A GENETIC AND BIOCHEMICAL ANALYSIS OF THE ROLE OF THE CCR4-NOT COMPLEX IN TRANSCRIPTION ELONGATION AND RECOVERY FROM DNA DAMAGE IN BUDDING YEAST

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ABSTRACT

Cells require proper gene transcription in order to produce functional proteins. DNA damage, one obstacle to transcription elongation by RNA Polymerase II (RNAPII), is repaired through pathways such as transcription-coupled repair (TCR). TCR begins when cell machinery senses that RNAPII has stalled on a gene. Before DNA-repair proteins act on the mutation site, RNAPII must be removed, perhaps through ubiquitin-dependent proteolysis of Rpb1, its large subunit.

Studies in budding yeast _S. cerevisiae_ have shown that the Rpb1 is degraded in response to DNA damage, but the complete mechanism behind RNAPII turnover remains unclear. The multisubunit Ccr4-Not complex is already known to act in transcription regulation via its roles in nuclear processes such as chromatin remodeling, DNA repair, transcription elongation and activation, and RNA processing and regulation. This complex has two major catalytic activities, deadenylation and ubiquitylation. Intrigued by the ubiquitin-ligase activity of Ccr4-Not, we investigate its role in Rpb1 degradation for TCR.

Our experiments indicate that Ccr4-Not subunits Ccr4 and Dhh1, which are involved in mRNA turnover, did not have a noticeable impact on Rpb1 degradation. However, Rpb1 levels in Ccr4-Not mutants under DNA-damaging conditions suggest that Not4, a ubiquitin ligase, does play a role in RNAPII turnover. Not4 possesses a Really Interesting New Gene (RING) domain, a zinc-finger motif responsible for the function of many E3 ubiquitin ligases. Our experiments indicate that the Not4 RING domain is responsible for this subunit’s role in Rpb1 turnover under DNA-damaging conditions. Further investigation will verify this and determine whether Not4 plays a direct or indirect part in Rpb1 ubiquitylation and degradation. By clarifying the role of
Ccr4-Not in the removal of RNAPII for TCR, our studies will shed light on the manner in which cells handle genomic damage.
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CHAPTER 1
INTRODUCTION

1.1 RNA Polymerase II and Transcription

There are three major RNA Polymerases in eukaryotes, labeled RNA Polymerase I, II, and III. Of these, RNA Polymerase II (RNAPII) is responsible for transcribing all protein coding genes from DNA to mRNA. RNAPII is also responsible for transcribing regulatory RNA molecules such as small snoRNA, miRNAs, snRNAs, and siRNAs (2). RNAPII varies in size depending on the organism, but in *S. cerevisiae* is 0.5 megadaltons and comprised of 12 subunits, Rpb1-Rpb12 (4, 17). Besides *RPB4* and *RPB9*, the genes coding for these subunits are necessary for life (4). Rpb1 and Rpb2 are the largest and most conserved subunits of RNAPII, and specific mutations in these genes indicate that Rpb1 and Rpb2 are necessary for both selecting the transcription start site and processing through obstacles during the elongation phase.

Prior to transcription, DNA enters a cleft comprised of Rpb1 and Rpb2 as it travels to the active site on Rpb1 (17). This cleft is porous so that nucleotides and elongation factors can access the site during transcription. Rpb1 and Rpb9 on one side of the cleft and Rpb5 on other form “jaws” that keep this DNA in position (Figure 1.1). Rpb1 also has a highly conserved C-terminal domain (CTD), which is composed of a Tyr-Ser-Pro-Thr-Ser-Pro-Ser repeat (4). The length of this repeat increases in accordance with the complexity of the organism, consisting of 26/27 repeats in *S. cerevisiae* and 52 repeats in humans. The CTD undergoes small-molecule modification during the course of transcription, which impacts its role as a docking site for both transcription regulators and RNA processing proteins. For example, phosphorylation of Ser5 on the CTD prompts the transition from transcription initiation to elongation. CTD phosphorylation
is also necessary for its association with RNA processing proteins, particularly those responsible for adding the 5’ cap to the mRNA and cleaving and polyadenylating the nascent transcript (57).

Other RNAPII subunits act in processes important for the fidelity and modification of the transcript. Rpb4/7 are functionally related putative factors in transcription initiation and mRNA export, decay, and translation (28). These factors are not as closely associated with the rest of the complex, and seem to shuttle between RNAPII molecules, associating in part with Rpb6. Rpb3 is involved in RNAPII assembly. Rpb9 appears to impact selection of the transcription start site, and also interacts with TFIIS in conjunction with Rpb6. In addition, Rpb9 and Rpb4 appear to act in transcription-coupled repair (TCR) pathways.

Impressively, RNAPII can unwind DNA, synthesize RNA, and self-correct by backing up and performing an excision reaction if it adds an incorrect ribonucleotide (4). However, proper transcription relies on the assistance of many other factors, from the recruitment RNAPII all the way to the termination of transcription. First of all, transcription-activating proteins are necessary to bind DNA and attract RNAPII to certain genes. Transcription occurs in three phases: initiation, elongation, and termination. Initiation begins with the help of general transcription factors (Figure 1.2). Firstly, the TBP (TATA-binding protein) of TFIIID binds the TATA box present in the promoter region of the gene to be transcribed, about 25 nucleotides upstream of the start site. TFIIID distorts the DNA, probably signaling other transcription initiation factors to assemble, along with RNAPII, into the transcription initiation complex. TFIIIB binds adjacent to the TFIIID TBP, recognizing the 7 nucleotide, cis-regulatory BRE element of the promoter just upstream of the TATA box. A protein complex called Mediator facilitates communication between RNAPII, transcription activating proteins, and general transcription factors. Other elements of the transcription initiation complex might bind in different orders, but additional
general transcription factors such as TFIIF, TFIIE, and TFIIH help the process along. TFIIF stabilizes the RNAPII-TBP and RNAPII-TFIIB interaction and helps attract TFIIE and TFIIH. TFIIE regulates and attracts TFIIE, which has both ATP-dependent DNA helicase and kinase domains. TFIIE unwinds DNA, allowing RNAPII to synthesize short, incomplete RNA transcripts. Once TFIIE phosphorylates Ser5 of the Rpb1 CTD, RNAPII can disengage from the other general transcription factors and enter the elongation phase. The Rpb1 CTD thus transitions between hypophosphorylated (II_A) and hyperphosphorylated (II_o) forms when it progresses from the initiation to the elongation phases. During elongation, RNAP unwinds the DNA and uses the principle of complementarity with the template DNA strand (A-U and C-G) to add bases 5’ -> 3’ to the growing RNA transcript. RNAPII adds these bases by hydrolyzing the high-energy phosphoanhydride bond of the ribonucleotide to synthesize the phosphodiester bond that attaches the correct base to the molecule. This unwound DNA-RNA hybrid site is 8-9 bps. During the elongation phase, RNAPII requires assistance from chromatin and histone-modifying enzymes to remove barriers to transcription. Other elongation factors utilize different mechanisms to assist RNAPII during the elongation phase (discussed subsequently). The transcription termination process involves destabilizing the DNA-RNA hybrid in the active site.

