Ubiquitin-mediated Regulation of RNA Polymerase I in *Saccharomyces cerevisiae*

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Abstract

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Ribosomes are key cellular components ensuring that cells correctly synthesize their protein cohort. Dysregulation of this process can lead to improper cell growth and is linked to the progression of several types of cancers, making understanding of this process of great interest to both researchers and clinicians. Ribosome biogenesis, the process by which ribosomes are produced, is largely controlled by regulating the rate-limiting step of rDNA transcription by RNA Polymerase I, which transcribes three of the four ribosomal RNAs within the cell. Although rDNA transcription is highly regulated by post-translational modifications in response to changing environmental conditions, little is known of how RNA Polymerase I activity is regulated. Our previous research identified a novel role for ubiquitin-mediated regulation of RNA Polymerase I in ribosome biogenesis. We discovered that ubiquitination of Rpa190, the largest subunit of RNA polymerase I, regulates growth and is modulated by the deubiquitinating enzyme Ubp10. This thesis explores the details of Rpa190 ubiquitination and provides an understanding of how ubiquitination regulates rDNA transcription using both genetic and pharmacological approaches. First, it identifies how ubiquitination of Rpa190 modulates active rDNA transcription. It also identifies the SCF^{Grr1} complex as a potential upstream ubiquitin pathway that regulates ribosome biogenesis. Second, it identifies that the ubiquitination of Rpa190 likely serves as the physiological axis affected by the pharmacology of BMH-21, a novel small molecule being considered for use as an anti-cancer therapeutic.

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Chapter I: Background and Significance

Regulation of Ribosome Biogenesis

Cellular physiology and health require highly orchestrated signaling pathways that adjust metabolic output according to environmental conditions. Cells have evolved mechanisms to increase or decrease signaling responses to changing environmental factors, nutrient access, or noxious stimuli. Much research has focused on understanding how signaling pathways regulate metabolic processes under specific conditions; however, every pathway involves the ribosome's ability to translate the necessary proteins for a given response. Ultimately, without sufficient ribosome production, all signaling pathways in the cell will be altered. Because ribosomes are responsible for translating every protein a cell needs to survive, the timely and managed production of ribosomes is a foundational process for cell growth, survival, and proliferation. Thus, ribosome biogenesis (RiBi) is one of the most important and energetically expensive processes in the cell (Thomson et al., 2013). The energy expenditure and cellular investment is significant: 60% of cellular transcription occurs on the ribosomal DNA (rDNA), 80% of all cellular RNA is ribosomal RNA (rRNA), and up to 2000 ribosomes are produced per minute when the cell is actively engaged in ribosome biogenesis (Henras et al., 2008; Warner, 1999). The complexity and energetics of ribosome biogenesis highlight the essential importance for the cell to control production of ribosomes.

The complexity of the ribosome biogenesis is also underscored by the number of proteins involved in the process. Eukaryotic ribosomes contain 4 ribosomal RNAs and 79-80 proteins (yeast and humans, respectively). The 60S large subunit of the yeast ribosome is comprised of 5S, 5.8S and 25S rRNAs and 46 ribosomal proteins (r-proteins), and the small 40S subunit of the ribosome incorporates 18S rRNA and 33 r-proteins (Fromont-Racine et al., 2003). Unlike traditional protein-complex assembly, construction of the ribosome occurs co-transcriptionally and the assembly process requires considerable coordination due to the complexity of its construction. RiBi requires coordination of all three RNA polymerases, over 250 *trans*-acting proteins, and orchestrated synthesis of hundreds of RNAs and their associated proteins to produce a single ribosome (Figure 1.1). RNA Polymerase II translates all

ribosomal proteins and RNA Polymerase III transcribes transfer RNA and 5S rRNA, which is part of the 60S ribosomal subunit.

The rate-limiting step in RiBi occurs in the nucleolus during rDNA transcription via RNA Polymerase I (RNAPI). RNAPI co-transcribes 5.8S, 18S, and 25S rRNAs (3 of the 4 ribosomal RNAs) as a single polycistronic transcript, known as the larger rRNA precursor 45S rRNA. As transcription progresses, the 45S precursor is matured into 5.8S, 18S, and 25S rRNA (Tollervey et al., 1993). Through both exo-and endo-nucleolytic digestions, the rRNA forms ball-like structures at the nascent 5' end of the transcript which can be visualized by chromatin spreads (Miller and Beatty, 1969). These ball-like structures are believed to form the 90S small subunit (SSU) processome complexes, which are the earliest pre-ribosome structures thought to contain approximately 80 ribosomal proteins, over 150 associated proteins, and small nucleolar RNAs (snoRNAs) (Bernstein et al., 2004).

The 90S SSU processome helps ensure correct folding, modification, and cleavage of rRNA. Once the 90S is formed, it is cleaved into the pre-40S and pre-60S pre-ribosomal particles and is exported from the nucleus to the cytoplasm for further maturation and assembly with other proteins (Vanrobays et al., 2001). Since ribosomes are assembled co-transcriptionally, this rate-limiting step ensures rRNA synthesis aligns with transcription of necessary ribosomal proteins for proper ribosome assembly (Turowski and Tollervey, 2015).

Research into the regulation of rDNA transcription as the rate-limiting step of RiBi has led to a better understanding of how the rDNA transcription process is regulated. Ribosomal DNA exists as a tandem repeated array (about 200 repeats in yeast) on the right arm of chromosome XII and is conserved amongst species; however, the number of repeats and length of rDNA genes differs between species (over 200 in mammalian cells on 5 different chromosomes) (Schneider, 2012; Wai et al., 2000). In yeast, rDNA transcription begins with RNAPI, the upstream activating factor (UAF), the TATA-binding protein (TBP), and the core factor (CF), all coordinately binding to the rDNA promoter. This signals for the RNAPI specific initiation factor Rrn3 to bind to the Rpa43 subunit of the RNAPI complex and initiate rDNA

transcription. Rrn3 is the main initiator of rDNA transcription; post-translational modifications of Rrn3, such as phosphorylation, regulate this process (Peyroche et al., 2000; Schneider, 2012).

Several small nucleotide ribosomal particles (snoRNPs), along with other ribosomal proteins and pre-ribosomal assembly factors, also modify rRNA co-transcriptionally; these modifications take place with ribosomal assembly to modify rRNA by removing transcribed spacers via endo- and exonucleolytic cleaves (Henras et al., 2015). There are two classes of snoRNPS (C/D and H/ACA) which can direct site specific 2'-O-ribsome methylation and pseudouridylation of rRNA (Kiss et al., 2004). Even with numerous studies into the regulation of RiBi, we still have much to learn about how this process is controlled, especially via post-translation modifications.

While examining the initiation of rDNA transcription *in vivo* is relatively straightforward, elongation of rDNA transcription is difficult to study and the requirements for elongation vary *in vitro* compared with *in vivo*. Unlike RNA Polymerase II, RNAPI is capable of arresting transcription alone and does not require trans-acting factors to clear transcriptional arrests during elongation (Kuhn et al., 2007). When RNA Polymerase II encounters an arrest, it "back-tracks" to reconfigure elongation by recruiting trans-acting factors to correct the misalignment (Ghavi-Helm et al., 2008). This property is a result of one to two RNA Polymerase II complexes transcribing any given gene at one time. which is not the case for RNAPI. RNAPI's ability to transcriptionally arrest is a direct result of multiple RNAPI complexes on the same strand of rDNA that are actively transcribing simultaneously (Russell and Zomerdijk, 2006). Due to this feature, a failure for one RNAPI complex to proceed can halt transcription by causing a buildup of all RNAPI complexes upstream of the stalled complex.

A key subunit of RNAPI, Rpa12, can promote cleavage during the transcriptional arrest to help resolve any transcription conflicts (Kuhn et al., 2007). Additional *in vitro* studies demonstrated that mutations in Rpa49 and Rpa43 (also subunits of RNAPI) induce elongation defects in rDNA transcription (Kuhn et al., 2007), suggesting an important function for these proteins in transcriptional arrest. Finally, the elongation inhibitor 6-azauracil has a pronounced growth effect in cells carrying a mutation in the

second largest subunit of RNAPI, Rpa135, which has elongation defects resulting from the prevention of loading incoming NTP molecules (Schneider et al., 2007).

Despite identification of some factors that regulate elongation, it is unclear how RNAPI is extracted from the chromosome upon transcription elongation failure. While mechanisms for RNA Polymerase II have been identified (e.g. ubiquitination of the c-terminal tail of RNAPII after DNA damage (Hsin and Manley, 2012)), mechanisms for RNAPI remain unknown (Wilson et al., 2013). Together, current knowledge in the field uncovered several factors that regulate elongation of rDNA transcription, but also revealed a greater need for understanding how RNAPI resolves transcription complexes after encountering transcriptional arrest. The next section introduces other molecular factors in ribosome biogenesis and specifically how they are involved in regulating ribosome biogenesis under pathophysiological conditions.

Ribosome Biogenesis in Cancer

One of the hallmarks of cancer is a cell's inability to curtail cell proliferation and maintain a differentiated state. RiBi involvement in cancer progression is of considerable interest because rapidly dividing cells need to upregulate the synthesis of ribosomes to meet the protein production needs of cell proliferation. Thus, there has been considerable focus on RiBi's role in cancer over the last century. In 1896, early histological observations revealed that cancer cells have large and irregularly shaped nucleoli compared to normal cells (Pianese, 1896). By 1936, nucleolar hypertrophy became an established marker for cancer (Quin et al., 2014). However, by the early 1960s, burgeoning research in the field revealed that normally proliferating cells also undergo nucleolar hypertrophy, establishing that RiBi is generally important for proliferating cells (Quin et al., 2014).

Research regarding RiBi's involvement in cancer development stalled until the 1990s when new ways to study rDNA transcription became available. A key method utilized silver staining to differentiate cancer cells from normal cells became the standard practice for cancer cell identification (Nagatani et al., 1991). Additionally, these techniques could identify the co-localization of ribosomal proteins during cellular stress. During interphase, ribosomal genes and proteins colocalize in tumor cells compared to normal cells (Derenzini et al., 1998). It was through these studies that the upregulation of rDNA transcription became an established marker of cancer cells compared to normally proliferating cells (Drygin et al., 2010). This information established the altered size and shape of nucleoli as a distinct hallmark for cancer cells, specifically in several breast and liver cancers, large cell carcinomas, and non-Hodgkin's Lymphoma (Quin et al., 2014). The next step was to determine how the regulation of rDNA at the molecular level translates to structural changes in the nucleoli.

While it has been established that a key hallmark of cancer cells is that they must upregulate rDNA transcription to maintain increased protein production and sustained proliferation (Russell and Zomerdijk, 2005), the molecular mechanisms that underlie this dysregulation of ribosome biogenesis and changes in nucleolar morphology observed in cancer cells are still unclear. Studies of tumor suppressor loss and activation of oncogenic proteins set the stage for subsequent research in understanding RiBi's

role in cancer progression. The well-known tumor suppressor p53 is known to inhibit RiBi and alter nucleolar morphology and p53 mutations or loss of expression occurs in almost 50% of primary cancers (Woods et al., 2015). Dysregulation of p53 activity was first identified in cancer progression through genotoxic studies, where it was discovered that the DNA damage response could activate p53 and therefore, decrease cell proliferation (Dai and Lu, 2004; Mills, 2013).

Exploitation of this mechanism became the foundation for early chemotherapeutic agents (discussed in Targeting the Nucleolus); however, these therapeutics also targeted normally proliferating cells as well as rapidly proliferating cancer cells (Hong et al., 2014; Stegh, 2012). Studies of MDM2 (an E3 ubiquitin ligase and a major negative regulator of p53) were crucial because they revealed p53 could be induced in absence of the DNA damage response (this mechanism is discussed in further detail in Ubiquitin Regulation in Ribosome Biogenesis) (Dai and Lu, 2004). Further pharmacological work demonstrated that inhibition of RNAPI (disruption of RiBi) induced p53-mediated senescence, autophagy and/or apoptosis (Vassilev et al., 2004). p53 is now known to act as part of the nucleolar surveillance pathway (Deisenroth and Zhang, 2010; Holmberg Olausson et al., 2012) suggesting that nucleolar disruption is one way p53 can become activated.

Additionally, the proto-oncogene MYC is another major factor in the regulation of RiBi (Campbell and White, 2014). MYC regulates nascent ribosome biogenesis through coordination of all three RNA polymerases (Calcagno et al., 2008; Wang et al., 2008; Westermann et al., 2008). MYC stimulates RNAPI activity by fostering initiation of transcription factors to the promoter to increase rRNA synthesis, stimulating RNA Polymerase II by increasing r-protein synthesis, and regulating RNA Polymerase III transcription by activating TFIIIB. Similar to p53, MYC is also upregulated in over 50% of cancers (Calcagno et al., 2008; Wang et al., 2008; Westermann et al., 2008). In 2008, MYC was found to cause upregulation of ribosome biogenesis and became an established marker of malignancy across several types of cancers (Calcagno et al., 2008; Wang et al., 2008; Westermann et al., 2008).

Another regulator of RNAPI in cancer progression is through the protein PTEN (phophatase and tensin homolog) (Ruggero and Pandolfi, 2003). Loss of PTEN increases RNAPI activity as a direct result of upregulation of the PI3Kα-AKT-mTOR, a pathway that is also known to effect coordination of all three RNA polymerases (Ruggero and Pandolfi, 2003). Altogether, the involvement of p53, MYC, PTEN in RiBi established a definable role for rDNA transcription (and, more broadly, RiBi) as a hallmark of cancer cell proliferation. While it is now understood that major cancer regulators play a key role in RiBi, there is still a need to understand how these regulators and RiBi factors are linked and how they could be exploited as potential therapeutic targets to mitigate cancer progression.

Pharmacological Targeting of the Nucleolus for Cancer

Recognition of the factors involved in regulating RiBi (e.g. p53, MYC, p53, PTEN) in rapidly proliferating cancer cells has resulted in a burgeoning area of cancer therapeutics aimed at targeting the nucleolus to modulate RiBi. A key issue in developing therapies is that factors such as p53, MYC, and PTEN are involved in many cellular pathways, making it difficult to develop therapeutics for factors that specifically alter RiBi. Due to this, more recent research has focused on targeting RiBi at the level of RNAPI action (Bywater et al., 2012; Drygin et al., 2011; Hannan et al., 2012). Several known mutations in r-proteins and ribosome assembly factors are causally linked to cancer progression (Ruggero and Pandolfi, 2003; Zhang and Lu, 2009). Targeting the nucleolus as an anti-cancer treatment (specifically targeting RNAPI) is associated with reservation as RiBi-targeted therapeutics could interfere with "housekeeping" functions of rDNA transcription. There are traditional chemotherapeutic agents that interfere with RiBi (Quin et al., 2014), such as cisplatin and 5-fluorouracil. A mass spectrometry screen of 36 chemotherapy drugs demonstrated that 21 of these drugs interfere with multiple stages of RiBi including rDNA transcription, early rRNA processing, and late rRNA processing (Quin et al., 2014). Development of a drug that does not interfere with normal housekeeping functions of the cell remains a work in progress.

Several drugs (both approved and in clinical trials) inhibit RNAPI either directly or indirectly (Hannan et al., 2013; Poortinga et al., 2015; Quin et al., 2014). Pan-transcription inhibitors, such as actinomycin D, have been used as cancer therapies since the 1970s, despite their lack of specificity for targeting proliferative cancer cells (Merkel et al., 2012; Perry and Kelley, 1970). Actinomycin D intercalates DNA and has some affinity for GC rich regions, which are extremely common in rDNA (Fetherston et al., 1984). Additional studies of actinomycin D established its ability to prevent elongation of RNAPI (Fetherston, et al., 1984), thereby inhibiting RNAPI transcription. However, actinomycin D also inhibits transcription of RNA polymerase II and III and therefore has little specificity (Bensaude, 2011; Casse et al., 1999). Cisplatin (another DNA intercalator introduced in 1972) is currently a standard of care for treatment of over 18 different types of cancers (Jung and Lippard, 2006). Cisplatin inhibits transcription similarly to actinomycin D, but through the DNA damage response without inducing RiBi

stress (Bruno et al., 2017; Jung and Lippard, 2006). While both cisplatin and actinomycin D are in current use, their lack of specificity for RiBi limits their use as a targeted cancer therapeutic for RiBi.

Structurally modifying some commonly used anti-cancer therapeutics has been a useful approach for developing drugs with greater specificity for modulating RiBi in cancer cells. Oxaliplatin, an analog of cisplatin, kills cells without inducing a DNA damage response (Bruno et al., 2017). The mechanism of oxaliplatin appears to be an induction of RiBi stress to kill cancer cells without slowing normally proliferating cells (Bruno et al., 2017). Two other drugs are in clinical trials that specifically target RNAPI transcription of rDNA: CX-5461 and CX-3453 DNA (Bywater et al., 2012; Drygin et al., 2011; Lee et al., 2017; Quin et al., 2016). CX-5461 is in Phase I clinical trials and prevents SL1 (the mammalian homolog of the yeast core factor CF) from binding to the rDNA promoter and recruiting RNAPI for transcription initiation (Bywater et al., 2012; Drygin et al., 2011; Lee et al., 2017; Quin et al., 2016). Promising preclinical data demonstrated that CX-5461 could kill B-cell lymphoma cells while sparing healthy B-cells (Quin, et al., 2014), suggesting it could be specific for targeting cancerous cells. CX-5461 also activates tumor suppressor p53 through the nucleolar surveillance pathway and induces cell death via autophagy (Deisenroth and Zhang, 2010; Woods et al., 2015). CX-3543, known as quaflorin, prevents rDNA transcription similar to CX-5461, in this case by preventing nucleolin 1, a nucleolar protein preferentially associated with unmethylated rRNA genes (Cong et al., 2012; Durut and Saez-Vasquez, 2015), from binding to rDNA resulting in an accumulation of RNAPI at the promoter (Pelletier et al., 2018; Quin et al., 2014; Woods et al., 2015).

Ubiquitination as a Regulator of Ribosome Biogenesis

Post-translational modifications such as phosphorylation, methylation and acetylation are crucial for the precise regulation of RiBi, but functional roles for most of these modifications remain poorly understood (Henras et al., 2008; Leary and Huang, 2001). One post-translational modification which plays a key role in this regulation is the ubiquitination of RNAPI (Shcherbik and Pestov, 2010; Stavreva et al., 2006). Ubiquitin is a small 8.5kDa protein that is covalently attached to a lysine on a target protein (Busch and Goldknopf, 1981). Ubiquitination of a target substrate can signal for degradation, changes in localization, or alterations in protein interactions (Ciechanover, 2006a). The regulation and specificity of substrate ubiquitination can be conferred two ways: through the specificity of the ubiquitination pathways and/or through the type of ubiquitin modification through single or multi-ubiquitin chains (Ronau et al., 2016). The linkage between ubiquitin moieties within their individual chains can alter the outcome of the target substrate (Ciechanover, 2006b).

Covalent attachment of ubiquitin to substrates occurs through an enzymatic cascade (Scheffner et al., 1995). The initial step occurs by a ubiquitin-activating enzyme (UBA or E1) forming a thioester linkage to ubiquitin by catalysis of an ATP molecule. The E1 transfers ubiquitin to a ubiquitin-conjugating enzyme (UBC or E2) through a similar mechanism. This charged ubiquitin is then transferred to a lysine on the target substrate through the action of a ubiquitin-protein ligase (UBPL or E3). In eukaryotes, there are 1-2 E1s, dozens of E2s, and hundreds of E3s, which are responsible for ubiquitinating their specific substrates. Because of the sheer number of E3s, one way the cell confers specificity for ubiquitination of the target substrates is through the binding specificity of the E3.

While the fate of a ubiquitinated protein is determined primarily by the E3 that recruits the substrate for ubiquitination, the fate is also guided by the architecture of the ubiquitin chain (Akutsu et al., 2016; Iconomou and Saunders, 2016). Ubiquitin has 7 lysine residues through which each lysine may form a chain with another ubiquitin molecule. Linkages through Lysine 48 (usually present with the connection of 4 our more ubiquitin moieties) signal a substrate for degradation by the proteasome (Chau et al., 1989; Thrower et al., 2000). Lysine 63 linkages are usually non-degradative and are thought to be

involved in DNA repair and the stress response (Spence et al., 1995). Lysine 11 linkages are associated with endoplasmic reticulum degradation (Xu et al., 2009); mono-ubiquitinated proteins usually result in altered localization or serve as an initial activation event (Sadowski et al., 2012). For example, histones are mono-ubiquitinated which serves as a precursor for chromatin activation (Cao and Yan, 2012; Meas and Mao, 2015). Transmembrane receptors can also be mono-ubiquitinated which can alter their subcellular localization to target the receptor for degradation by the lysosome (Drake et al., 2006).

Recent literature has also demonstrated the importance of mixed linkages (Akutsu et al., 2016; Nakasone et al., 2013). For example, mixed change linkages have distinct signaling properties: linkage selective receptors and DUBs can recognize different patterns of K48 and K63 linkages in tri-UB branches resulting in distinct signaling and chain processing respectively (Nakasone et al., 2013). Between the vast number of E3s and the various permutations of ubiquitin chain architecture, considerable diversity exists in ubiquitin signaling pathways.

The role of ubiquitin's role in RiBi initially came from its fusion to ribosomal proteins that must be processed before incorporation of the proteins into the ribosome (Finley et al., 1989). Of the four ubiquitin genes in yeast, three of them are fused to ribosomal proteins. The small ribosomal subunit protein S27a (Rps31 in yeast, fused to UBI1) and L40 (Rpl40A/B in yeast, fused to UBI2 and UBI3 respectively) are expressed as fusions with an N-terminal ubiquitin moiety (Finley et al., 1987; Lacombe et al., 2009; Ozkaynak et al., 1987). Ubiquitin is not released from these proteins until after ribosomal assembly, indicating ubiquitin’s importance as a checkpoint during ribosome biogenesis (Kressler et al., 2010). Many r-proteins also require ubiquitination (typically mono-ubiquitination) for efficient ribosomal assembly (Sung et al., 2016). Ubiquitination also signals for degradation of a surplus of r-proteins when rDNA transcription is dysregulated (Zhou et al., 2015).

Several RiBi complexes are known to be ubiquitinated (Finley et al., 1989); similarly, several factors in the ubiquitin pathway have been discovered to be involved in regulating RiBi (Stavreva, et al., 2006). For example, in humans, a large RNAi screen revealed that the E3 CLR4 is involved in the

assembly of the 40S ribosomal subunit (Badertscher et al., 2015). Another E3 involved in ribosome biogenesis in humans is Mdm2, wherein its function is inhibited when bound to large subunit r-proteins RpL5, RpL11, and RpL23 (Dai and Lu, 2004; Dai et al., 2008). Binding of Mdm2 to the r-proteins controls the stability of p53 (Dai et al., 2008). During RiBi, free surplus of RpL5, RpL11, and RpL23 is relatively low and Mdm2 is free to target p53 for ubiquitination and degradation (Dai and Lu, 2004; Dai et al., 2008). When rDNA transcription is halted, there is an increase in excess r-proteins, which bind Mdm2 and prevent its interaction with p53, thus preventing its proteasomal degradation (Dai and Lu, 2004; Dai et al., 2004; Davydov et al., 2004; Li et al., 2002; Vassilev et al., 2004; Zhang et al., 2003). In cancer cells where RiBi is increased, the activity of Mdm2 is directed at ubiquitinating p53, thereby reducing its levels through degradation (Brooks and Gu, 2006; Li et al., 2002). The decrease in p53 levels in part leads to the continued production of ribosomes that support the excessive proliferation of cancer cells.

