ubiquitylated proteins (*Chapter Three)*, and activation of transcription (*Chapter Four*).

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