The Pennsylvania State University

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Eberly College of Science

Department of Biochemistry and Molecular Biology

ROLES OF CCR4-NOT IN UBIQUITINATION AND THE LAST RESORT MECHANISM OF RNA POLYMERASE II DEGRADATION

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by

Abhinaya Srikanth

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The thesis of Abhinaya Srikanth was reviewed and approved* by the following:

Joseph C. Reese  
Professor of Biochemistry and Molecular Biology  
Thesis Adviser

David S. Gilmour  
Professor of Biochemistry and Molecular Biology  
Co-Chair Graduate Studies

Scott E. Lindner  
Assistant Professor of Biochemistry and Molecular Biology

*Signatures are on file in the Graduate School
Abstract

Several studies have explored the relationship between the cellular transcription machinery, the stress response mechanism and mRNA decay factors. Despite the identification of multiple factors that function in linking the three responses together, studies are constantly being conducted to explore the extent of cellular responses to various stress conditions. Cells mitigate the effect of stress damage by balancing transcription and mRNA decay effectively.

The understanding of the transcription machinery covers various aspects of activation, regulation, and repression under normal cell conditions, as well as proofreading and repair response during a stress or damage-induced arrest. However, studies are still revealing new mechanisms for these responses and the proteins involved in performing these functions. One such response is the selective ubiquitination and degradation of the largest subunit of RNA polymerase II (Rpb1) upon DNA damage. Previous published work by Jesper Svejstrup’s laboratory showed the processing of Def-1 and the Def-1 dependent ubiquitination and degradation of Rpb1 in response to DNA damage induced arrest of PolII.

As a follow-up to this work, it was shown that this degradation is a last-resort mechanism and can be reversed by deubiquitination factors (DUBs) like Ubp3 and its cofactor Bre5.

In this thesis, I have explored the role of one such protein complex, the Ccr4-Not complex, in transcription, mRNA decay and DNA damage response. The Ccr4-Not complex has previously been implicated as a transcriptional elongation factor and Ccr4 is the primary deadenylase in yeast. In my study, I explored a novel function for Not4, one of the components of the complex, in the stress-induced ubiquitination of Rpb1. Not4 is required for the turnover of Rpb1 under UV-induced stress and the absence of Not4 leads to a loss of ubiquitination of PolII under 4-NQO stress. Furthermore, the Ccr4-Not complex was also shown to have a physical interaction with the DUBs that reverse the ubiquitination of Rpb1. These results indicate a previously unknown role for the Not4 module of the Ccr4-Not complex that is required for the ubiquitination of Rpb1 and a possible interaction with the DUBs Ubp3/Bre5 to set up a novel stress-response process that will be discussed in the following chapters of this thesis.
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Scope and significance of this project

The purpose of this project is to understand the specific function of Not4 in the regulation of RNA PolII under stress-induced polyubiquitination and degradation. The Ccr4-Not complex is a myriad of functionally related proteins that link transcriptional functions with mRNA degradation. As discussed in later sections, this complex opens doors to inter-connectivity of stress-related Not4-dependent ubiquitination of the Rpb1 subunit of RNA Polymerase II. This previously unknown role of Not4 in the ubiquitination of Rpb1 will add to the collection of functions of the complex as well as solve a piece of the connectivity that links the transcription of genes to the decay of mRNA and to the stress response of the cell.

Many studies have been done in all of these fields – transcription regulation, mRNA decay factors, stress response and overlapping factors. Work in our lab has shown that when pausing or other events impede transcription, the Ccr4-Not complex promotes elongation. If the block is not overcome, PolII either assists in transcription-coupled repair or disengages from the elongation site. The detachment of PolII requires the removal of the largest subunit of PolII – Rpb1 – by ubiquitination and degradation by the proteasome.

Initial studies were conducted to understand the effect of 4-Nitroquinoline (4NQO) stress on the turnover of Rpb1. In wild-type cells treated with 4NQO, Rpb1 was degraded over the course of the experiment. The same experiment was conducted with mutants of the Ccr4-Not complex and it was observed that in the absence of not4 (in a Δnot4 strain), the turnover of Rpb1 was not observed. Not only was Δ not4 impairing the turnover of Rpb1, the effect was found to be contributed by the RING domain of Not4, which points to a functional role of Not4 in the turnover of Rpb1. This initial observation led to the experiments conducted in this thesis that aim to bridge a gap between the stress response and the Rpb1 degradation factors of the cell.
Chapter 1: Background

The central dogma of Molecular biology describes the progression of genetic information from the DNA to the messenger RNA and ultimately results in the production of the protein that carries out the required function for the cell. In order to successfully complete this data transfer from DNA to protein, several key proteins must work together efficiently. However, it is also essential for the cell to regulate the process at every level to ensure the balance of proteins, and for appropriate cellular response to stimuli. Additionally, if an error in this mechanism occurs, the result could prove disastrous for the cell. Therefore, equally important are the proofreading and repair factors that ensure the correct message is being relayed and the cell can effectively respond to damage or stress.

Cell response to DNA damage and stress has been actively studied due to the significance of the response in maintaining genome integrity and cell survival. Cells have evolved multiple mechanisms to ensure continued transcription following a stress or damage. Gene transcription by the RNA Polymerase II (PolII) is affected by numerous events such as stalling, pausing, arrest or even backtracking of PolII. To overcome these hurdles, cells have specialized proteins and factors that assist Polymerase in completing transcription successfully. One such multi-functional protein complex, the Ccr4-Not complex, is conserved among eukaryotes and is composed of nine core subunits. Between these subunits, the complex covers several functions such as transcription initiation, deadenylation, mRNA turnover, transcription coupled nucleotide excision repair (TC-NER), deubiquitination and others (Reese 2013).
1.1 Yeast as a model organism

The experimental methods conducted in this thesis benefit from using a model organism that is efficient and easy to manipulate. The model organism selected is *Saccharomyces cerevisiae*, or Baker's yeast, a single-celled eukaryote with most cellular processes functionally conserved among higher eukaryotes. The yeast genome consists of 5770 genes that are organized on 16 chromosomes. The entire genome is sequenced and a complete database is available online at [www.yeastgenome.org](http://www.yeastgenome.org).

Apart from the ready availability of gene information, mutants, and inferred protein sequences, yeast are easy and inexpensive to grow with a doubling time of 90 minutes for wildtype cells. They can also grow in a haploid condition, which makes them ideal for knockout genetic manipulations that would otherwise be difficult in a diploid state. There are numerous mutants and tagged strains commercially available and others are easily made in the laboratory. Yeast can be made with several identifying markers and can hence have multiple gene deletions and tags within the same strain. These methods will be employed for conducting the experiments described in this thesis and will be further discussed in each chapter.
1.2 The Ccr4-Not Complex

The primary complex in this study is the Ccr4-Not complex. It is a highly conserved, multi-functional ~1MDa protein complex that is composed of core protein components with 9 subunits and a larger ~1.5MDa complex with additional protein subunits. The 9 core subunits are Ccr4, Caf1, Caf40, Caf130, and Not1-5 as shown in figure 1.1 (Collart & Panasenko 2012), while additional proteins like Dhh1, Dbf2, Caf4, Caf16, and Btt1 form the larger 1.5MDa complex (Reese 2013). The complex functions to provide multiple levels of gene regulation, in several crucial cellular processes like transcriptional initiation and elongation, deadenylation, mRNA decay and ubiquitination.

In addition to co-purifying with RNA PolII, the Ccr4-Not complex also interacts with other elongation factors, and mutations in the Ccr4-Not subunits were sensitive to elongation inhibitors 6-Azauracil (6-AU) and mycophenolic acid (Reese 2013). Ccr4 and Pop2/Caf1 are the primary deadenylases in yeast and act as a functionally separate unit with Caf1 acting as the link between Ccr4 and the rest of the complex (Bai et al. 1999). Ccr4 is a member of the exonuclease III family of proteins, and as a deadenylase, has a 3′-5′ exonuclease activity on the Poly-Adenylation (Poly A) tail found on mRNAs, while Caf1 contains an RNaseD/DEDD domain and also has a 3′-5′ exonuclease activity (Chapat & Corbo 2014). Although initially discovered for its role in activating ADH2 (Alcohol Dehydrogenase 2) in media containing a non-fermentative carbon source, Ccr4 was soon shown to be a regulator of transcription as well as mRNA decay (Kruk et al. 2011).

Another significant function of the complex is ubiquitination by the Not4 protein, discussed in further detail later. This subunit contains a RING domain with E3 ubiquitin ligase activity, which is known to have several targets such as the histone demethylase Jhd2, and leads to its degradation by the proteasome (Mersman, Du, et al. 2009). Furthermore, the first four NOT genes, NOT1-4, were identified as negative regulators of TATA-less promoters (Collart & Struhl 1994).

The complex has also been linked to the cellular stress response in a regulatory manner. In a study conducted by Traven et al. (Traven et al. 2010) it was observed that in cells lacking the Pop2/Caf1 subunit of the Ccr4-Not complex, a deletion of Puf5 (an RNA binding protein that promotes mRNA deadenylation and associates with the Ccr4-Pop2
subunits) resulted in the increased sensitivity of the cell to DNA replication stress. Puf5 and Pop2 were shown to act in the same genetic pathway and the repression of HO endonuclease mRNA by Puf5 was eliminated in the absence of Pop2 (Goldstrohm et al. 2006). Another subunit, Dhh1, a DEAD-box RNA helicase, co-purifies with Pat1 (a component of p-bodies) and results in the nuclear localization of Pat1 upon UV stress. In the absence of dhh1 this nuclear localization was abolished (Bahassou-Benamri et al. 2013). Dhh1 was also implicated in cell-cycle DNA-damage checkpoint recovery at the G1/S phase by doing a quality control check on mRNA (Bergkessel & Reese 2004) and cells lacking Dhh1 were defective in release from arrest at the checkpoint.
Figure 1.1: Subunits of the Ccr4-Not complex color coded according to their functions. The deadenylases, Ccr4 and Caf1 indicated in blue with Caf1 acting as the link between Ccr4 and the rest of the complex. Not4, the RING E3 ubiquitin ligase is indicated in yellow and Dhh1 the RNA helicase in purple. With the exception of Dhh1, the rest of the proteins shown are core subunits.
Adapted from: (Collart & Panasenko 2012)
Table 1. Components of CCR4–NOT in Yeast and Human Cells. Table taken from: (Chapat & Corbo 2014)

<table>
<thead>
<tr>
<th>Saccharomyces cerevisiae</th>
<th>Homo sapiens</th>
<th>Domain</th>
<th>Position Inside the Complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not1</td>
<td>CNOT1</td>
<td>HEAT repeats MIF4G</td>
<td>Scaffold</td>
</tr>
<tr>
<td>Not2</td>
<td>CNOT2</td>
<td>NOT-box</td>
<td>'NOT' module C-terminal part of Not/CNOT1</td>
</tr>
<tr>
<td>Not3</td>
<td>CNOT3</td>
<td>NOT-box</td>
<td></td>
</tr>
<tr>
<td>Not4</td>
<td>CNOT4</td>
<td>RING E3 Ligase</td>
<td>Ubiquitination Module</td>
</tr>
<tr>
<td>Not5</td>
<td></td>
<td>NOT-box</td>
<td></td>
</tr>
<tr>
<td>Ccr4</td>
<td>CNOT6/CCR4a</td>
<td>EEP, LRR</td>
<td>'Deadenylase' module central part of Not/CNOT1</td>
</tr>
<tr>
<td></td>
<td>CNOT6L/CCR4b</td>
<td>EEP, LRR</td>
<td>—</td>
</tr>
<tr>
<td>Pop2/Caf1</td>
<td>CNOT7/CAF1a</td>
<td>DEDD</td>
<td>Deadenylase</td>
</tr>
<tr>
<td></td>
<td>CNOT8/POP2/CAF1b</td>
<td>DEDD</td>
<td>—</td>
</tr>
<tr>
<td>Caf40</td>
<td>CNOT9/hRcd1</td>
<td>Armadillo repeats</td>
<td>Central part of Not/CNOT1</td>
</tr>
<tr>
<td>Caf130</td>
<td>—</td>
<td>—</td>
<td>C- and N-terminal part of Not1</td>
</tr>
<tr>
<td>—</td>
<td>CNOT10</td>
<td>—</td>
<td>N-terminal part of CNOT1 (distinct module)</td>
</tr>
<tr>
<td>—</td>
<td>C2ORF29/CNOT11</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Dhh1</td>
<td>Rck/p54/DDX6*</td>
<td>DEAD-box</td>
<td>Central part of Not1/CNOT1</td>
</tr>
</tbody>
</table>
1.3 RNA Polymerase II, transcription and decay

The Ccr4-Not complex was initially identified associating with the RNA Polymerase II complex in cells and was hence described as an elongation factor. However, the association of the Ccr4-Not complex with PolII offers an ideal location for the protein to access the DNA, RNA, and all the associated proteins that facilitate the multitude of functions conducted by the Ccr4-Not complex.

RNA Polymerase II (PolII) is a very large protein complex – 0.5 MDa in yeast – composed of 12 subunits and essential for cellular function as indicated in figure 1.2. It is an enzyme that transcribes DNA protein-coding templates to make messenger RNAs that are translated into proteins. It also transcribes several regulatory elements such as small snoRNA, miRNAs, snRNAs, and siRNAs that are not translated into proteins (Woychik & Hampsey 2002). Its function dictates cellular differentiation, adaptation to environmental changes, response to stress and general maintenance of the cell. Therefore regulation of PolII occurs at multiple levels. The first step is during initiation when PolII assembles with multiple general transcription factors such as TFIIB, TFIID, TFIIE, TFIIF and TFIIH, to form the pre-initiation complex. This allows PolII to access the open promoter DNA and initiate RNA transcription (Sainsbury et al. 2015).

With the exception of Rpb4 and Rpb9, all other PolII subunits are necessary for the survival of the cell. The largest subunits, Rpb1 and 2 are highly conserved and form the central clamp around DNA for transcription. Rpb1 also has a highly conserved C-terminal tail (CTD), which is composed of the repeating sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser (YSPTSPS) (Arndt & Kane 2003). The number of repeats varies from organism to organism. In yeast, there are 26-27 repeats while in humans there are 52 repeats in the CTD. This sequence is very important as it undergoes post-translational modifications during gene transcription and impacts the function of other factors and modifiers that are recruited to its docking sites (Feaver et al. 1991). Two of the most significant modifications are the phosphorylation of Ser2 and Ser5. PolII enters the preinitiation complex in an unphosphorylated state. During transcription initiation, the CTD has a predominantly Ser5 phosphorylation signature. This changes to a Ser2 phosphorylation during elongation (Cramer 2004).
Of the 12 subunits of PolII, 10 form the core of polymerase, while two – Rpb4 and Rpb7 – form a dissociable heterodimer that has been linked to multiple post-transcriptional processes such as mRNA export, translation and decay (Harel-Sharvit et al. 2010; Choder 2004). The Rpb4/7 sub-complex has also been implicated in promoting deadenylation of certain mRNA transcripts in the cytoplasm (Lotan et al. 2005). Additionally, it was discovered that in order for the Ccr4-Not complex to bind to RNA PolII Elongation Complexes (EC) in vitro, the interactions needs to be stabilized by the sub-complex Rpb4/7 (Babbarwal et al. 2014).