There are two main pathways in eukaryotic organisms; poly(A)- and sen1-dependent termination (56). Sen1-dependent termination occurs on mostly non-coding RNAs. Poly(A) termination occurs on most protein coding genes, and is a two-step process facilitated by proteins bound to the Rpb1 CTD. The core sequence (AAUAAA) recognized by cleavage and polyadenylation-stimulation factor (CPSF) not only directs cleavage and polyadenylation of the nascent transcript, but also signals RNAPII to stop transcription. The transcript is cleaved between the core sequence and a G-U rich region bound by cleavage-stimulation factor (CstF).
The upstream cleavage product receives a poly(A) tail and is processed further, and the downstream product is degraded. The mechanism of RNAPII detachment from DNA is complicated and not fully known. This basic outline of transcription varies from event to event by the presence of different regulatory proteins, environments, and obstacles RNAPII encounters during the process.

1.2 Transcription Elongation

Elongating RNAPII frequently stalls as it transcribes a gene, even when reading through a virtually error-free template. Elongation factors help RNAPII along, and become even more important when RNAPII encounters obstacles to transcription such as mistakes in the RNA transcript or DNA template that cause it to arrest completely. A variety of methods are used to provide support for the role of candidate species in transcription elongation. Some factors are identified by their activities \textit{in vitro} on purified DNA complexes, or in cells by chromatin immunoprecipitation (2). Others are characterized by their mutant phenotypes such as sensitivity to drugs like mycophenolic acid (MPA), hydroxyurea (HU), or 6-azauracil (6-AU), all of which lead to slow-growth phenotypes in cells deficient in elongation factors. Still others are recognized by synthetic lethality when eliminated in conjunction with known elongation factors (59). TFIIS, a well-studied elongation factor, acts when 3’ cleavage of the transcript is necessary to fix an incorrect addition or extreme misalignment of the RNA transcript (35). When stalled on a gene, RNAPII can move forward and backward across the DNA, threading the transcript back and forth through the exit channel. When the transcript is displaced from the active site to a secondary funnel, TFIIS can insert its acidic C-terminal domain into the funnel and activate the nuclease activity of RNAPII to remove the incorrect part of the transcript (Figure 1.3).
Other elongation factors promote transcription through barriers found on the DNA template. These barriers include features of chromatin structure such as nucleosomes, as well as DNA damage in the form of abasic sites, bulky lesions, or a helix distorted by some other means. DNA damage may arise through replication error or environmental stress. The heterodimer Spt4/5 is another important elongation factor which helps maintain the DNA-RNA transcription bubble and might also stabilize the elongating transcript in conjunction with Rpb4/7 (43). Some elongation factors associated with chromatin remodeling include FACT (facilitates chromatin transcription), which destabilizes nucleosomes, and SWI-SNF, which helps remodel nucleosomes in an ATP-dependent manner so that RNAPII can transcribe through the DNA (Figure 1.4, 2). The PolII-associated factor complex (Paf1c), another elongation factor, promotes activator and histone-acetyltransferase-dependent transcription by recruiting Rad26/Bre1, which monoubiquitylate H2B (32, 36). Monoubiquitylated H2B recruits other proteins that help RNAPII transcribe through the gene. In addition, Paf1c associates with TFIIS and possibly recruits it to RNAPII and stabilizes it on the complex. Experiments in RAD26 and DST1 (TFIIS) single and double mutants suggest that Rad26, the yeast homologue of human transcription coupled repair factor Cockayne’s syndrome B (CSB), promotes transcription elongation independently from TFIIS by enabling RNAPII to add another ribonucleotide when stalled at a lesion (37, 64).

The THO/TREX complex is thought to promote elongation by preventing the formation RNA–DNA hybrids (R-loops) by either binding the mRNA or assembling heterogeneous nuclear ribonucleoproteins (hnRNPs) onto the transcript (39). The Ccr4-Not complex, which will be discussed subsequently, is another factor that promotes elongation by a mechanism separate from TFIIS (35). Other elongation factors include TFIIF, elongin, elongator, the 19S proteasome, and
other chromatin-remodeling, DNA repair, and RNA processing proteins (4, 14). The identity, mechanisms, and interactions of elongation factors lie waiting to be discovered. The ways in which the cell contends with transcriptional stalling and arrest comprise an area rich with opportunity for investigation.

1.3 Transcription-coupled repair

The cell preferentially repairs DNA damage on the transcribed strand over the nontranscribed strand, even if both strands are equally accessible (44). This is because completely arrested RNAPII, more so than transiently paused RNAPII, indicates the presence of damaged DNA and prompts the recruitment of repair factors for transcription-coupled repair (TCR). TCR is a conserved process that utilizes different DNA repair pathways, most notably nucleotide excision repair (NER) and base excision repair (BER).

NER involves the use of a few proteins that repair many different types of damage, such as bulky lesions like those induced by ultraviolet radiation and the DNA-damaging agent 4-nitroquinoline 1-oxide (4NQO). In yeast, Rad4/Rad23 first recognize the lesions, then the Rad25 and Rad3 helicase subunits of TFIIH unwind the DNA in an ATP-dependent manner. Rad14 and single-stranded DNA binding protein RPA (replication protein A) bind the DNA and allow for endonucleases Rad2 and Rad1/Rad10 to make single-stranded breaks to remove the damage whereupon DNA polymerase can fill in the missing sequence and DNA ligase can seal the nicks (25).

BER is different from NER in that it involves many different proteins, each of which recognizes a specific type of damage. However, some of the base alterations and oxidative lesions that are substrates for BER are also NER substrates. In the general BER process, DNA
glycosylase removes the damage, then an apurinic/apyrimidinic (AP) endonuclease recognizes the abasic site and cleaves the phosphodiester bond upstream of the lesion, leaving a strand break with normal 3’ and abnormal 5’ ends. DNA polymerase β replaces the abasic residue with the correct nucleotide and DNA ligase seals the nicks (66).