It is important to note that substrate ubiquitination can be reversed through the action of deubiquitinating enzymes (DUBs), of which approximately 20 exist in budding yeast and 100 in humans (Reyes-Turcu et al., 2009). DUBs remove the covalent bond between the ubiquitin and the target substrate, allowing for the maintained stability of the target substrate, recycling of the free pool of ubiquitin, and freeing of the proteasome of ubiquitin chains to ensure proper function (Wilkinson, 1997). DUBs are also responsible for the proofreading of substrate ubiquitination to prevent improper ubiquitin chains being formed (Wilkinson, 2009). In RiBi, DUBs are responsible for cleaving ubiquitin from r-proteins to ensure proper ribosome formation (Amerik and Hochstrasser, 2004). For example, in humans, HAUSP (herpesvirus-associated ubiquitin-specific protease) deubiquitinates both Mdm2 and p53 to properly maintain ubiquitination of p53 and in turn regulate p53-dependent autophagy (Lim et al., 2015). Another example, in yeast, is Ubp3 (along with cofactor Bre5), responsible for degradation of the 60S large ribosomal subunit (Hinnebusch, 2009).

By combining what we currently understand about RiBi regulation (in normal and proliferating cancer cells) with the known roles of ubiquitination in this process, this thesis contributes to the current knowledge of how ubiquitination regulates ribosome biogenesis and builds upon our previous work

demonstrating that ubiquitination of Rpa190 is important for regulating its stability (detailed in chapter II). This body of work expands on our knowledge of when ubiquitination of Rpa190 occurs during the transcription process, identifies a specific SCF complex involved in regulating Rpa190 ubiquitination, and identifies the ubiquitination sites for Rpa190 stability, which are controlled by the yeast DUB Ubp10. I then present evidence for the importance of Rpa190 ubiquitination in mediating the pharmacological effects of the novel DNA intercalator, BMH-21. This work concludes by discussing our expanding knowledge regarding the molecular factors involved in the ubiquitin-mediated regulation of RNAPI.

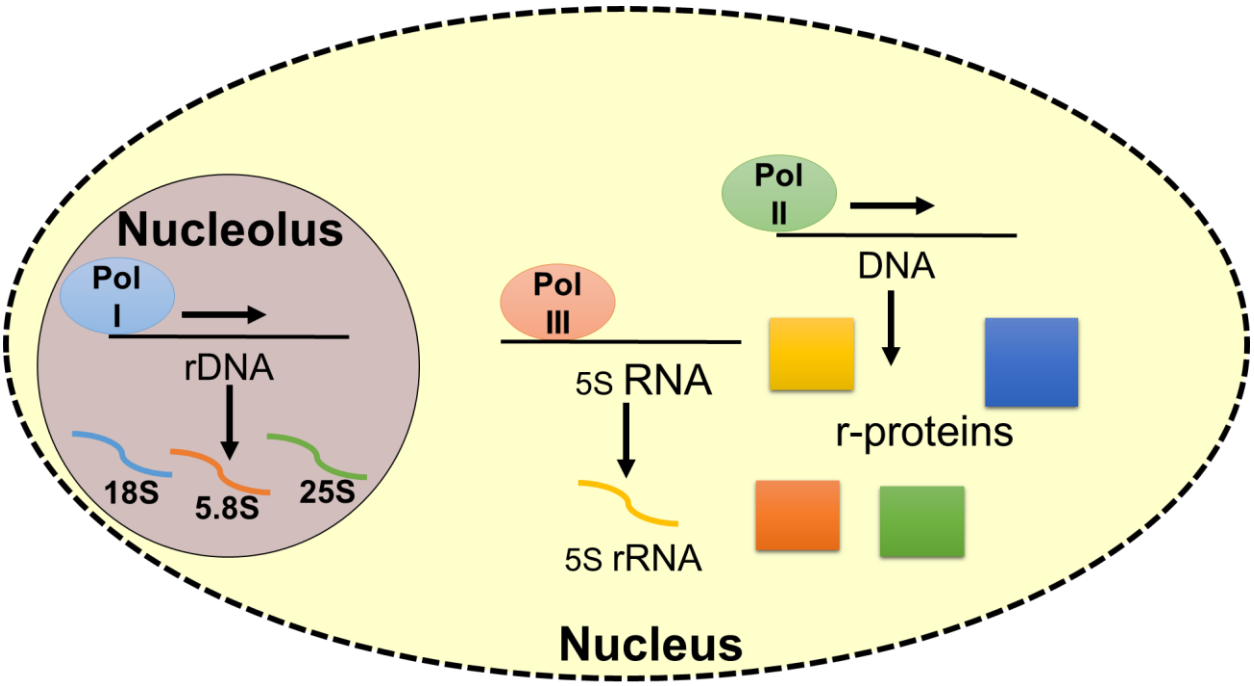


Figure 1.1 - Yeast ribosome biogenesis requires coordination by all three RNA polymerases

RNA polymerase II transcribes all ribosomal proteins. RNA polymerase III transcribes the 5S rRNA that makes up the large subunit of the ribosome and transfer RNA that is required for protein translation. RNA Polymerase I transcribes the 18S, 5.8S, and 25S which make up the large and small subunits of the ribosome and is the rate limiting step in ribosome biogenesis.

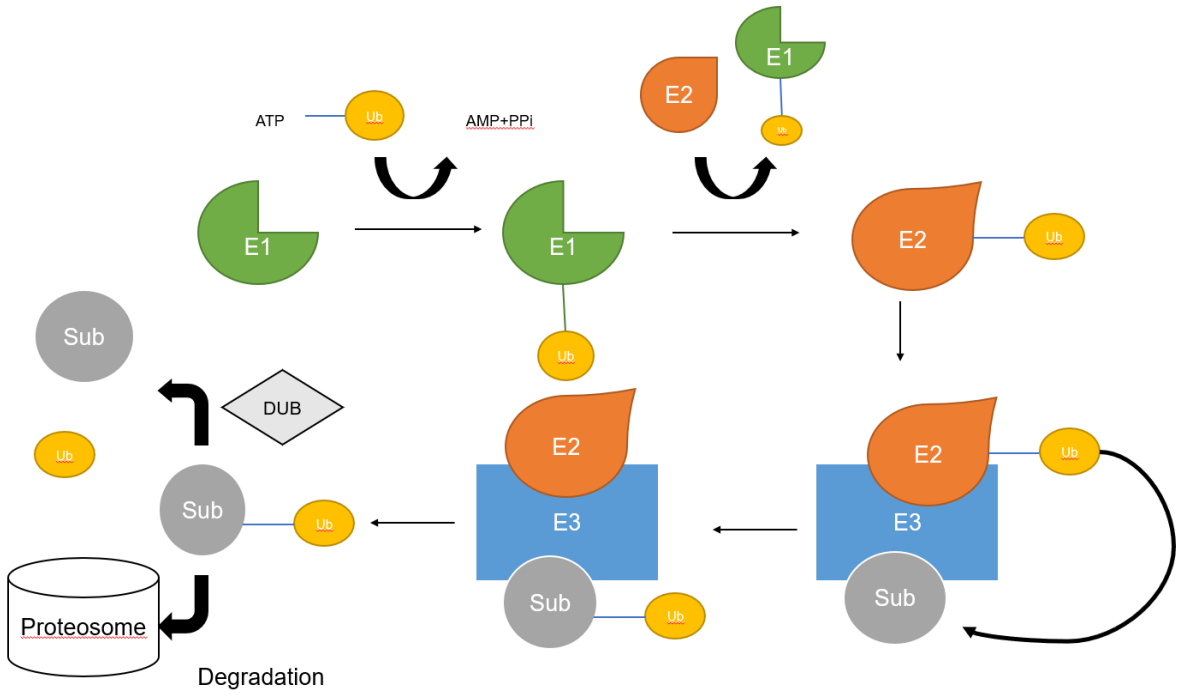


Figure 1.2 - Ubiquitination cascade

The cascade begins with the E1 (ubiquitin-activating enzyme) hydrolyzing a molecule of ATP for the ubiquitin to attach to the E1. Once the ubiquitin is attached to the E1, the ubiquitin is then transferred to the E2 (ubiquitin-conjugating enzyme). Once the ubiquitin is conjugated to the E2, the E3 (ubiquitin ligase) aids in recruiting the substrate targeted for ubiquitination. Once the ubiquitin is transferred from the E2 to the substrate, the substrate is released from the E3 and is either sent to the proteasome for degradation or the ubiquitin is removed from the substrate via deubiquitinating enzyme (DUB) and substrate remains intact.

Chapter II: Understanding the Ubiquitin-mediated Regulation of Rpa190

Introduction

Our group's previous research discovered additional ubiquitin regulatory mechanisms involved in RiBi, specifically during the process of rDNA transcription. We demonstrated a novel role for the yeast DUB Ubp10 during this RiBi and identified ubiquitin-mediated regulation of the largest subunit of RNA Polymerase I. Ubp10's only previous known role was to deubiquitinate histone H2B in silent chromatin through interaction with the silent-information regulatory protein Sir4 (Gardner et al., 2005), which binds to the silent mating-type loci and the telomeres in yeast (Gardner et al., 2005). However, deletion of *UBP10* caused a slow-growth phenotype in yeast independent of Sir4's ability to bind Ubp10 (Richardson et al., 2012), indicating that Ubp10 regulated growth through an alternate pathway. Additionally, when Ubp10 is rendered inactive via a mutation to one of its catalytic residues, the slow-growth phenotype persists (Richardson et al., 2012), demonstrating that the catalytic activity of Ubp10 is responsible for maintaining proper growth in cells. This work also showed that Ubp10 colocalized with Nop58, a nucleolar marker, which indicated that Ubp10 was localized to the nucleolus (Richardson et al., 2012).

Because Ubp10 regulates growth and localizes to the nucleolus, we hypothesized that Ubp10 may be playing a role in ribosome biogenesis (Richardson et al., 2012). To test this, we studied the impact Ubp10 had on production of ribosomal component transcripts. Deletion of *UBP10* leads to a decrease in the production of 25S and 18S ribosomal subunit transcripts (Richardson et al., 2012). In order to uncover potential factors that link how Ubp10 regulated ribosome biogenesis, the Gardner lab performed a mass spectrometry screen to identify proteins that interacted with Ubp10 (Richardson et al., 2012).

While several ribosome biogenesis factors were identified as Ubp10-interacting proteins, we became especially interested in one: Rpa190, the largest subunit of RNAPI. In yeast, depletion of Rpa190 directly correlates to a significant drop in rRNA synthesis and r-protein synthesis (Wittekind et al., 1990). Based on this information, we wanted to determine the relationship between Rpa190 and Ubp10.

Since Ubp10 is a deubiquitinating enzyme, we suspected that deletion of *UBP10* would result in increased ubiquitination and degradation of Rpa190 (Richardson et al., 2012). This process is also conserved in humans: we demonstrated that USP36 (the human homolog of Ubp10) could also serve as a functional analog to deubiquitinate Rpa190 in yeast (Richardson et al., 2012).

While we uncovered a novel role for Ubp10 (in addition to discovering the ubiquitination of Rpa190), many of the regulators of this process remained unclear. It was also unclear, outside of the deletion of *UBP10*, what potential role ubiquitination of RNAPI may serve. Building upon this seminal work from our lab, we sought to determine several regulatory factors that control ubiquitination of Rpa190, in addition to understanding more about the purpose of this ubiquitination event through identification of the ubiquitination sites.

The following results section details the understanding of this mechanism of regulation by determining the following: if ubiquitination of Rpa190 was a regulator during transcription, the ubiquitin-protein ligase (E3) involved in recruiting RNAPI to be ubiquitinated, and the site(s) of Rpa190 ubiquitination. We completed analysis identifying ubiquitin's role in Rpa190 during active rDNA transcription, identified a novel E3 ligase involved in recruiting Rpa190 for ubiquitination, and determined the sites of Rpa190 ubiquitination. In addition, we sought to determine conditions where Rpa190 ubiquitin may play a role in rDNA transcription. These analyses revealed a more complete landscape of the context of Rpa190 ubiquitination and new areas for understanding the regulation of ribosome biogenesis.

Results

Rpa190 ubiquitination occurs on chromatin

We previously discovered that deletion of the deubiquitinating enzyme *UBP10* results in the ubiquitination of Rpa190, a slow growth phenotype, and ribosome biogenesis defects (Richardson et al., 2012). Given this information, we wanted to determine if Rpa190 ubiquitination occurred in association with chromatin. Ubp10 has been shown to have a role in active chromatin: in the absence of *UBP10*, gene expression in both silent and active chromatin is altered (Gardner et al., 2005). Additionally, Ubp10 is also known to deubiquitinate histone H2B on active chromatin (Gardner et al., 2005). Understanding where Rpa190 ubiquitination occurs will provide insight into whether ubiquitination occurred during rDNA transcription or if it controlled Rpa190 levels after RNAPI disengaged from chromatin. If Rpa190 ubiquitination occurs on chromatin, we hypothesized it might be involved in controlling initiation, elongation, or termination of the rDNA transcription process. Together with previously published data, we hypothesized that Rpa190 ubiquitination might occurring during active rDNA transcription.

To assess whether or not Rpa190 ubiquitination was associated with chromatin, cell fractionation was conducted using *UBP10* and *ubp10Δ* cells. Cells expressing endogenous ubiquitin carrying an 8-His tag were spheroplasted for thirty minutes to degrade the cell wall for gentle lysis, and the total lysate was taken from each treated strain. The soluble and insoluble fractions of these cells were then separated by differential centrifugation, with the insoluble fraction containing the chromatin. Ubiquitinated proteins were purified using metal affinity purification. Each fraction (total, soluble and insoluble) was diluted in denaturing lysis buffer, and ubiquitinated proteins were purified using metal affinity purification. To assess proper purification of each fraction, Pgk1 was used as a marker for the soluble fraction and histone H2B was used as a marker for the insoluble/chromatin fraction. Because Ubp10 is known to deubiquitinate histone H2B, and that ubiquitinated histone H2B occurs on chromatin (Gardner et al., 2005), ubiquitinated histone H2B was also used as a marker for a ubiquitinated chromatin protein that is regulated by Ubp10 activity.

In *UBP10* cells, approximately 60-70% of Rpa190 localized to the insoluble/chromatin fraction (Figure 2.1), with nearly all the ubiquitinated Rpa190 species localized to the insoluble/chromatin fraction (Figure 2.1). In *ubp10*Δ cells, there is a decrease in steady-state levels Rpa190 consistent with previous data (Richardson et al., 2012). This decrease in stability corresponds with the increase in ubiquitination of Rpa190, which also occurs exclusively on the insoluble/chromatin fraction (Figure 2.1). The chromatin protein histone H2B also demonstrated exclusive localization to the insoluble/chromatin fraction, consistent with previous data demonstrating that histone H2B ubiquitination is part of active transcription by RNA Polymerase II (Tanny et al., 2007). Together, these data support the hypothesis that Rpa190 ubiquitination occurs on chromatin and is likely part of regulating RNAPI transcription.

Genetic manipulation of SCF^{GRR1} partially restored Rpa190 steady-state levels in ubp10Δ cells

Next, we wanted to identify potential ubiquitin pathway components that are involved in Rpa190 ubiquitination, specifically the ubiquitin ligase (E3). Ubiquitin ligases are important for transfer of the ubiquitin to its substrate, leading to targeted signaling events. There are 54 known E3s (HECT, Ubox, and Ring) in yeast, therefore, querying each would be an inefficient method to determine which of these might be the E3. Fortunately, previously unpublished data from our lab provided an insight into a potential E3 involved in this process. Previous overexpression of *URA3* 2-micron library constructed from genomic fragments (obtained from Marian Carlson) by Daniel Gottschling and Richard Gardner (unpublished, data not shown), identified 4 constructs that were able to restore the slow growth phenotype by spot test when transformed into *ubp10*Δ cells. These plasmids contained the following pRG370: *CDC53*, *RPL41B*, *YDL133W* and *LYS21*; pRG371: *UBA1* (E1), *SAC1* (partial), *TRP3*, *SNR64*, and *STE6* (partial); pRG372: *UBC4* (E2), *RPG1* (partial), *SEC18*, *SPT7*, *tD(GUC)B*, and *tR(UCU)B*; pRG373: *GRR1*, *YJR089W* (partial), *JSN1*, *BUD4*, *FIP1* (partial). All four of these plasmids contain components of the SCF complex, a multiprotein E3 ubiquitin ligase complex that ubiquitinates proteins for proteasome degradation: *UBA1* (E1), *UBC4* (E2), *CDC53* (cullin E3), and *GRR1* (F-box protein).

Based on this information, we tested to see if these overexpression plasmids (containing SCF complex components that rescued the slow-growth phenotype in *ubp10*Δ cells) would also stabilize

Rpa190 in these same strains (since the slow-growth phenotype is linked to Rpa190 stability (Richardson et al., 2012)). We transformed the previously identified constructs into *UBP10* and *ubp10*Δ cells and examined steady-state levels of Rpa190 via Western analysis. In *ubp10*Δ cells, overexpression of plasmids containing *UBA1*, *UBC4*, *CDC53*, and *GRR1* restored steady-state levels of Rpa190 (Figure 2.2). Since Uba1 and Ubc4 (E1 and E2, respectively) are involved in initiating several various ubiquitin cascades, it is unlikely that either enzyme confers particular specificity for Rpa190. Cdc53, the cullin component of the SCF ubiquitin ligase, is also involved in the recruitment of several substrates for ubiquitination and is also likely to lack specificity for Rpa190. Cdc53 is also essential; it regulates key cell cycle-dependent processes, likely not conferring specificity for Rpa190 or rDNA transcription alone.

Of the remaining SCF complex components we tested, we suspected Grr1 potentially confers the most specificity, either through a direct or indirect mechanism, for Rpa190. It is possible that Grr1 may aid in the recruitment of Rpa190 for ubiquitination directly or Grr1 aids in recruitment of another substrate that regulates the ubiquitination of Rpa190. Grr1 is an interesting F-box protein in that it has been shown to regulate glucose transport and ubiquitinates several proteins involved in carbon catabolite repression, glucose-dependent divalent cation transport, morphogenesis, and sulfite detoxification (Avram and Bakalinsky, 1996; Benanti et al., 2007; Entian and Zimmermann, 1980). These processes are known to regulate RiBi through various cellular signaling mechanisms (Blondel et al., 2005; Gonzalez et al., 2014; Horak and Wolf, 2005; Pasula et al., 2010). Grr1 is localized to the nucleus; its removal from the nucleus prevents glucose transport (Horak and Wolf, 2005). Given the specialized role of Grr1 in regulating conditions that are known to alter ribosome biogenesis and that Grr1 is localized to the nucleus, we examined Grr1's potential role in regulating Rpa190 stability.

Because *GRR1* overexpression partially restored steady-state levels of Rpa190 and is tied to growth, we wanted to determine if overexpression of *GRR1* could rescue the slow growth phenotype in *ubp10*Δ cells using dilution spot tests. We isolated *GRR1* from the yeast *URA3* overexpression library plasmid, cloned it in a *URA3* 2μ expression plasmid, and expressed it in *UBP10* and *ubp10*Δ cells (Figure 2.3). An empty vector containing *URA3* only was also expressed in these cells as a control. Spot tests of

10-fold dilutions of yeast revealed that *GRR1* overexpression restores growth in *ubp10*Δ cells but does not enhance growth in wild-type cells. This restoration in growth in *ubp10*Δ cells seen in this spot test corresponds with the partial restoration of Rpa190 levels seen in our previous assays.

Since *UBP10* expression is responsible for maintaining Rpa190 stability and *GRR1* overexpression can restore steady-state levels and slow growth in *ubp10*Δ cells, we wanted to confirm that the stability of Rpa190 could be restored if *GRR1* was overexpressed. For ubiquitination to properly occur in the cell, components of the pathway often must be expressed in the correct stoichiometry (Pierce et al., 2013). Therefore, overexpression of any one of the components of a multiple component E3 could result in an improper ubiquitination cascade and thereby decrease ubiquitination of substrates. To test this hypothesis, *GRR1* was overexpressed in *UBP10* and *ubp10*Δ cells and a cycloheximide-chase assay was performed to examine the stability of Rpa190 (Figure 2.4). *GRR1* overexpression partially restored stability of Rpa190 in *ubp10*Δ cells and Rpa190 stability remained stable in *UBP10* expressing cells, indicating that the SCF complex (and specifically Grr1) is potentially one of the ubiquitination cascades regulating Rpa190 stability, either through a direct or indirect mechanism.

We then wanted to determine that the stability of Rpa190 by overexpression of *GRR1* was due to a decrease in Rpa190 ubiquitination. We expected that overexpression of *GRR1* would reduce ubiquitination of Rpa190. To test this, *GRR1* was overexpressed in *UBP10* and *ubp10*Δ cells and ubiquitin-affinity assays were performed, wherein we isolate the entire ubiquitin proteome using Tandem Ubiquitin Binding Entities (TUBES) and probe for the presence of Rpa190 in the purified ubiquitinated protein proteome via Western analysis. As expected by *GRR1* overexpression leading to increased stability of Rpa190 in *ubp10*Δ cells, overexpression of *GRR1* also decreased Rpa190 ubiquitination in *ubp10*Δ cells, though the effect was partial (Figure 2.5). In contrast, overexpression of *GRR1* in *UBP10* cells did not alter ubiquitination of Rpa190. The partial restoration of stability, the decrease in ubiquitination of Rpa190 when *GRR1* is overexpressed, and the rescue of the slow-growth phenotype to approximately wild-type levels cumulatively indicate that Grr1 likely plays a major role regulating Rpa190 ubiquitination.

Identification of Rpa190 ubiquitination site

Although we implicated SCF^{Grr1} as a potential regulator, a key issue in interpreting these experiments is that alterations to Grr1 would affect other pathways in the cell and might not confer absolute specificity for regulating RNAPI. Therefore, our next goal was to determine the ubiquitination sites within Rpa190. Ubiquitin is typically attached to lysines on a target substrate; however, Rpa190 contains 133 lysines and thus mutagenesis of each lysine residue would be a labor-intensive and time-consuming process to identify possible ubiquitination sites.

Our next approach was to use a pharmacological approach that targeted the mTOR pathway. The mTOR pathway is known to regulate all 3 RNA Polymerases (Gentilella et al., 2015). In yeast, mTOR specifically regulates RNA Polymerase I by allowing TIF-IA and Rrn3 binding to the promoter to initiate rDNA transcription. The mTOR inhibitor, rapamycin, prevents these initiation components from binding to the promoter and directly inhibiting ribosome biogenesis (Hannan et al., 2003; Mayer et al., 2004). This correlates with our previously published data which shows that Rpa190 stability is maintained even after 200 nM treatment of rapamycin (Richardson et al., 2012). Based on this information, we suspected acute and chronic treatment with rapamycin may decrease ubiquitination of Rpa190. By using mass spectrometry, we hypothesized that this approach may uncover peptides indicating the location of Rpa190 ubiquitination.