In order to understand the role that various factors play in association with PolII, it is necessary to review some of the significant steps in the three phases: Initiation, Elongation and Termination. Initiation is carried out by a host of general transcription factors and begins with a TATA-binding protein (TBP) interacting with the TATA box in the promoter region of a gene. A general transcription factor, TFIID alters DNA to prepare for transcription and assemble the other factors. Another factor TFIIH unwinds DNA and phosphorylates Ser5 of the CTD promoting the elongation phase (Woychik & Hampsey 2002). In the elongation phase, RNA PolII encounters frequent pauses, backtracks and arrests due to a variety of reasons. Elongation factors assist in the continuation of transcription, such as TFIIS, an elongation factor that causes PolII to cleaves the transcript in the 3’ end to rescue PolII from backtracking in the event of an error or misalignment. This allows PolII to thread back and forth to readjust and recover from the arrest. In the event of DNA damage or other barriers like a nucleosome, another important factor, Spt4/5 helps in maintaining the elongation bubble as well as stabilizing the emerging transcript (Martinez-Rucobo et al. 2011).

Cellular response and function is a complex coordination of proteins that interact with each other. The synchronization of RNA PolII with the Ccr4-Not complex to facilitate transcription elongation is a means by which the cell could further regulate the transcription of a gene under precise conditions. In this thesis I explore the mechanism behind one particular cellular response involving the Ccr4-Not complex and RNA PolII, and DNA damage induced arrest of PolII. The requirement of the Ccr4-Not complex is undeniable, and the mechanism and purpose of the response is explored in the following chapters.
Figure 1.2: Crystal structure of Pol II. (a) The polymerase core has been shown divided into three subassemblies, the Rpb1 subassembly containing Rpb1, Rpb5, Rpb6 and Rpb8, the Rpb2 subassembly containing Rpb2 and Rpb9, and the Rpb3 subassembly containing Rpb3, Rpb10, Rpb11 and Rpb12. The fourth subassembly consists of Rpb4 and Rpb7 protruding from the core (PBD ID 1I6H). (b) Cartoon of the 12 subunits of eukaryotic RNA Polymerase II.

Figure taken directly from: (Wild & Cramer 2012).
1.4 Stress response in the cell

When the cell encounters a stress or damage to the DNA, the cell utilizes two main mechanisms of response. The first is a transcription-coupled repair mechanism – Transcription Coupled Nucleotide Excision Repair (TC-NER) – that is initiated by the recruitment of several factors like Rad4/Rad23 to recognize the lesion (Schärer 2007), repair factors like Rad1/Rad10, and Rad26 to repair the UV damage. This usually occurs in actively transcribed genes and is triggered by the stalled PolIII (Svejstrup 2002) (Figure 1.3). Alternatively, the cell has a General Genome Repair (GGR) mechanism that addresses damage in locations not actively transcribed (Nouspikel 2009).

In the event that the TC-NER mechanism is insufficient to resolve the DNA damage, cells possess a back-up mechanism to overcome the problem. In yeast, upon DNA damage, a small protein – Def1 – that is normally found in the cytoplasm undergoes ubiquitination and is partially processed in a proteasome-dependent manner, to produce a functionally active protein. It is then internalized into the nucleus where it forms a complex with Rad26 on the damaged site and recruits factors that ubiquitinate and degrade the largest subunit of PolII – Rpb1. This results in the disintegration of the Polymerase complex and frees up the DNA for GGR (Wilson et al. 2013; Woudstra et al. 2002). This process salvages the other subunits of PolIII, which are recycled for further use.

In the Def-1 initiated last resort response, Rpb1 is monoubiquitinated by Rsp1 (as shown in figure 1.3) and subsequently polyubiquitinated by the Elongin-Cullen complex. The Elongin-Cullen E3 ligase complex (Ela1-Elc1) interacts directly with Def-1 via a CUE (named after domain homology with Cue1 – coupling of ubiquitin to ER degradation) domain-dependent manner and is recruited to PolII along with Ubc4/5 (Ubiquitin conjugating enzymes) (Wilson et al. 2013). Rpb1 is then degraded by the proteasome and the damage caused to the DNA is repaired by GGR.
Figure 1.3: Molecular mechanism for degradation of transcriptionally stalled PolII. Upon DNA damage the transcribing complex becomes irreversibly stalled. Blocking of transcription might lead to cell death (C). To ensure continuation of transcription, the damage is either repaired by TCR (A) or as a “last resort” mechanism RNAPII is polyubiquitylated and degraded by the proteasome, an action which allows the GGR pathway to repair the damage (B). Image unaltered from: (Karacasili 2010).
1.5 The Polyubiquitination code and proteasome-mediated degradation

To understand the mechanism of the stress response and ubiquitin-mediated degradation of Rpb1, it is necessary to appreciate the function that ubiquitin plays in the cell. A newly translated protein often undergoes several post-translational modifications that allow the cells to control the protein’s function. Ubiquitin is one such modifier that varies the fate and function of the target protein to which it is appended, depending on the site of ubiquitination as well as the number of ubiquitin moieties appended. This ubiquitin code is read by Ubiquitin reading proteins, which carry out the function dictated by the code. Ubiquitination of a protein can result in multiple outcomes such as cell cycle regulation, DNA repair, membrane protein trafficking, and proteasomal degradation of proteins (Finley et al. 2012).

Ubiquitin is a 76 amino acid polypeptide that forms a chain through one of its 7 internal lysine residues. It can also mark a target protein when it is appended to a lysine residue on the target protein. Most of the ubiquitination marks result in the degradation of the target protein but the primary mark that degrades proteins is through the K48 mediated poly-ubiquitination. The process of ubiquitination occurs as a cascading event when an E1 (Ubiquitin activating enzyme) is charged with carboxylate moiety at the C-terminus of ubiquitin (Ub) linked to the catalytic cysteine of E1 by a thioester bond. Following this, an E2 protein (Ubiquitin conjugating enzyme) associates with the E1 and ubiquitin is transferred to the E2. This charged E2 then interacts with an E3 (Ubiquitin ligase) and the ubiquitin moiety is either directly transferred to the target protein, or via the E3 as an intermediate (Komander & Rape 2012; Heride et al. 2014). Figure 1.4 shows the transfer of a ubiquitin moiety from an E1 to an E2 and then to the substrate via the E3. In yeast, the machinery has a hierarchical organization with only one E1 (Uba1), 11 E2s (Ubc 1-8, 10, 11, 13) and 60-100 E3s. This is because the E3 proteins recognize the target proteins and are hence specific to the targets (Finley et al. 2012).

Ubiquitination can result in multiple outcomes depending on the number of ubiquitin moieties attached – monoubiquitination or polyubiquitination – as well as the site of ubiquitination. Polyubiquitination occurs when ubiquitin is transferred from a charged E2 to a lysine on the target protein and subsequent ubiquitin molecules are attached to
lysine 48 on the preceding ubiquitin to form a multi-ubiquitin chain. This chain acts as a message for the proteasome machinery to degrade the targeted protein (Finley et al. 2012; Heride et al. 2014).

The proteasome is a 2.5 MDa protease with 33 distinct subunits and is highly conserved among all eukaryotes. Its primary function is to degrade proteins marked with the lysine 48 chain ubiquitination. It is organized into two subunits, the 26S core particle that contains the proteolytic active sites, and the 19S regulatory particle that recognizes the substrate. The core is composed of two α and two β rings of seven subunits each. The α rings flank the β rings in a α-β-β–α structure, with the protease active sites present in the β rings. The Regulatory particle is comprised of a lid structure containing the ATPase subunits Rpn3, -5-9, and -11, -12, as well as a base that contains the ATPases Rpt1-6 and non-ATPases Rpn1,-2,-10 (Glickman et al. 1998). Rpn10 contains a hydrophobic patch that binds to Ubiquitin chains (Fu et al. 1998). Proteins entering the proteasome core are unfolded and a DUB (Deubiquitinating Enzyme) removes the ubiquitin marks for recycling.

This understanding of the ubiquitin pathway can be applied to exploring the role of the Ccr4-Not complex in the ubiquitination and proteasome-mediated degradation of Rpb1. As discussed in the following section, Not4 is a RING E3 ubiquitin ligase and it is functionally relevant in the stress-response degradation of Rpb1.
Figure 1.4: Graphical representation of protein ubiquitination. Ubiquitin activation by E1 in an ATP-dependent manner followed by transfer of the ubiquitin to the ubiquitin-conjugating enzyme (E2), and finally covalent attachment to substrate proteins via ubiquitin ligases (E3). A single ubiquitin molecule generates monoubiquitinated proteins and repeated rounds of ubiquitination results in multi- or polyubiquitinated proteins. Unaltered image from: (Finley et al. 2012).
1.6 Not4 as a RING E3 ubiquitin ligase

Yeast cells have 44 different E3s that contain a RING (Really Interesting New Gene) domain. As previously mentioned, the RING domain binds to the E2 and transfers the ubiquitin to the target by inducing subtle changes to the E2.

The ubiquitination module of the Ccr4-Not complex is the Not4 protein. Not4 is an E3 ubiquitin ligase with a RING domain on the N-terminus, a central RRM (RNA Recognition Motif) and an unstructured C-terminus (Bhaskar et al. 2015). Not4 functions with ubiquitin conjugating enzymes Ubc4/5, and has been reported to ubiquitinate multiple targets like Jhd2, the H3K4 histone demethylase in yeast (Douglas P Mersman, Du, et al. 2009) and Cdc17, the catalytic subunit of pol-α in yeast (Haworth et al. 2010) Not4 is also required for the stress-induced transcription of RNR (Ribonucleotide Reductase) genes – RNR2, 3 and 4. It was observed that the TATA Binding Protein (TBP) and PolII are not recruited to the RNR genes in the absence of Not4 (Mulder, Inagaki, et al. 2007; Mulder et al. 2005).

The function of Not4 as an E3 in the process of ubiquitination of Rpb1 is unclear. However, it is known that Rpb1 is degraded through a proteasome-dependent manner. Not4 is also known to associate with the proteasome to regulate levels of H3K4 trimethylation (Laribee et al. 2007). Loss of not4 was shown to decrease global and gene-specific H3K4 trimethylation. This was demonstrated by Not4’s ability to polyubiquitinate Jhd2, and target Jhd2 for proteasome-mediated degradation (Douglas P Mersman, Harmeyer, et al. 2009). Another substrate for the ubiquitination activity of Not4 is Egd2, a complex that is associated with the ribosomal exit tunnel (Panasenko et al. 2006) and its ubiquitination is required for regulation of nascent protein targeting.

The presence of multiple substrates for the ubiquitination activity of Not4 and its association with the proteasome could indicate that Rpb1 is also a substrate for Not4 under DNA damage induced arrest. However, Not4 may also function indirectly in the turnover of Rpb1 by ubiquitinating an accessory protein like Def1. Def1 is known to be proteolytically processed in the cytoplasm, in response to Rpb1 arrest and is required for the ubiquitination of Rpb1. Not4 also targets other substrates in the cytoplasm such as Rsp7A, a small ribosomal protein. Another notable substrate is a ribosome-associated chaperone protein NAC (Nascent polypeptide-Associated Complex) that plays a role in removing
translational arrest products by Not4-mediated degradation by the proteasome (Dimitrova et al. 2009).

Multiple studies have also shown the possible role of Not4 in cotranslational quality control (QC). Although Not4 does not ubiquitinate the arrest products, it accumulates in response to factors that induce translational QC (Halter et al. 2014), and Not4 deletion leads to an accumulation of polyubiquitinated and aggregated protein deposits (Panasenko 2014).
1.7 Rescue of PolII by Ubp3/Bre5 Deubiquitinating enzyme

Although the process of RNA Polymerase II arrest and ubiquitination of Rpb1 is a process to recover the remaining proteins in PolII and repairing the damage to the DNA, the cell possesses a method to rescue polymerase as a last resort mechanism. Deubiquitinases (DUBs) are a large group of proteases that play an antagonistic role to the ubiquitin machinery and reverse the fate of proteins designated for destruction by the proteasome. DUBs also function with the proteasome to release ubiquitin moieties from proteins ready for degradation. As previously discussed, RNA PolII undergoes ubiquitination and degradation as a last-resort mechanism in response to DNA damage. Yeast Ubp3 targets PolII as its substrate to rescue it from possible degradation (Kvint et al. 2008). However, Ubp3 also plays a role in Nucleotide Excision Repair (NER) by targeting Rad4 for degradation. Rad4 and Rad23 function to detect and respond to DNA damage. Ubp3 was shown to be a negative regulator of a cellular response to UV irradiation by interacting with Rad4, however the actual degradation of Rad4 may require additional factors (Peng Mao & Smerdon 2010). It is possible that Ubp3/Bre5 may also play a role in rescuing PolII from degradation by targeting factors that ubiquitinate and degrade PolII.

Ubp3 plays a crucial role in removing ubiquitin chains from PolII. This function rescues Rpb1 from proteasome-mediated proteolysis in the event of DNA damage that cannot be repaired effectively through TC-NER. Deletion of ubp3 results in higher levels of ubiquitinated PolII that are also degraded faster upon UV treatment (Kvint et al. 2008). An x-ray crystal structure of Ubp3, as shown in figure 1.5, reveals that the protein forms a hetero-tetrameric complex between Bre5 and Ubp3. Bre5 consists of a nuclear transport factor 2-like domain (NTF2) at the N-terminus and a RNA Recognition Motif (RRM) at the C-terminus. Ubp3 consists of a UCH (ubiquitin carboxyl-terminal hydrolase) catalytic domain on the C-terminus and a Bre5 binding domain close to the N-terminus. Bre5 forms a homodimer through the NTF2 domain and associates with Ubp3 via the Bre5 binding site (Li et al. 2007).

Other functions of Ubp3 include transcription initiation, silencing, protein degradation and retrograde transport (Moazed & Johnson 1996; Solé et al. 2011; McCullock et al. 2006). Ubp3 interacts with transcription factor TFIID, as well as ubiquitin ligase
Bul1/Rsp5. Ubp3 was found to promote cellular resistance to DNA damaging agents in a way that is genetically antagonistic to Bul1 gene (Bilsland et al. 2007). Similarly, it is also involved in the degradation of Rad4, a protein that responds to DNA damage by UV directly and facilitates Nucleotide Excision Repair (NER) (Peng Mao & Smerdon 2010). It is not yet clear if this function is a genome-wide effect or if Ubp3 targets particular genes in a stress-specific way. However, the role of Ubp3 and Bre5 is not restricted to just DNA damage repair, Ubp3 also acts as a transcriptional activator of Gal4 and Gcn4 by preventing the degradation of TATA-binding protein Tbp1/Spt15. The authors showed that upon induction of the Gal1 and His3 genes, Ubp3 is recruited to the promoter of both genes and is required to activate the genes (Chew et al. 2010a). In addition to this, Ubp3 is recruited to osmoresponse genes to modulate transcriptional initiation as well as elongation (Solé et al. 2011).
Figure 1.5: Structure of Ubp3 and Bre5 showing functional domains. (a) Domain structure of Ubp3 and Bre5 showing important sites – Bre5 binding domain, catalytic site, NTF2 domain and RNA Recognition Motif. (b) Ribbon structure of the interaction between two units of Bre5 (shown in two shades of blue) with the two units of Ubp3 (shown in shades of red). (c) A space-filling model showing the Bre5 homodimer and the N-terminus of Ubp3.