The NER and BER pathways are complex but relatively well studied, whereas there is still much to discover of the mechanism of recognition of RNAPII stalled on a gene and recruitment of repair factors. One major problem TCR must overcome is the removal of stalled RNAPII from the site of DNA damage. It has been proposed that the first course of action in contending with arrested RNAPII involves elongation-factor-mediated backward displacement of the complex to remove RNAPII from the DNA-damaged site, thus exposing it to repair proteins. If RNAPII is unresponsive to attempts of elongation factors to displace it, the cell then turns to pathways leading to Rpb1 degradation and RNAPII dissociation to expose the damaged site (64, 42, 66, 74).

Many different models for RNAPII removal have been suggested (Figure 1.5). Arrested RNAPII itself initiates DNA damage recognition, and, in conjunction with other TCR factors, the recruitment of repair proteins (21). There are several well-known TCR factors including Rad26 and Rad28, Spt4/5, the Paf1 complex, and general transcription factors, however, additional TCR factors remain to be discovered. Rad26, which is required for TCR but not NER, plays a role in recruiting the BER pathway. It is a member of the SNF2 family of DNA-dependent ATPases and acts in chromatin remodeling as well as TCR. Most SWI/SNF-like complexes are involved in altering histone-DNA contact, therefore, it is possible that Rad26 promotes elongation through rearrangement of the DNA-RNAPII interface. There is also evidence that Rad26 assists TFIIH in transitioning from the DNA repair mode back to a transcribing mode (8, 72). Interestingly,
deleting *RAD26* prevents TCR, but does not confer UV sensitivity to the cell while deleting *RAD28* causes UV sensitivity, but does not result in TCR defects. Rad26 and Rad28 do not form a stable complex in the cell (8, 72). These results indicate some role for Rad28 and Rad26 in DNA damage repair, but the exact nature of their relationship is unknown. The elongation factor Spt4 is implicated in TCR by its ability to modulate Rad26 activity (31). Paf1c plays a role in TCR, but it is uncertain whether this role involves histone modification (21). Some general transcription factors and elongation factors have roles in both transcription and DNA repair. For instance, TFIIH is required for both NER and BER.

Other TCR factors such as Def1, Rsp5, and the Elongin-Cullin complex (see Discussion) play roles in the removal of RNAPII from the active site by Rpb1 degradation via the ubiquitin-proteasome pathway. It has been shown that Rpb1, but not other RNAPII subunits, are degraded in response to DNA damage, although Rbp4 and Rpb9 have been implicated in TCR (21, 42). Presumably, once arrested RNAPII triggers the need for Rpb1 degradation, many TCR pathways act in parallel to target Rpb1 for degradation to free the DNA template for repair, each pathway responding to different regulatory factors. Thus, TCR pathways often converge in labeling Rpb1 for proteasomal-mediated degradation by ubiquitylation.

**1.4 Ubiquitin-dependent degradation**

The ubiquitin-proteasome pathway acts in many cell processes, including cell division, differentiation, stress response, signaling pathways, DNA repair, transcription regulation, and organelle biogenesis (23). The pathway involves using the 76 amino acid molecule ubiquitin (Ub) to target a particular substrate for degradation in the 26S proteasome by using three enzymes, E1, E2, and E3 (Figure 1.6). First, a Ub-activating enzyme (E1) activates the C-
terminus of a ubiquitin molecule in a two-step, ATP-dependent reaction which proceeds through a covalent AMP-Ub intermediate (2). At the end of this reaction, Ub is attached via a high-energy thioester linkage to a cysteine side chain on E1. Next, this Ub molecule is transferred to the cysteines on a ubiquitin-conjugating enzyme (E2). This E2-Ub conjugate is then bound by a ubiquitin ligase (E3) which binds degrons (degradation signals) on a substrate, and enables the E2 to catalyze the formation of an isopeptide bond between its activated Ub and the N-terminus of a Lys residue on the substrate.

There are two main types of E3s, those with a HECT (homologous to the E6-AP COOH terminus) domain and those with a RING (Really Interesting New Gene) domain. HECT domain E3s have a catalytic role as Ub is transferred from the E2 to a conserved active site internal Cys on the E3 before being attached to the N-terminus of a substrate Lys residue (63). An example of a HECT E3 is E6-associated protein (E6-AP), which targets p53 for rapid degradation under certain conditions (63). In contrast to HECT E3s, RING-finger E3s catalyze the direct transfer of Ub from the E2 to the substrate (10, 62). RING finger E3s bind their E2s via the RING domain. The structure of RING domains involves a cross-brace of conserved Cys and His residues that bind two Zn$^{2+}$ ions (Figure 1.7). RING domains are present on single and multisubunit molecules. Mdm2 is an example of a monomeric protein with a RING domain involved in the regulation of p53 (11). The APC is a large, multisubunit complex that also has a RING domain, and degrades cell-cycle regulators (30).

Polyubiquitin chains are comprised of Ub molecules covalently linked from the C-terminus on one Ub to a Lys on the next. After the addition of the first Ub molecule by a specific E2/E3 complex, another E2/E3/Ubiquitin-E2-variant (UEV) complex carries out chain elongation (58). UEVs lack the active site Cys of E2s. The number and structure of Ub
molecules added to a substrate determines its fate (Figure 1.8). Substrate monoubiquitylation seems to signal for cellular localization but not degradation (30). Chains containing at least four Ub molecules are necessary for efficient binding to the proteasome (69). The relationship between Ub-chain length and proteasome-binding is not additive, since chains of four Ubs bind 100 times better than chains of two, but there is only a 10-fold increase when eight more Ubs are added. Though the poly-Ub chain is enough for the proteasome to bind the substrate, some E3s such as Dsk2 facilitate this process by associating with both the proteasome and substrate (23).