In collaboration with Judit Villen and Sam Entwisle (UW, Genome Sciences), a mass spectrometry screen was performed to determine if rapamycin altered Rpa190 ubiquitination after acute and chronic translation arrest (Figure 2.8). Stable-isotope labeling of amino acids in cell culture (SILAC) were used to label lysine residues in yeast by supplementing media with light (+0 Da), medium (+4 Da) and heavy (+8Da) lysine. Yeast were treated with 200 nM rapamycin for 0 (light), 30 minutes (medium) or 3 hours (heavy). Proteins were extracted from each sample and digested with trypsin. Peptides (containing lysines modified with the tryptic diglycine remnant of ubiquitin) were enriched using

immunoaffinity enrichment and analyzed using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

Two distinct peptide regions of Rpa190 were identified in the mass spectrometry screen and both showed a decrease in ubiquitination after acute and chronic treatment of rapamycin. We examined the individual peptides from the screen to determine which (if any) of these lysines could be used for further mutagenesis studies in aiding the identification of Rpa190 ubiquitination sites. This experiment revealed ubiquitination on two peptide regions of Rpa190: the region containing lysines K405, K408, and K410, and the region containing K1374, K1376, and K1377 (Figure 2.5). Ubiquitination at both regions decreased after 30-minute and 3-hour rapamycin treatment, relative to total Rpa190 protein levels (Figure 2.6). The identification of these regions allowed us to narrow down possible ubiquitination sites in Rpa190 to examine.

Given the identification of two lysine-rich peptides as potential regions containing Rpa190 ubiquitination sites, we wanted to determine if either of these two regions contained the actual sites of Rpa190 ubiquitination that are regulated by Ubp10. While Gly-Gly residues (the residues left after the isolated proteins were digested by trypsin) were identified on K408 and K1376, it is known that surrounding ubiquitination sites can also be ubiquitinated due to the promiscuity of E3s. Thus, we mutated the following two lysine clusters to arginine in both regions of *UBP10* and *ubp10*Δ cells to prevent any promiscuous ubiquitination: K405R, K408R, and K410R (Rpa190^{K405,8,10R}), as well as K1374R, K1376R, and K1377R (Rpa190^{K1374,6,7R}).

We performed ubiquitin-affinity assays as previously described to assess Rpa190 ubiquitination in either *UBP10* or *ubp10*Δ cells expressing either normal Rpa190, Rpa190^{K405,8,10R}, or Rpa190^{K1374,6,7R} (Figure 2.10). As expected, we saw no change in overall Rpa190 ubiquitination in *UBP10* cells with either normal Rpa190, Rpa190^{K405,8,10R}, or Rpa190^{K1374,6,7R}. In *ubp10*Δ cells expressing either normal Rpa190 or Rpa190^{K1374,6,7R}, overall Rpa190 ubiquitination levels remained high. In *ubp10*Δ cells expressing Rpa190^{K405,8,10R}, very little to no Rpa190 ubiquitination was observed. Overall, the region containing

K405, K408, and K410 appears to be the region of Ubp10-controlled ubiquitination in Rpa190. Moving forward, the K405, K408, and K410 Rpa190 mutant will be referred to as Rpa190^{3KR}.

Rpa190 ubiquitination-deficient mutant rescues the slow-growth phenotype of ubp10Δ cells

After the identification of the Rpa190 ubiquitination sites, we wanted to determine if the expression of Rpa190^{3KR} in *ubp10*Δ cells could rescue the slow-growth phenotype since the slow growth appear to be due primarily to the reduction in Rpa190 steady-state levels (Richardson et al., 2012). We performed spot tests of 10-fold dilutions of yeast and bioscreen assays to determine growth and calculate doubling time intervals (doubling time of yeast between OD 0.2 to OD 0.4), respectively (Figure 2.11). Both spot tests and bioscreen assays were performed at 30ºC (72h and 48h, respectively). Ubiquitination-deficient Rpa190^{3KR} was able to rescue the slow-growth phenotype of *ubp10*Δ cells, demonstrated by both spot tests and bioscreen assays.

Doubling time intervals calculated from bioscreen assays displayed a significant increase in doubling time of *ubp10*Δ cells compared to *UBP10* cells expressing wild-type Rpa190 (mean doubling time 105.6 minutes versus 84.1 minutes respectively, $p<.0001$ using One-way ANOVA, Bonferonni Post-Hoc), which corresponds with the spot test results and our previously published data (Richardson et al., 2012). In Rpa190^{3KR} ubiquitination-deficient mutants, doubling times were not significantly different between *UBP10* and *ubp10*Δ cells (means 84.4 minutes and 88.7 minutes, respectively). Additionally, there was no significant difference between both Rpa190^{3KR} ubiquitination-deficient mutants and *UBP10* cells expressing wild-type Rpa190. These collective data support the idea that the slow rate of growth of *ubp10*Δ strains occurs primarily through the unregulated ubiquitination of Rpa190.

To confirm that Rpa190 stability was also maintained in the Rpa190 ubiquitination-deficient strains, we performed cycloheximide-chase assays as previously described. We hypothesized (based on ubiquitination and growth assays) that Rpa190 stability would be maintained in the of Rpa190^{3KR} ubiquitination-deficient strains. In line with previous growth and ubiquitination data, Rpa190 stability is maintained, even in the absence of Ubp10 function (Figure 2.12). These data revealed that these

ubiquitination sites are the sites where Ubp10 deubiquitinates Rpa190, leading to regulation of Rpa190 stability.

No single Lysine was sufficient for Rpa190 Ubiquitination

Because we had mutated three lysines in the Rpa190^{3KR} ubiquitination-deficient mutant, we wanted to determine if any of these individual lysines were sufficient to serve as the ubiquitination site on Rpa190. Given that the lysine 408 residue was identified in the mass spectrometry experiment as the ubiquitination site within that peptide, we hypothesized that lysine 408 might be the sole site responsible for Rpa190 ubiquitination. To determine if a single lysine was the required ubiquitination site, we mutated each individual lysine in this region and performed cycloheximide-chase assays to examine Rpa190 stability and performed TUBE pulldowns to probe for Rpa190 ubiquitination (Figure 2.13). To our surprise, cycloheximide assays revealed that both Rpa190^{K408R} and Rpa190^{K410R} remained stable even in the absence of *UBP10*, indicating that either or both of these sites may be the ubiquitination site and that ubiquitination of Rpa190 may be promiscuous. Lysine 405 mutated alone was insufficient to maintain stability of Rpa190 in *ubp10*Δ cells, indicating that this is not a site of Rpa190 ubiquitination when the other 2 residues are intact.

Based on this information, we wanted to examine the ubiquitination pattern of each of these individual mutants, especially for Rpa190^{K408R} and Rpa190^{K410}. We hypothesized that Rpa190 ubiquitination would be absent in these two individual mutants, which would indicate that Rpa190 ubiquitination is indiscriminate at this location. Again, to our surprise, ubiquitin pulldowns revealed that ubiquitination of Rpa190 remains in the Rpa190^{K408R} and Rpa190^{K410} mutants, but to a lesser extent than in *ubp10*Δ cells expressing wild-type Rpa190. While the ubiquitination and degradation data for the Rpa190^{K408R} and Rpa190^{K410} mutants do not seem to exhibit the same pattern as the Rpa190^{3KR} mutant, the ubiquitination pattern we see in both of these single mutants may be insufficient to cause a decrease in the overall stability of Rpa190 that is detectable via cycloheximide-chase assay. Therefore, we decided to continue all future experiments using the Rpa190^{3KR} ubiquitination-deficient mutant as this was the only version that eliminated Rpa190 ubiquitination.

Manipulating rDNA copy number did not alter RNA Polymerase I ubiquitination

It remains unclear what role ubiquitination of Rpa190 might play in regulating rDNA transcription. Our previously published research demonstrated that only deletion of *UBP10* led to increased ubiquitination of Rpa190 (Richardson et al., 2012). Rapamycin treatment, amino acid and glucose deprivation, as well as DNA damage were unable to induce ubiquitination of Rpa190 in wild-type *UBP10* cells (Richardson et al., 2012). At this point, we began to examine other conditions where Rpa190 ubiquitination may occur in wild-type cells. One condition we tested was manipulation of copy numbers of rDNA. Research has demonstrated that cancer cells are known to have lower copy numbers of rDNA, despite higher proliferation, compared to non-cancerous cells (Gibbons et al., 2015; Sharifi and Bierhoff, 2018; Wai et al., 2000; Xu et al., 2017). These same observations are also seen in budding yeast: cells with lower copy numbers of rDNA exhibit higher rates of growth compared to normal copy number cells, suggesting a potential advantage for these cells under DNA replication stress (Salim et al., 2017).

Based on this information, we wanted to determine if manipulation of copy number of rDNA could induce conditions where we would observe increased ubiquitination of Rpa190 even with Ubp10 activity intact (based on the assumption that cells produce the same number of RNA polymerase I needed for proper rDNA transcription). We hypothesized that cells with a lower copy number of rDNA would experience increased RNA polymerase I stalling during transcription due to the increased likelihood of more RNA polymerase I transcribing rDNA simultaneously (Figure 2.12). This increase in stalling might lead to an increase in Rpa190 if ubiquitination is a response to stalling. To test this hypothesis, we used two strains (kindly provided by David Schneider, University of Alabama - Birmingham) where cells had either a “high” copy number of rDNA (143 copies, normal in yeast is between 150-200) or “low” copy number of rDNA (25 copies) (Hontz et al., 2008). Additionally, we also sought to test if deletion of *UBP10* could induce any further ubiquitination of Rpa190 in either of these strains, similar to what we have previously seen.

Deletion of UBP10 in rDNA copy number manipulated strains caused a slow growth phenotype

As an initial step, we examined the qualitative growth of the rDNA copy number manipulated strains with and without *UBP10* deleted. Dilution spot tests demonstrated no qualitative difference in growth between 143 and 25 rDNA copy number strains, and deletion of *UBP10* resulted in a slow-growth phenotype in both 143 and 25 rDNA copy number strains as anticipated (Figure 2.13). While dilution spot tests provided a qualitative growth measure, we wanted to assess quantitative growth rates in these strains by utilizing bioscreen assays. Cells were grown to saturation overnight and two microliters of saturated culture were resuspended in 148 µL YEPD and grown at constant shaking for 48 hours at 30ºC. Doubling time intervals were calculated for these strains.

As with the spot tests, we observed no significant difference between strains with 143 copy number and 25 copy number of rDNA; however, when *UBP10* was deleted, we saw a significant difference between doubling time intervals between 143 and 25 rDNA copy number strains (Figure 2.14, $p<.05$ One-Way ANOVA, Bonferonni post-hoc multiple comparison tests). Thus, ubiquitination of Rpa190 might be playing a more necessary role in conditions where rDNA copy numbers are reduced. While this result does not directly answer whether or not RNA polymerase I is stalling during transcription due to the lack of significant difference in growth rate between 143 and 25 rDNA copy number strains, it does indicate that Ubp10 may be playing a more prominent role in growth when rDNA copy numbers are reduced.

Deletion of UBP10 does not induce further ubiquitination in lower copy number rDNA strains compared to higher copy number strains

Given the bioscreen data, we wanted to examine ubiquitination of Rpa190 in these strains. Based on our hypothesis, we would expect to see an increase in Rpa190 ubiquitination in the 25 rDNA copy number strain versus the 143 rDNA copy number strain. In addition, we would expect to see a further increase in ubiquitination of Rpa190 in our 25 rDNA copy number strain compared to our 143 rDNA copy number strain when *UBP10* was deleted. To detect Rpa190, we transformed Rpa190-3HA on a *URA3* 2 micron plasmid to facilitate examination of Rpa190 ubiquitination by Western analysis.

Examination of Rpa190 levels and ubiquitination revealed little ubiquitinated Rpa190 in both the 143 and 25 rDNA copy number strains with *UBP10* intact (Richardson et al., 2012). We were unable to detect a significant difference in the amount of ubiquitinated Rpa190 between the two strains where *UBP10* was deleted. While the bioscreen data supported our hypothesis, it is possible that Ubp10 has an additional role in these strains that is revealed by rDNA copy number variation.

Discussion

While we have previously identified a novel role for Ubp10-controlled Rpa190 ubiquitination in regulating RiBi (Richardson et al., 2012), many of the regulatory factors involved in this process remain undiscovered. The studies detailed here uncovered new regulatory factors involved in regulating ubiquitination of Rpa190. We have demonstrated ubiquitination of Rpa190 occurs on chromatin, discovered an involvement of the SCF^{Grr1} complex as an upstream factor potentially involved in ubiquitinating Rpa190, and identified a region containing three lysine residues that serves as the area for Ubp10-controlled ubiquitination of Rpa190. These data provide a new glimpse into the regulation of Rpa190 ubiquitination. One remaining question is: at what point during rDNA transcription does Rpa190 become ubiquitinated and when does Ubp10 act? Further work will be necessary to determine if Rpa190 ubiquitination serves as a proper checkpoint during rDNA transcription.

One insight into the functional purpose of Rpa190 ubiquitination could be through the examination of RNA Polymerase II in yeast. During DNA damage (Verma et al., 2011) or polymerase inhibition (Wilson et al., 2013), the largest subunit of RNA Polymerase II, Rbp1, is mono-ubiquitinated by the E3 Rsp5. Subsequently, Rbp1 is either deubiquitinated by Ubp2 (which resolves the transcription conflict and transcription can continue) or Rbp1 is further ubiquitinated by Elc1-Cul3 complex through a K48 polyubiquitin chain that initiates proteasome degradation of RNA Polymerase II (Karakasili et al., 2015). These polyubiquitin chains can be deubiquitinated by Ubp3 (Karakasili et al., 2015), suggesting a secondary checkpoint for regulation of Rbp1.

Because of the degree of homology between Rpa190 and Rbp1, a similar mechanism may exist for ubiquitination of RNAPI. Evidence for this comes from our identification of SCF^{Grr1} complex components (and more specifically Grr1 itself) in regulating the ubiquitination of Rpa190. We have shown that overexpression of Grr1 could partially restore Rpa190 steady-state levels and reduce the ubiquitination of Rpa190 in *ubp10*Δ cells. The partial rescue of the *ubp10*Δ phenotype could be attributed to checkpoint regulation similar to Rsp5 and Elc1-Cul3's regulation of ubiquitination of Rbp1. It is possible that the SCF^{Grr1} complex initiates a polyubiquitination event similarly to Elc-Cul3 on Rbp1. Further work

will be necessary to determine if parallel mechanisms exist between the ubiquitination of Rpa190 and Rpb1.

We can begin to understand at some level the purpose of Rpa190 ubiquitination based on the location of the identified ubiquitination sites on Rpa190. All three lysines identified as the ubiquitination sites are contained in the clamp region of the RNA polymerase I complex (Fernandez-Tornero et al., 2013), which is responsible for RNAPI's ability to bind to rDNA (Fernandez-Tornero et al., 2013). As rDNA is transcription occurs and the 90S small subunit processome (SSU) forms, a conformational change may occur, causing the clamp region to open up and reveal these sites for ubiquitination (Perez-Fernandez et al., 2007). When the rRNA exits the polymerase, there is a lid loop region shortly upstream of where the ubiquitination sites have been identified (Fernandez-Tornero et al., 2013), indicating that ubiquitination might serve as a regulator as rRNA exits. Additional structural studies with ubiquitinated Rpa190 may shed light on how modification of this region affects rDNA transcription.

There are several factors to consider when evaluating our hypothesis that ubiquitination of Rpa190 might play a role in our rDNA copy number manipulated strains. While our spot tests and bioscreen data suggest that ubiquitination of Rpa190 may play a role in rDNA copy number variation, our ubiquitination experiment did not confirm this hypothesis. One aspect to consider is that our ubiquitination experiments used Rpa190 that was overexpressed on *URA3* 2 micron plasmid. It is known that expression of a 2 micron plasmid in yeast can vary from 40-60 copies in a haploid cell. Due to this, it is possible that expression of Rpa190 is not consistent in both 143 and 25 rDNA copy number strains. It is unknown if cells with varying copy numbers of rDNA results in changes to RNA Polymerase I levels. Further experiments would need to be conducted by genomically tagging Rpa190 in both strains and performing ubiquitination experiments under these expression conditions.

Acknowledgments

A significant portion of this work is due to contributions from previous members of the Gardner Lab, as well as collaborators at the University of Washington. The chromatin isolation assay (Figure 2.1) and initial query of the two potential ubiquitin sites (Figure 2.10) were performed by former graduate student Ben Reed. Mass spectrometry of the ubiquitin proteome was performed by Sam Entwisle (Villen Lab, Genome Sciences UW). Mutations of the single lysines were created by former undergraduate Ruth Groza.

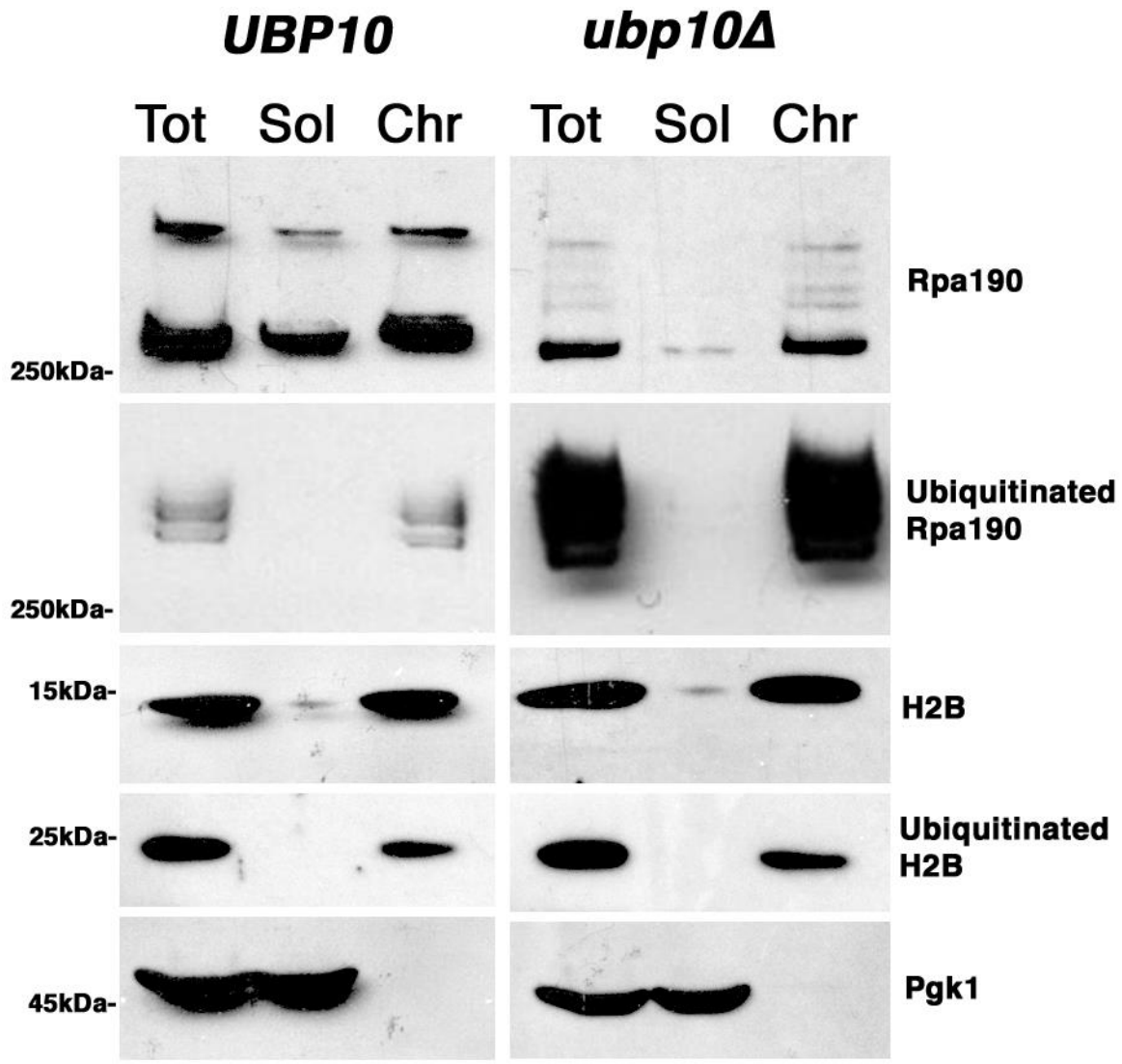


Figure 2.1 - Rpa190 ubiquitination occurs on chromatin

UBP10 or *ubp10*Δ cells were grown to mid-log phase and spheroplasted for 30 minutes in isotonically balanced buffer using zymolyase to digest the cell wall. 40% of spheroplasted cells were removed and lysed in hypotonic solution (Tot). Remaining 60% of cells were spheroplasted and lysed in additional hypotonic solution. Soluble (Sol) and insoluble (Chr) fractions were separated by different centrifugation. Ubiquitinated proteins were purified from each fraction (Tot, Sol, Chr). Levels of Rpa190-3HA (anti-HA), histone H2B (anti-H2B), and PGK1 (anti-PGK1) were determined via Western analyses. Blots are representative images. Experiment was performed with 3 biological replicates.

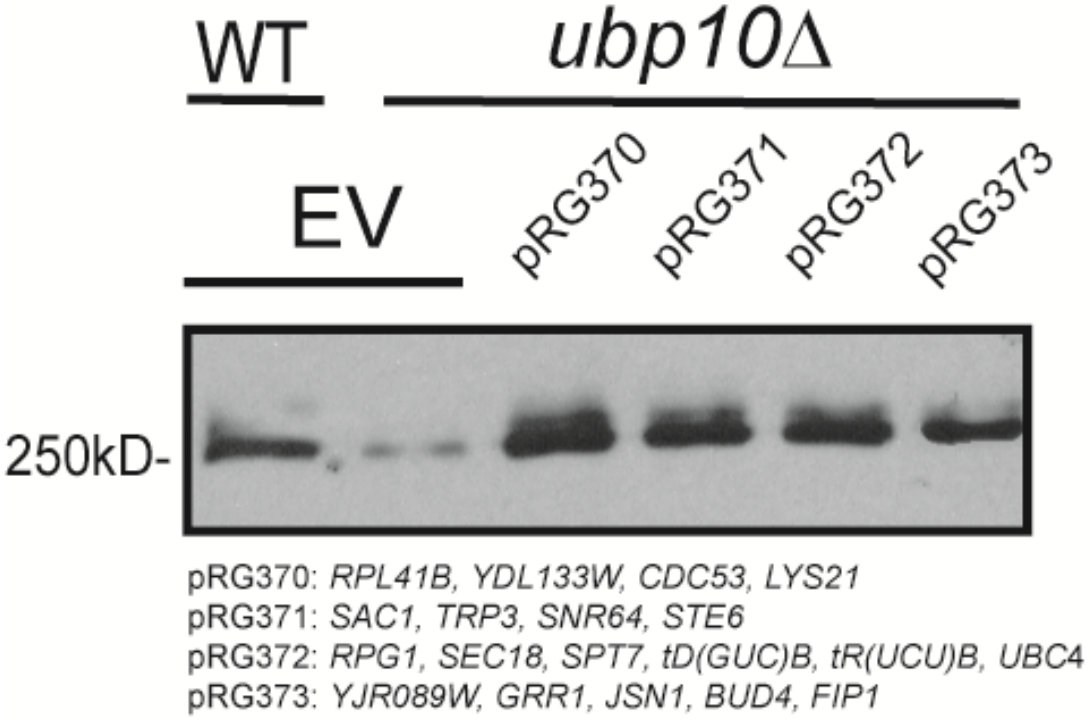


Figure 2.2 - Genetic manipulation reveals SCF F-box components restore Rpa190 steady-state levels in *ubp10*Δ cells

UBP10 (WT) and *ubp10*Δ cells were transformed with either an empty *URA3* vector (pRG16), pRG370, pRG371, pRG372, or pRG373. All plasmids were *URA3* 2-micron expression plasmids. 5mL of cells at a density of ~1.8 x10^7 were harvested and lysed in 200 uL of SUMEB with 100 µL of glass resin. Rpa190-3HA (anti-HA) was visualized via western blot analysis. Blot is a representative image. Experiment was performed with 3 biological replicates.