Images unaltered from: (Li et al. 2007)
1.8 Turnover of Rpb1 under 4NQO stress

In previous experiments conducted in the lab (Libert & Reese 2014), the turnover of Rpb1 upon UV stress was observed and the results of this experiment were documented in yeast strains expressing either wild-type or deletion mutants in the Ccr4-Not complex members. The hypothesis was that Rpb1 turnover would be diminished as compared to wild-type strains, in the absence of not4, under 4NQO stress. The experimental results supported the hypothesis, suggesting a previously undiscovered role for Not4. Other subunits of the Ccr4-Not complex were also tested and did not show this effect.

More significantly, the experiment was repeated with additional mutants in the not4 RING domain by introducing plasmids containing variants of not4 into a Δnot4 background; a complete deletion of the RING domain deactivates the function of not4 completely, and a point mutant in the RING domain (I64A) which was identified as a key residue for functionality of the RING domain. It was observed that the RING domain deleted Not4 protein impaired the turnover of Rpb1 nearly as much as the deletion of not4 itself, while the RING domain point mutant (I64A) showed an intermediate phenotype. The gel experiment is shown in figure 1.6, where the cell extracts from each strain (wild-type, Δnot4, Δccr4, Δdhh1) were treated with 100μg/μL cycloheximide to inhibit further protein biosynthesis. Each lane represents a time-point in the course of the 4NQO treatment from 0 min or untreated sample, to 30, 60, 90 and 120 min of treatment with 6μg/μL 4NQO, alongside an untreated sample at the final time point. The samples were probed for Rpb1 levels using an antibody to Rpb1. The results from this experiment are graphed in figure 1.7. To further confirm the role of Not4 in the turnover of Rpb1, a plasmid containing Not4 was substituted in the Δnot4 strain. The turnover of Rpb1 was recovered nearly as well as wild-type implying that Not4 has a functional role in the stress-response turnover of Rpb1 (figure 1.7). Error bars were unavailable for this data.

This novel role of Not4 in the turnover of Rpb1 under 4NQO stress was a key factor that led to questions that are addressed in this thesis. This experiment launched a number of hypotheses regarding the role of the Ccr4-Not4 complex in a complicated stress-response mechanism.
Figure 1.6: Turnover of Rpb1 under 4NQO stress. Cells were grown in YPD and treated in log phase growth. At time 0 min., all samples were treated with 100 ug/mL cycloheximide (CHX) and indicated cells with 6 ug/mL 4NQO. Rpb1 levels dropped in wt, Δccr4, and Δdhh1 strains over the course of the experiment. Δnot4 strains were defective in this turnover of Rpb1.

Figure unaltered from: (Libert & Reese 2014).
**Figure 1.7: Graphical representation of Rpb1 turnover under 4NQO stress:** The top graph shows the turnover of Rpb1. Δnot4 strains have a nearly consistent level of Rpb1 compared to wildtype strain and others. The sudden increase in Rpb1 levels in the Δdhh1 strain may be due to experimental inconsistencies. The second graph shows the rescue of the Δnot4 phenotype by incorporating a plasmid containing wt Not4, Not4 with a deletion in the RING domain, and a point mutation in the RING domain (I64A). Results show that the RING domain is required for the turnover of Rpb1. Error bars are unavailable for this data. Extracted unaltered from: (Libert & Reese 2014)
Chapter 2: Investigating the association of the Ccr4-Not complex with DUB Ubp3/Bre5

2.1 Introduction

Protein-protein interactions facilitate a quick and effective response for the cell. The interaction can change depending on external stimuli and internal responses. The purpose of this study is to understand how Ubp3/Bre5 interacts with the Ccr4-Not complex and PolIII, and the implication of this association. In the case of PolIII, there are several factors that are connected to the complex at each stage in transcriptional initiation, elongation, arrest and transcription termination. As previously described, the Ccr4-Not complex interacts with the transcription machinery for various functions, as does the DUB Ubp3/Bre5. In order to understand how these two protein complexes function together, it is necessary to know how they associate with each other and the polymerase. This association could shed some light on the method of recruitment of these factors during stress response.

The association of the Ccr4-Not complex to Ubp3 was first visualized in a preliminary experiment (unpublished work Babbarwal, V) where the deletion of Not4 resulted in the pull down of several proteins such as Rad26, Ubp3, Def1 and Cdc48 with the Ccr4-Not complex. Rad26 as described in earlier sections is a factor that functions in TC-NER and also associates with Def1 in the event of a last-resort degradation of Rpb1 in DNA damage response. Def1 is ubiquitinated and processed in the cytoplasm and internalized into the nucleus where it is required for the ubiquitination of Rpb1 (Marcus D Wilson et al. 2013). Cdc48 is a cell cycle regulator that is thought to play a significant role as a proteasome adapter in the ubiquitin-proteasome system (UPS). Its functions vary from ER-associated protein degradation (Stolz et al. 2011), control of E3 ligase activity for enzyme regulation (Yen et al. 2012) and even during mitochondrial stress response (Heo et al. 2010). Interestingly, Cdc48 also interacts with Ubp3 to function in starvation-induced ribophagy (Ossareh-Nazari et al. 2010). The DUB Ubp3 has been previously shown to interact with the transcription machinery where ubp3 mutant cells are defective in expression of osmoreponsive genes (Solé et al. 2011), however its association to the Ccr4-Not complex is novel. The information provided in this thesis does not confirm a direct
association between Ubp3/Bre5 and the Ccr4-Not complex, and it is likely the interaction could be mediated by other proteins (like Cdc48). Cdc48 may not be directly linked to the ubiquitination of Rpb1, but instead may associate with factors that lead to Rpb1 destruction.

Additionally, according to the domain structure of Bre5, there is a defined RNA recognition motif (RRM), which does not have any known RNA targets. Ubp3 itself may have an unidentified RNA binding domain and was identified in a study looking at RNA-binding proteins (Tsvetanova et al. 2010). As previous experiments have shown that Ubp3 and Bre5 form a complex together and associate with RNA PolII, it is reasonable to speculate that the interaction may be through the emerging RNA transcript, or may be stabilized by the RNA or perhaps the deubiquitination function requires interaction with mRNA.

To assess the interaction of Ubp3 and its cofactor Bre5 with the Ccr4-Not complex, protein immunoprecipitations were performed with strains containing tagged versions of Ubp3 and Bre5, as well as deletions of either of the two genes in the complex. Furthermore, to investigate recruitment of Bre5 to mRNA, RNA immunoprecipitations (RIP) were also performed with the tagged strains to investigate the recruitment of Ubp3 and Bre5 to specific mRNA. The purpose of these experiments was to determine whether the interaction with the Ccr4-Not complex might regulate the recruitment of Ubp3/Bre5 to mRNA. Specific mRNA candidates were chosen based on previous work that identified RNAs that associate with the Ccr4-Not complex (J.E. Miller, J.C. Reese, unpublished) under stressed and unstressed conditions.

The details of the interaction of these proteins with each other and to mRNA could answer a number of questions about the mode of function of Ubp3/Bre5 and the Ccr4-Not complex, and lead us to ask new questions about the purpose of the interaction. One model is that Ubp3/Bre5 could use the emerging transcript as a scaffold to be recruited to PolII. The recruitment could also be a method of selectivity for determining which mRNAs are to be degraded or maintained during a stress response. These experiments and the observations are presented in the upcoming sections of this chapter.
2.2 Materials and Methods

2.2.1 Strain generation

Ubp3 and Bre5 were tagged using common yeast tagging methods via PCR amplification of a tagging cassette. Using a pFA6a plasmid with different markers (figure 2.1) (Bähler et al. 1998), Ubp3 was tagged with a 13-Myc tag and Bre5 with a 3-HA tag. Further, strains were created with deletions in Bre5 and Ubp3 to form combinations – a Δbre5 Ubp3-Myc strain and a Δubp3 Bre5-HA strain. Along with these, I also made strains with deletions of proteins in the Ccr4-Not complex to explore the relationship between the complex and the DUBs. For this experiment, I made Δccr4 and Δnot4 mutants with the tagged Ubp3-Myc strain.

2.2.2 Yeast Transformation

Yeast cell transformation of the tagging cassettes was performed to generate the desired mutant strains. An overnight culture of BY4741 strain of *Saccharomyces cerevisiae* was seeded and then grown to an OD600 of 1.0. The cells were harvested and washed in ddH₂O, then resuspended in 1X lithium acetate (0.1 M Lithium Acetate). For the transformation step, 1-3ug of cassette DNA, 50ug salmon sperm carrier DNA, and 50ul yeast cell suspension were mixed in a 1.5ml sterile microfuge tube. 300ul PLATE (1 ml v/v 10X lithium acetate, 1ml v/v 10X Tris-EDTA (TE) pH 8.0 at 21°C, 8ml 50% v/v PEG 3350) was added and the tube was vortexed for 10 sec. After incubation at 30°C for 30 min., the tubes were placed at 42°C for 15 min. The cells were then spun at 4300 X g for 2 min, resuspended in YPD, and incubated in a sterile glass tube on a roller for 4-5 hours. Around 200uL of the cells was plated on each of four selective plates and incubated at 28°C for 2-4 days. Colonies were re-streaked onto selective plates and PCR tested using two sets of primers. One set was designed to anneal to the insert to verify the strains, and the other was designed to anneal to the ORF to confirm that the strains contain the desired gene deletions.

2.2.3 Harvesting cells and protein isolation.

Cells were inoculated into 200ml rich media YPD and grown at 30°C until an OD600 of 1.0-2.0 was reached. Cells were then centrifuged at 4000 X g, 5 min in a SLC6000 rotor at
4°C. Cells from each bottle were resuspended in 3mls ice-cold dH2O containing 1 mM Benzamidine-HCl and 0.25 mM PMSF and combined into a single 10ml screw-top tube and were further centrifuged at 1500 X g or 10 minutes at 4°C. The pellet was resuspended in 2 volumes of 0.2M NaCl Whole Cell Extract buffer (WCE) (25 mM Tris-HCl pH 7.5 at 21°C, 0.2 M NaCl, 5 mM MgCl2, 2 mM EDTA, 10% v/v glycerol, 0.1% v/v triton-X100 – protease inhibitors were added fresh 3µg/mL of leupeptin and aprotinin, 2ug/mL pepstatin A, 1ug/mL chymostatin, 1 mM benzamidine-HCl, 1 mM PMSF). Acid-washed glass beads were added in equal volume as WCE and tubes were vortexed rapidly using Vortex Genie-II for 30 secs with 2 min rest on ice for a total of 8 times. Cell suspension was transferred to a fresh tube using a Pasteur pipet and avoiding the beads. Additional 2mL of WCE was added to the beads to wash them and this was combined with the extract and this was centrifuged in the J6 centrifuge for 15 minutes at 1300 X g SS-34 rotor at 4°C. The supernatant was transferred into a fresh 10ml screw top tube and centrifuged in a SS34 rotor at 38000 X g for 30 min at 4°C. The final supernatant was carefully removed and used for protein analysis and immunoprecipitation.

2.2.4 Bradford assay to determine protein concentrations

A standard curve was prepared using 0, 2, 4, 8, 15, and 20mg/mL BSA mixed with 1mL 1X Bio-Rad Protein Assay Dye Reagent (dye, phosphoric acid, methanol) (Bio-Rad #5000006). OD595 readings were plotted into a graph using Microsoft Excel. OD 595 readings from 1µL sample mixed with 1mL 1X Bio-Rad Protein Assay Dye Reagent were made. Using the standard curve graph to generate an equation for the calculation of protein concentration from OD measurements, concentration of proteins in the unknown samples was determined by interpolation from the graph.

2.2.5 Protein Immunoprecipitation

1.0-2.0mg of protein of each extract was brought to a final volume of 1mL in 0.15 M NaCl IP buffer (25 mM Tris-HCl pH 7.5 at 21°C, 0.15 M NaCl, 5 mM MgCl2, 2 mM EDTA, 10% glycerol, 0.1% NP40 – protease inhibitors added as before). Extracts were incubated on ice for 10 min and were then centrifuged at 13000 X g, 4°C for 10min. The supernatant was transferred to fresh tubes and 2µL of polyclonal antibody (1.0mg/mL concentration raised
in rabbit) was added to each tube (antibody depends on the assay – detailed information is provided in the results section). The mixture was incubated at 4°C with end-over-end mixing (Labquake) for about 1-3 hrs. The tubes were centrifuged again for 10 min at 13000 X g, 4°C and the supernatant was transferred to a fresh tube. 30μL of a 50% slurry of protein A sepharose beads (washed thoroughly in 0.3 M NaCl IP buffer) was added and again the tubes were incubated at 4°C overnight with end-over-end mixing. The following morning, the tubes were centrifuged at 60 X g (lowest speed), 4°C for 2 min, and the supernatant was carefully removed. The beads were washed three times with 1.0mL IP buffer 0.15 M NaCl by mixing for 5 min at 4°C end-over-end (labquake) and centrifuging at low speed (60 X g 2 min) and removing the wash solution each time. After the final wash, 40μL of 1.5X SDS loading buffer was added and the sample was heated at 90-100°C for 4°C min. Supernatant was separated from the beads by spinning at 600 X g and transferred to a fresh tube.

2.2.6 SDS-PAGE and protein transfer

Standard 6% SDS-PAGE gels were run and transferred according to the protocol described in published literature (Mahmood & Yang 2012).

2.2.7 Western blotting and protein detection by fluorescence

The de-stained membrane was blocked for 1 hr in 5% milk solution (5g powdered milk, 100mL TBST) then incubated with a primary antibody at a 1:1000 dilution in 2% milk solution overnight at 4°C. The primary antibody used was mouse monoclonal 8WG16 (manufactured by Covance) for detecting Rpb1. After washing twice for 5 min with 2% milk solution, the membrane was incubated at room temperature in darkness with Cy5-labeled anti-mouse (8WG16) secondary antibodies at a 1:3000 dilution for 1-2 hrs. The membrane was washed once for 5 min and twice for 10 min in 2% w/v milk solution, then twice for 5 min in TBST before being scanned on a Typhoon 8600 at a PMT voltage of 500 V to detect fluorescence.
Figure 2.1: Integrative plasmid containing kanMX marker and 3HA or 13Myc tag. These plasmids were used to generate tagged Bre5 and Ubp3 respectively. Image extracted from: (Bähler et al. 1998)
2.2.8 Cell extract preparation for RIP

1L yeast cultures of each strain were grown at 30°C overnight in YPD (2% peptone, 1% yeast extract and 2% dextrose) supplemented with 0.02 mg/ml adenine sulfate to an OD600 of approximately 0.9. Formaldehyde was added to ~1% w/v final concentration and incubated for 15 minutes, and the cultures were quenched with glycine ([final concentration] = 136 mM) for 5 minutes. Cells were harvested by centrifugation (4000 X g for 5 mins at 4°C) in SLC6000 rotor and the pellet was resuspended in 20ml ice-cold STE (TE plus 50mM NaCl) containing protease inhibitors (0.5mM phenylmethylsulfonyl fluoride (PMSF); 1mM benazamide-HCl) and transferred to a sterile 50 ml centrifuge tube. Samples were spun in a J6 rotor at 2000 X g for 15 minutes, the supernatant was removed once again and the pellet was resuspended in 18ml cold STE. Cells were aliquoted into 24 microfuge tubes (0.9ml each) and spun at 10K for 2 minutes at 4°C.