The ubiquitin-proteasome pathway has a hierarchical structure (Figure 1.9) as there is only one E1 in yeast (Uba1), eleven E2s (Ubc 1-8, 10, 11, 13), and many different E3s that bind multiple substrates, all heading to the 26S proteasome. The 26S proteasome is a 2.5 MDa complex that is present in the cytosol and the nucleus, making up nearly 1% of cell protein (Figure 1.10). The active site is contained in the 20S core particle (CP), which 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. Each end of the 20S CP is covered by a 19S regulatory particle (RP). This particle is comprised of a lid, which contains the non-ATPase subunits Rpn3, -5-9, and -11, -12, and a base, which contains the ATPases Rpt1-6 and non-ATPases Rpn1,-2,-10 (22). Rpn10 has a hydrophobic patch that binds to Ub chains (20). This is important because the function of the 19S particle is to act as a gate, unfolding properly ubiquitylated substrates and allowing them to pass into the CP. The 26S proteasome keeps whole substrate bound until it is digested into short peptides. In this manner, the ubiquitin-proteasome pathway plays a major role in many cellular processes.

1.5 The Ccr4-Not Complex
1.5.1 Overview of Ccr4-Not structure and function

Ccr4-Not is a conserved, multisubunit complex involved in regulating different stages of gene expression such as transcription, mRNA processing, translation, and protein degradation. It exists in \( \sim 1 \) and \( \sim 2 \) MDa states. The 1 MDa form consists of nine core subunits, Ccr4, Caf1, Caf40, Caf130, and Not1-5, while the larger form may also contain Dhh1, Dbf2, Caf4, Caf16, and Btt1 subunits (Figure 1.11, 7, 18). The NOT genes were identified in a screen for mutants with increased expression of compromised HIS3 gene, which is involved in histidine biosynthesis. Mutations in NOT genes mutations caused increased transcription from the constitutive TATA-less promoter over the regulated TATA-containing promoter, indicating that NOT genes act in repressing the TATA-less promoter (15). This is the basis for their name, NOT (Negative on TATA-less). Not1 is the only essential subunit in yeast, it forms an important scaffold and most other subunits interact with its C-terminus, although Caf1 interacts near its center (40). Although Not2 is the smallest subunit, it is important for complex integrity (61).

The function of the Ccr4-Not complex is dependent on its subunits appropriately associating with one another, as deleting each subunit had a unique effect on both the integrity of the complex and gene expression (5). For example, a NOT2 deletion resulted in the upregulation of exosome genes, while NOT3 and NOT5 deletions resulted in the upregulation of genes involving vacuolar function and the cell wall, respectively. Continuing in this vein, a CAF1 deletion resulted in the upregulation of genes involved in oxidative phosphorylation and respiratory-chain phosphorylation whereas a CCR4 deletion resulted in the downregulation of genes encoding histones. These results show that the genes regulated by Ccr4-Not are rarely dependent on multiple subunits of the complex, indicating that Ccr4-Not is very modular in structure. Ccr4-Not has many different functions, but its association with RNAPII is an
important feature for many of its nuclear functions associated with transcription regulation and mRNA processing. Though the CTD of Rpb1 is necessary for many transcription regulators to bind, Ccr4-Not does not need it to associate with RNAPII (35). Although Ccr4-Not subunit and RPB4 mutants share phenotypes such as stress sensitivity, as well as impaired transcription, TCR, and mRNA turnover, Rpb4/7 is not necessary for Ccr4-Not to associate with RNAPII. These results suggest that Ccr4-Not is interacting with the core of RNAPII (35).

The Ccr4-Not complex has two major enzymatic activities, ubiquitylation and deadenylation, which are associated with the C- and N-terminus of the Not1 scaffold, respectively. Ubiquitylation is performed by the Not4 subunit, a novel C4C4 E3 ubiquitin ligase (3, 29). Deadenylation is performed by the Ccr4-Caf1 subunits. Caf1 does not seem to have catalytic activity but is structurally important for deadenylation. Ccr4, the catalytic subunit in this function, contains an EEP (endonuclease-exonuclease-phophatase) 3’ exoribonuclease domain which functions as a 3’->5’ poly(A) RNA and ssDNA exonuclease, preferentially digesting poly(A) regions (71). It is the major cytoplasmic deadenylase in yeast and the rate-limiting step for mRNA decay. Several studies indicate that Ccr4 plays some direct or indirect role in transcription elongation (19, 60). Another subunit associated with both transcription elongation and the deadenylation function of Ccr4-Not is Dhh1, which associates with the N-terminus of Not1 as part of the larger ~2MDa complex rather than the ~1MDa core (35, 41). Dhh1 is a DEAD-box containing putative RNA helicase. It has been associated with mRNA decapping and regulation, especially the shift between translation and decay. Consistent with the role of Ccr4-Not in DNA damage repair, its crosslinking across the RNR3 gene increases with increases in DNA damage (35). When CCR4 or CAF1 is deleted, the association of Dhh1 with RNAPII decreases greatly. Likewise, mutated NOT2 or deleted NOT4 nearly eliminates the
association. Therefore, Dhh1 likely plays structural or catalytic roles in other Ccr4-Not functions via association with core subunits.

In addition to acting in transcription elongation and TCR, as discussed subsequently, the nuclear roles of Ccr4-Not include DNA repair, transcription activation, and RNA processing. Ccr4-Not indirectly regulates DNA remodeling via histone modification (47). Ccr4-Not subunits play contrasting roles in the DNA damage response regarding ribonucleotide reductase (RNR) gene transcription (35). RNR genes are necessary to increase dNTP levels in the cell so that it can repair damaged DNA. The Ccr4 subunit positively regulates RNR DNA binding repressor Crt1, while the Not4 subunit recruits general transcription factors to RNR genes (51, 76).

Ccr4-Not was first implicated in transcription as a negative regulator of RNAPII transcription. Early studies suggested that it acts as a universal transcription regulator by interacting with promoter DNA and impacting TFIIID/TBP function (6, 38). Later studies involving genomics and chromatin immunoprecipitation DNA sequencing (CHIP-seq) show that Ccr4-Not plays a greater role in regulating transcription of stress-response genes by the SAGA histone acetyltransferase complex/TBP than the housekeeping genes transcribed by TFIIID/TBP (5, 18, 73).

In addition to acting in the transcription process, Ccr4-Not plays a role in modifying mRNA transcripts. Ccr4-Not interacts with nuclear mRNA export machinery, acts in mRNA degradation, and may serve to reactivate “over-adenylated” mRNA (47). Ccr4-Not’s obvious role in RNA processing stems from the deadenylase activity of the Ccr4-Caf1 subunits, but NOT2/4/5 were shown to be required for deadenylation-independent decapping of EDC1 mRNA, a protein that acts in translation during heat stress (50). It has been suggested that Ccr4-Not plays a role in linking DNA damage with downstream post-transcriptional events in the cytoplasm via
mRNA regulation. Ccr4-Not plays a role in many steps of gene expression including DNA repair, transcription activation, and RNA processing and regulation, however, its roles in transcription elongation and TCR are most pertinent to the experiments detailed in this thesis.