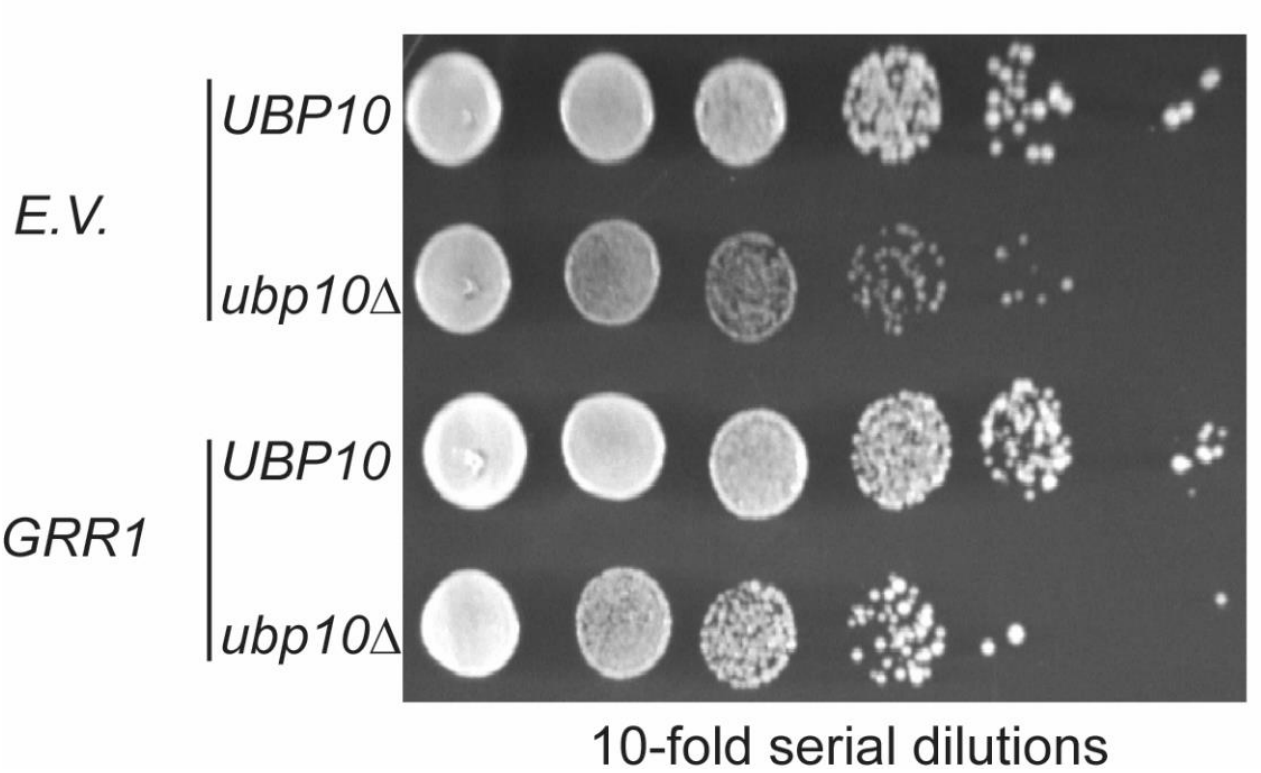


Figure 2.3 - *GRR1* overexpression recovers slow growth in *ubp10*Δ cells

UBP10 and *ubp10*Δ cells containing either empty vector (E.V.) or *GRR1* were plated on uracil-deficient plates in 10-fold dilutions and incubated for 3 days at 30ºC.

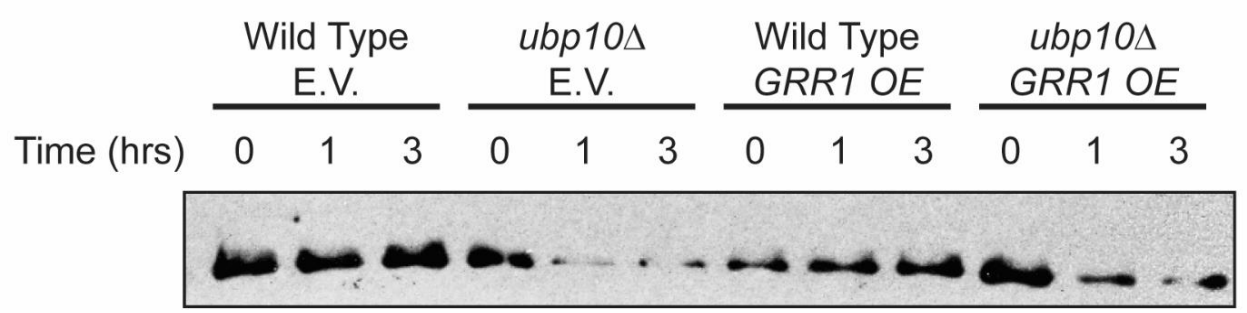


Figure 2.4 - GRR1 overexpression partially restores Rpa190 stability in *ubp10*Δ cells

Cycloheximide-chase degradation assay of *UBP10* or *ubp10*Δ cells expressing either empty vector or *GRR1 URA3* overexpression plasmid. Hours after cycloheximide addition are indicated above each lane. Western analysis of whole-cell extracts was performed using anti-HA antibodies. Blot is a representative image. Experiment was performed with 3 biological replicates.

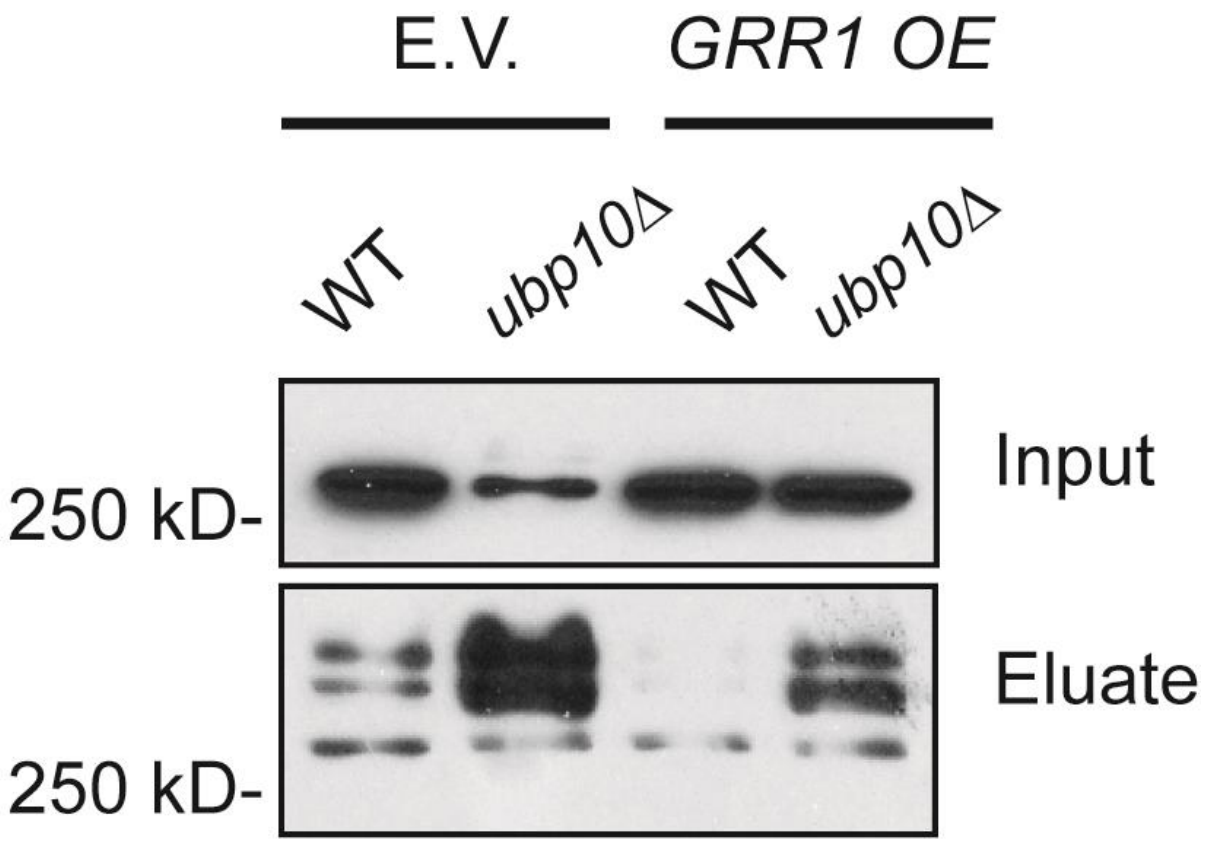
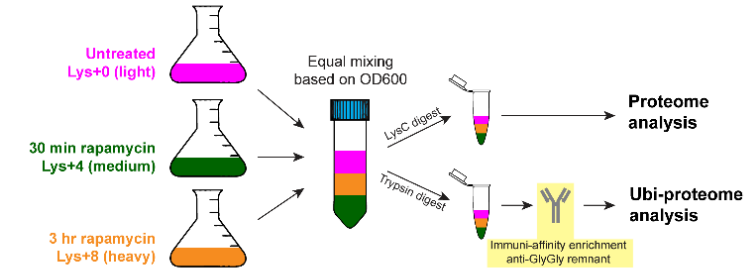


Figure 2.5 - *GRR1* overexpression reduces Rpa190 ubiquitination in *ubp10*Δ cells

Ubiquitinated proteins from of *UBP10* (WT) or *ubp10*Δ cells expressing either empty vector or GRR1 URA3 overexpression plasmid were isolated via affinity purification using magnetic TUBEs (Tandem Ubiquitin Binding Entities). Levels of Rpa190-3HA (anti-HA) in input and eluate were determined by Western analysis. Blot shown is a representative image. Experiment was performed with 3 biological replicates.



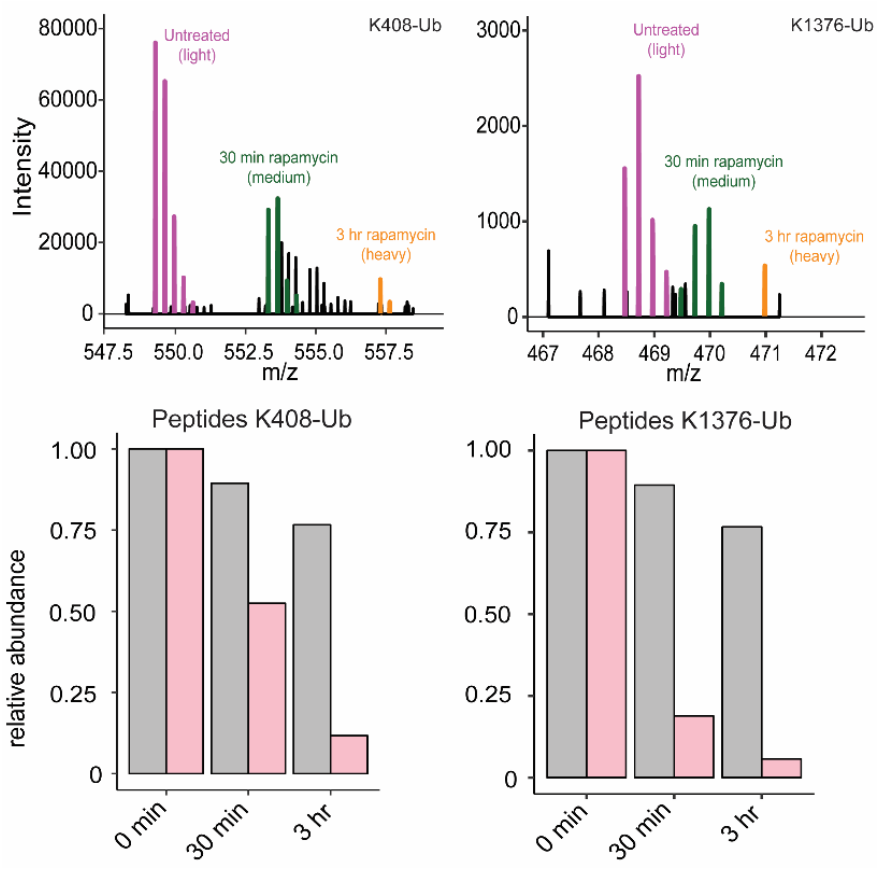


Figure 2.6 - Rapamycin treatment reduces Rpa190 ubiquitinated peptides by mass spectrometry

Yeast were grown in either light, medium, or heavy medium and treated with vehicle or rapamycin for 30 minutes or three hours as indicated. Equal mixing based on OD600 was used to combine each group. LysC digest was used for whole proteomic analysis and trypsin digest was used for the ubiquitin proteome analysis after Gly-Gly affinity enrichment.

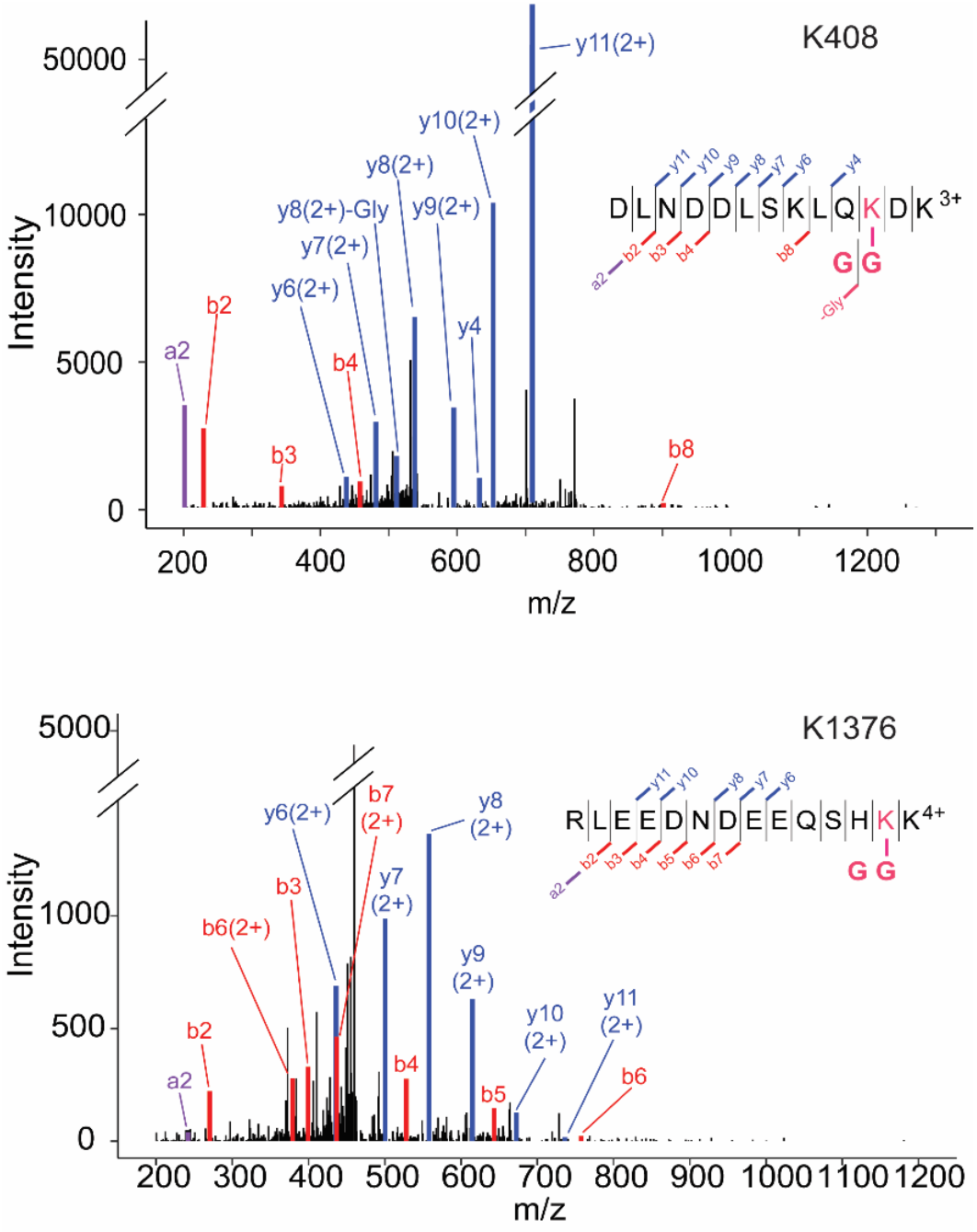


Figure 2.7 - Identification of Rpa190 ubiquitinated peptides

Examination of individual peptides after acute and chronic rapamycin treatment. Two individual lysines on Rpa190 contained a Gly-Gly residue indicating ubiquitination: K408 and K1376.

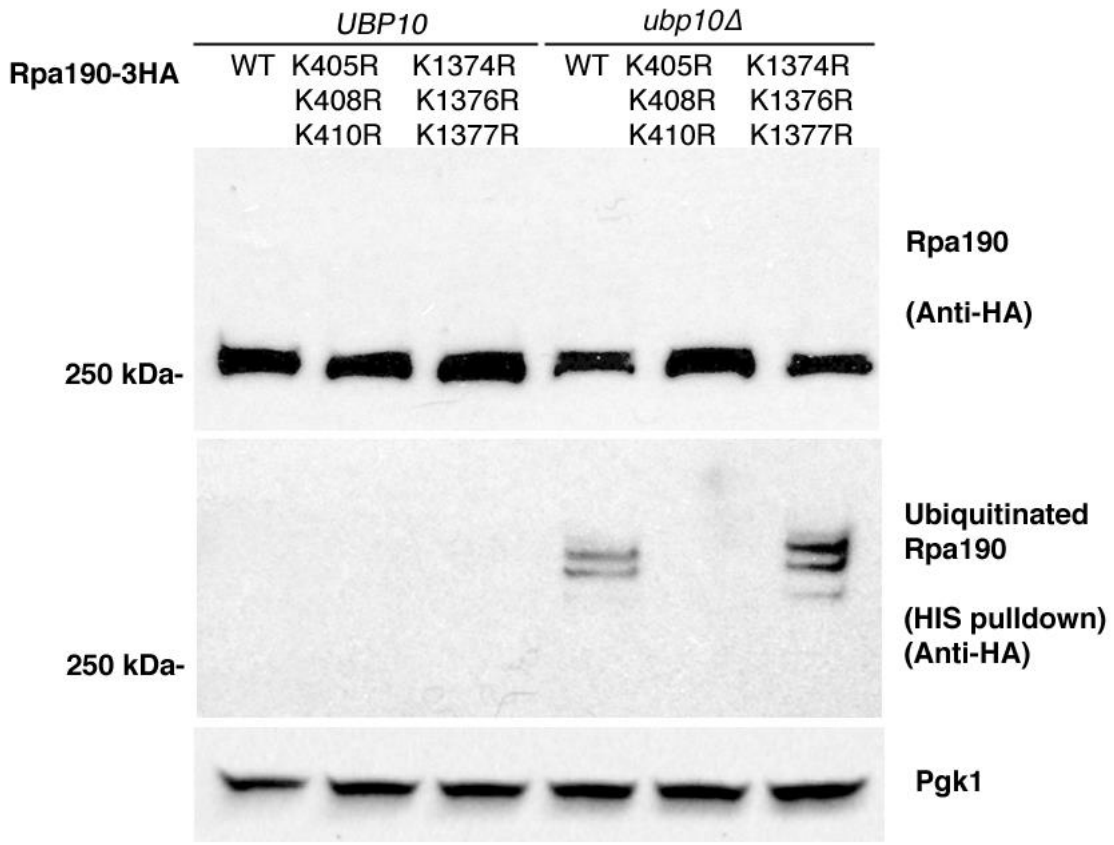


Figure 2.8 - Mutation of lysines 405, 408 and 410 eliminate Rpa190 ubiquitination

Ubiquitinated proteins from *UBP10* or *ubp10*Δ cells expressing either Rpa190, Rpa190^{K05,8,10R} (K405R, K408R, and K410R), or Rpa190^{K1374,6,7R} (K1374R, K1376R, 1377R) were isolated via affinity purification using agarose TUBE purification. Rpa190-3HA (anti-HA) was visualized via western blot analysis. Blots are representative images. Each experiment was performed with 3 biological replicates.

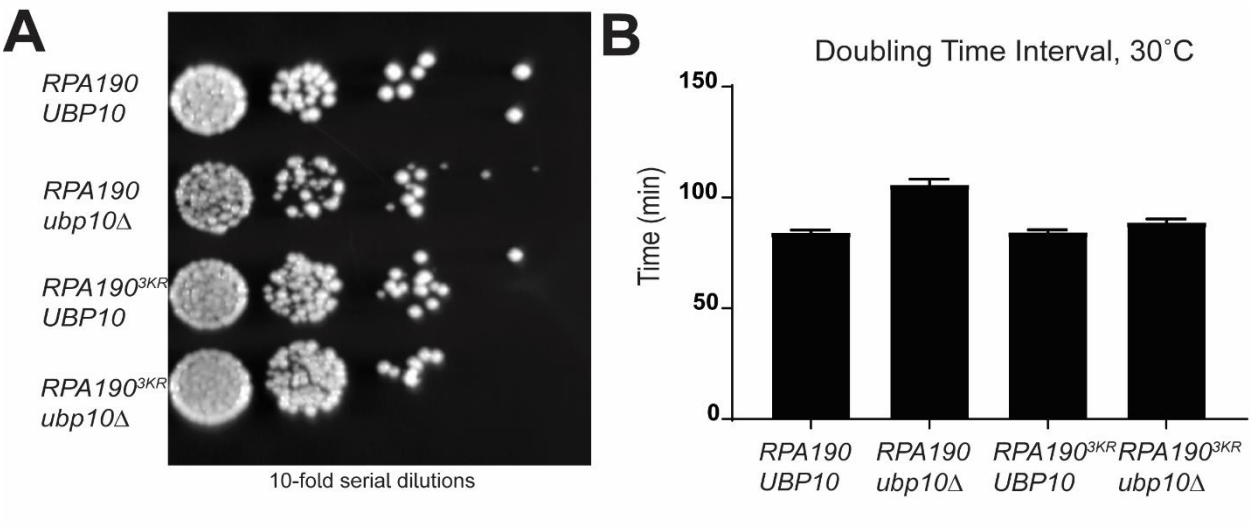


Figure 2.9 - Rpa190 ubiquitination-deficient mutant rescues the slow growth phenotype of *ubp10*Δ cells

UBP10 or *ubp10*Δ cells expressing either Rpa190 or Rpa190^{3KR} were diluted 10-fold and plated on rich media for 3 days at 30ºC. The bioscreen analysis was performed on these same strains to calculate doubling time interval. Three technical replicates were used for each biological replicate (N=3). Error bars represent SEM.