Each cell pellet was resuspended in cold 250μl FA-lysis buffer (50 mM HEPES/KOH pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate) and complete protease inhibitors were added just prior to use (2ug/ml leupeptin; 3ug/ml aprotinin; 2ug/ml pepstatin A; 1ug/ml chymostatin; 1mM benzamidine-HCl, 0.5mM PMSF). To the suspension, 300ul of glass beads were added and vigorously vortexed 4 times by hand for 40 sec each sample, and placed on ice between bursts. Tubes were placed on the shakehead vortexer and continuously shaken on high speed at 4°C for an additional 45 mins. Two hundred microliters of FA Lysis buffer was added to each tube and the extract separated from the beads by centrifuging. Total cell extract volume was combined for each sample and brought to about 1.8mls by adding lysis buffer if needed.

The sample was sonicated to liberate the RNA from the crosslinked material. Sonication also breaks up RNA into smaller fragments. Samples were placed in a 15ml polystyrene tube and covered with metal caps. Tubes were placed in ice-cold water in the Bioruptor and set to 2 pulses (30 sec on and 30 sec off). Cell suspension is clarified by centrifuging at 13000 X g for 15 mins at 4°C. Supernatant was transferred to a fresh eppendorf tube and centrifuged again for 30 mins at 13000 X g and 4°C. Clarified extract was analyzed for protein concentration using Bradford’s assay as described earlier.
2.2.9 RNA Immunoprecipitation

2.5mls of WCE was diluted with equal volume of FA Lysis buffer and placed on ice for 5 mins. 500ul of 10X MgCl2/CaCl2 (25mM/5mM) solution was added and mixed. To each tube, 250ul RNAse-free DNase I (concentration 4U/ul) was added and the solution was mixed gently by inversion. Samples were incubated for 90 minutes at 30°C (waterbath) and mixed gently by inversion a few times. To stop the DNase treatment after the incubation, 250ul of RNAse free 0.5 M EDTA was added. Sample was aliquoted into 4 microfuge tubes and spun at 13000 X g for 10 min at 4°C. Supernatants were removed and divided into two parts. Two 100ul aliquots were set aside as input samples (each 1/60 of the total) and the rest was incubated overnight with 30ul of 50% protein A sepharose-antibody complex, prepared independently by incubating with lysis buffer-washed beads with 20ul of antibody per 200ul of beads for 2-3 hrs on end-over-end rotation. Beads were pelleted by centrifugation (268 X g 2min 4°C), aspirated and washed as follows.

Samples were washed with 1ml of FA-lysis buffer once with 5 min incubation, and twice more with 10 min incubations. This was followed by two 5 min washes in 1ml of FA-wash 2 (0.5 M NaCl, 50 mM HEPES/KOH pH 7.5 at 21°C, 1 mM EDTA, 1% v/v Triton X-100, 0.1% w/v sodium deoxycholate; 0.5 M NaCl, 0.5 M PMSF, 1mM benzamidine-HCl). Finally, beads are washed in 1ml Wash buffer 3 (0.25 M LiCl; 1% v/v NP-40; 1% w/v sodium deoxycholate; 1 mM EDTA; 10 mM Tris-HCl, pH 8.0 at 21°C, 0.5 M PMSF, 1mM benzamidine-HCl) for 5 min and then 2 min incubation. Ice-cold 1X TE pH 8.0 was added to the beads and washed for 5 mins. An additional 0.5 ml TE was added, the tubes mixed gently, and spun to remove the liquid immediately. Immune complexes were eluted by adding 450ul of Elution Buffer (25 mM Tris, pH 7.5 at 21°C, 1 mM EDTA, 0.2 M NaCl, 0.5% w/v SDS) to beads, vortexed briefly to mix and incubated at 65°C for 20 minutes. Tubes were vortexed gently and spun at 600 X g for 3 min. Input samples were processed simultaneously with the IP and washed as above.

Three microliters of 10 mg/ml Protease K was added to the IP samples and incubated at 65 °C for 4-5 hours to digest protein and to reverse the protein-nucleotide cross-linking. To the 100ul of input samples 350ul of elution buffer and 10ul of 10% SDS was added, followed with 3μl of 10mg/ml Protease K and incubated at 65°C for 4-5 hours.
To this, 400ul Acid-phenol (pH 4.8)/chloroform (1:1) was added, vortexed for 15 sec, spun for 4 minutes at 13K, and 380ul of aqueous phase was recovered. RNA was precipitated by adding 10ul of 2mg/ml glycogen, 20ul of 3 M NaOAc (pH 5.0) and then 1ml 100% ethanol. Tubes were inverted multiple times to mix well and placed at -80°C for 1-2 hours. Sample was pelleted by centrifuging at highest speed for 8-10 mins and then washed once with ice-cold 75% v/v ethanol. Pellets were resuspended in DEPC treated water and the concentration was calculated using a Nanodrop spectrophotometer.

To remove any additional DNA, a second DNase treatment was done by adding 8ul DEPC water, 10ul 10X (MgCl₂-CaCl₂ buffer) DNase I buffer, 1ul RNasin (RNase Inhibitor), 2.5ul 10 U RNase-free DNase I and incubated at 37°C for 20 mins. Sample was acid phenol chloroform-isoamyl alcohol (PCIAA) treated and RNA was precipitated in the presence of 20ul of 3 M sodium acetate and 10ug glycogen with 550ul 100% ethanol. Tubes were incubated at -20°C overnight. The tubes were spun at maximum speed for 8-10 mins and the pellets were washed once with 80% v/v ethanol and air-dried. The final RNA pellet was resuspended in DEPC-treated water and once again the concentration was measured spectrophotometrically. The procedure is outlined in figure 2.2.

2.2.10 Real-Time Reverse Transcriptase (RT) PCR

For the quantitative measurement of RNA immunoprecipitating with the proteins, 4ul of each RNA sample was incubated with random primers (0.01ug/ul, (Promgea #C1181)) in a 30ul total volume for 10 minutes at 70°C. The samples were then briefly transferred to room temp and then incubated on ice for 5 minutes. A master mix containing ddH₂O, AMV (Avian Myeloblastosis Virus) buffer, 1mM dNTPs, and 1 unit/ul AMV RT (Promega #M510F) was added to the reaction containing RNA and primers. The samples were then incubated at 42°C for 1 hour to synthesize cDNA from the RNA template. cDNA was then serial diluted so that there were two different concentrations in order to later measure amplification efficiency of the real time reaction. A master mix of forward and reverse primers, and Quanta PerfeCTa SYBR Green SuperMix was added to 4ul of diluted cDNA (in duplicate) in a 96-well plate. The Applied Biosystems StepOnePlus instrument was used to carry out Real-Time PCR. To measure the enrichment, %IP was measured as the average value $2^{(Ct \text{ MeanInput}-Ct \text{ MeanIP})}$ between both dilutions.
IP with Myc and HA was done in the above listed strains and the RNA was isolated after reversing the cross-linking overnight as described in the methods. A standard control, no-RT (Reverse Transcriptase) PCR was done to determine that the RNA samples were not contaminated with DNA. This was used as a template for PCR to amplify a random house-keeping gene (PDA1) with high copy number and run out on a standard agarose gel (1.2%). Once the sample was determined to be free of DNA, 4ul of the RNA was loaded in two different concentrations 1:1 and 1:2 in DEPC water and loaded in technical triplicate to increase accuracy of PCR. The input samples were also diluted 1:5 and 1:10 and 4ul was loaded in triplicate alongside the IP samples on the same 96 well PCR plate. A master mix as described in the earlier section was prepared with primers to SKN7 or PYK1 and 8ul added to each well. Three wells were loaded with 4ul DPEC water as negative control and combined with the master mix.

From the PCR results, \( C_T \) (Cycle Threshold) was recorded and the data was analyzed in Microsoft Excel. The mean \( C_T \) of triplicates is taken and efficiency of PCR is calculated between the two concentrations of each sample. From the mean \( C_T \) the %IP is estimated by first measuring:

\[
C = 2^{(IN \ C_T - IP \ C_T)}
\]

Using this value with the appropriate dilution factor (\( D_{IN} \) or \( D_{IP} \)) to calculate %IP:

\[
%IP = C(D_{IP} / D_{IN}) \times 100
\]

The %IP for each sample is calculated from the \( C_T \) values of the IP for the strain and the Input \( C_T \) values and adjusted for the dilution factor of the two sets. The %IP for each sample is plotted on a graph, in the order: Wild-type or negative control, Dhh1 or positive control, dual tagged Ubp3-13Myc Bre5-3HA, \( \Delta bre5 \) Ubp3-13Myc, \( \Delta ubp3 \) Bre5-3HA. The %IP of each strain allows us to compare the difference between immunoprecipitation of a particular sample or protein versus the Input or background sample. The antibody used determines which protein is studied and the %IP shows the percent over background immunoprecipitated with the mRNA.
Formaldehyde Crosslinking protein to DNA & RNA

Cell lysis with bead beating and sonication

Dnase treatment I

Protein A conjugated primary antibody

Bead washing and protein-RNA elution

Reverse crosslinking proteinase K and Dnase treatment II

RNA analysis by Realtime RT PCR

cDNA
Figure 2.2: Outline of the RNA Assay: Cells are cross-linked using formaldehyde and lysed. Nucleotide-protein conjugates are released by sonication and DNA is removed by DNase treatment. Protein-A beads conjugated to primary antibody is used to target the protein-of-interest and pull down the protein crosslinked to RNA. By reversing the crosslinking, and an additional DNase treatment, RNA is released from the protein of interest. RT and Realtime PCR is used to generate cDNA and determine quantitative analysis of the RNA-protein interactions.
2.3 Results

The purpose of this study was to explore the extent of interaction between Ubp3, Bre5, the Ccr4-Not complex and RNA PolII. Understanding the protein-protein association is key to constructing a model for how Not4 functions in the ubiquitination and degradation of Rpb1 under 4NQO stress. The assay for protein IP is qualitative and serves to show the physical interaction of the proteins immunoprecipitated and the proteins probed for. The assay does not account for direct or indirect association of the proteins; hence the result is incomplete. The hypothesis is that the Ccr4-Not complex may also play a role in the last-resort rescue of Rpb1 by association with Ubp3 and Bre5. This implies that the role of Not4 is not just as a RING E3 ubiquitin ligase, but also as a regulator of stress response to DNA damage by controlling whether PolII will resume transcription, or be dismantled by GGR specific factors.

To explore the interaction between the Ccr4-Not complex and the Ubp3/Bre5 complex, the samples were RNA Immunoprecipitated (RIP). This method is used for the identification of mRNAs that associate with Ubp3 and Bre5. If Ubp3 and Bre5 are recruited to the same mRNA as Ccr4, it could shed some light on the method of interaction of the two protein complexes. Ubp3/Bre5 could associate with the Ccr4-Not complex or to PolII through the mRNA.

2.3.1 Analyzing Ubp3’s association with its cofactor Bre5

The first step is to determine if the tagged proteins, Ubp3 and Bre5, still interact with each other in an immunoprecipitation. Using the dual tagged strain, protein immunoprecipitation (IP) was performed on one of the two proteins – Ubp3 or Bre5 – followed by a western blot for the other. Immunoprecipitation was set up to either Myc (for Ubp3) or HA (for Bre5) and the blot was incubated with antibody to Bre5 (HA) or Ubp3 (Myc) respectively, to observe the interaction of Bre5 to Ubp3 and vice versa. It is expected to see a band for Bre5 in the assay with Ubp3 and a band for Ubp3 in the assay with Bre5, confirming that the two proteins do associate with each other.

Figure 2.3(a) shows the blot probed for Bre5-3HA with an anti-HA antibody in the top row and Ubp3-13Myc probed with an anti-Myc antibody in the bottom row. The Inputs from each sample are to the left and the IPs are to the right. In figure 2.3(a), it is evident
that both Ubp3 and Bre5 immunoprecipitated with each other in the dual tagged strain in lane 8. As expected, none of the other IP lanes (5-7) show any protein bands because the strains lack Bre5 or Ubp3. The corresponding input lanes show the protein concentration in each strain loaded directly from the cell extract without performing an IP. The inputs in the lower panel appear to show a decreased abundance of Bre5 in the ubp3 mutant despite adjusting the concentrations loaded using the values from the Bradford’s assay. This effect was observed on multiple replicates and may indicate a possible decrease in the expression, or stability of Bre5 in the absence of Ubp3.

The input samples show a number of smaller bands below the primary Ubp3 protein band which may be partially degraded species of Ubp3. The banding pattern seen in the top panel of input samples (2.3a lanes 3, 4) but not in the IP with Bre5 (lane 8) suggests that the degradation of Ubp3 could be in the C-terminus region (opposite to the tag), and appears to affect the binding of Bre5 interaction with Ubp3 (refer figure 1.5 a for Ubp3 and Bre5 interaction).

2.3.2 Protein Immunoprecipitation with RNA PolII

Once Ubp3 and Bre5 are shown to be associating with each other, the next step of verification is to confirm that they associate with RNA PolII as previously observed and what the effect of a deletion of one of the two proteins (Ubp3 and Bre5) would be on this interaction. Following similar methods as for the previous immunoprecipitations, WCE was incubated with PolII antibody (8WG16) and western blotted for Ubp3 or Bre5. Additionally, the assay was done in mutants strains Δccr4 and Δnot4 to determine if these proteins play any role in the interaction.

From the blots in figure 2.3b, both Ubp3 and Bre5 immunoprecipitated with PolII (figure 2.3 b lane 12), and in the absence of Bre5, Ubp3 is still recruited to PolII (lane 11). On the contrary, in the absence of Ubp3, no Bre5 was detected in the IP with PolII (lane 10). This could suggest that Ubp3 might be essential for the stabilization of the recruitment of Ubp3-Bre5 complex to RNA PolII. It appears that neither Ccr4 nor Not4 are required for the recruitment of Ubp3-Bre5 complex to RNA PolII (lanes 8 and 9). Only Ubp3 was tagged in these strains and it is assumed that Bre5 is also recruited to PolII with Ubp3. This does not rule out other interactions via the Ccr4-Not subunit. The analysis requires more significant
experimentation with other members of the Ccr4-Not complex to determine the role it plays in the association, as well as to determine if the association is direct or indirect.