1.5.2 Ccr4-Not in transcription elongation and transcription-coupled repair

Studies showing genetic interactions between Ccr4-Not and Nhp6, a high mobility group protein involved in recruiting FACT and other chromatin remodeling complexes to nucleosomes during elongation, first provided evidence for the role of Ccr4-Not in transcription elongation (9). Consistent with its role in transcription elongation, Ccr4-Not is recruited to actively-transcribing genes (59). Furthermore, mutants in CCR4 and NOT1/2/4/5 subunits showed sensitivity to elongation inhibitors 6-AU and MPA (19). Likewise, double mutants in Ccr4-Not subunits known elongation factors Paf1c, FACT, TFIIS, and Spt4/5 exhibit synthetic lethality (47). Ccr4-Not subunits co-purify with elongation factors such as Paf1c and others (13).

Kruk, et. al. present evidence for one model of Ccr4-Not in elongation, namely that the complex directly binds elongating RNAPII and reactivates arrested RNAPII by a mechanism distinct from TFIIS, dependent on the length of the nascent transcript (35). This suggests that Ccr4-Not reactivates arrested RNAPII rather than stimulating elongation rate, possibly by trapping stalled RNAPII in its forward motions and preventing backward movements.

Another possible role of Ccr4-Not in elongation stems from studies of its mutant phenotype in gene length-dependent accumulation of mRNA (GLAM) assays (49). GLAM reporter genes have GC-rich regions which hamper elongation by forming R-loops. Mutations in Paf1c, and TRO/TREX elongation factors show impaired elongation through GLAM reporter genes, similar to Ccr4–Not subunit mutations (21, 39, 59). Ccr4–Not binds to the mRNA transcript in elongation complexes, so it could prevent R-loop formation in a manner similar to
the TRO/TREX complex. Mutations in factors preventing R-loops (PAF1c, *HPR1*) tend to have hyperrecombination phenotypes due to the transcription-associated recombination (TAR) associated with R-loops. Since Ccr4-Not subunit mutations do not evince this characteristic, preliminary evidence does not support an R-loop binding role for Ccr4-Not in transcription elongation. Despite these early results, closer examination is necessary to eliminate R-loop prevention as a possible mechanism for Ccr4-Not in transcription elongation.

Evidence from both genome-wide analyses and genetic and molecular studies implicates Ccr4-Not in TCR (21). This makes sense because several transcription elongation factors have been shown to act in TCR, and Ccr4-Not is recruited to actively transcribing genes. Gaillard, et. al. utilized the fact that mutations in genes involved in TCR exhibit increased UV sensitivity in the absence of global genome (GG)-NER to support the hypothesis that Ccr4-Not subunits are involved in TCR. *RAD7* is a gene essential for GG-NER. Double mutants in *RAD7* and *NOT3, NOT4, NOT5, CCR4*, or *CAF1* exhibit greater UV sensitivity than single mutants in either component, which shows that multiple Ccr4-Not subunits might act in TCR. Further analysis of DNA repair in *not5Δ* mutants revealed that the transcribed strand was repaired much less efficiently than in wild type cells, information which more directly links this subunit with TCR. Experimental evidence implicates the Ccr4-Not in TCR, but it is unclear if its mechanism of action is linked to its role as an elongation factor or even if it plays a direct role in the process. Further investigation to characterize the nature and importance of the actions of different Ccr4-Not subunits in TCR will give more insight onto the part it plays in this process.

### 1.5.3 The Not4 subunit

The Not4 subunit is part of the core Ccr4-Not complex, associating with the C-terminus of Not1. Not4 contains a C4C4 RING E3 ubiquitin ligase domain as well as an RNA recognition
motif (RRM). The caf1A and not4A mutants have similar phenotypes, indicating that Not4 might be important for the association Caf1 with the complex (27). Deletion of NOT4 results in the accumulation of poly-Ub newly synthesized proteins and destabilization of the 26S proteasome (27).

Not4’s major function is to act as an E3 ubiquitin ligase, and it plays a role in protein quality control separately from Ccr4. Its E2 ubiquitin-conjugating enzymes are Ubc4 and 5. The first Not4 substrate discovered is the nascent-associated polypeptide complex (NAC), which binds nascent peptides at the ribosome. The NAC associates with both the ribosome and the proteasome, and thus might act in protein quality control (54). This theory is consistent with the fact that the Not4 RING domain also associates with the proteasome, via Rpt6. In vivo, Not4’s ubiquitylation of the NAC is dependent on other Ccr4-Not subunits. A second substrate of Not4, histone H3 lysine 4 (H3K4) demethylase Jhd2, has been identified (45). Jhd2 controls histone methylation and gene expression, so regulation of its levels by Not4 undoubtedly affects transcription. Other substrates of Not4 have been identified, namely the transcription activator Yap1, cyclin C (Srb11 mediator subunit), cdc17 (DNAPα catalytic subunit), and Rsp7 (ribosomal protein) as well as other Ccr4-Not subunits (16, 27, 59). Synthetic genetic array (SGA) analysis of NOT4 and Not4 RING-mutants show that there are more substrates for Not4 to be found, as it identified genes associated with ubiquitylation, transcription, the DNA damage response, and other processes (52).

1.6 Scope and Significance of the Project

Transcription elongation and TCR play important roles in enabling cells to produce functional proteins and therefore maintain health and vitality. TCR also helps with DNA repair,
which is important in avoiding genetic damage that may lead to cancer and other pathologic conditions. Cockayne’s syndrome, a rare disease resulting from a lack of CSA or CSB, provides a classic example of the importance of TCR in humans. Patients exhibit sensitivity to sunlight, short stature, and premature aging (53, Figure 1.11). The cause of Cockayne’s syndrome is known but its mechanism is still not understood and there is no cure. Since TCR is so essential to maintaining health, it is important to both study the mechanisms of known factors, and investigate novel pathways that act in this process.