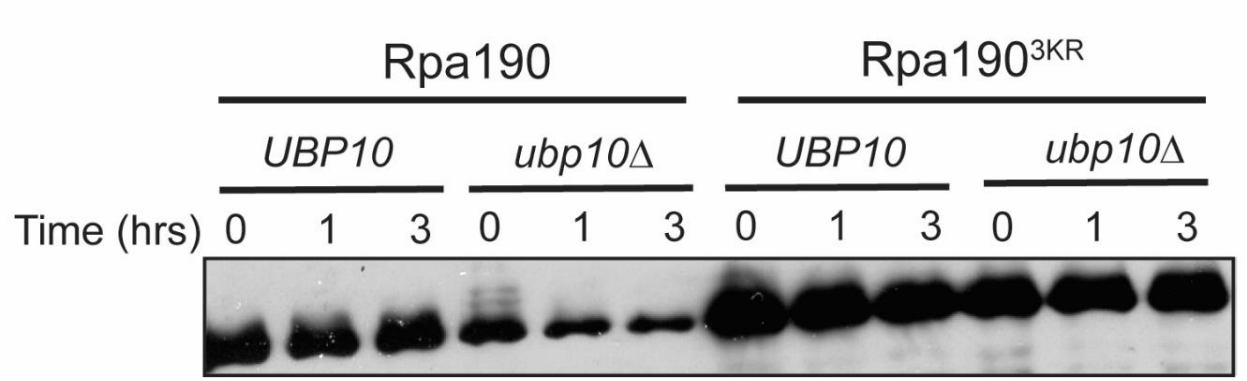


Figure 2.10 - Rpa190 ubiquitination-deficient mutant restores Rpa190 stability in *ubp10*Δ cells

Cycloheximide-chase degradation assay of *UBP10* or *ubp10*Δ expressing either Rpa190-3HA or Rpa190^{3KR}-3HA. Hours after cycloheximide addition are indicated above each lane. Western analysis of whole-cell extracts was performed using anti-HA antibodies. Blots are representative images. Each experiment was performed with 3 biological replicates.

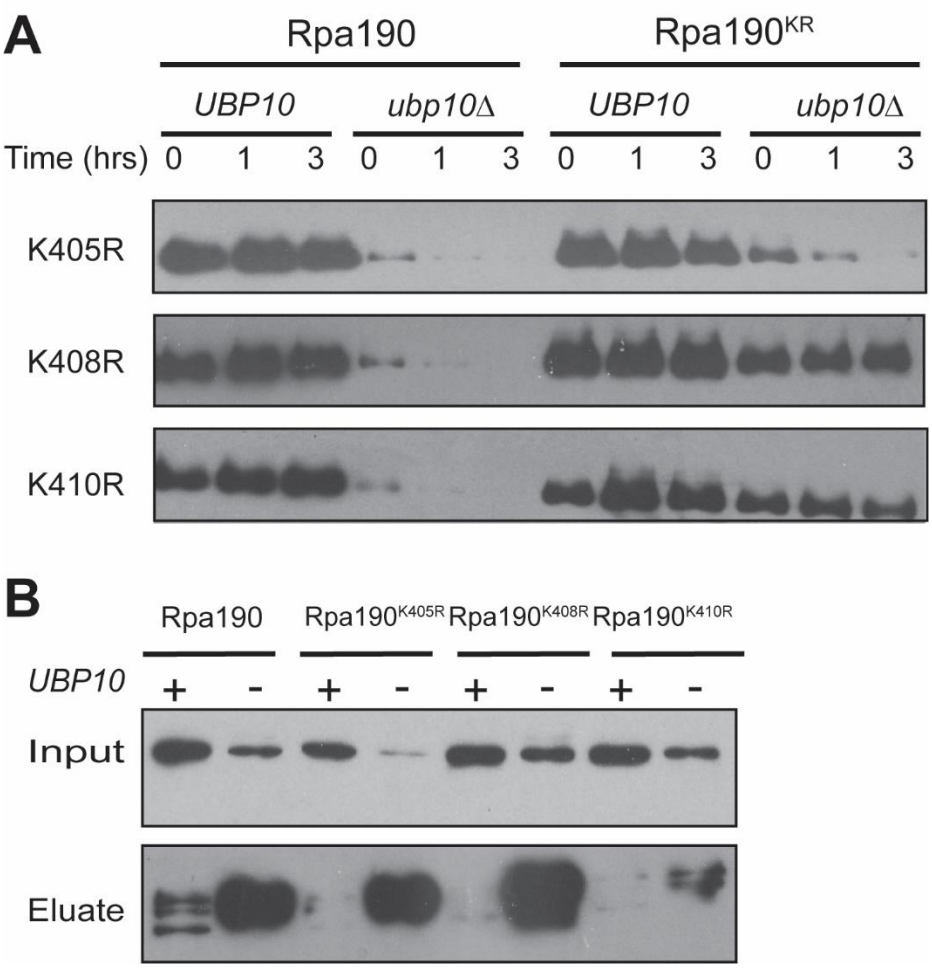


Figure 2.11 - No single lysine mutant is required for Rpa190 ubiquitination

(A) Cycloheximide-chase degradation assay of *UBP10* or *ubp10*Δ cells expressing either Rpa190-3HA or Rpa190^{K405R}-3HA, or Rpa190^{K408R}-3HA, or Rpa190^{K405R}-3HA. Hours after cycloheximide addition are indicated above each lane. Western analysis of whole-cell extracts was performed using anti-HA antibodies.

(B) Ubiquitinated proteins from *UBP10* (WT) or *ubp10*Δ cells expressing either Rpa190-3HA or Rpa190^{K405R}-3HA, or Rpa190^{K408R}-3HA, or Rpa190^{K405R}-3HA were isolated via affinity purification using magnetic TUBEs (Tandem Ubiquitin Binding Entities). Levels of Rpa190-3HA (anti-HA) in input and eluate were determined using Western analysis. Blots are representative images. Each experiment was performed with 3 biological replicates.

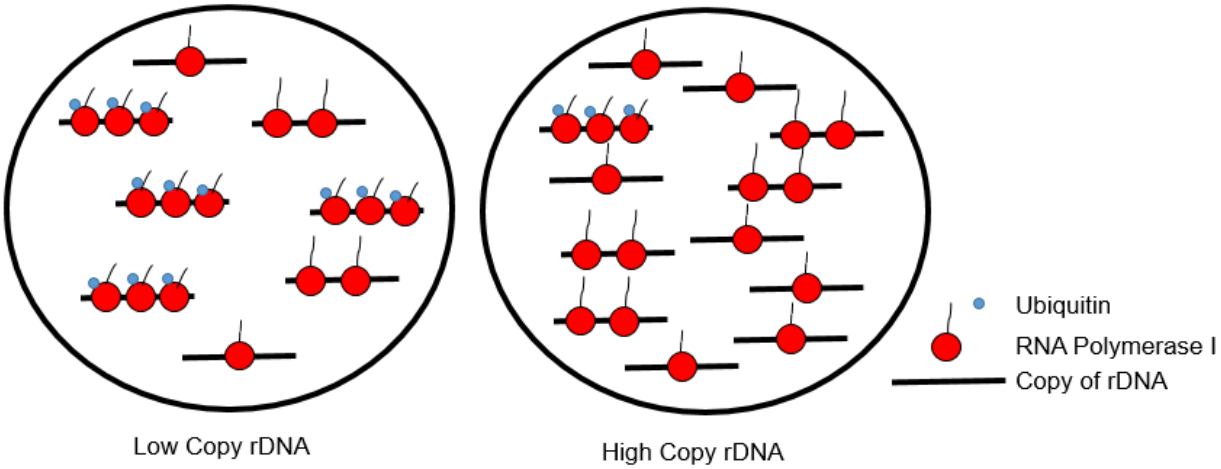


Figure 2.12 - Model for induction of RNA Polymerase I stalling by rDNA copy number manipulation

Assuming the same number of RNA Polymerase I is expressed in each cell, there will be more RNA Polymerase I complexes actively transcribing on low copy numbers of rDNA versus high copy numbers of rDNA. In low copy number cells, RNA Polymerase I has a greater chance of stalling during transcription compared to high copy numbers. This may induce ubiquitination of RNA Polymerase I (specifically Rpa190 ubiquitination) at a greater proportion in low rDNA copy number cells versus in high rDNA copy number cells.

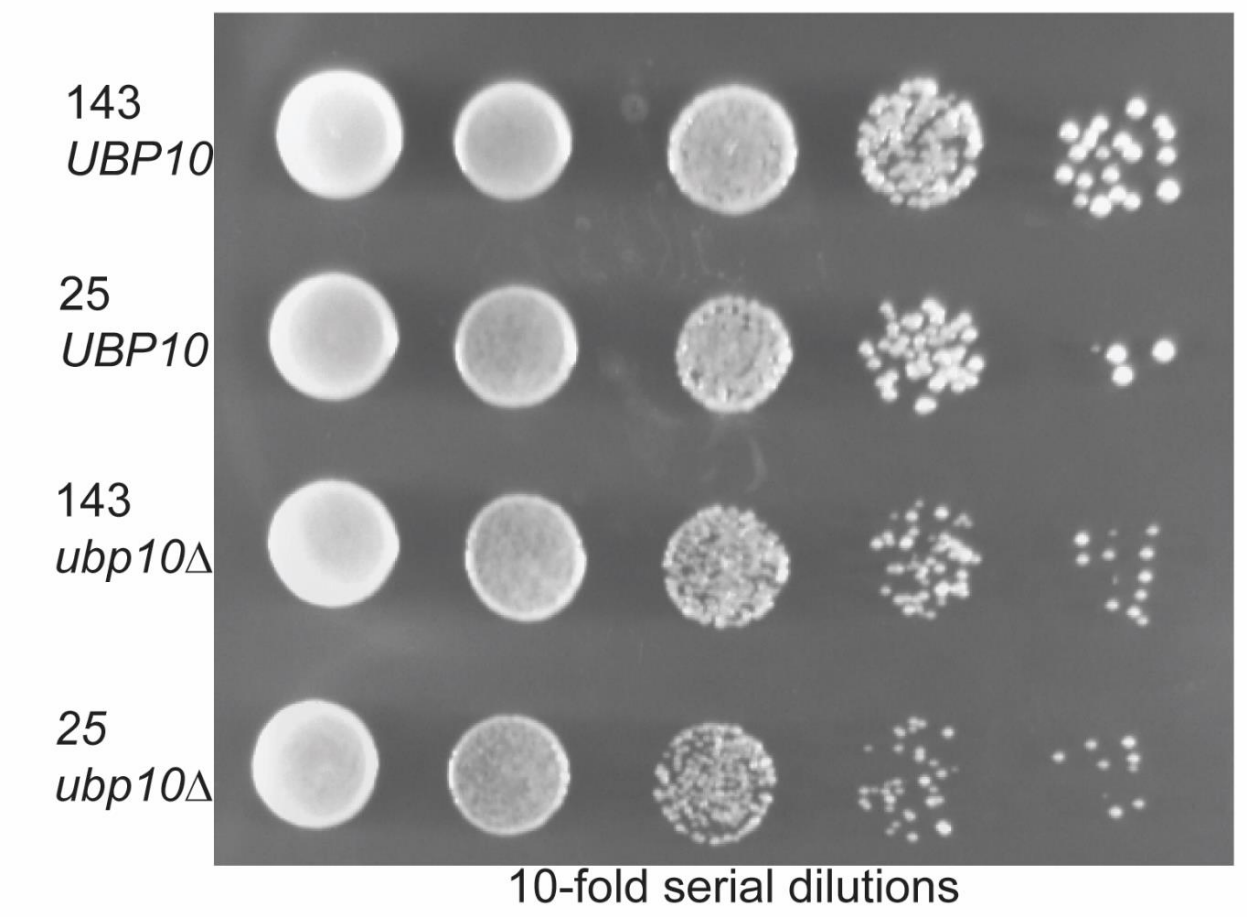


Figure 2.13 - Deletion of *UBP10* in rDNA copy number manipulated strains causes a slow growth phenotype

UBP10 or *ubp10*Δ cells expressing either 143 of 25 copy numbers of rDNA were diluted 10 fold and plated on rich media for 3 days at 30ºC. Experiment was performed with 3 biological replicates.

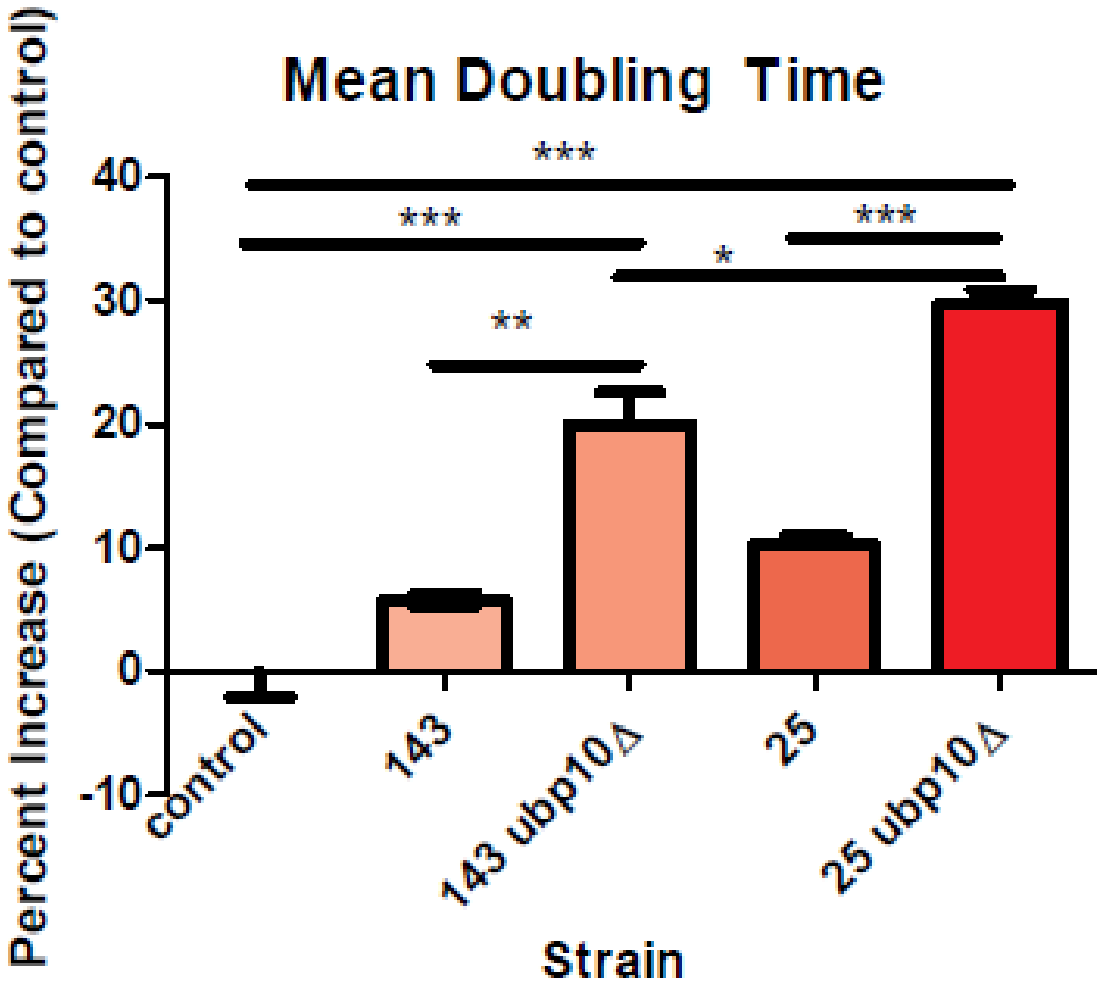


Figure 2.14 - Deletion of *UBP10* in rDNA copy number manipulated strains induced slower growth in lower copy number strain

Bioscreen assays were performed on wild-type cells (control) and *UBP10* or *ubp10*Δ cells expressing either 143 or 25 rDNA copy numbers. Cells were grown at constant shaking at 30ºC for 48 hours. Three technical replicates were used for each biological replicate (N=3). Error bars represent SEM.

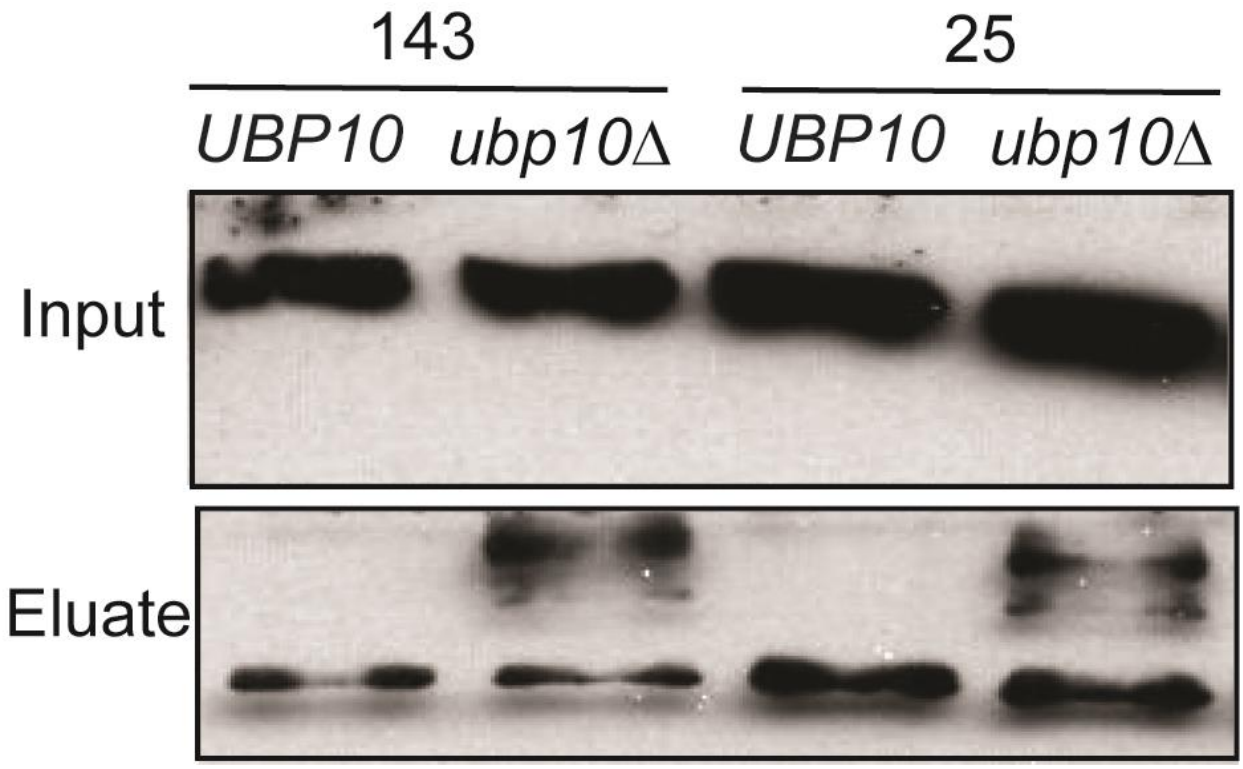


Figure 2.15 - Deletion of *UBP10* does not induce further ubiquitination in lower copy number rDNA strains compared to higher copy number strains

Ubiquitinated proteins from lysates derived from *UBP10* or *ubp10*Δ cells expressing either 143 or 25 rDNA copy numbers were isolated via affinity purification using agarose TUBEs (Tandem Ubiquitin Binding Entities). Levels of Rpa190-3HA (anti-HA) in input and eluate were determined using Western analysis. Blots are representative images. Each experiment was performed with 3 biological replicates.

Chapter III: The Ubiquitination State of Rpa190 Modulates the Pharmacological Effects of BMH-21

Introduction

Targeting RNAPI activity has emerged as a new and promising area of drug discovery and has been a focus in cancer drug development during the past decade (Drygin et al., 2010; Peltonen et al., 2014; Quin et al., 2014). Several drugs such as cisplatin and actinomycin D have been used since the 1970s to slow down the rate of transcription; however, these drugs are not specific for RNA Polymerase I transcription (Bensaude, 2011; Casse et al., 1999). In 2014 BMH-21, a DNA intercalator thought to be specific for inhibiting rDNA transcription (due to its affinity for binding to the GC-rich specific sequences of rDNA) showed promise in slowing growth of cancer cells in culture (Peltonen et al., 2014). BMH-21 has been shown to decrease the steady-state levels of RPA194 (mammalian Rpa190 homolog) via ubiquitination and proteasomal-mediated degradation (Peltonen et al., 2014). Recent research has begun to reveal more about the mechanism of action of BMH-21. rDNA transcription initiation factors such as Rrn3, TAF1, and UBF restore stability of RPA194 in the presence of BMH-21, suggesting that initiation of rDNA transcription is necessary for BMH-21 activity (Wei et al., 2018). However, deletion of RPA135, the subunit necessary for elongation of rDNA, restored stability of RPA194 in the presence of BMH-21(Wei et al., 2018). This data suggests that BMH-21 is altering active elongation and not initiation of rDNA transcription in human cells.

The pharmacological action of BMH-21 also appears to be conserved in budding yeast. BMH-21 has been shown to inhibit rRNA synthesis in yeast (Wei et al., 2018). These data also indicate that BMH-21 is altering elongation in yeast: cells that contain elongation-deficient mutations in Rpa135 and Rpa190 show decreased proliferation after treatment with BMH-21, whereas cells that contain mutations in the rDNA transcription factor Rrn3 (which block initiation of transcription) showed no change in growth upon BMH-21 treatment (Wei et al., 2018). From these results, it has been hypothesized that BMH-21's pharmacological action is likely through the inhibition of rDNA transcriptional elongation in budding yeast (Wei et al., 2018), similar to humans. rDNA transcription analyses in yeast confirms this effect: *in vitro* transcription assays of wild type cells demonstrated that BMH-21 caused a 20% reduction in transcript products after 10 minutes in addition to causing a decreased rate of transcription (Wei et al., 2018). In

summary, current knowledge of BMH-21 pharmacology indicates that one of BMH-21's primary pharmacological actions may occur at the level of elongation during rDNA transcription, most likely by inducing ubiquitination of Rpa190/RPA194.

Despite these recent insights, the importance of Rpa190/RPA194 ubiquitination in eliciting the pharmacological effects of BMH-21 has yet to be revealed. Since the slow-growth effects of BMH-21 in cancer cell culture mimic that of a *ubp10*Δ phenotype in yeast, and BMH-21 increases RPA194 ubiquitination in human cell culture (Peltonen et al., 2014; Wei et al., 2018), we were interested in determining how modulation of Rpa190 ubiquitination in yeast might alter the pharmacological outcomes of BMH-21 in yeast. Therefore, we used the Rpa190 ubiquitination-deficient mutants we developed in Chapter 2 to explore how altering Rpa190 ubiquitination modulates the effects of BMH-21. In particular, we hypothesized that loss of Rpa190 ubiquitination would mitigate the inhibition that BHM-21 has on the growth of yeast cells.

Results

BMH-21 does not slow growth in ubiquitination-deficient Rpa190 compared to wild-type Rpa190

Given published data indicating that human cell growth in cell culture was inhibited by BMH-21 addition (Peltonen et al., 2014) we wanted to determine if BMH-21 would similarly alter growth in yeast cells and, if so, whether or not cells that are deficient for Rpa190 ubiquitination would be refractory to BMH-21 effects. There are three possible hypotheses:

1. Loss of Rpa190 ubiquitination rescues induced slow growth in *UBP10* and *ubp10*Δ cells when treated with BMH-21. The would indicate that BMH-21's effect is dependent on Rpa190's ability to be ubiquitinated. This would suggest that BMH-21 is inducing ubiquitination of Rpa190 faster than the rate which Ubp10 can remove ubiquitin. This would lead to slow growth in *UBP10* cells after BMH-21 treatment. By removing the ubiquitination site, BMH-21 could no longer induce ubiquitination of Rpa190 and the slow growth phenotype would be rescued. In *ubp10Δ* cells where we already observe a slow-growth phenotype, addition of BMH-21 would lead to a further ubiquitination of Rpa190, compounding the slow growth.