2.3.3 Detect protein associations with the Ccr4-Not Complex

To complete the association analysis, the final piece of the puzzle is to understand how Ubp3-Bre5 interacts with the Ccr4-Not complex and what the impact of deleting protein components will be on this interaction. Because the Δccr4 and Δnot4 strains only have a tagged Ubp3 protein, this experiment only looked at the interaction of the complex (Ubp3-Bre5) as a whole assuming the two proteins stayed associated with each other. Polyclonal antibodies to Not4 of the Ccr4-Not complex were used to immunoprecipitate the Ccr4-Not complex and either Myc or HA antibodies were used to visualize Ubp3 or Bre5 respectively by western blot.

In the assay with Not4, (figure 2.3c) interestingly, both Ubp3 and Bre5 were detected in the dual tagged strain, but in the absence of Bre5 only a faint band for Ubp3 was detected in the blot (lane 11 figure 2.3c). Bre5 immunoprecipitates with Not4 even in the absence of Ubp3 (lane 10 figure 2.3c). Additionally, in the Ubp3-13Myc Δccr4 strain, Ubp3 was still detectable indicating that Ccr4 is not necessary for the interaction of Ubp3-Bre5 with the Ccr4-Not complex. This result shows that Bre5 may be necessary for the association of Ubp3 to the Ccr4-Not complex, and Bre5 either recruits Ubp3 to the Ccr4-Not complex or stabilizes the interaction.

The immunoprecipitation assay only accounts for association of the proteins, but does not determine if the association is direct or indirect through another protein or other factor. Using just the information from the gels, if the association is direct, a model can be postulated from the interaction pattern. The overall proposed association, based on only the results from the assay is described in figure 2.4, where Ubp3 is required for the DUB to be recruited to polymerase, and Bre5 is necessary to bridge the gap with Ccr4-Not. This figure is incomplete since Bre5 may still associate with PolII, and the interactions could be indirectly mediated by other proteins or nucleic acids.
2.3 a

IP: Bre5: HA
Western: Ubp3:Myc

IP: Ubp3:Myc
Western: Bre5:HA

2.3 b

IP: 8WG16 (Rpb1)
Western: Ubp3-Myc

Western: Bre5-HA
2.3 c

Figure 2.3: Protein immunoprecipitation using tagged strains for Ubp3 and Bre5. Input samples to the left indicate the concentration of each protein in the strains. (a) Interaction of Ubp3 with Bre5. Ubp3 was tagged with a 13-Myc tag and Bre5 with a 3-HA tag. Strains lacking Ubp3 and Bre5 do not show the association. (b) IP with antibodies against polymerase shows Ubp3 and Bre5 associating with polymerase. (c) IP with antibodies to Not4 of the Ccr4-Not complex also shows association of Ubp3 and Bre5.
Figure 2.4: Diagram showing the proposed model for interaction of Ubp3/Bre5 with PolII and the Ccr4-Not complex. From the gels in the protein immunoprecipitation assay, Ubp3 is seen associating with PolII, irrespective of deletion of not4 or ccr4. Although it is not known whether Bre5 independently interacts with PolII, in this model, it is assumed that its interaction is through Ubp3. Previous studies have already shown that the Ccr4-Not complex associates with PolII, and the IP assays in this thesis indicate that Bre5 associates with the Ccr4-Not complex even in the absence of ubp3.
2.3.4 Recruitment of proteins to mRNA

Since the protein immunoprecipitation does not account for indirect association, it is possible that Ubp3-Bre5 may interact with PolII or the Ccr4-Not complex via another protein, or even by recruitment to mRNA. To test the theory that Ubp3 and Bre5 are recruited to RNA, an altered method for protein immunoprecipitation was used to detect relative RNA recruitment using RNA Immunoprecipitation (RIP). In this method, mRNA is crosslinked to proteins and nucleic acids (both DNA and RNA) using formaldehyde and isolated from cell cultures and purified. The RNA is broken into fragments associated with RNA bound proteins and these proteins are immunoprecipitated using antibodies specific to their tag. Once precipitated, the crosslinking is reversed and the RNA is isolated. This RNA is reverse transcribed to cDNA using reverse transcriptase. Using Real-Time quantitative PCR, the RNA copies for a selected gene are quantified and measured. The more an mRNA is enriched for in a particular immunoprecipitate, the more significant the recruitment of the protein is to the mRNA. By immunoprecipitating tagged proteins from the different strains: Ubp3-13Myc, Bre5-3HA, Δubp3 Bre5-3HA, Δbre5 Ubp3-13Myc, it can be determined if Ubp3 or Bre5 or both associate with mRNA, and if either protein is necessary for the interaction. Because it is not known which mRNA in particular they may target (if any), the samples were experimentally assayed for mRNAs that are enriched by RIP experiments involving components of the Ccr4-Not complex.

In previous experiments performed in the laboratory, RIP was done for components of the Ccr4-Not complex – like Ccr4 and Dhh1 – and followed up with genome-wide sequencing of the mRNA to determine the mRNA targets for Ccr4 and Dhh1 (J. E. Miller, J.C. Reese, paper in review). The experiment identified several prominent targets for Ccr4 and Dhh1 among the mRNA transcripts, among which two were also tested in this study – SKN7 and PYK1. These mRNAs were chosen because they were highly enriched targets for Ccr4. Because it is possible that Ubp3/Bre5 may interact with the Ccr4-Not complex, these targets were good initial candidates to establish whether Ubp3 and/or Bre5 are recruited to mRNA. To estimate the recruitment of Ubp3 and Bre5, Dhh1 was used as a positive control because it is highly recruited to both mRNA studied in this thesis. Untagged wild-type samples were used as a background measure.
In three independent experiments, the results showed a few consistent results regarding the recruitment of Ubp3 to \textit{SKN7} and \textit{PYK1}. However, the experiment does not have a positive control for an HA-tagged RIP, hence the results for RIP with anti-HA for Bre5 recruitment is inconclusive. Because the tags used for detecting both proteins are different, 13Myc on Ubp3 and 3HA on Bre5, the output between the two antibodies vary considerably. Additionally, the efficiency of the antibodies, Myc or HA, could determine the efficiency of the RIP, however, the amount of antibody used was determined to be saturated. The structure of Ubp3-Bre5 dimer could also alter the positioning of the tag and the accessibility of the antibody. The experiment is not designed to compare the exact recruitment of Ubp3 and Bre5, but instead to simply determine if the proteins are recruited to mRNA. The error bars on the graph are calculated from the standard deviation across three replicates performed for each of the strains.

From figure 2.5a and 2.6a, the bar graphs show the results of the RNA IP with anti-Myc to \textit{PYK1} and \textit{SKN7} respectively. Dhh1 as the positive control (indicated in red) for the RIP showed statistically significant recruitment (p-values 0.034 and 0.017) to \textit{PYK1} and \textit{SKN7} respectively (t-test p-values are listed in table 2). The recruitment of Ubp3 to \textit{PYK1} in the dual-tagged strain (grey bar figure 2.5a) is also significant (p-value 0.017), implying that protein complex as a whole may be recruited to the \textit{PYK1} mRNA. Ubp3 does not appear to be significantly recruited to \textit{PYK1} in the \textit{Δbre5} strain (2.5a, yellow bar, p-value 0.148).

In figure 2.6a, recruitment of Ubp3 to \textit{SKN7} is not statistically very significant in either the dual tagged strain (grey bar) or the \textit{Δbre5} mutant strain (yellow bar) (p-values 0.064 and 0.088). This result may be a biologically important phenomenon regarding the recruitment of Ubp3 to specific mRNA (such as \textit{PYK1}) and not to others (like \textit{SKN7}).

The recruitment of Bre5-HA to \textit{PYK1} and \textit{SKN7} is shown in figures 2.5b and 2.6b with the p-values and standard errors listed in table 2. In the dual tagged strain, Bre5 does not show a statistically significant recruitment to \textit{PYK1} (2.5b grey bar, p-value 0.060). The recruitment is also not significant in the \textit{Δubp3} strain (2.5b dark blue bar, p-value 0.155). Due to the lack of a positive control, the recruitment of Bre5 cannot be inferred from the RIP experiments. Similarly, in figure 2.6b, Bre5 is not significantly recruited to \textit{SKN7} in
either the dual tagged (grey bar, p-value 0.059) or the \( \Delta ubp3 \) mutant strains (dark blue bar, p-value 0.440).

The full scope of this project was not attained, but the experiments have revealed a possible specific recruitment of Ubp3/Bre5 to certain mRNA in a Bre5-dependent manner that may help explain the protein’s function better. The difference in recruitment to the two mRNA, as well as the role of Bre5 in the recruitment need to be further explored. Additional experimentation with expansion to other target mRNA and stress induced RIP procedures may expand upon the genome-wide recruitment of Ubp3 and Bre5 to mRNA, as well as the role of Bre5 and Ubp3 in identifying mRNA.
Table 2. Statistical analysis for RIP with *PYK1* and *SKN7*.

<table>
<thead>
<tr>
<th>mRNA IP - antibody</th>
<th>% IP</th>
<th>Standard error</th>
<th>T-test p values</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PYK1 - Myc</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wildtype</td>
<td>1.663483608</td>
<td>4.05013132</td>
<td>0</td>
</tr>
<tr>
<td>Dhh1:Myc</td>
<td>26.60451337</td>
<td>3.816128681</td>
<td>0.034919562</td>
</tr>
<tr>
<td>Ubp3:Myc Bre5:HA</td>
<td>24.74532598</td>
<td>5.921914282</td>
<td>0.017189921</td>
</tr>
<tr>
<td>Δbre5 Ubp3:Myc</td>
<td>42.26902168</td>
<td>11.42898272</td>
<td>0.148348464</td>
</tr>
<tr>
<td>Δubp3 Bre5:HA</td>
<td>6.390076158</td>
<td>3.261459592</td>
<td>0.12492601</td>
</tr>
<tr>
<td><strong>PYK1 - HA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wildtype</td>
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<td>0.306823078</td>
<td>0</td>
</tr>
<tr>
<td>Dhh1:Myc</td>
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<td>0.0</td>
<td>0.332640948</td>
</tr>
<tr>
<td>Ubp3:Myc Bre5:HA</td>
<td>9.654530083</td>
<td>3.724662054</td>
<td>0.06016574</td>
</tr>
<tr>
<td>Δbre5 Ubp3:Myc</td>
<td>2.760567699</td>
<td>0.638473238</td>
<td>0.00975957</td>
</tr>
<tr>
<td>Δubp3 Bre5:HA</td>
<td>9.062466494</td>
<td>3.26107344</td>
<td>0.155699447</td>
</tr>
<tr>
<td><strong>SKN7 - Myc</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wildtype</td>
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<td>1.625451355</td>
<td>0</td>
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<td>7.895574033</td>
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<tr>
<td>Ubp3:Myc Bre5:HA</td>
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<td>18.95356103</td>
<td>0.064954902</td>
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<tr>
<td>Δbre5 Ubp3:Myc</td>
<td>18.35589584</td>
<td>10.13602043</td>
<td>0.088364468</td>
</tr>
<tr>
<td>Δubp3 Bre5:HA</td>
<td>4.859551045</td>
<td>2.038253023</td>
<td>0.424224192</td>
</tr>
<tr>
<td><strong>SKN7 - HA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wildtype</td>
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<td>0</td>
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<tr>
<td>Dhh1:Myc</td>
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<td>0</td>
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<tr>
<td>Ubp3:Myc Bre5:HA</td>
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<td>0.065473554</td>
<td>0.059304907</td>
</tr>
<tr>
<td>Δbre5 Ubp3:Myc</td>
<td>1.271837056</td>
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<td>0.013203349</td>
</tr>
<tr>
<td>Δubp3 Bre5:HA</td>
<td>5.973404012</td>
<td>1.940091062</td>
<td>0.440246479</td>
</tr>
</tbody>
</table>

The table shows the RIP samples immunoprecipitated with the tag Myc or HA and quantified for the mRNA *PYK1* or *SKN7*. The %IP values show the relative percent of recruitment of the sample when compared to input samples. The standard error was calculated from three independent experiments and the p-values were calculated statistically using students t-test.
Figure 2.5: Results showing the recruitment of Ubp3 and Bre5 to PYK1 mRNA. The Y-axis shows the percent IP. The Wildtype strain acts as the background signal and the Dhh1-Myc strain was used as a positive control based on previous work that identified the interaction of Dhh1 with PYK1 mRNA. (a) In an IP with anti-Myc, dual tagged Ubp3 and Bre5 show high enrichment with PYK1 mRNA (third bar). Even in the absence of Bre5, the recruitment is not diminished (fourth bar). (b) In the reverse, with anti-HA antibodies, the recruitment of Bre5 to mRNA is significant in the dual tagged strain (third bar) and somewhat significant in the Δubp3 mutant (fifth bar).
Figure 2.6: Results showing the recruitment of Ubp3 and Bre5 to SKN7 mRNA. The Y-axis shows the percent IP using the untagged wildtype strain as background. Dhh1-Myc strain was used as a positive control based on previous work that identified the interaction of Dhh1 and SKN7 mRNA. (a) In the IP using anti-Myc antibody, Ubp3 shows very high enrichment to SKN7 mRNA in the dual tagged strain (third bar). In the absence of Bre5, the recruitment of Ubp3 is slightly diminished but still significant (fourth bar). (b) In the IP with anti-HA, there is no significant enrichment in any of the samples.
2.4 Discussion

Protein-protein associations and study of their recruitment to other factors help to understand how these factors interact with each other and carry out functions within the cell. The purpose of this section was to determine how the Ccr4-Not complex interacts with the Ubp3/Bre5 complex, and to begin to understand how this interaction regulates the function of both protein complexes.

The results shown in figure 2.3 outline the basic physical association of Ubp3 and Bre5 to each other, the Ccr4-Not complex, and to RNA PolII. Because tagging proteins can alter their structure, interfere with function and affect interaction with other proteins, protein immunoprecipitation was performed with known functional associations. Ubp3 and Bre5 are known to form a complex together and this complex is considered essential for their functions. The protein immunoprecipitations indicate that the tagged Ubp3 and Bre5 associate with each other as expected. The only odd phenomenon observed in several blots was the decrease in the intensity of the band for Bre5-3HA when Ubp3 was deleted in the input samples (figure 2.3a input lane 3). This may indicate a decrease in relative abundance of Bre5 in the Δubp3 mutant but the inference is inconclusive since it was not consistent in figure 2.3b.

The presence of partially degraded species of Ubp3 (seen in all the input samples but not immunoprecipitated) could be biologically relevant to understand the recruitment of the Ubp3/Bre5 complex to PolII as well as the function of Ubp3. The C-terminus of Ubp3 contains the catalytic domain (figure 1.5 a) and there could be a role for Bre5 in preventing the cleavage of the C-terminus. Bre5 may dissociate from Ubp3 to allow its cleavage and inhibit its function as a DUB.

The self-association experiment was followed up with an inquiry into the interaction of Ubp3/Bre5 with the RNA PolII. In addition to the strains used previously, two more strains with mutants in the Ccr4-Not complex were included. The dual purpose of this experiment was to determine what role Ubp3 and Bre5 played in the interaction with PolII, as well as the possibility of Ccr4 and Not4 affecting this interaction. Despite the possibility of a decreased abundance of Bre5 in the Δubp3 Bre5-3HA strain, it was determined that Bre5 is not essential for the interaction of Ubp3 with PolII, and neither are
Not4 and Ccr4. Although it cannot be concluded whether Bre5 interacts with PolII, it is likely that Ubp3 can interact with PolII independent of Bre5. As discussed in the results section, the assay does not conclude whether the association of Ubp3-Bre5 with PolII is direct or indirect through another protein or mRNA.