Already shown to act in transcription elongation and play some role in TCR, the Ccr4-Not complex is an excellent candidate for further examination. Moreover, it is conserved from yeast to human as detailed in table 1.1. This investigation focuses on the role of specific Ccr4-Not subunits in transcription elongation and then TCR via RNAPII turnover induced by Rpb1 degradation. These studies provide evidence for the role of Not4 in Rpb1 degradation by the ubiquitin degradation pathway, and conclude with models detailing possible mechanistic explanations for the role of Ccr4-Not in RNAPII turnover in conjunction with TCR. By providing evidence to support the role of Ccr4-Not in TCR, these results help to expand our knowledge of possible TCR pathways. Greater understanding of TCR will facilitate comprehension of how the misregulation of these processes leads to diseases and cancers in humans.
Figure 1.1: Schematic of the DNA-binding site of RNAPII

(A) Schematic depicting DNA entering a cleft formed by Rpb1 and Rpb2 where it proceeds to the active site on Rpb2. The DNA template strand is light blue, the non-template strand is green. (B) View into the cleft of RNAPII. Rpb1 and Rpb5 crosslink on one side of the cleft, and Rpb2 on the other. Rpb4/7 are not shown.

Figure 1.1: Schematic of the DNA-binding site of RNAPII. Taken from (26). (A) Schematic depicting DNA entering a cleft formed by Rpb1 and Rpb2 where it proceeds to the active site on Rpb2. The DNA template strand is light blue, the non-template strand is green. (B) View into the cleft of RNAPII. Rpb1 and Rpb5 crosslink on one side of the cleft, and Rpb2 on the other. Rpb4/7 are not shown.
Figure 1.2: Transcription initiation and elongation. Taken from (1). The TBP of TFIIID binds the TATA box, and other transcription factors assemble along with RNAPII. Eventually, TFIIH phosphorylates Ser5 of the RNAPII CTD, allowing it to escape the initiation complex and enter elongation phase.
Figure 1.3: TFIIS Mechanism

Figure 1.3: TFIIS Mechanism. Taken from (67). TFIIS stimulates the nuclease activity of RNAPII so that incorrect portions of the transcript are removed, the transcript is realigned, and transcription continues.
Figure 1.4: Factors that promote RNAPII transcription elongation

The PAF transcription elongation complex recruits Rad6/Bre1, which monoubiquitylates histone H2B. Ub-H2B recruits other activities, such as the 19S proteasome, to transcribed genes. During transcription elongation, Ub-H2B stimulates H2A/H2B dimer removal by FACT, thus facilitating efficient RNAPII elongation through chromatin. SWI-SNF is an ATP-dependent nucleosome-remodelling complex that helps RNAPII transcribe through nucleosomes (gray). Spt4/5 and the THO/TREX complex are elongation factors that help stabilize the nascent mRNA transcript. Rad26 prompts stalled RNAPII to transcribe through a lesion by adding another ribonucleotide (see text for references).
Figure 1.5: Proposed Mechanisms for RNAPII Removal in TCR

(A)-(F) show possible mechanisms for RNAPII removal from the site of a lesion so that DNA repair factors can fix the damage. (C) represents a possible role for CSB in TCR, and (F) represents the ubiquitin-mediated RPB1 degradation that prompts the dissolution of RNAPII.
Figure 1.6: The ubiquitin-proteosome pathway

Ubiquitin (Ub) is first activated by a Ub-activating enzyme (E1). Next, it is transferred to a Ub-conjugating enzyme (E2) which associates with an E3 Ub-ligase, allowing for the transfer of Ub to a Lys residue on the substrate. Elongation of the Ub chain results in degradation of the substrate by the 26S proteasome.
Figure 1.7: The structure of a C4HC3-type RING domain

Figure 1.7: The structure of NOT4, Cbl, IEEHV, and RAG1 RING domains. Taken from (29). A conserved α-helix is colored red, and the other helices/helical turns are yellow. The β-sheet is green. Conserved zinc ions are magenta balls, others are gray balls (present in RAG1). Residues that coordinate zinc ions are yellow for cysteine and blue for histidine. NOT4 is a C4C4 RING domain, Cbl, RAG1, and IEEHV are C3HC4 RING domains.
Figure 1.8: The pattern of ubiquitylation on a substrate determines its fate. While mono-Ub might signal histone regulation or cellular localization (see text), poly-Ub in a certain manner will target a species for degradation by the 26S proteasome.

Figure 1.9: The ubiquitin-proteosome pathway has a hierarchical structure. In yeast, cells, there is one E1, eleven E2s, and many E3s that each may have several substrates, each being targeted to the 26S proteasome.
Figure 1.10: The structure of the 26S proteosome

![Diagram of the 26S proteosome](image)

**Figure 1.10: The structure of the 26S proteosome. Taken from (78).** The 26S proteosome is composed of the 20S core particle, which contains α and β subunits. The catalytic site is present on the β subunits. The CP can be surrounded by 19S regulatory particles (RP) which recognize poly-Ub substrates targeted for degradation and unfold them as they enter the CP.

Figure 1.11: The Ccr4-Not Complex

![Diagram of the Ccr4-Not Complex](image)

**Figure 1.11: The Ccr4-Not Complex. Adapted from (16).** The nine core subunits along with the additional subunit Dhh1 are pictured here, along with some of the known functions of the subunits.
Figure 1.12: Individual with Cockayne’s Syndrome

![Figure 1.12: Individual with Cockayne's Syndrome. Taken from (53). Patients with this rare autosomal recessive disorder exhibit sensitivity to sunlight, short stature, and premature aging.](image)

Table 1.1: Homologs of Elements of Transcription Elongation, Ccr4-Not, and the Ubiquitylation-degradation pathway in Yeast and Human

<table>
<thead>
<tr>
<th>Yeast</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Transcription Elongation</strong></td>
<td></td>
</tr>
<tr>
<td>TFIIS</td>
<td>TFIIS</td>
</tr>
<tr>
<td>Spt4/5</td>
<td>DSIF</td>
</tr>
<tr>
<td>FACT</td>
<td>FACT</td>
</tr>
<tr>
<td>Rad26</td>
<td>CSB</td>
</tr>
<tr>
<td>Rad28</td>
<td>CSA</td>
</tr>
<tr>
<td>Def1</td>
<td>unknown</td>
</tr>
<tr>
<td><strong>Ccr4-Not</strong></td>
<td></td>
</tr>
<tr>
<td>Not4</td>
<td>CNOT4</td>
</tr>
<tr>
<td>Caf1</td>
<td>CNOT7, CNOT8</td>
</tr>
<tr>
<td>Ccr4</td>
<td>CNOT6, CNOT6L</td>
</tr>
<tr>
<td>Caf40</td>
<td>CNOT9</td>
</tr>
<tr>
<td>Dhh1</td>
<td>Rck, p54, DDX6</td>
</tr>
<tr>
<td>Not5</td>
<td>CNOT3 (considerable but not complete homology)</td>
</tr>
<tr>
<td><strong>Ubiquitylation degradation pathway</strong></td>
<td></td>
</tr>
<tr>
<td>E1</td>
<td>Uba1</td>
</tr>
<tr>
<td>E2</td>
<td>Ubc4, Ubc5</td>
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<tr>
<td>E3</td>
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<tr>
<td>Rsp5</td>
<td>hRPF1</td>
</tr>
<tr>
<td>Cul3</td>
<td>CUL3</td>
</tr>
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</table>
CHAPTER 2
MATERIALS AND METHODS