2. Loss of Rpa190 ubiquitination rescues slow growth only in *ubp10*Δ cells when treated with BMH-21. This would indicate that BMH-21's effects are dependent on Rpa190's ability to be ubiquitinated but only in the absence of Ubp10. If Ubp10 is present in cells and can deubiquitinate Rpa190, then any induction of slow growth by BMH-21 would not be exaggerated. In *ubp10Δ* cells, we already observe a slow-growth phenotype and would expect this to be exaggerated in *ubp10*Δ cells treated with BMH-21. This could be recovered by removing Rpa190's ability to be ubiquitinated.

3. Loss of Rpa190 ubiquitination has no effect in cells treated with BMH-21. This would suggest that the slow growth effects we see in BMH-21 treated cells are caused by a factor independent of Rpa190 ubiquitination status.

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To test our hypotheses, we performed bioscreen growth assays in four different yeast strains (Rpa190/*UBP10*, Rpa190/*ubp10*Δ, Rpa190^{3KR}/*UBP10*, and Rpa190^{3KR}/*ubp10*Δ). Cells were grown in the

presence of vehicle (DMSO) or 25µM of BMH-21 at 30ºC for 48 hours. Dosing of BMH-21 was established based on dose-response experiments that we conducted (Figure 3.1) and previously published literature of BMH-21 activity in yeast (Peltonen et al., 2014). Each strain has 3 biological replicates with each biological replicate containing 3 technical replicates. BMH-21 caused a significant increase in doubling time in all four of strains when compared to the addition of vehicle control (Figure 3.2 Two-way ANOVA, Bonferonni Post-Hoc p<.0001). Surprisingly, BMH-21 had the same slight growth-slowing effect on both ubiquitination-deficient Rpa190^{3KR} cells and wild-type Rpa190 cells when *UBP10* was intact (Figure 3.2). However, in *ubp10*Δ cells, loss of Rpa190 ubiquitination rescued the slow-growth phenotype, indicating that at 30ºC, BMH-21's activity is dependent on Rpa190 ubiquitination.

Ubiquitination-deficient Rpa190^{3KR} remains stable in BMH-21-treated cells

Because loss of Rpa190 ubiquitination rescued the growth slowing effects of BMH-21 on *ubp10*Δ cells, we hypothesized that ubiquitination-deficient Rpa190 was stable under these conditions. We therefore wanted to determine if BMH-21 could still alter stability in of the ubiquitination deficient Rpa190^{3KR}. Previously published literature in both yeast and mammalian cell models show that BMH-21 decreases Rpa190 and RPA194 stability, respectively (Peltonen et al., 2014; Wei et al., 2018). To test this hypothesis, we pre-treated cells with 50 µM BMH-21 for one hour and performed cycloheximide-chase assays to examine Rpa190 stability over time. BMH21 increased the rate of Rpa190 degradation in *UPB10* cells. Ubiquitination-deficient Rpa190^{3KR} was stable in all cells whether treated with BMH-21 or not (Figure 3.3). These results suggest that the activity of BMH-21 on Rpa190 stability is dependent on the ability of Rpa190 to be ubiquitinated and that part of the slow-growth effect we see with BMH-21 is due to overall transcription deficits caused by BMH-21.

BMH-21 effects on Rpa190 Ubiquitination

Previously published data suggest that BMH-21 induced ubiquitination causes proteasomal degradation of RPA194 (Peltonen et al., 2014). Thus, we wanted to examine if BMH-21 would induce ubiquitination in the Rpa190^{3KR} ubiquitination-deficient strains. We pretreated all four strains with 50 µM

BMH-21 for one hour and purified the ubiquitinated protein proteome to examine Rpa190 ubiquitination. BMH-21 causes a decrease in the ubiquitination of Rpa190 in *ubp10*Δ cells, likely due to the rapid turnover of Rpa190 through proteasomal degradation (Figure 3.3). In ubiquitination-deficient Rpa190^{3KR} cells, Rpa190 remains stable, and we see no further ubiquitination of Rpa190 (Figure 3.3). This suggests that the decrease in Rpa190 stability we see after BMH-21 treatment is due to inducing Rpa190 ubiquitination.

Ubiquitination-deficient Rpa190^{3KR} partially rescues slow-growth at low temperatures after BMH-21 treatment

Previous research established that RiBi is altered at lower temperatures in addition to causing defects in rRNA processing (Nogi et al., 1993; Schneider et al., 2006; Wittekind et al., 1988; Yano and Nomura, 1991). Temperature can also cause mechanistic defects in rDNA transcription: at lower temperatures it is more difficult to unwind rDNA, causing replication fork conflicts and inhibiting RNAPI from being able to actively transcribe (Mettrick and Grainge, 2016). In our previously published work, higher temperatures were shown to decrease ubiquitination of Rpa190 in the absence of *UBP10* (Richardson et al., 2012). With what we know regarding temperature and its influence on growth and ubiquitination, we wanted to understand whether low temperatures would alter growth upon manipulating Rpa190 ubiquitination and treating with BMH-21. We hypothesized that lower temperatures would increase Rpa190 ubiquitination and provide us with a condition where we could examine how manipulating Rpa190 ubiquitination and treating with BMH-21 might influence cells that were wild-type for *UBP10*.

We performed bioscreen assays at temperatures known to cause RiBi defects (17ºC and 25ºC) (Lewinska et al., 2010; Sykes and Williamson, 2009; Woolford and Baserga, 2013). At both 17ºC and 25ºC, we see a significant increase in doubling time for all strains treated with BMH-21 compared to vehicle (Figure 3.4). In the presence of BMH-21 at 25ºC, we observe similar growth patterns to 30ºC, where both ubiquitination-deficient Rpa190^{3KR} strains' growth rates are similar to wildtype Rpa190 strains expressing *UBP10* (no statistical difference was observed between these three strains) (Figure 3.4).

This suggests that 25ºC may not be sufficient to induce RiBi defects where ubiquitination of Rpa190 and the pharmacology of BMH-21 could be discerned. When we lowered the temperature to 17ºC, both ubiquitination-deficient Rpa190^{3KR} strains display advantageous growth when compared to wild-type Rpa190 strains (Two-way ANOVA, Boferonni Post-Hoc $p<.0001$ for both strains compared to Rpa190/*UBP10*) (Figure 3.4). In the presence of BMH-21, the doubling time interval of Rpa190 strains is over twice as slow compared to both ubiquitination-deficient Rpa190^{3KR} strains, and there was no statistically significant difference between either ubiquitination-deficient Rpa190^{3KR} strain (Figure 3.4). These data convey an important role for ubiquitin regulating in ribosome biogenesis: in conditions where RiBi is known to be altered, any perturbation of this process, such as by BMH-21, is mitigated if Rpa190 is unable to be ubiquitinated. This suggests that ubiquitination of Rpa190 serves as a regulator for the proper continuation of rDNA transcription.

Discussion

Insight into BMH-21's pharmacological action is important to evaluate it as a promising anti-cancer therapeutic. BMH-21 was initially identified from a small molecule library from the National Cancer Institute and is currently in preclinical development as an anti-cancer therapeutic (Peltonen et al., 2014). BMH-21 has been shown to activate p53 in the absence of DNA damage and causes ubiquitination of RPA194 in mammalian cells (Colis et al., 2014). Additional research has shown that BMH-21 causes elongation defects in both yeast and mammalian cells (Wei et al., 2018). We have a general understanding of BMH-21 pharmacological properties; it is a GC-rich DNA intercalator (Colis et al., 2014; Peltonen et al., 2014). However, the underlying molecular mechanisms of BMH-21's physiological actions were unclear. In this study, we revealed new insights into the cross-section between Rpa190 ubiquitination and BMH-21 pharmacological action.

Our current knowledge demonstrates BMH-21's pharmacological action is conserved in yeast and in humans. Previous research in both yeast and humans demonstrated that BMH-21 acts to prevent elongation of RNAPI, potentially due to its intercalating properties (Peltonen et al., 2014; Wei et al., 2018). Combined with what we know about how Rpa190 ubiquitination, we were able to utilize yeast as a model system for understanding how BMH-21 altered Rpa190 ubiquitination to elucidate more about its molecular mechanism. The results from this work provide us with a foundation for conducting future studies in human cell models.

It was unclear until this point whether BMH-21 ubiquitination of RPA194/Rpa190 was the driving force behind the slow growth effects we see after BMH-21 treatment, or if its intercalating properties (to halt rDNA transcription) were enough to slow the growth of cells. We sought to determine this by manipulating temperature condition during growth. Research from the Nomura group demonstrated that ribosome biogenesis in *Escherichia coli* is altered at lower temperatures (Nomura et al., 1969). Under such lower temperature conditions, BMH-21 induces ubiquitination at the same sites where Ubp10 deubiquitinates Rpa190, and the activity of BMH-21 is dependent on the ubiquitination status of Rpa190. While BMH-21 significantly slows the growth of cells in our Rpa190^{3KR} ubiquitination-deficient mutants,

they nevertheless grow almost twice as fast than Rpa190/*UBP10* cells. These data reveal that BMH-21's ability to slow cell growth is largely based on the ubiquitination status of Rpa190.

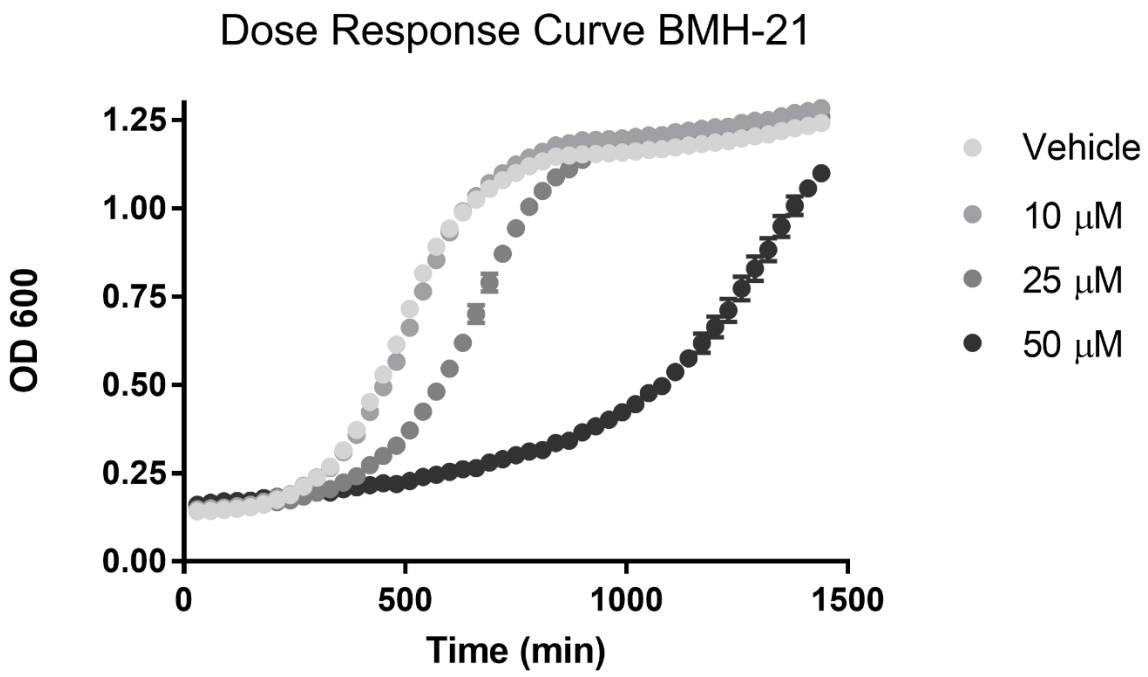

Figure 3.1 - Dose response curve for BMH-21

A bioscreen analysis was performed in UBP10 cells in presence of vehicle (DMSO), 10, 25 or 50μM BMH-21 for 48 hours to calculate doubling time interval. Three technical replicates were used for each biological replicate (N=3). Error bars represent SEM.

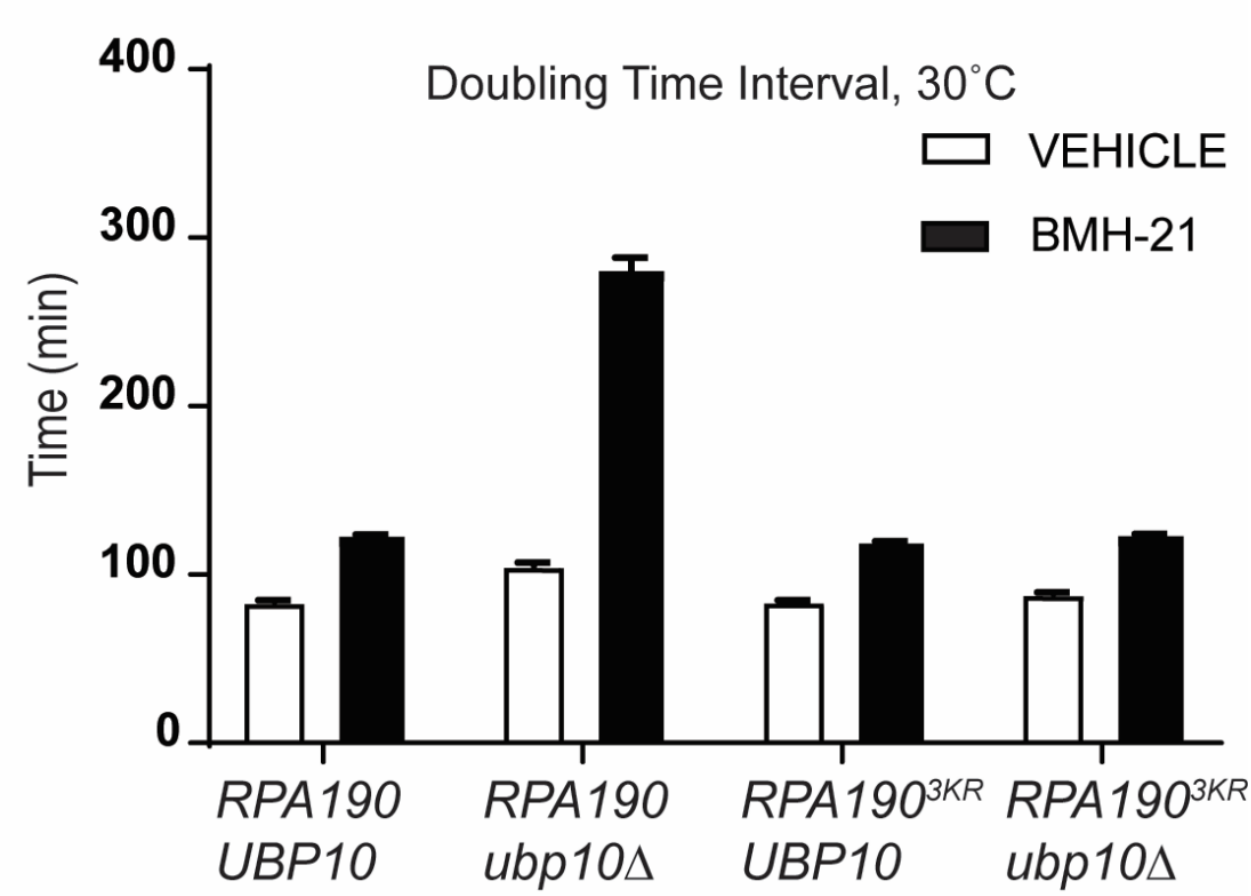

Figure 3.2 - BMH-21 does not slow growth in ubiquitination-deficient Rpa190 cells compared to wild-type Rpa190 cells

A bioscreen analysis was performed in each of the 4 indicated strains in presence of vehicle (DMSO) or 25µM BMH-21 for 48 hours to calculate doubling time interval. Three technical replicates were used for each biological replicate (N=3). Error bars represent SEM.

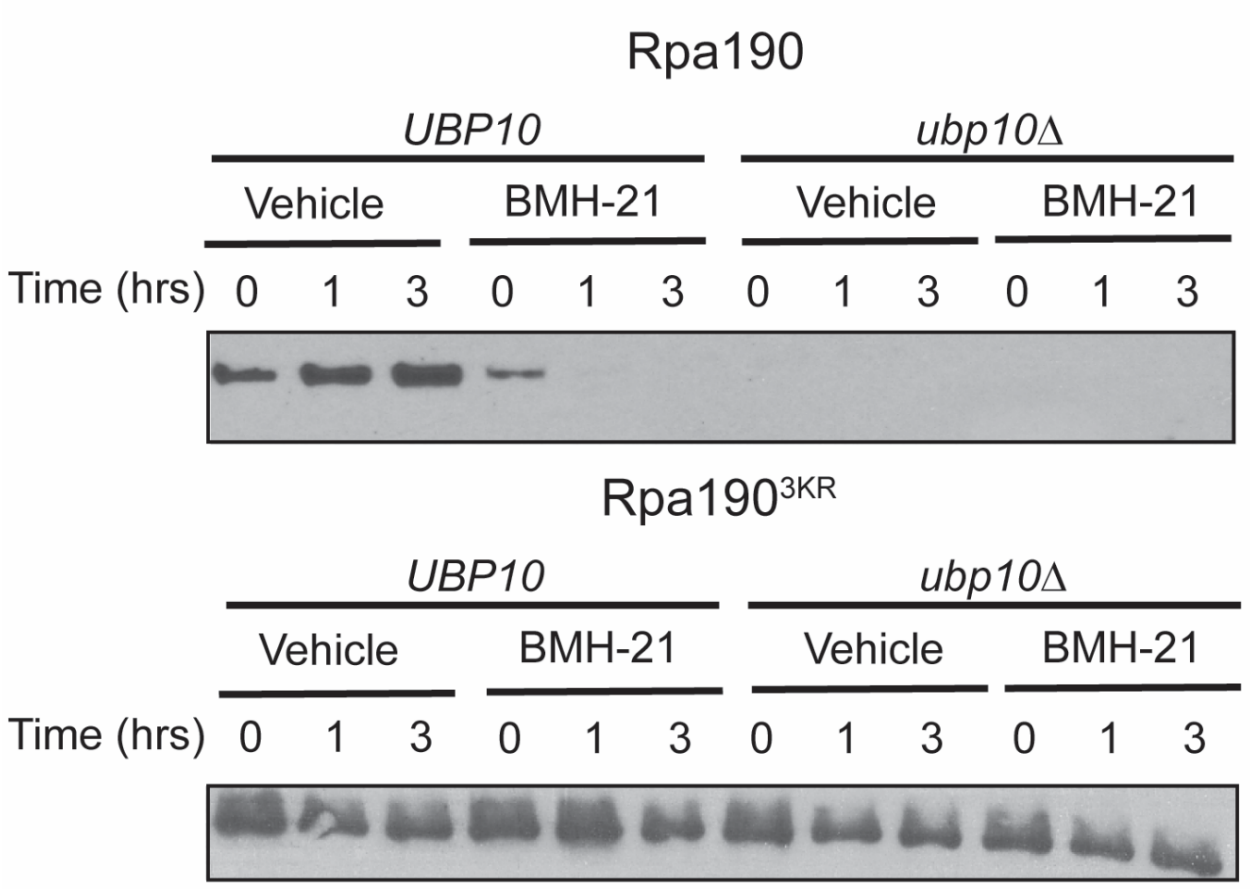

Figure 3.3 - Ubiquitination-deficient Rpa190 is stable in BMH-21 treated cells

Cycloheximide-chase degradation assays of *UBP10* or *ubp10*Δ cells expressing either Rpa190-3HA or Rpa190^{3KR}-3HA. Cells were treated with either vehicle (DMSO) or 50μM BMH-21 for one hour prior to cycloheximide addition. Hours after cycloheximide addition are indicated above each lane. Western analysis of whole-cell extracts was performed using anti-HA antibodies. Blots are representative images. Each experiment was performed with 3 biological replicates.

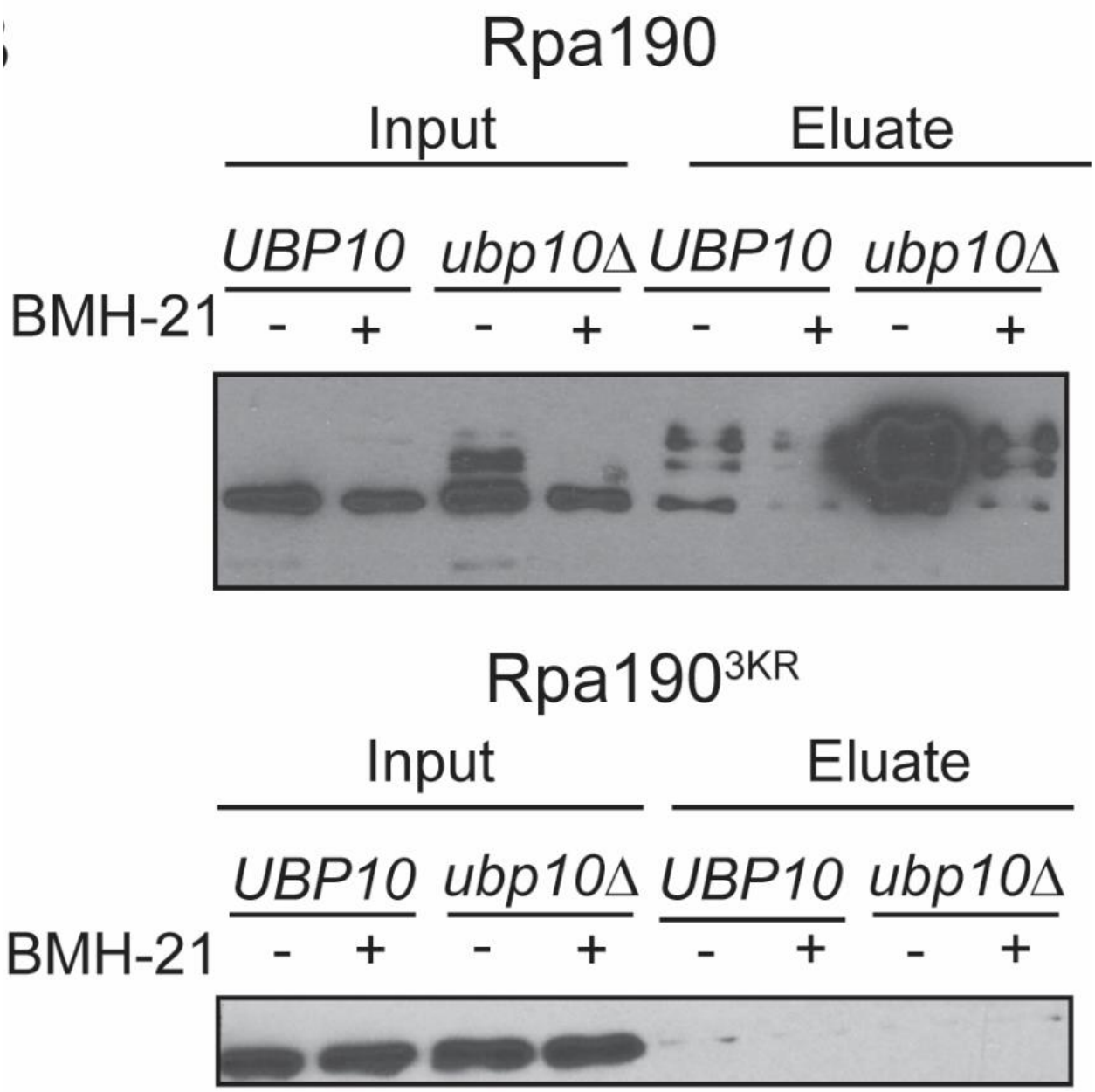

Figure 3.4 - Ubiquitination of Rpa190 is required for BMH-21 activity

Ubiquitinated proteins from lysates of *UBP10* (WT), *ubp10*Δ cells expressing Rpa190-3HA, or Rpa190^{3KR}-3HA were isolated via affinity purification using magnetic TUBEs (Tandem Ubiquitin Binding Entities). Cells were treated for one hour with either vehicle (DMSO) or 50 µM BMH-21 for 1 hour prior to lysis. Levels of Rpa190-3HA (anti-HA) in input and eluate were determined using Western analysis. Blots are representative images. Each experiment was performed with 3 biological replicates.