One hypothesis was that Ubp3/Bre5 could be recruited to RNA PolII via the Ccr4-Not complex. Although it is not possible to entirely delete the complex (as it is essential for cell survival), Not4 and Ccr4 were selectively deleted to observe the outcome on the recruitment of Ubp3/Bre5 to PolII. While neither protein affected the recruitment of Ubp3, these strains do not contain a tagged Bre5 for observation. To follow up on the interaction with the Ccr4-Not complex, the assay was performed with Not4 and probed for Bre5 and Ubp3. It appears that Ubp3 may not associate with Not4 robustly in the absence of Bre5, but Bre5 does associate with Not4 even in the absence of Ubp3. This result could indicate a role for Bre5 in the recruitment or stabilization of Ubp3/Bre5 to the Ccr4-Not complex. Again, the association is not confirmed to be a direct one since the IP is done with the entire cell extract and could be mediated by other factors.

Because Bre5 and Ubp3 associate with both PolII and the Ccr4-Not complex, it is interesting to explore the possibility that the Ubp3/Bre5 complex is also being recruited to mRNA. The Ccr4-Not and PolII associate with mRNA and it is likely that mRNA may bridge some of the interactions between these various proteins. Additionally, the RRM on Bre5 and previous studies on Ubp3 suggest a link with mRNA. By selecting mRNA that Ccr4 is already known to bind, it is possible to see if Ubp3 and Bre5 also followed similar recruitment patterns. Although the assay does not determine if the recruitment of Ubp3/Bre5 to mRNA is direct, it provides insight into the possible functions of the complex. Both PYK1 and SKN7 are prominent targets for Ccr4 recruitment under stressed and unstressed conditions. The idea is to follow this experiment with a genome-wide study, similar to the one conducted with Ccr4, and look at similarities in the recruitment of the two complexes (discussed in chapter 4).

The recruitment of Ubp3 to PYK1 is significant as indicated by the p-values in table 2 but less so for SKN7. The Δbre5 mutant does not appear to significantly decrease the recruitment of Ubp3 to PYK1. These observations may indicate that Ubp3’s association with PYK1 is independent of Bre5 yet its association with SKN7 may require Bre5. It suggests a
dynamic relationship between Ubp3 and Bre5 which may assist in the identification of specific mRNA and recruitment to mRNA in a stress-specific manner.

On the other hand, Bre5 does not appear to be recruited to SKN7 and PYK1 in a similar manner. Bre5 is not significantly recruited in any of the strains, indicating that there may be a different mechanism for the recruitment of Ubp3 and Bre5 to mRNA. It is possible that Ubp3 may be recruited to PolII or to the Ccr4-Not complex through mRNA or may be selectively recruited to particular mRNA to identify the transcript that needs to be upregulated. Ubp3 could function in a similar, antagonistic way to the Ccr4-Not complex by targeting mRNA that are essential for stress response and countering the ubiquitination of Rpb1 on those transcripts. This is an efficient method for the cell to regulate the transcription of genes necessary for survival, while terminating the transcription of other genes that are not essential. It is still unclear what the role of Bre5 is in the recruitment of Ubp3 or in the identification of mRNA for stress-regulation.
Chapter 3: Not4’s function as an E3 RING ubiquitin ligase in PolII degradation

3.1 Introduction

The response to DNA damage depends on several factors such as the severity of the damage, the type of damage, the location and the effect of the response. As mentioned earlier, in extreme cases when the damage is not repaired by transcription-coupled machinery, cells resort to a mechanism wherein the largest subunit of RNA Polymerase II is ubiquitinated and degraded in a proteasome-dependent manner. This disengages the elongation complex (EC) from the DNA with minimal degradation of other proteins, giving way to the general genomic repair machinery to fix the damage.

Several factors have been implicated in the process of ubiquitination of Rpb1. In yeast, most notable are Rad26 and Def1 (Woudstra et al. 2002). Def1 is itself ubiquitinated and processed by the proteasome upon DNA damage and transported into the nucleus wherein it interacts with Rad26 and assists in the ubiquitination of Rpb1 (Wilson et al. 2013). However, Def1 is not a part of the ubiquitination machinery, hence it must interact with an E1, E2 and/or E3 to facilitate ubiquitination of itself and Rpb1. According to Wilson et al (Wilson et al. 2013), Def1 recruits the Elongin-Cullen complex, which is an E3 ubiquitin ligase shown to mediate the ubiquitination and degradation of PolII under UV stress (Woudstra et al. 2002; Yasukawa et al. 2008). However it is likely that the mechanism of response may vary depending on the damage and other factors. Hence it is likely that the proteins involved in the response may also differ. One protein that may be involved with the process of ubiquitination of RNA PolII is Not4. As a RING E3 Ubiquitin ligase that is already associated with the Elongation Complex and part of the Ccr4-Not complex, it is possible that Not4 may play a role in ubiquitination.

Because Not4 has a role in the turnover of Rpb1 as described in previous sections of this thesis, it is important to know if this is due to its role in ubiquitination of Rpb1, or due to an alternate function in the turnover of Rpb1. To explore the role of Not4 as a ubiquitin ligase in conjugating ubiquitin moieties to Rpb1, immunoprecipitation experiments were done to determine if the polymerase was undergoing ubiquitination in the presence and
absence of Not4 as well as other subunits of the Ccr4-Not complex. These experiments are discussed in detail in this chapter.
3.2 Materials and Methods

3.2.1 Strains and conditions

In order to effectively visualize ubiquitin substrates, all strains previously made were transformed with a plasmid containing a HA tagged ubiquitin species – pRG426 (2u URA3). Strains were already lacking pdr5, a pleiotropic drug resistance transporter that confers multi-drug resistance in yeast (Balzi et al. 1994). The absence of pdr5 ensures that any drug-induced stress will be more efficient, without the drug being transported out of the cell. Addition of MG132, which is a proteasome inhibitor, prevents the degradation of proteins upon stress-induced ubiquitination. Strains were created with the Δpdr5 background and the following gene deletions were made by standard approaches in the Ccr4-Not complex – Δnot4, Δnot5, Δdhh1 and Δccr4.

3.2.2 Stress-inducing chemicals

A peptide aldehyde, MG132 or carbobenzyl-Leu-Leu-Leu-aldehyde, was one of the first classes of proteasome inhibitors developed and is the most widely used (Kisselev & Goldberg 2001). These aldehyde inhibitors are slow binding but reversible. MG132 functions by inhibiting the chymotrypsin-like site; the pharmacophore region interacts with a catalytic residue to form a covalent adduct while the peptides associate with the active site in the binding pocket of the enzyme. MG132 acts as a proteasome inhibitor to ensure that the protein is not degraded before it can be observed.

4-Nitroquinoline (4NQO), one of the chemicals that is used for stress induction, is a mimic of UV damage on cells. It is a quinolone derivative that is a known tumorigenic compound and induces DNA lesions. These lesions are normally repaired by the cell’s Nucleotide Excision Repair (NER) machinery (La Voie et al. 1983).

6-Azauracil (6AU) is a transcription elongation shut-off drug that works by decreasing GTP and UTP pools (Exinger & Lacroute 1992). This method is a very effective way of halting the Elongation machinery and mimicking a stress-induced arrest.

3.2.3 Cell Growth and MG132/4NQO Treatment.

Yeast cultures were inoculated into 50mL minimal nitrogen base media with amino acid mix lacking Uracil and were grown at 30°C until an OD600 of 1.0. Samples were as
follows: Wildtype Δpdr5, Δnot4 Δpdr5, Δccr4 Δpdr5, Δnot5 Δpdr5 and a positive control of a proteasome mutant pre1,2. The proteasome mutant inhibits the proteasome and was used as a positive control for the visualization of ubiquitinated proteins and to check the efficiency of MG132. For each of these samples, four separate 50mL cultures were grown, except for the proteasome mutant where only two cultures were grown because these strains do not require the proteasome inhibitor MG132. Two sets of samples were treated with 50uM MG132 per mL of sample for 1 hour to inhibit the function of the proteasome. One of these MG132 treated samples and a third sample was further treated with 6ug/mL 4-Nitroquinoline 1-oxide (4NQO) for 1 hour after the MG132 treatment to cause the DNA damage-induced arrest of RNA PolII. The fourth sample was left untreated as control. For the proteasome mutant, one flask was treated with 4NQO and the other was untreated. Cultures were harvested as described earlier. Assays were set up as described in earlier sections.

### 3.2.4 6AU treatment

Cells were grown in a 50mL culture to an OD600 of 1.0 and 100ug/ml of 6-Azauracil (6-AU) was added for 2 hours to one set of cultures to inhibit transcription elongation by decreasing the GTP (Guanosine Triphosphate) and UTP (Uridine Triphosphate) production. The other culture was left untreated as control. Cells were harvested by the TCA extract preparation.

### 3.2.5 Trichloroacetic Acid (TCA) extract preparation

This procedure was used instead of the IP extract preparation to prepare wild type, Δnot4, Δccr4, and Δnot5 cell extracts following 6-AU treatment. All steps were carried out at room temperature unless otherwise indicated. Cells were grown and treated with 6-AU to inhibit transcription elongation, 10 mL of culture was removed and spun 10 min at 600 X g. The pellet was resuspended in 1 mL 20% v/v TCA, spun for 2 min 600 X g, aspirated and resuspended in 200ul 20% v/v TCA. Two hundred and fifty microliters of glass beads were added and the tubes vortexed twice for one minute before being placed on a shakerhead vortexer for 10 min. An additional 400ul 5% v/v TCA was added before the lysate was separated from the beads by centrifugation. The tube was spun 2400 X g for 10 min, then the pellet was washed in 1mL 0.25 M Tris pH 7.4 at 21°C. The tubes were spun at 2400 X g
for 5 min, the supernatant aspirated, then the pellet was resuspended in 100ul 0.25 M Tris pH 7.4 at 21°C and 150uL 3X SDS-PAGE loading buffer. Finally, the samples were boiled 4-5 min, spun at 11000 X g for 10 min, and 200-300ul of supernatant was transferred to a new tube.

### 3.2.6 SDS Gel running and soaking

Six percent acrylamide gels were poured as previously described and run in 1X Tris-glycine SDS at 30 mA per gel for 45min. Because heavily ubiquitinated proteins tend to move slowly through gels, they are difficult to transfer onto membranes. To increase the efficiency of the transfer, gels were soaked for 20 mins in a soaking buffer with 1% SDS, 5% 2-mercaptoethanol, 63 mM Tris-HCl, pH 6.8 prior to setting up transfer (Mimnaugh & Neckers 2005). After soaking, the gel was transferred onto a 0.2um reinforced nitrocellulose membrane using the tank transfer method (1X Tris Glycine transfer buffer - 25 mM Tris, 190 mM glycine 20% methanol).

### 3.2.7 ECL Visualization

These nitrocellulose membranes were blocked in 5% w/v milk in TBST for 1 hour and set in primary antibody overnight at a 1:1000 dilution in 2% w/v milk in TBST as previously described. The blot was washed twice for 5 min in 2% w/v milk and TBST and incubated in 1:1000 dilution of HRP-(horseradish peroxidase) conjugated secondary anti-mouse antibody for 1 hour. The blot was washed again twice in 2% w/v milk solution and twice in TBST.

For the ECL (Enhanced Chemiluminescence), a kit from Thermo Scientific was used. To generate the substrate, 5ml of reagent A and 5ml of reagent B were mixed and the blot was incubated in this solution for 1 min with gentle tilting and shaking. The blot was then removed and drained of excess liquid and placed on a glass plate with the protein side up and the blot was covered with a thin plastic film wrap. The plate was then exposed to x-ray film in a dark room for multiple exposures (30sec, 1, 3, 5 and 10 mins) depending on the intensity of the bands.
3.3 Results

3.3.1 MG132 treatment in TCA extracted samples

A preliminary set of samples including a wildtype, Δccr4, Δdhh1, Δnot4, Δnot5 and pre1,2 (proteasome mutant) was grown under the conditions described in the previous section (3.2.3) and a second set of these samples (except for pre1,2) was treated with proteasome inhibitor MG132 to prevent the degradation of Rpb1 and allow the observation of the ubiquitinated proteins. These cell cultures were not immunoprecipitated with antibodies to either ubiquitinated proteins or PolII, but were extracted by TCA protocol and directly run on a gel to detect the quantity of PolII protein in each sample, the effect of addition of MG132, and extent of ubiquitination and degradation of PolII. Ubiquitinated proteins have higher molecular weights and run slower than the native protein on the gel. Polyubiquitinated species like Rpb1 are expected to show a streak or ladder above the native protein. It is predicted that the assay for the detection of ubiquitinated proteins would show a smearing of higher molecular weight proteins indicating ubiquitinated Rpb1 under 4NQO stress induced conditions. From the experiment shown in the introductory figure 1.7 the Δnot4 strain should be deficient in the ubiquitination of Rpb1 under the same 4NQO stress when compared to the other strains. As a positive control for the addition of MG132 to disable the proteasome function, the blot includes a proteasome mutant pre1,2 to prevent the degradation of poly-ubiquitinated proteins.

The panels in figure 3.1 show the same blot under two different exposures. The blot in the top panel was exposed to the film for 30 seconds and the bottom for 1 minute. Wild-type BY4741 sample was run without any proteasome inhibitor or 4NQO added. The remaining samples: wildtype, Δccr4, Δdhh1, Δnot4 and Δnot5 each contain a pdr5 deletion that prevents drug export and increases the effectiveness of MG132 as reported in various studies (Balzi et al. 1994; Yen et al. 2012; Haworth et al. 2010). One set of these samples is treated with MG132 to observe the effect of the proteasome inhibitor on the mutants. Finally, the last sample is the pre1,2 used as a positive control for proteasome inhibition.
Figure 3.1: **TCA procedure on samples grown without MG132, and treated with MG132.** Wild-type BY4741 sample was run without any proteasome inhibitor added. Both panels are the same set of samples with different exposures (30sec and 1min). Gels show the level of PolII in the different samples using the antibody 8WG16.
The bands seen below the primary Rpb1 band are likely to be partially degraded species of Rpb1 and the addition of MG132 to the samples was not sufficient to prevent this. However, it appears that the addition of MG132 may increase the visibility of the higher species that are modified species of Rpb1. Since this assay looks at all Rpb1 modifications, it is not conclusive because Rpb1 undergoes modifications other than ubiquitination (Chen et al. 2009).

The amount of Rpb1 in each of the samples varies due to the approximation of the volume of TCA samples added to the gel. The second panel (higher exposure) shows the amount of smearing of the protein, predicted to be modified species of Rpb1. Because the effect of MG132 and the pdr5 deletion appears to assist in the visualization of the polyubiquitinated species, this drug was used in subsequent experiments to visualize polyubiquitinated Rpb1. The blot shows that although the levels of Rpb1 are inconsistent, there is no significant loss of Rpb1 in the mutant strains.