2.1 Materials

All media were standard. Reagents and media materials came from BD Biosciences, American Bioanalytical, Sigma-Aldrich, VWR, or GE Healthcare. YPD consists of 10 g/L yeast extract, 20 g/L bacto-peptone, and 2% dextrose. Synthetic complete (SC) minimal media consists of 8 g/L yeast nitrogen base with ammonium sulfate, 10% 10X desired amino acid mix, and 2% dextrose. Luria broth (LB) media consists of 10 g/L bacto-tryptone, 5 g/L yeast extract, and 10 g/L NaCl, pH 7.0. All optical density (OD) measurements were taken on a Thermo Scientific Genesys 20.

2.2 Creating NOT4, CCR4, DST1, and CAF40 KO cassettes in the pAG25 plasmid

First, PCR was performed to generate NOT4, CCR4, DST1, and CAF40 knockout cassettes using the NatMX4 resistance cassette, which confers resistance to clonNAT-Nourseothricin (CLONAT) through the nourseothricin N-acetyl-transferase gene (NAT1). Primer sequences for each knockout cassette can be found in Table 2.2, and a map of the pAG25 plasmid in Figure 2.1. 2.5 ul DMSO was added per 0.4 ul of plasmid template in the PCR mix, because of the G-C rich nature of the NAT1 cassette. The PCR product was purified using a phenol- chloroform isoamyl (PCIAA) extraction and ethanol precipitation, and 5 ul of the final product was visualized using ethidium bromide (EtBr) in a 1.5% agarose gel. 100 bp and 1 kb Fermentas GeneRulerTM DNA ladders were used.

2.3 Deletion of NOT4, CCR4, and CAF40 from BY4742
Yeast cell transformation of the four knockout cassettes into BY4742 was performed to generate the desired mutant strains. An overnight culture of BY4742 was seeded and then grown to an OD600 of 1.0. The cells were harvested and washed in ddH2O, then 1X lithium acetate. For the transformation step, 1-3 ug cassette DNA, 50 ug salmon sperm carrier DNA, and 50 ul yeast cell suspension were mixed in a 1.5 ml sterile microfuge tube. 300 ul PLATE (1 ml 10X lithium acetate, 1 ml 10X Tris-EDTA (TE) pH 8.0, 8 ml 50% 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 8,000 rpm for 2 min, resuspended in YPD, and incubated in a sterile glass tube on a roller for 4-5 hours. Next, the cells were spread on plates containing 100ug/mL CLONAT.

2.4 Verification of NOT4, CCR4, and CAF40 deletions in BY4742

After colonies appeared, four large and four small from each knockout were restruck onto selective media, and genomic DNA was isolated from cells that grew the second time. Genomic DNA was isolated by resuspending a pinhead-sized amount of yeast cells in 50 ul digestion buffer (800 ul 1.2 M sorbitol, 200 ul 0.5 M EDTA pH 8.0, 1 ul 14 M β-mercaptoethanol, 40 ul 10 mg/ml Zymolyase) and incubated at 30oC for 20-30 min. After spinning at 5,000 rpm for 3 min., the supernatant was aspirated and the pellet resuspended in 120 ul TE. 200 ul glass beads and 120 ul phenol-chloroform isoamyl alcohol (PICAA) was added and the tubes were vortexed for 1 min. at the highest speed. Next, the tubes were spun at 14,000 rpm for 5 min. and 75 ul of the supernatant was transferred to a new tube and added to 80 ul CIAA. After vortexing for 10 sec. and spinning 2 min. at the highest speed, 40 ul of the supernatant was transferred to a fresh tube and 1 ul used for PCR. The mutants were verified through two sets of PCR. The first used
ORF diagnostic primers that primed within the deleted ORF. The second set of primers included an upstream diagnostic and a downstream nat1. The PCR products were analyzed using EtBr in a 1.5% agarose gel.

2.5 Mating, sporulation, tetrad dissection, and replica plating to isolate double mutants in DST1 and CAF40, CCR4, or NOT4

2.5.1. Mating

Δdst1, Δcaf40, Δccr4, and Δnot4 single mutants were grown overnight in YPD. The next day, 5 mL cultures of the Δdst1, Δcaf40, Δccr4, or Δnot4 single mutants were seeded in YPD to an OD600 of 0.2. The cultures were allowed to mate for 4-6 hrs, before being streaked onto plates containing 100 μg/mL of either CLONAT and G418. Single colonies were restruck once more and then transferred to YPD plates.

2.5.2. Sporulation

Mated cells were taken from a fresh YPD plate and grown in YPD with 4% dextrose to an OD600 of around 0.8, whereupon 0.5 ml of culture was removed, added to 10 ml ddH2O, and spun 3200 rpm for 5 min. The pellet was resuspended into two different sporulation cultures, one with 2 ml SPM (1 g potassium acetate and 1 ml 20% sterile-filtered raffinose in 100 mls ddH2O, pH 7.0) and the other with 2 ml SPM + trace amino acids (1 ml 10X His, Leu, and Ura amino acid mix in 50 ml SPM media). These cultures were incubated at 23°C on a roller for 4 days then moved to 30°C for 2-4 days before they were checked for tetrads by light microscopy.

2.5.3. Tetrad Dissection

20 ul sporulation culture was added to 20 ul 1 mg/mL zymolyase 20T in 1 M sorbitol and incubated 8-10 min. at 30°C. 20 ul of this mixture was run down a YPD plate in a stripe (Fig.
2.2). After drying slightly, the plate was placed facedown on a dissection apparatus and a fine needle was used to dissect tetrad spores (Fig. 2.3).