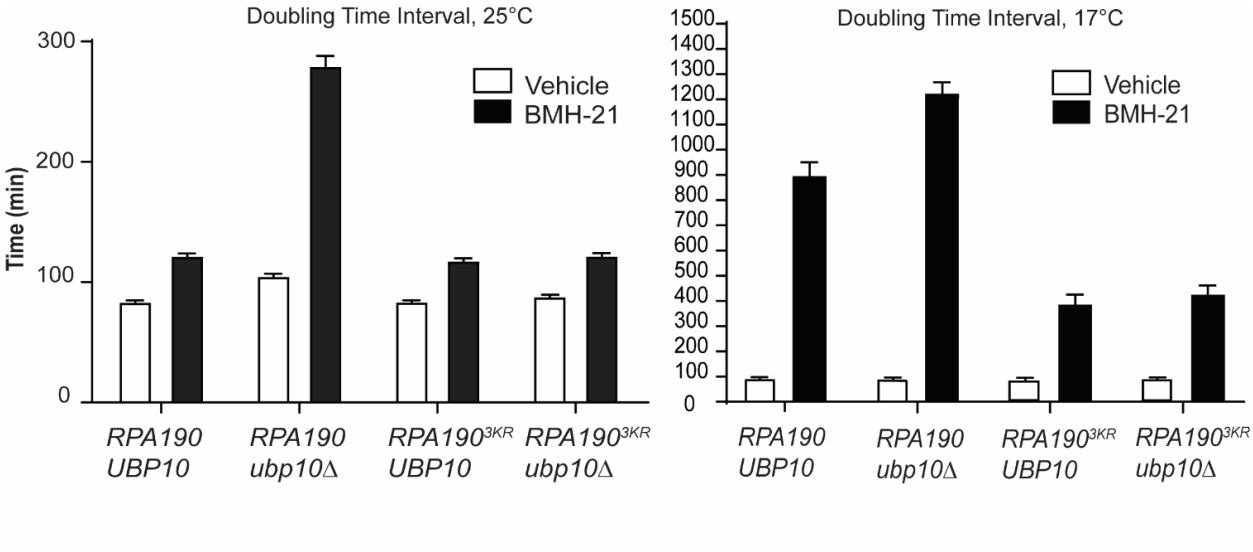

Figure 3.5 - Ubiquitination-deficient Rpa190 partially rescues slow growth at lower temperatures during BMH-21 treatment

Bioscreen assays were performed on the indicated strains using either vehicle (DMSO) or 25 μM BMH-21 for 48 hours at either 17ºC or 25ºC to calculate the doubling-time interval. Three technical replicates were used for each biological replicate (N=3). Error bars represent SEM.

Chapter IV: The Dynamic Deubiquitination of RNA Polymerase I is Conserved in Human Cells

Introduction

Deubiquitinating enzymes have become a new target for trying to moderate the growth and proliferation of cancer cells (Farshi et al., 2015). Several deubiquitinating enzymes are known to be upregulated in cancer cells (Hussain et al., 2009); the hypothesis is that actively proliferating cells need to increase their amount of protein production, leading to an increase in potential ubiquitinated substrates (He et al., 2017). By increasing deubiquitinating enzymes levels, this can lead to a decrease in overall ubiquitinated proteins, resulting in the maintained of stability of necessary proteins needed for continued cell proliferation in cancer cells. In humans, there are 100 known DUBs; over 20 have been implicated in cancer development either through association with the DNA damage response or through modulating gene regulation through histone H2A/B ubiquitination (Pinto-Fernandez and Kessler, 2016). This body of work has led to the development of DUB inhibitors, none of which are currently FDA approved.

USP36 is one DUB potentially implicated in tumorigenesis; it has been shown to be upregulated in ovarian cancer (Li et al., 2008). USP36 has been shown to be localized to the nucleolus and can regulate c-MYC to alter the growth of cancer cells (Sun et al., 2015). Additionally, USP36 has been shown to upregulate both the Akt and ERK pathways to increase cell proliferation of cancer cells (Kim et al., 2018). USP36 was found to be essential for maintaining nucleolar structure and function: depletion of USP36 leads to a decrease in rDNA transcription, underdeveloped nucleolar morphology, and a reduction in cytoplasmic ribosome formation, ultimately leading to a decrease in cellular proliferation (Endo et al., 2009a; Endo et al., 2009b).

Previous research by our group has demonstrated that USP36 serves as a functional homolog to of Ubp10 to deubiquitinate Rpa190 (Richardson et al., 2012). Expression of USP36 in *ubp10*Δ cells can rescue the slow-growth phenotype, restore stability of Rpa190, and decrease ubiquitination of Rpa190. Given this information, we wanted to determine if USP36 also regulated RPA194 in human cells similar to how Ubp10 mediates ubiquitination of Rpa190 in yeast. Establishing such conservancy would provide us with a framework for understanding not only the mechanism by which USP36 regulates RPA194, but

would also strengthen the use of yeast as a model for understanding how ubiquitination of Rpa190/RPA194 regulates proper RiBi.

In this chapter, we establish a potential role for USP36 in regulating cell growth by modulating the steady-state levels of RPA194, similar to Ubp10's regulation of Rpa190 (Richardson et al., 2012). We demonstrate that that siRNA of USP36 in HeLa cells decreased steady-state levels of RPA194, resulting in a slow-growth phenotype similar to that which is seen when *UBP10* is deleted in yeast. Similar to our previously published yeast cell model (Richardson et al., 2012), we also demonstrate that overexpression of RPA194 could rescue the slow-growth phenotype in HeLa cells when USP36 is depleted. We have established that USP36 regulation of RPA194 occurs at the protein level and siRNA of USP36 does not alter mRNA levels of RPA194. Together, this information provides us with a new foundation to explore the ubiquitin-mediated regulation of the largest subunit RNAP I in both yeast and human cell models.

Results

Depletion of USP36 by siRNA decreased RPA194 steady-state protein levels

We previously demonstrated that deletion of *UBP10* in yeast results in a slow-growth phenotype caused by an increase in ubiquitination and a decrease in stability of Rpa190 (Richardson et al., 2012). Additionally, we also demonstrated that expression of human USP36 in *ubp10*Δ cells rescued the slow-growth phenotype and stability of Rpa190 was restored through a decrease in Rpa190 ubiquitination (Richardson et al., 2012). Since USP36 could serve as a function homolog to Ubp10, we wanted to determine if USP36 could regulate stability of RPA194 in human cells similar to how Ubp10 regulated Rpa190 in yeast. To do this, we performed three separate siRNA experiments to knockdown USP36 levels in HeLa cells. Cells were treated with either scrambled or 100 nm of siUSP36 siRNA for 24 hours and whole cell lysates were harvested to probe for both USP36 and RPA194 levels. In all three siRNA experiments, USP36 levels were shown to decrease by about 83% on average compared to scrambled siRNA (Figure 4.1). This resulted in approximately a 52% decrease in the amount of RPA194 levels. Thus, USP36 depletion resulted in a reduction in RPA194 steady-state levels similar to how deletion of *UBP10* in yeast cells resulted in a decrease in steady-state levels of Rpa190 (Richardson et al., 2012).

Depletion of USP36 by siRNA caused a slow-growth phenotype that is rescued by overexpression of RPA194

Overexpression of Rpa190 in yeast rescued the slow-growth phenotype of *ubp10*Δ cells (Richardson et al., 2012). Therefore, we wanted to determine if overexpression of RPA194 in HeLA cells could rescue the reduced proliferation when USP36 levels were reduced. Three different siRNA experiments were performed by treating cells with either scrambled or 100 nM of siUSP36. After siRNA transfection for 24 hours, control cells and siUSP36 cells were plated and MTS assays were performed to look at growth of cells at 0 hours, 24 hours, 48 hours, and 72 hours post-transfection. For RPA194 over expression experiments, RPA194 was transfected post-siUSP36 treatment. In cells treated with siUSP36, we see a decrease in cell growth compared to cells treated with scrambled siRNA that is prominent 72 hours post-transfection. This slow growth is recovered in siUSP36 treated cells with

RPA194 over expressed. These data taken together indicate that USP36 mediates growth in cells through regulation of RPA194 similarly to Ubp10 regulation of Rpa190.

Depletion of USP36 by siRNA did not alter RPA194 mRNA levels

Given that depletion of USP36 altered RPA194 steady-state protein levels and altered cell proliferation dependent on RPA194 expression, we wanted to confirm that this was not a result of USP36 indirectly regulating mRNA levels of RPA194. To verify this was not the case, we performed qRT-PCR of USP36 and RPA194 mRNA levels on cells transfected with either scrambled or 3 different siUSP36. In cells treated with siUSP36 constructs, we observed a significant reduction in USP36 mRNA as expected (Figure 4.3). However, we observed no significant difference in the amount of RPA194 mRNA. This indicates that depletion of USP36 does not lead to a decrease in RPA194 mRNA levels and that USP36 is most likely regulating RPA194 protein stability, likely through modulating ubiquitination of RPA194.

Discussion

While we previously established that USP36 could serve as a functional homolog for Ubp10 in budding yeast, it was unclear whether USP36 could regulate RPA194 similar to how Ubp10 regulates Rpa190. The preliminary data in this chapter demonstrates that USP36 likely does regulate RPA194 in human cells through controlling RPA194 stability. Similar to deletion of *UBP10* in yeast, depletion of USP36 led to reduced proliferation that was rescued by over expression of RPA194. This mirrored what we previously observed when overexpression of Rpa190 in ubp10Δ cells rescued the slow-growth phenotype(Richardson et al., 2012).

Although these results have established a foundation for a conserved mechanism between budding yeast and humans in regulating RNAPI through dynamic ubiquitination/deubiquitination, there is much to be learned about how USP36 regulates RPA194 in human cells. Despite our attempts to purify the ubiquitinated protein proteome in mammalian cells, we have yet to establish if siRNA of USP36 results in an increase in RPA194 ubiquitination. It is possible that RPA194 ubiquitination is mediated by other DUBs and that RPA194 has evolved to be regulated by more than one DUB.

For example, RNAPI in yeast is also known to be ubiquitinated by ubiquitin ligase Rsp5 and is mediated by Ubp2 and Ubp4 in response to zinc starvation (Lee et al., 2013). There are also several DUBS, in humans and yeast, that are known to be involved in DNA replication regulation (Kee and Huang, 2016). For example, Ubp8 (USP22 in humans) is part of the SAGA complex and is known to regulate DNA transcription through ubiquitin-mediated regulation of histone H2B (Kee and Huang, 2016). Additionally, there are several DUBS known to regulate the cell cycle in humans which is tightly linked to RiBi; Usp1, Usp3, Usp11, Usp 28 and Usp44 are all known to regulate various aspects of the cell cycle in humans (Farshi et al., 2015; He et al., 2017; Hussain et al., 2009; Pfoh et al., 2015; Reyes-Turcu et al., 2009).

It is not known whether the sites of ubiquitination in RPA194 are conserved with those in Rpa190. Based on NCBI protein BLAST, Rpa190 and RPA194 share only 53% homology. In the region we

previously established as the site of ubiquitination of Rpa190, this same position in RPA194 contains a conserved lysine residue; however, without a structure of RPA194, it is impossible to determine if this conserved position exists in the same clamp region as in Rpa190. Further studies will need to be conducted in order to determine if USP36 can deubiquitinate RPA194 in the same region where Ubp10 deubiquitinates Rpa190. While we have established that USP36 can serve as a functional homolog in yeast, it is unclear if RPA194 can serve as a function homolog for Rpa190. One method to confirm this hypothesis this is by expressing RPA194 in yeast to determine in the absence of *UBP10* if RPA194 can be ubiquitinated. If expression of RPA194 in *UBP10* cells results in steady-state levels of RPA194 and in *ubp10*Δ cells we see an increase in ubiquitination and a decrease in stability of RPA194, it is possible that RPA194 ubiquitination may occur at the same site similar to Rpa190.

Acknowledgements

A significant portion of this work is due to our collaborators at the University of Washington. Kyung-Soon Lee performed siRNA, MTS assays and qRT-PCR experiments.

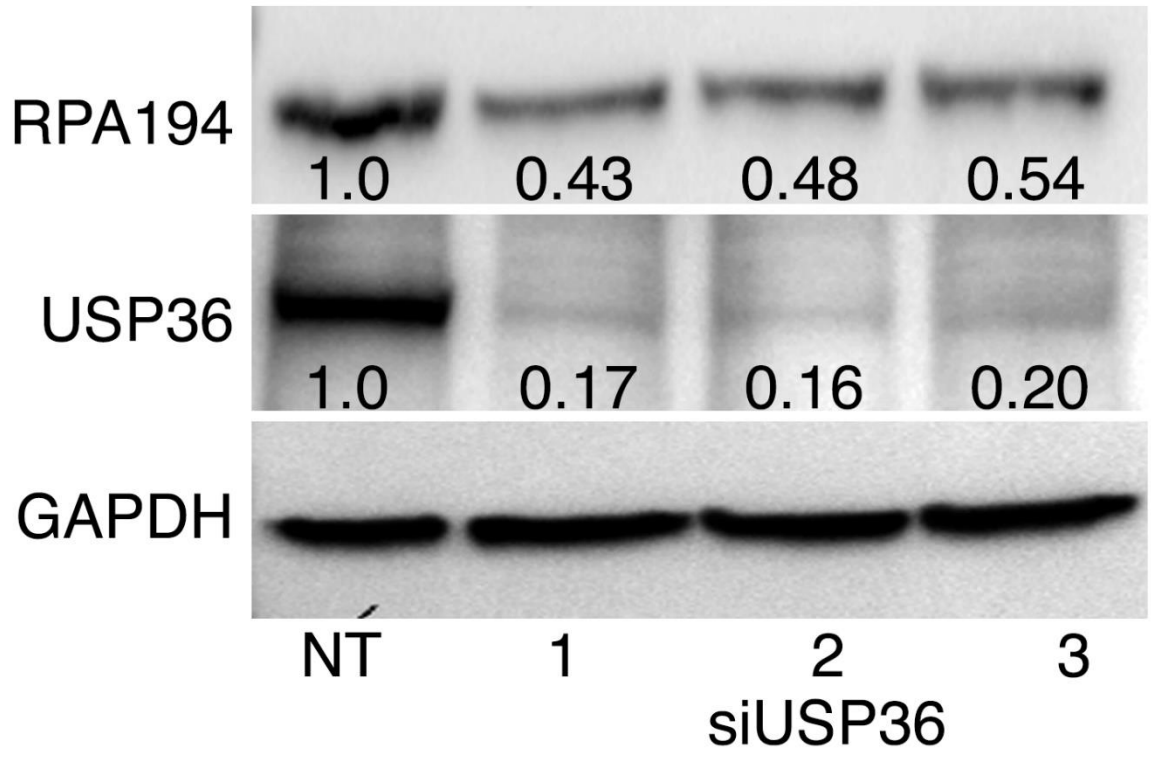

Figure 4.1 - siRNA of USP36 decreases RPA 194 levels

HeLa cells were transfected with either 100 nM scrambled siRNA (NT) or three different siUSP36 (1,2, and 3) for 24 hours in serum free media. Media was washed off 24 hours and replaces with fresh DMEM for 24 hours. Cells were harvested and RPA194 and USP36 levels were probed via Western analysis. GAPDH was used as a positive control.

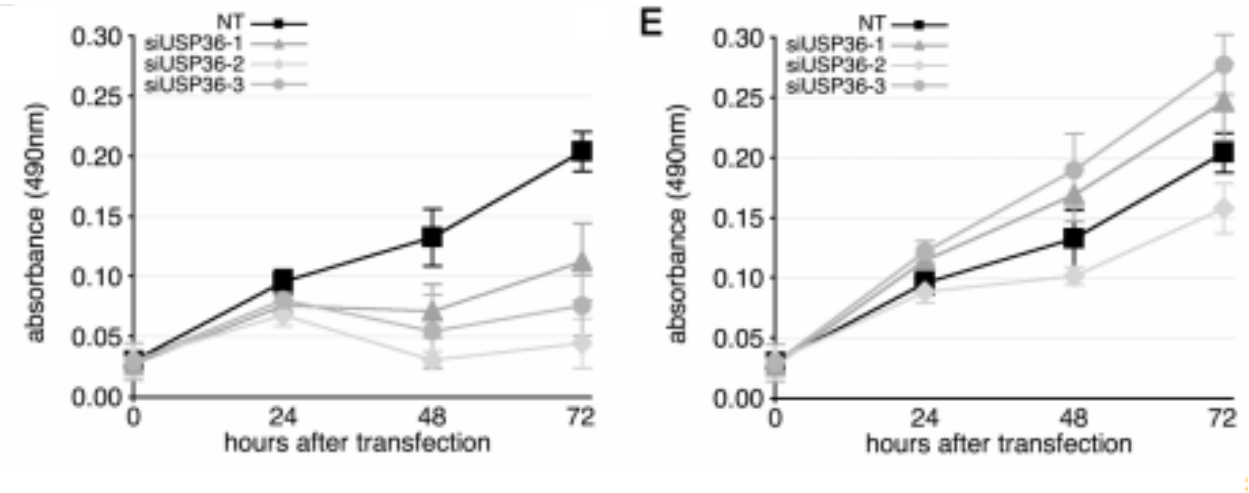

Figure 4.2 - siRNA of USP36 causes a slow growth phenotype and is rescued with RPA194 overexpression

HeLa cells were transfected with either 100 nM scrambled siRNA (NT) or three different siUSP36 (1,2, and 3) for 24 hours in serum free media. Media was washed off 24 hours and replaced with fresh DMEM to recover for 6 hours. CellTiter 96® AQu$_{eous}$ One Solution Cell Proliferation Assay was performed according to manufacturer's instructions.

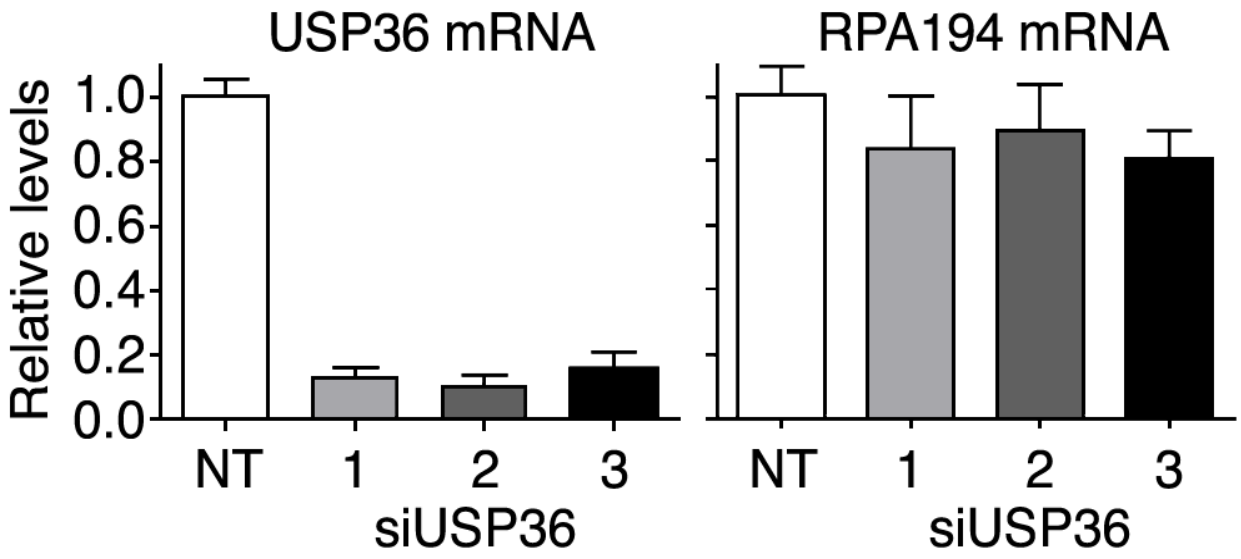

Figure 4.3 - siRNA of USP36 does not alter mRNA levels of RPA194

HeLa cells were transfected with either scrambled (NT) or siUSP36 (1-3) for 24 hours in serum free media. Cells recovered in fresh DMEM for 6 hours. qRT-PCR was performed using PowerUp™ SYBR® Green on ABI 7500 Fast Machine.

Chapter V: Broader Implications

Conclusions

When embarking on this project, we were determined to clarify the conditions inducing Rpa190 ubiquitination and how this ubiquitination event regulates rDNA transcription. There was an identifiable gap in the general knowledge of internal and external factors that induced Rpa190 ubiquitination in addition to the other molecular mechanisms that regulated this process. We have closed some of these gaps through identification of a ubiquitin cascade that regulates Rpa190 ubiquitination.

We then utilized pharmacological approaches to identify a role for of Rpa190 ubiquitination. These results not only provided us with an understanding of the sites of Rpa190 ubiquitination but revealed the ubiquitination of Rpa190 is necessary for the pharmacology of BMH-21 in yeast. It was also unclear whether this ubiquitin-mediated regulation of Rpa190 was conserved in humans. Our data shows that ubiquitin-mediated regulation of RNA Polymerase I is conserved in humans: USP36 regulated stability of RPA194 and siRNA of USP36 results in a slow growth phenotype akin to deletion of *UBP10* in yeast. While the biological relevance of Rpa190 ubiquitination remains to be determined, key findings of this research set the stage for asking specific questions in future work.

SCF Complex Regulation of Ribosome Biogenesis

This work has identified a role for the SCF^{Grr1} complex in modulating Rpa190 for ubiquitination. The involvement of the SCF^{Grr1} complex suggests certain conditions Rpa190 may be ubiquitinated. In our previous research, we attempted to identify conditions where Rpa190 ubiquitination occurred without the deletion of *UBP10* to help us identify either environmental or stress conditions that could induce Rpa190 ubiquitination (Richardson et al., 2012). Neither amino acid or glucose deprivation were shown to decrease Rpa190 stability (Richardson et al., 2012). Previous literature demonstrated that Grr1 regulates glucose transport and other nutrient dependent processes that regulate RiBi (Horak and Wolf, 2005; Pasula et al., 2010).

Our previously published research, in addition to the results of this thesis, suggests that regulation of Rpa190 ubiquitination may be more complicated than merely removing essential nutrients. RiBi is an energetically expensive process that can quickly turn on and off when conditions are less than ideal; it is only activated when cells must generate more ribosomes (Lempiainen and Shore, 2009). Therefore, it is possible that mutations in Grr1 causing improper glucose transport, in addition to applying nutrient deprivation conditions, may induce Rpa190 ubiquitination. Experiments addressing these hypotheses will need to be conducted in the future in order to determine the SCF^{Grr1} complex's role in regulating RiBi.