### 3.3.2 MG132 and 4NQO treatment for Immunoprecipitated samples

To observe the effectiveness of the antibody to detect Rpb1, an immunoprecipitation assay was performed with the 4H8 antibody (Mouse monoclonal to RNA polymerase II CTD repeat YSPTSPS phospho S5) and probed for Rpb1 using the 8WG16 antibody. This antibody 4H8 was selected because it recognizes both the phosphorylated and unphosphorylated forms of the RNA polymerase II (Biolegend Cat. # 904001 Application notes). In figure 3.2, the two panels show the different strains under MG132 treatment, 4NQO treatment, and both Mg132 and 4NQO added. As described earlier, addition of 4NQO mimics UV damage on the DNA, causing PolIII to be arrested and Rpb1 to be ubiquitinated. The wildtype and pre1,2 samples were loaded in duplicates because the samples were divided into two gels and these two sets of samples act as a transfer control. All the samples were loaded in equal concentrations, determined from the Bradford's assay. The panels show the quantity of Rpb1 appears to be slightly decreased on addition of 4NQO, possibly because of the effect of 4NQO leading to the degradation of Rpb1. However, the primary purpose of this blot is to verify that the Δnot4 is not deficient in Rpb1 concentrations. From the lower panel of figure 3.2, lanes 7-10, it appears that the concentration of Rpb1 is relatively consistent in the Δnot4 strain.
The purpose of adding MG132 was to inhibit the proteasome and allow for a better visualization of ubiquitinated species. To fully solubilize MG132 in the culture, it was dissolved in 1 mL cell culture medium and placed in a hot water bath at 40°C for 15 minutes and added to the flasks on the shaker. Ultimately MG132 was not essential for the detection of polyubiquitinated species, perhaps due to the efficiency of the 4H8 antibody in detecting the ubiquitinated species and the Δpdr5 mutation in the strains.
Figure 3.2: 4H8 immunoprecipitation probed with 8WG16 antibody showing the levels of Rpb1. Each strain was treated with MG132 or 4NQO or both chemicals and immunoprecipitated with 4H8 antibody. The blot was then incubated with 8WG16 antibody. The blots show the amount of Rpb1 antibody being pulled down by the 4H8 antibody and levels of the protein under each circumstance.
In figure 3.3, immunoprecipitation with anti-Rpb1 antibody 4H8 was performed and the blots were probed with (4H8). The major band that is visible in all the lanes was identified as Rpb1, while the bands below it are possibly partially degraded products of Rpb1. It is likely that the antibody recognizes the polyubiquitinated Rpb1 species that are visible as a smearing and banding pattern above the primary Rpb1 band. The smearing pattern shows some interesting information about the different strains as well as the chemicals added. As expected, the presence of 4NQO increases the appearance of these polyubiquitinated species. Unfortunately, addition of the proteasome inhibitor MG132 does not improve the detection of polyubiquitinated species, but the detection of these ubiquitinated species was not hindered in the absence of MG132.

The wildtype and Δnot5 strains show similar patterns, with an increase in polyubiquitinated species upon addition of 4NQO. The Δccr4 strain, on the other hand, shows an overall decrease in the Rpb1 levels (lanes 7-10), but in higher exposures (not shown here), this strain also shows the presence of polyubiquitinated species upon addition of 4NQO. Interestingly, the Δnot4 strain does not have any visible smearing of proteins above the primary band (lanes 21 – 24). This could be the expected result of loss of polyubiquitination of Rpb1 in the not4 mutant. The 8WG16 antibody blot (figure 3.2) does not detect these higher bands possibly because 8WG16 may be weaker at detecting Rpb1 than the 4H8 antibody.
Figure 3.3: Immunoprecipitation of samples with 4H8 antibody, and probed with 4H8 antibody. Samples treated with MG132, 4NQO or both, were immunoprecipitated with the 4H8 antibody and probed with the same antibody. Loading concentrations were adjusted by values calculated from the Bradford's assay.
To follow up on the verification of overall Rpb1 protein concentrations in the strains, Figure 3.4 shows the input samples treated with MG132, 4NQO or both chemicals and probed with the anti-Rpb1 antibody (4H8). The input samples are not immunoprecipitated with any antibody and are just loaded with equal concentration of cell extract for each strain. There is a recurrence of wildtype and pre1,2 samples in the two gel columns because all the samples did not fit into one gel and these repeated samples act as a control for transfer of samples from the gel to the membrane. In both exposures, the higher order modified Rpb1 species is visualized in each sample set, there appears to be an increase in the modified Rpb1 species (higher molecular weight and smearing in the gel) with the treatment of the cells with 4NQO in the presence of MG132. However, in the Δnot4 strains, there is a marked absence of the higher molecular weight species in any of the lanes. Additionally, the Δccr4 strain also appears to show modification of Rpb1 to a lesser degree than wild-type or Δnot5 strains.
Figure 3.4: Sample inputs probed with 4H8 antibody, treated with MG132, 4NQO and both chemicals. Inputs were loaded with equal concentrations determined from Bradford’s assay. The blots were probed with 4H8 antibody. The top panel shows the blots exposed for 30 secs and the bottom for 1 min.
To confirm that the smearing observed in the inputs is ubiquitinated Rpb1, immunoprecipitations were performed using the 4H8 antibody for Rpb1 (figure 3.5) and anti-HA antibody for ubiquitinated proteins (figure 3.6), and probed with the anti-HA antibody and anti-Rpb1 (4H8) antibody respectively. All the strains contained the ubiquitin-HA plasmid that over-expressed Ub-HA. Under normal growth conditions, proteins are regularly ubiquitinated and degraded as part of house-keeping within the cell. Consequently, all strains except for the Δnot4 strain showed ubiquitination of proteins in strains that were left untreated, as seen in figure 3.5, lanes 1, 3, 11, 15 and 17.

In figure 3.5, the immunoprecipitation with 4H8 antibody pulls down Rpb1 and other proteins associated with it. Probing with anti-HA visualizes all the ubiquitinated proteins that have immunoprecipitated, in addition to Rpb1. Therefore, it is not conclusive from this IP alone that the protein modifications seen in the blot are indeed polyubiquitinated Rpb1. In figure 3.6, all ubiquitinated proteins were immunoprecipitated with the anti-HA antibody and the blot was probed for Rpb1 using the 4H8 antibody. The smearing in this blot also shows the ubiquitinated species associated with Rpb1 that is detected by the antibody and it is comparable to the blot in figure 3.5 indicating that the smearing seen in figure 3.5 could also be Rpb1 poly-ubiquitination.

In both blots, there appears to be an increase in the smearing of ubiquitinated Rpb1 in the wildtype and not5 mutant strains upon 4NQO addition (lane 4, 12 and 18 from both figures 3.5 and 3.6). The ccr4 mutant shows intermediate effect with lesser smearing overall (lanes 7 – 10) and the not4 mutant does not appear to show any higher order ubiquitinated species of Rpb1 (lanes 21 – 24). MG132 acts as a proteasome inhibitor and should allow for better detection of ubiquitinated proteins before they are degraded upon treatment with 4NQO, but the effect was not visible in the samples (figure 3.5 and 3.6 lanes 6, 10, 14, 20).

Both the IP and Input samples (figures 3.4, 3.5 and 3.6) show higher molecular weight proteins (presumably polyubiquitinated species of Rpb1) upon 4NQO addition in at least three replicates (only one replicate of each strain is shown in each figure). Interestingly, Δccr4 strains showed an intermediate phenotype for the ubiquitination judging by the amount of smearing detected in lanes 7-10 figures 3.4, 3.5 and 3.6. The amount of smearing was decreased in all replicates of Δccr4 when visibly compared to the
wildtype or *not5* mutant samples, but was not absent as in the case of the Δ*not4* strain. The results imply that Not4 is required for the poly-ubiquitination of Rpb1 upon 4NQO stress and the absence of Ccr4 may affect the role of Not4 in some way. Alternatively, Ccr4 may also play a supporting role in this function. The absence of Not5 does not have any significant effect on the ubiquitination of Rpb1. These results are further discussed in Chapter 4.
Figure 3.5: Immunoprecipitation of strains with 4H8 antibody and probed with anti-HA antibody. Samples were loaded on the gel in equal concentrations deduced from the Bradford’s Assay. Blots were probed with anti-HA antibody to detect ubiquitinated species. Each strain contains a plasmid-expressed ubiquitin tagged with HA. Samples were treated with MG132, 4NQO or both.
Figure 3.6: Immunoprecipitation of strains with HA antibody and probed with 4H8 antibody. Samples were run on a gel with equal concentrations deduced from the Bradford’s Assay. Blots were probed with 4H8 antibody to detect ubiquitinated Rpb1 species. Each strain contains a plasmid-expressed ubiquitin tagged with HA and the strains were treated with MG132, 4NQO or both drugs.
The results of the immunoprecipitation indicate a very interesting phenomenon in the ubiquitination of Rpb1. The assay identifies any Ubiquitin-HA bound proteins and the presence of a band in each of the Δnot4 strains (lanes 21-24 figure 3.5) could indicate that the Rpb1 detected is possibly mono-ubiquitinated PolII and is identified via the HA tagged ubiquitin bound to it. It has already been shown before that the process of ubiquitination of Rpb1 occurs in a two-step process; first a single ubiquitin mark is added onto Rpb1 which serves multiple purposes as described in the introduction section 1.5, and then Rpb1 is polyubiquitinated either at the same site, or a different one.

Alternatively, the presence of a band in the Δnot4 corresponding to Rpb1 could be because the IP was done under native conditions and Rpb1 could be pulled down indirectly through a different subunit of PolII that may be ubiquitinated. Studies have shown the ubiquitination of Rpb2, Rpb7, Rpb6 and Rpb7 detected in a large-scale post-translational modification (PTM) screen in yeast (Beltrao et al. 2012).
3.3.2 6AU treatment

In order to understand how Not4 functions, the strains were tested using another chemical 6-Azauracil (6-AU). As described in section 3.2.4, 6-AU leads to a reduction of intracellular GTP levels which blocks transcriptional elongation. Adding 6-AU serves two purposes: On the one hand, it prevents new production of Rpb1 that could alter the result showing the degradation of Rpb1, and on the other hand it acts as a stress itself by arresting PolIII on the DNA, causing a situation similar to DNA damage induced stress. The purpose of the experiment was to observe if the ubiquitination of Rpb1 could be detected upon 6-AU treatment, and if Not4 was required for this ubiquitination as well.

The experiments for 6AU treatment were done using TCA precipitation and only observed the amount of ubiquitinated smearing in each strain. As shown in figure 3.7, strains show an increased smearing upon addition of 6AU in lanes 4 and 10. The band seen in between lanes 2 and 3 is an overflow of sample from lane 2. The proteasome mutant in lanes 1 and 2 do not show much increase in ubiquitination upon addition of 6-AU. The smearing below the Rpb1 band may be partially degraded products due to the addition of 6-AU. However, both the Δnot4 strain and the Δccr4 strain showed little or no smearing (lanes 6 and 8); indicating that once again Not4 is required for the ubiquitination and subsequent degradation of Rpb1. Additionally, it appears that the Δccr4 also is deficient in the ubiquitination and degradation of Rpb1, implying that Ccr4 may also play a role in this function, as observed in the experiments conducted with 4NQO. Not5 on the other hand does not affect the functioning of Not4 since the smearing of ubiquitinated proteins in the Δnot5 strain is similar to that of the wild-type strain.
Figure 3.7 TCA precipitation of 6AU treated cells to study ubiquitination of Rpb1 in mutants in the Ccr4-Not complex. The order on the blot from left to right: Proteasome mutant, wildtype Δpdr5, Δpdr5 Δnot4, Δpdr5 Δccr4 and Δpdr5 Δnot5 with and without 6AU. Smearing in the lanes may be an indication of ubiquitinated species as the smearing is increased in the samples with 6AU treatment.
3.4 Discussion

The main hypothesis of this thesis was that Not4 is required for the stress-induced poly-ubiquitination of Rpb1. The results from this chapter indicate a lack of poly-ubiquitinated species in the absence of Not4, signifying that the hypothesis is valid. While Not4 may not be the only protein involved in this process, it is likely that Not4 plays a functional role either by directly ubiquitinating Rpb1 or indirectly ubiquitinating another factor that is responsible for this process. One alternative hypothesis mentioned previously was that Not4 could lead to the proteasome-mediated processing of Def1, which causes it to be internalized into the nucleus where it aids in the ubiquitination of Rpb1.

It is also suggested from the results that Ccr4 may also play an accessory or supporting role in the ubiquitination and degradation of Rpb1, resulting in a decrease of poly-ubiquitinated species in the Δccr4 strains. In addition to its primary role as a deadenylase in the cell, deletion of Ccr4 also lead to an increased density of RNA PolII along the gene. This was observed to a larger extent in the Δnot4 strain implying that both Ccr4 and Not4 assist PolII in traversing the gene during transcription (Kruk et al. 2011). Surprisingly, deletion of Not5 did not have any significant effect on the ubiquitination of Rpb1, although deletion of Not5 has been shown in other studies to be lethal when combined with the deletion of Not4 (Villanyi et al. 2014). Not5 also plays a role in transcription regulation as well as in the tri-methylation of histone H3 K4 (Collart et al. 2013) indicating that its role may be connected with Not4 (Mulder, Brenkman, et al. 2007).

The mechanism of the stress-mediated ubiquitination and degradation is in response to DNA damage induced arrest of the transcription machinery. To replicate those conditions experimentally, the strains were treated with 4NQO. This proved to be effective in preliminary experiments (shown in the introduction figure 1.7) where Rpb1 turnover was decreased in Not4 mutants when compared to wild-type strains. This line of investigation was pursued by tagging Ubiquitin with a HA tag to visualize it better. Immunoprecipitating with anti-HA antibody resulted in an overabundance of ubiquitylated species being pulled down and an inefficiency to detect Rpb1. Hence the only reasonable way to identify ubiquitininated Rpb1 is to immunoprecipitate Rpb1 using the 4H8 antibody and then probe for ubiquitinated species.
This technique proved to be effective and showed a lack of poly-ubiquitination of Rpb1 not only in the IP but also in the inputs themselves, indicating that the loss in poly-ubiquitination was not an artifact of the IP process. Addition of the proteasome inhibitor MG132 also increased the detection of poly-ubiquitinated Rpb1 before it was degraded by the proteasome. This too did not have any change in the Not4 mutant. The requirement of Not4 is undeniable, however the function is still unclear.