Temperature mediates Rpa190 ubiquitination status during rDNA transcription

Two conditions revealed the importance of Rpa190 ubiquitination: temperature and pharmacological inhibition of rDNA transcription by BMH-21. Initially, we suspected that our ubiquitination-deficient $Rpa190^{3KR}$ mutants would be more susceptible to such conditions where ribosome biogenesis is altered. If Rpa190 ubiquitination serves as a checkpoint during rDNA transcription, then removing the ability of Rpa190 to be ubiquitinated conditions might slow the growth of these cells. Surprisingly, our data demonstrated that losing the ability to ubiquitinate Rpa190 provides an advantageous effect on rDNA transcription, especially at lower temperatures and in the presence of BMH-21. This work illustrates the significance for which ubiquitin acts as a regulator of the transcription process, especially under conditions of altered RiBi. This resulted in two important conclusions: ubiquitin may serve as a regulator of RiBi during kinetic restraints, such as temperature, and ubiquitin plays a role in the pharmacology of BMH-21.

Temperature regulation of rDNA transcription has been studied for decades, beginning in the 1960s with *E. coli* (Guthrie et al., 1969; Nomura et al., 1969; Traub and Nomura, 1968, 1969), followed by studies performed in *S. cerevisiae* (Nogi et al., 1993; Nogi et al., 1991; Wittekind et al., 1988; Yano and Nomura, 1991). Temperature is an environmental stressor that alters the ability of rDNA to unwind and promote successful transcription (Shen and Skibbens, 2017). At lower temperatures, less kinetic energy is available to unwind rDNA to promote rDNA transcription (Mettrick and Grainge, 2016), which promotes

ubiquitination of several r-proteins and rRNAs (Sung et al., 2016; Zhou et al., 2015) . This suggests that temperature is a key kinetic barrier in the proper development of ribosome formation. In the case of proper rRNA synthesis, researchers discovered a temperature-sensitive mutation in Rpa12 (a subunit of RNAPI), a subunit vital for effective transcription (Nogi et al., 1993; Prescott et al., 2004). However, this is only applicable at high temperatures (37ºC), indicating its role in transcription at certain high kinetic energy environments (Nogi et al., 1993). This is directly tied to Rpa190 levels: at permissive temperatures, depletion of Rpa190 is seen when Rpa12 is absent (Nogi et al., 1993). Because our data showing the importance of Rpa190 ubiquitination in RiBi and the ties to temperature dependence of Rpa12 expression and function, ubiquitination of Rpa190 likely plays a key regulator in assuring proper rDNA transcription.

Conservation of Ubiquitin-Mediated RNA Polymerase I Regulation

One important aspect to consider is how temperature influences on ubiquitination in yeast translates to a condition human cell. In other words, what is the equivalent human condition to temperature stress in yeast? Temperature stress in yeast has been extensively studied and is a physiological concern for yeast in the wild in terms of adaptation (Finley et al., 1987; Mager and Ferreira, 1993; Strassburg et al., 2010). While yeast in nature will experience vast temperature changes, human cells are much less adaptive to temperature changes (Watanabe and Okada, 1967). For example, the mouse lymphoma line, LY5178Y, grow significantly slower at 34ºC compared to 37ºC (Watanabe and Okada, 1967).

One way to understand this parallel could be through how cells handle replication fork conflicts. For example, temperature changes are known to induce replication fork conflicts and have been studied in budding yeast (Labib et al., 2000; Mettrick and Grainge, 2016). In order to apply the parallel condition in humans, we must look for a condition that induces replication fork conflicts in human cells that can mimic temperature stress yeast experience. Given that drugs such as BMH-21 and D-actinomycin can induce replication fork conflicts (Colis et al., 2014; Guy and Taylor, 1978; Peltonen et al., 2014) and this thesis demonstrates that ubiquitination of Rpa190 is important for the pharmacology of BMH-21, this

collective information may reveal the role of how ubiquitination of RPA194 mediates replication fork conflicts. Future work will need to be conducted to examine rDNA transcription in mammalian cells in response to such stresses in order to understand the role ubiquitin serves in mediating RNA Polymerase I ubiquitination.

Therapeutic Considerations of targeting the Rpa190/RPA194 dynamic ubiquitination axis

When considering BMH-21 as an effective anti-cancer therapeutic, one must consider the high mutation rate among cancer cells (Duesberg et al., 2000). There are several instances where mutant cancer cells mutations occur after treatment with an anti-cancer drug, most notably the kinase inhibitor family of therapeutics (Bhullar et al., 2018). Together with this consideration and the data in this thesis, mutations in the ability for Rpa190 ubiquitination will likely reduce the effectiveness of BMH-21 and other DNA intercalators in development. Focusing on efforts to maintain ubiquitination of Rpa190 will ensure successful pharmacological effects of BMH-21 and other GC-rich specific DNA intercalators.

Another consideration for BMH-21 as a therapeutic is dosing. Recent studies demonstrated that low doses of the DNA intercalator actinomycin D are successful in treating chronic lymphocytic leukemia as well as early and late stage myelomas (Merkel et al., 2012). One explanation is that lower therapeutic doses can target actively proliferating cancer cells while still preserving normally proliferating cells. Alternatively, cells may be less susceptible to mutation and/or drug resistance at lower doses than at higher doses. For example, lower therapeutic doses of actinomycin D were shown to re-induce p53 activation in patients with ST-EPN-RELA ependymoma through promotion of Mdm2 (Tzaridis et al., 2016). Therefore, treatment at lower therapeutic doses may be successful in maximizing effectiveness of BMH-21; because it is a DNA intercalator like actinomycin D. Since actinomycin D and BMH-21 have overlapping pharmacological properties, these considerations will be useful in future studies to develop effective DNA intercalator therapeutics.

A Model for Ubiquitin-Mediated Regulation of RNA Polymerase I

The work in this thesis can be summarized into a model for how ubiquitination of Rpa190 mediates rDNA transcription under normal conditions and during BMH-21 treatment. Our research has demonstrated that the SCF^{Grr1} complex aids, either directly or indirectly, in the ubiquitination of Rpa190. Building upon our previously published literature (Richardson et al., 2012), under normal conditions Ubp10 can deubiquitinate Rpa190 to maintain the stability of RNAPI. Under these conditions, we hypothesize that rDNA transcription is sustained resulting in normal growth in cells (Figure 5.1). When *UBP10* is deleted, ubiquitination of Rpa190 is persistent and Rpa190 degraded leading to a decrease in ribosome synthesis.

While previous research demonstrated that BMH-21 induces slow growth in both yeast and human cells (Peltonen et al., 2014; Wei et al., 2018), we sought to test the effects on Rpa190 ubiquitination in yeast. The work in this thesis demonstrates that, in the presence of Rpa190, BMH-21 causes a slow growth defect and is further exacerbated when *UBP10* is deleted in these cells. The slow-growth phenotype observed in *ubp10*Δ cells is compounded with the addition of BMH-21 treatment, likely due to increase in ubiquitination of Rpa190 and BMH-21's intercalator properties (Figure 5.2). Under conditions where Rpa190 can no longer be ubiquitinated, our data demonstrated that these cells grow at roughly the same rate; however, some slow growth is seen. We hypothesize this is due to BMH-21's properties as an intercalator, physically inhibiting rDNA transcription. Our model for understanding BMH-21 pharmacological action centers around the fact that much of the slow-growth phenotype we see caused by BMH-21 is due to Rpa190 ubiquitination.

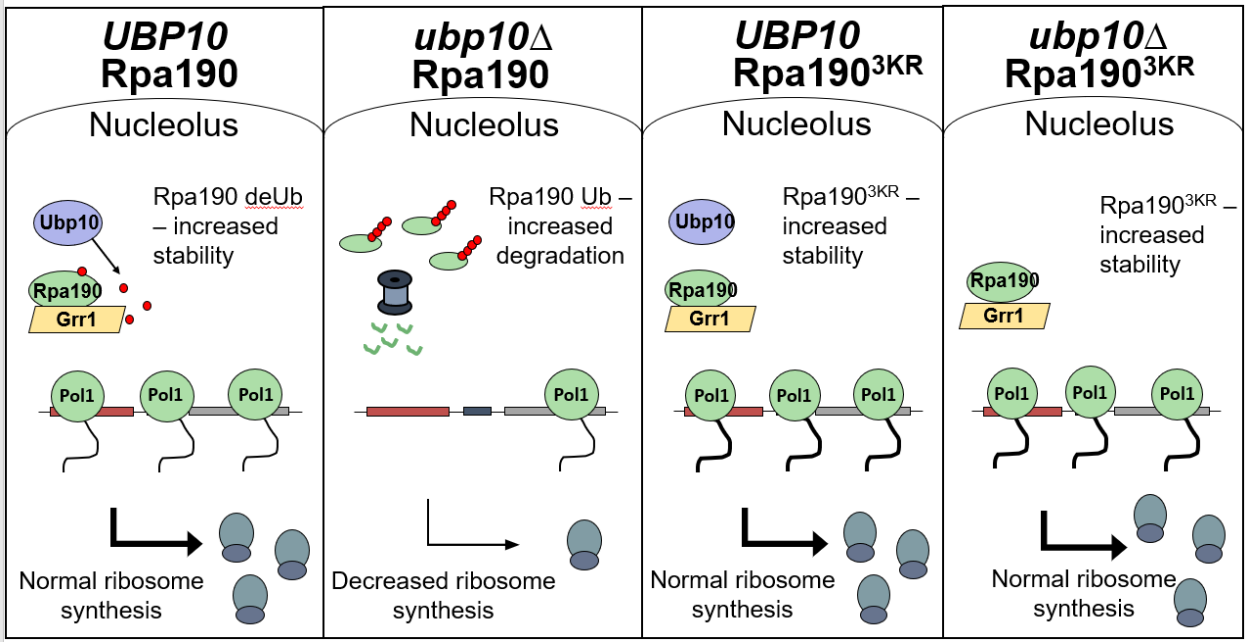

Figure 5.1 - A model for ubiquitin mediated regulation of RNA Polymerase I

The SCF^{Grr1} complex aids in the recruitment for Rpa190 ubiquitination. In *UBP10* cells, Rpa190 stability is maintained and normal ribosome synthesis occurs. In *ubp10*Δ cells, Rpa190 ubiquitination is persistent and a decrease in ribosome synthesis is seen. In both ubiquitin-deficient strains ($Rpa190^{3KR}$), we see normal growth compared to wild type cells likely resulting in normal ribosome synthesis.

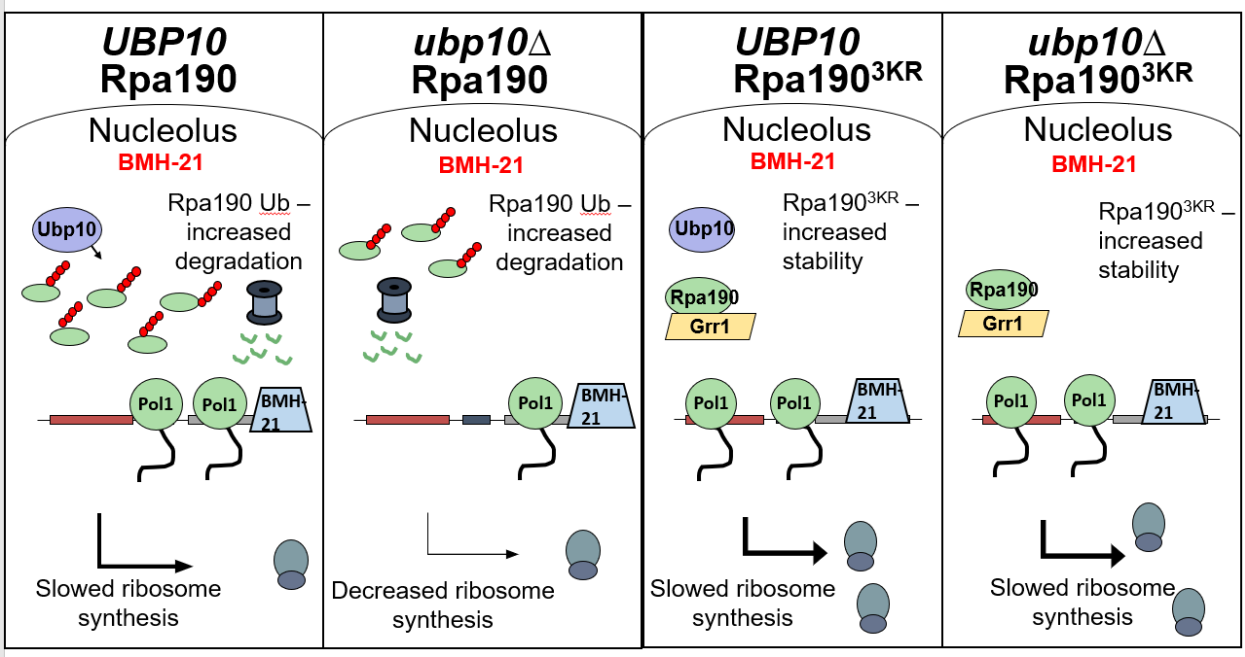

Figure 5.2 - Ubiquitin-meditated regulation of RNA Polymerase I is influenced by BMH-21

In *UBP10* cells, BMH-21 treatment causes slow growth due to an increase in ubiquitination of Rpa190 as well as halting rDNA transcription likely resulting in slow ribosome synthesis. BMH-21 exacerbates the slow growth seen in *ubp10*Δ cells by inducing further ubiquitination of Rpa190 likely resulting in a further reduction of ribosome synthesis. In both ubiquitin deficient strains, BMH-21 causes slow growth on par with its effect in wild type cells, likely due to its intercalator properties. This likely results in slowed ribosome synthesis in both conditions.

Materials and Methods

Yeast strains and plasmids

Yeast strains used in this study derived from BY4741 (Brachmann et al., 1998) are RGY5836 (Rpa190, *UBP10*), RGY5837 (Rpa190^{3KR}, *UBP10*), RGY5840 (Rpa190, *ubp10*Δ::*LEU2*), RGY5841 (Rpa190^{3KR}, *ubp10*Δ::*LEU2*), RGY6267 (Rpa190^{K405R}), RGY6268 (Rpa190^{K408R}), RGY6269 (Rpa190^{K410R}), RGY6270 (Rpa190^{K405R}, *ubp10Δ::LEU2*), RGY6271 (Rpa190^{K408R}, *ubp10Δ::LEU2*), and RGY6272 (Rpa190^{K410R}, *ubp10Δ::LEU2*). Yeast strains used in this study derived from W303 (Voth et al., 2005) are RGY2970 (W303 control), RGY5850 (143 rDNA copy number), and RGY5851 (25 rDNA copy number). Standard yeast growth media and yeast genetics were used (Gunthrie and Fink, 1991). Standard cloning protocols were used to construct the *GRR1* overexpression plasmid pRG4298 (*GRR1*, *URA3*, 2 micron).

Chromatin Isolation

Cells were grown to an optical density of 0.5 and spheroplasted for 30 minutes. Fractionation was conducted using sucrose fractionation as described by Keogh et al. 2006.

Ubiquitinated protein Purification

BY4742 cells (400 mL cultures) were treated with rapamycin for 3 hours or 30 minutes during log phase growth. OD600 was measured directly before harvest. Equal amounts of each culture condition were harvested using vacuum filtration through filter paper, followed by rapid freezing in a single plastic conical tube in liquid nitrogen. Lysis was performed by vortexing the frozen filter papers in lysis buffers until no cells remained on the paper. The lysis buffer contained 8M urea, 50 mM Tris pH 8.2, Roche complete mini EDTA-free protease inhibitor cocktail, phosphatase inhibitors (50 mM β-glycerophosphate, 1mM sodium orthovanadate, 10 mM sodium pyrophosphate, 50 mM sodium fluoride). Yeast cell walls were disrupted by rapid shaking for 3 x 1 min with 0.5 mm diameter silica beads. Beads were removed by centrifuge filtration (1,000xg in a small table-top centrifuge) through a small hole, poked with a heated 21G needle, in the bottom of the centrifuge tube. The lysate was then clarified by centrifugation at 9,000 x g for 8 minutes. Protein concentration was measured at this stage using the BCA Assay (Pierce).

Protein cysteine residues were then reduced using 5 mM dithiothreitol (DTT) for 45 min at 55°C and alkylated using 15 mM iodoacetamide for 30 min at room temperature in the dark. Excess iodoacetamide was quenched with an additional 5 mM DTT at room temperature for 15 min. LysC was used to digest peptides for proteome analysis, and trypsin was used for ubiquitination analysis.

Trypsin digestion of gel slices for MS/MS

For trypsin digestions, samples were diluted 5-fold in 50 mM pH 8.2 Tris and digested with trypsin at a 1:260 enzyme to substrate ratio for 16 hours at 37°C. For LysC digestions, samples were diluted 4-fold in 50 mM pH 9 Tris then digested with trypsin at a 1:150 enzyme to substrate ratio for 16 hours at room temperature. Trypsin and LysC digests were quenched by adding 10% trifluoroacetic acid to a pH of <2. Due to precipitated debris at the low pH, peptide samples were clarified by centrifugation at 3,500 x g at 4°C. Samples were then desalted using Waters Sep-Pak tC18 cartridges (50 mg packing material), dried by vacuum centrifugation, and stored at -20°C until further use. For proteome analysis, LysC peptides were fractionated using four cut-out layers of Empore SDB-XC material in a Stage-tip configuration (Ai et al.). Six fractions were generated by eluting in 0.1% NH_4OH and increasing concentrations of acetonitrile (0%, 5%, 10%, 15%, 20%, 80%). Fractions were evaporated by vacuum centrifugation, and then re-dissolved in 4% formic acid, 3% acetonitrile prior to MS analysis. Fractions were analyzed using 90-minute gradients on an Orbitrap Velos (Thermo) coupled to an Easy nLC II (Thermo). The liquid chromatography method contained a 61:30 minute, linear gradient from 6% to 30% acetonitrile in 0.15% formic acid.

For ubiquitination analysis, 0.5 mg of dried peptides were dissolved in 0.5 mL of ice-cold immuno-affinity purification (IAP) buffer (50 mM MOPS-NaOH, pH 7.2, 10 mM Na_2HPO_4, and 50 mM NaCl). Anti-diglycyl (anti-KGG) antibodies loaded onto protein A agarose beads (Cell Signaling Technology) were prepared by washing with cold IAP buffer. 10 mg of tryptic peptides and 45 µL of bead slurry were used in the enrichment. Peptides and anti-KGG beads were incubated on a rotating mixer for 1 hour at 4°C and washed five times with 1 mL ice-cold IAP buffer, followed by two washes with 1 mL water. Diglycine-modified peptides were eluted two times with 0.15% trifluoroacetic acid for a total of 100 µL. Eluted

peptides were desalted and fractionated identically to the LysC peptides above, using four cut-out layers of Empore SDB-XC material. Samples were re-dissolved in 4% formic acid, 3% acetonitrile prior to MS analysis. MS/MS acquisition for the ubiquitination analysis was performed the same as for the whole proteome analysis, except the linear chromatography gradient was 54 minutes, from 8%-32% acetonitrile.

MS/MS Analysis:

MS/MS acquisition for whole proteome analysis was performed as follows: full-scan MS was acquired in an Orbitrap between 300-1500 m/z at 60,000 resolution. MS/MS acquisition was performed in a linear ion trap using collision-induced dissociation with 35% normalized collision energy. The 20 most abundant precursor ions were fragmented and analyzed. Peptides were identified from ubiquitination data using Maxquant [19029910], which generated heavy/light and medium/light ratios for each ubiquitination site. Whole proteome data was searched using Comet [23148064], filtered using Percolator [17952086], and quantified using ThunderQuant, an in-house developed software for peptide quantification that relies on MS1 peak area integration. Peptide quantifications were pooled into protein quantifications, where the mean peptide ratio for each protein was taken to represent the protein. All ratios of each type (i.e. heavy-light and medium-light) were normalized to the median ratio in that run.

Degradation Assays

Cycloheximide-chase degradation assays were performed as previously described (Gardner et al., 2005a). Cultures were grown at 30ºC to ~1x10^7 cells/ml. Cycloheximide was added to a final concentration of 50 µg/ml and cells were further incubated for 0-3 hours. BMH-21 was added (25µM final concentration) 1 hour prior to cycloheximide addition where indicated. Cells were lysed at the appropriate time point in 200 µl SUMEB with 10 mM PMSF and 100 µL acid washed glass resin and vortexed for 5 minutes at room temperature. Cells were incubated at 65ºC for 10 minutes. The lysate was removed from resin and clarified via centrifugation at 14k rpm for 2 minutes.

Bioscreen assays

Cells were grown in 5 ml cultures overnight until saturation. 2 µL of saturated culture was added to 148 µL of YEPD containing DMSO or specified concentration of BMH-21 (Sigma-Aldrich, SML1183) and plated in a 100 well honeycomb microplate (Bioscreen C). Cells were incubated at the specified temperature for 48 hours using Bioscreen C Analyzer and OD was read every 30 minutes with continuous shaking. Doubling time interval calculations were generated using Yeast Outgrowth Data Analyzer (Kaeberlin Lab, University of Washington).

Ubiquitinated Rpa190 analyses

Cells expressing Rpa190-3HA were grown in 50 mL cultures to ~$1.8x10^7$ cells/ml. Harvested cells were lysed at 4 ºC in 1 mL TUBE Lysis Buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 1mM EDTA, 1% NP-40, 10% glycerol with 1mM PMSF and 100mM NEM) and 1.5 mL acid washed glass resin, vortexed for 20 minutes at 4ºC. The supernatant was removed and clarified via centrifugation at 14k rpm for 10 minutes. Protein concentration was determined via Bradford Assay (Bio-Rad) and normalized to lowest protein concentration. Proteins were incubated on 20 µL of magnetic TUBE Beads (LifeSensors, UM401M) overnight at 4ºC. Beads were washed 3 times for 5 minutes in 1mL of TUBE Buffer. Protein was eluted with 30µL of SUMEB (1% SDS, 8M Urea, 10mM MOPS, pH 6.8, 10mM EDTA, 0.01% bromophenol blue) and incubated at 65ºC for 10 minutes. Proteins were separated on 8% SDS-PAGE gels and transferred to nitrocellulose. Rpa190-3HA was visualized with anti-HA (Sigma, H9658) antibody.

siRNA

HeLa cells were plated between 50-70% (150k cells/well) confluency in 6-well plates. 100 nM of either scrambled or siUSP36 (Ambion) were incubated with RNAiMAX for 24 hours in serum free media. Media was removed and cells were left to recover in DMEM for 6 hours. Cells were harvested and lysed using NETN Buffer (250mM NaCl, 5mM EDTA pH 8.0, 50 mM Tris-HCl pH 8.0, 0.5% NP-40) with protease and phosphatase inhibitors at 1:1000 (Sigma P8340 and Sigma 542627). USP36 (Proteintech 14783) and RPA194 (Cell Signaling, 24799) were visualized via western blot.

MTS Assay

CellTiter 96® AQ$_{ueous}$ One Solution Cell Proliferation Assay was used according to manufacturer's instructions.

qPCR

cDNA was generated using SuperScript® IV Reverase Transcriptase according to manufacturer's instructions. RNA was generated using RNeasy® Mini Kit. qPCR was performed using PowerUp™ SYBR® Green Master Mix (Applied Biosystems) and ran on ABI 7500 Fast Machine.

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