Furthermore, the Δnot4 mutant revealed an interesting detail regarding the process of ubiquitination of Rpb1. One suggestion from the result is that Rpb1 is ubiquitinated even in the Δnot4 strain, but the lower molecular weight of the band implies it may be a mono-ubiquitinated protein. Therefore, while it can be deduced that Not4 is required for the polyubiquitination of Rpb1, Not4 is not the only E3 ubiquitin ligase that places its ubiquitin mark on the polymerase. Alternatively, Rpb1 could be pulled down indirectly with another subunit of PolII that is ubiquitinated. Post-translational modifications of Rpb2, Rpb7, Rpb6 and Rpb7 were detected using mass-spectrometry and it was identified that these proteins are also ubiquitinated (Beltrao et al. 2012).
Chapter 4: Discussion of results

The results shown in the experiments described in this thesis suggest a new dynamic in the coordination of cellular response to DNA damage. All the proteins described have multiple functions that overlap. This thesis develops a new role for this complex in the stress-responsive degradation of the largest subunit of RNA Polymerase II. The Ccr4-Not complex’s previously identified functions in transcriptional elongation and mRNA decay suggest that its new role may be one part of an intricate response that is well-regulated by the cell.

The Ccr4-Not complex has been identified as a key player in multiple cellular functions ranging from transcriptional elongation to ribophagy and mRNA decay. The different subunits of the complex coordinate their functions with each other. The deletion of one subunit can lead to the alteration of the function of another subunit. Ccr4 is not only the primary deadenylase, but also has secondary phenotypes in transcription elongation. One such phenotype was observed (Kruk et al. 2011) in both the Δccr4 and Δnot4 strains, and resulted in the build-up of RNA PolII along the gene. This build-up could trigger the recruitment of other cellular response factors such as the GGR or TCNER factors. The result of PolII collection along the transcript may be because of the loss of polyubiquitination of Rpb1 due to the deletion of Not4 as seen in the results in this thesis. The partial decrease in polyubiquitination of Rpb1 in the Δccr4 strain that was also seen in the western blot experiments could be a secondary effect where Ccr4 assists in promoting elongation, and loss of Ccr4 results in the stalling or arrest of PolII along the transcript. The Ccr4-Not also functions in the proteasome-mediated degradation of Yap1 (Gulshan et al. 2012), ubiquitination of Jhd2 (Douglas P. Mersman et al. 2009) and modulates Ubc4p/Ubc5p-mediated stress responses in vivo (Mulder, Inagaki, et al. 2007). These functions of the Ccr4-Not complex not only position it in a strategic way to assist in stress response at the transcriptional level, but also indicate that the complex has been previously identified in stress response functions. This suggests that the hypothesis and results presented in this thesis could add to the previously known functions of the Ccr4-Not complex and broaden the understanding of both the complex and other regulatory factors involved in cellular stress response.
Multiple studies have shown the role of Ubp3 in the removal of ubiquitination marks from several proteins. In addition to Rpb1 (Kvint et al. 2008), Ubp3-Bre5 also functions in transcriptional activation by reversing the ubiquitination of Tbp1 (TATA-binding protein 1) (Chew et al. 2010b). It plays a role in heat resistance by destabilizing misfolded proteins (Öling et al. 2014). However, Ubp3 also plays an antagonistic role in the negative regulation of several factors. It plays a role in UV resistance by targeting Rad4 for degradation. Ubp3 interacts with the 26S proteasome and loss of Ubp3 results in the UV resistance in cells (P. Mao & Smerdon 2010). Ubp3 is also involved in ribophagy and is required for specific pathways that activate under nitrogen starvation in yeast (Ossareh-Nazari et al. 2014). It was shown that Ltn1, an E3 ligase, acts antagonistically to Ubp3. Ubp3 is itself a target for phosphorylation under osmostress, and is recruited to osmoreponsive genes to modulate transcription (Solé et al. 2011).

Although the proteins studied in this thesis all function independently of each other, they are interconnected more than just by physical interaction. RNA PolII interacts with Ubp3/Bre5 and the Ccr4-Not complex physically and functionally. The Ccr4-Not complex as described earlier, assists in transcription and mRNA decay, and from the results shown in this thesis, Not4 is also essential for Rpb1 poly-ubiquitination. PolII also interacts with the Ubp3/Bre5 complex which functions to reverse the poly-ubiquitination of Rpb1. This suggests that there may be a coordinated degrade/rescue mechanism between the Ccr4-Not complex and Ubp3/Bre5. Both Ubp3/Bre5 and Not4 play opposing roles in the degradation of Rpb1, and both proteins are recruited to PolII either independently or dependent on some tertiary factor such as mRNA, or an as yet undiscovered protein. It is also possible that the stress-induced arrest of PolII results in a mass recruitment of many proteins including Ubp3/Bre5.

Several studies have looked into the coordination of stress-response and gene transcription (de Nadal et al. 2011) and specific gene expression responses to stress were seen in cultured human cells (Murray et al. 2004). Even with the studies done in the lab with the Ccr4-Not complex (Miller, Reese, unpublished) it was observed that the deadenylase Ccr4 is recruited to a group of mRNA under unstressed conditions, and a specific subset of mRNA under stressed conditions. It is predicted that a similar recruitment of Ubp3-Bre5 to specific mRNA may exist, leading to a coordinated promotion
of gene-transcription by deubiquitinating Rpb1. This specific stress response may be initiated by targeting mRNA required for the specific response and the recruitment of Ubp3-Bre5 to PolII may be through the identification of specific mRNA.

To follow up on the idea that the recruitment of these proteins to PolIII may have mRNA as the common denominator, the recruitment of these protein complexes to a common mRNA was explored. Interesting, Ubp3 is recruited to one of the mRNAs studied that the Ccr4-Not complex is also highly recruited to (as measured by the associated protein – Dhh1). With the previous deduction of the coordinated roles of Ubp3/Bre5 and the Ccr4-Not complex, it is likely that the coordination could be intermediated through mRNA. In our other studies on the Ccr4-Not complex, it was discovered that Ccr4 is selectively recruited to a certain subset of mRNA under unstressed conditions, and this subset is diminished upon hydrogen-peroxide induced stress. The hypothesis is that since Ccr4 is the primary yeast deadenylase, it targets mRNA that are non-essential for the cell upon stress. In order for the cells to recover from the stress, Ccr4 is no longer recruited to mRNA that are necessary for cell survival. This led to a potential similar hypothesis for Ubp3/Bre5, except in the opposite direction. Ubp3/Bre5 rescues Rpb1 from degradation upon stress-induced arrest. When the cell encounters stress, it requires an up-regulation of certain mRNA and a down-regulation of others (Gasch 2002). Ubp3/Bre5 could identify the essential mRNA and be selectively recruited to rescue PolII and resume transcription, while the Ccr4-Not4 complex could assist in this regulation by targeting the corresponding down-regulated mRNA for degradation. Simultaneously, Not4 could facilitate the termination of transcription on these mRNA by targeting Rpb1 for degradation.

It is also likely that the interaction with RNA could be independent of its role in polymerase rescue from degradation. Both Ubp3 and Bre5 have been known to be involved in ribophagy as discussed earlier (Kraft et al. 2008). This function may require an association with RNA to regulate the degradation of mature ribosomes. Alternatively, because the immunoprecipitation assays indicated that Ubp3/Bre5 may be associating with the Ccr4-Not complex (figure 2.3), their interaction may also be independent of the polymerase. Ccr4-Not plays various roles in the cytoplasm and Ubp3/Bre5 could have alternative functions in mRNA decay. Figure 4.1 shows some possible interactions of Ubp3/Bre5 with RNA and the predicted function of these interactions.
While Not4 was shown to be necessary for the polyubiquitination of Rpb1, it was also observed that Rpb1 appears to be monoubiquitinated even in the absence of Not4 suggesting that the mechanism of ubiquitination and degradation of Rpb1 may be a multi-step process involving other ubiquitin ligases. Proteins are ubiquitinated for several reasons and functions as discussed earlier, and Rpb1 showed monoubiquitination under unstressed conditions, implying that this ubiquitination may be independent of Not4, but may be required for the subsequent polyubiquitination by Not4. One proposed theory is that the Elongin-Cullen complex, which was shown to also be required for the Def-1 mediated degradation of Rpb1 (Marcus D. Wilson et al. 2013), may place the initial ubiquitin mark on Rpb1, and Not4 may be responsible for the poly-ubiquitination mark. Both proteins function with the same substrate but under different conditions. The mono-ubiquitin mark by the Elongin-Cullin complex may serve as a target for Not4, which would then target the Rpb1 for degradation.
4.1 Proposed Model

In the model described below, two possible interactions of Ubp3/Bre5 with the Ccr4-Not complex are illustrated. In the first image, the interaction of Ubp3/Bre5 with the Ccr4-Not complex and mRNA is described by the bridging of the two with Ubp3/Bre5. Additionally, Ubp3/Bre5 recruitment to RNA Polymerase II is described as a recruitment via either the Ccr4-Not complex, or the mRNA transcript.

It is possible that the interaction of Ubp3/Bre5 to the Ccr4-Not complex could be through the recruitment to the emerging mRNA transcript. The prior studies with the Ccr4-Not complex show a large group of mRNA that Ccr4 is recruited to, and this set of mRNA changes during the cellular stress response. One possibility is that under stress-response, the Ccr4-Not complex is recruited to mRNA either to control the degradation of the mRNA or to regulate the transcription by Rpb1 degradation. Subsequently, Ubp3/Bre5 may also be recruited to specific mRNA along with the Ccr4-Not complex to prevent the degradation of Rpb1. It is also possible that the recruitment of Ubp3 and Bre5 may be independent of mRNA, or could vary depending on other factors. The necessity of Bre5 for the recruitment of Ubp3 to mRNA also needs to be further explored. The recruitment of Ubp3 to SKN7 and PYK1 from figure 2.5 and 2.6 showed that Ubp3 may be recruited to certain mRNA in a Bre5 dependent manner, and not to others.

Another alternative shown in the second figure is that the association of Ubp3/Bre5 to the Ccr4-Not complex may be independent of its role with RNA PolII. The Ccr4-Not complex plays multiple roles, such as deadenylation, which requires association with mRNA. It is possible that Ubp3/Bre5 may be recruited to mRNA at this stage to counter the deadenylation. This too may be in a mRNA specific manner, and could act as a stress response in the cell, preventing certain mRNA from being degraded.

A final alternative is that the recruitment of Ubp3/Bre5 and the Ccr4-Not complex may be entirely independent of each other. Ubp3/Bre5 may be recruited to PolII via the mRNA during stress response, or in its role in ribophagy (Kraft et al. 2008). Additionally, Ubp3/Bre5 could also play an as yet unstudied role by its association with mRNA.
Figure 4.1: Diagram showing a possible model of association of Ubp3/Bre5 to RNA.
(a) From the immunoprecipitation studies shown in figure 2.3, it is likely that Ubp3-Bre5 associates with both PolII and the Ccr4-Not complex. Ubp3 is also recruited to mRNA, so this figure incorporates both interactions to produce a hypothetical interaction of Ubp3-Bre5 with polymerase through mRNA transcript. (b) Bre5 was also shown to be required for the association of Ubp3-Bre5 complex with the Ccr4-Not complex. Both SKN7 and PYK1 are targets for the Ccr4-Not complex, and Ubp3 is also recruited to these mRNAs. This recruitment is hypothesized in the second figure.
4.2 Future Studies

The association of Ubp3/Bre5 with PolII and the Ccr4-Not complex could be indirectly mediated by other proteins or nucleic acids. Treating the samples to RNase or DNase and repeating the protein immunoprecipitation assays would determine if the association is indirectly through RNA or DNA. To uncover the full potential of the proposed hypothesis regarding the selective recruitment of Ubp3/Bre5 to mRNA, it is necessary to conduct a genome-wide analysis of the recruitment of Ubp3 to mRNA and compare these results with the set of results from Ccr4 recruitment. Following this up with a stress-induced assay would shed some light on the cell’s stress response. Additionally, if the degradation or rescue of Rpb1 is dependent on the mRNA transcript, future experiments could be conducted using purified proteins – RNA PolII, Ubp3/Bre5 and the Ccr4-Not complex – in an in-vitro study to understand the effect of each protein on the ubiquitination and deubiquitination of Rpb1. This assay would also confirm if Not4 can directly ubiquitinate Rpb1.

Furthermore, since it has been shown in previous studies that the Elongin-Cullin complex (Ela1-Elc1) can also ubiquitinate Rpb1 under stress-induced arrest (Marcus D Wilson et al. 2013), it is necessary to pursue the functional difference between the Elongin-Cullin complex and Not4. It is likely that the method or site of ubiquitination is different in either case; or the type of stress-induced arrest could determine which protein complex induces the ubiquitination and degradation of Rpb1. It is also likely that the Ubp3/Bre5 complex may interact with the Elongin-Cullin complex. A ubiquitin mark on one residue of Rpb1 could recruit the Ccr4-Not complex to PolII, while a ubiquitin mark on another residue could recruit the Ubp3/Bre5 complex instead. More research needs to be done in order to understand the functions of these protein complexes.
**Tables**

**Table 3: Primer sequences used for tagging and deleting Ubp3 and Bre5**

<table>
<thead>
<tr>
<th>Protein tagged/deleted</th>
<th>Primer location</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tagging Bre5</td>
<td>5’ end primer plus strand</td>
<td>5’-AATGGAACACGTTCATAGAAAGCAA CCCCTAAAAAGAAAGGAGCCGGATCCCCGG GTTAATTAA-3’</td>
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<tr>
<td></td>
<td>3’ end primer minus strand</td>
<td>5’-ATACAGTTTCTTTTCAATTTTCTAG ATTTTTAATTAGCGGGTTTGAATTGAGCT CGTTTAAAC- 3’</td>
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<tr>
<td>Deleting Bre5</td>
<td>5’ end primer</td>
<td>5’-GCATTTGAAGTCATACCTCGAATAGA AGTATCAAATAAAGAAACCGGATCCCCGG GTTAATTAA – 3’</td>
</tr>
<tr>
<td></td>
<td>3’ end primer</td>
<td>5’-TTTTATTATTATTATTATTCTTTT TAAAAGGCTTTGTTGAGAATTGAGCCT CGTTTAAAC- 3’</td>
</tr>
<tr>
<td>Deleting Ubp3</td>
<td>5’ end primer</td>
<td>5’-ACCATCATCCAGGTACCCTTTTCTTTT CCATCATCATATTAAAAACCGGATCCCCGG GTTAATTAA – 3’</td>
</tr>
<tr>
<td></td>
<td>3’ end primer</td>
<td>5’-CTATATTATTATTATTATTGTATTTCTCT ATAAATACCCCACTCGAATTGAGCT CGTTTAAAC- 3’</td>
</tr>
<tr>
<td>Tagging Ubp3</td>
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<td>5’-TCTGATTCGAGGACTGCTATTATTTTA ATGTATAAAAGAGAAATCGGATCCCCGG GTTAATTAA</td>
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<td></td>
<td>Downstream</td>
<td>5’-CCAAAGCATAAAGTGTAACTCTGTCTCT GTGTCTGTCTCTATTCTGAAATTGAGCT CGTTTAAAC</td>
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### Table 4: List of strains generated or used in the study

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<tr>
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<th>Details</th>
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<td>MAT alpha Δubp3::kanMX BRE5-3HA::HIS3 leu2Δ0 met15Δ0 ura3Δ0</td>
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<tr>
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<tr>
<td>JR1588</td>
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<tr>
<td>JR1552</td>
<td>MAT a his3Δ1; leu2Δ0; MET15?; ura3Δ0 not5Δ::HIS3;</td>
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References