2.5.4 Replica Plating

Spores from tetrad dissection were allowed to grow on YPD plates and were restruck once on YPD plates. Sterile velvet was used to transfer the yeast onto diagnostic plates to test for certain characteristics (Table 2.3). Plates were read and scored 24 hrs after replica plating.

2.6 Spot plating of Ccr4-Not and TFIIS single and double mutants

Strains JR1538A through JR1585A were analyzed by spot plating (Table 2.1). All plates were grown at 30°C, except for one YPD and one SC plate grown at 37°C. Growth conditions included YPD, YPD+25 mM hydroxyurea (HU), YPD+50 mM HU, SC, SC + 5 ug/mL Mycophenolic acid (MPA), SC + 10 ug/mL MPA, and SC + 15 ug/mL MPA. After overnight growth in YPD, samples of OD600 1.0, 0.1, and 0.01 were generated and 2 ul of each was pipetted onto the appropriate plate. Images of YPD and SC plates grown at 30° and 37°C were taken at 24 and 48 hr timepoints. Images of 25 and 50 mM HU + YPD plates were taken for 24, 48, and 72 hrs. Images of 5, 10, and 15 ug/mL MPA + SC plates were taken at 24, 48, 72, and 96 hr. timepoints.

2.7 Growing cells, extract preparation, and Bradford assay for Rpb1 degradation analysis under 4NQO treatment

2.7.1 Growing and harvesting cells

Strains JR1466-JR1469 were used for this analysis (Table 2.1). Cells were grown overnight in YPD then seeded to an OD600 of 0.2 in 60 mL YPD. Once the cells reached on OD600 of 0.8- 1.0, 10 mL of cells were removed as a 0 min timepoint. The cultures were treated
with 6 ug/mL 4-Nitroquinoline 1-oxide (4NQO) and 100 ug/mL cycloheximide (CHX) unless noted otherwise. 10 mL of each culture was removed, treated with 100 ug/mL CHX only, and harvested 120 min post-treatment. Subsequent timepoints of 4NQO- and CHX-treated cells were harvested at 30, 60, 90, and 120 min post-treatment. After removal from culture, the cells were centrifuged 3200 rpm, 4°C. Next, media was removed and cells were divided into two tubes and resuspended in 1.4 mL ice-cold phosphate-buffered saline + 1mM EDTA pH 7.0 (0.5 mM phenylmethanesulfonylfluoride (PMSF), 1mM benzamidine-HCl, and 2 mM N-ethylmaleimide (NEM) were added right before use). Cells were spun 8,000 rpm, 3 min, the liquid was aspirated, and the cells were stored at -80°C on crushed dry ice.

2.7.2 Extract preparation

This preparation was used in wild type, Anot4, and Alsml mutants for the experiment detailed in section 4.2. Each cell pellet was resuspended in 250 uL lysis buffer (150 mM Tris-acetate [pH 7.5], 50 mM NaCl, 20% glycerol, 2 mM EDTA, 5 mM Dithiothreitol (DTT), 2 mM NEM, 20 uM lactocystine (Boston Biochemicals), 0.1% Triton X-100, and protease inhibitors [2 ug/ml leupeptin; 3 ug/ml aprotinin; 2 ug/ml pepstatin A; 1 ug/ml chymostatin; 1 mM benzamidine-HCl, 0.5 mM PMSF]). Approximately 300 ul of glass beads were added and the tubes were vigorously vortexed 4 times, 30 sec each sample, placed on ice between bursts. Then, the tubes were continually shaken at high speed, 4°C on a shakehead vortexer for another 30 min. Next, 200 ul lysis buffer was added and the tubes vortexed for 20 sec. Lysate was separated from the beads by centrifugation and both 2 mM MgCl2 and 100U benzonse were added. Cells were sonicated for 2 min (30 sec on, 30 sec off) in a diagenode bioruptor and then clarified by centrifuging at 14,000 rpm for 15 min at 4°C. Supernatant was then spun at 14,000 rpm 30 min at 4°C before being stored at -80°C.
2.7.3 Bradford assay to determine protein concentrations

A standard curve was prepared using 2.5, 5, 10, 15, 20, 30, and 40 mg/mL BSA mixed with 1 mL Bio-Rad Protein Assay Dye Reagent (dye, phosphoric acid, methanol). OD595 readings were plotted using Microsoft Excel. OD 595 readings from 1 uL sample mixed with 1 mL Bio-Rad Protein Assay Dye Reagent were analyzed my means of the standard curve to determine protein concentrations.

2.8 Trichloroacetic acid (TCA) whole extract preparation

This procedure was used instead of the above extract preparation and Bradford standard curve protocols (2.7.2-3) to prepare wild type, Adhh1, Acr4, and Anot4 cells for the experiment detailed in section 4.1 as well as the Not4 RING domain mutants utilized in section 4.3. All steps were carried out at room temperature unless otherwise indicated. Cells were grown and treated with 4NQO and CHX as in 2.7.1, and timepoints were taken in the same manner. OD600 readings were taken at each timepoint and reagent volumes were adjusted (the subsequent protocol if for 10 mL of culture with an OD600 of 1.0). At the appropriate timepoint, 10 mL of culture was removed and spun 10 min at 3,200 rpm. The pellet was resuspended in 1 mL 20% TCA, spun for 2 min 3,000 rpm, and resuspended in 200 ul 20% TCA. ~250 uL glass beads were added and the tubes vortexed twice for one minute before being shaken on a shakerhead vortexer for 10 min 400 ul 5% TCA was added before the lysate was separated from the beads by centrifugation. The tube was spun 6,000 rpm for 10 min, then the pellet resuspended in 800 ul 0.5M Tris pH 7.4. The tubes were spun at 6,000 rpm for 5 min, then resuspended in 100ul 0.5 M Tris pH 7.4 and 150 uL 3X SDS-PAGE loading buffer. Finally, the tubes were boiled 4-5 min, spun at 15,000 rpm for 10 min, and the top 200-300 ul of sample was transferred to a new tube.
Samples were stored at -80°C. Before loading, they were boiled 4 min, and spun at 15,000 rpm for 10 min. 5 ul of each sample was run on an SDS-PAGE gel.

### 2.9 SDS-PAGE and protein transfer

6.5% acrylamide gels were poured. 10 mL of separating and stacking gel solutions were made for each gel. Separating gel solution consisted of 2.5 mL 4X separating gel buffer, (1.5 M Tris-HCl pH 8.8 and 0.4% SDS) 2.17 mL 30% acrylamide, 5.33 mL non-sterile H2O, 30 ul 25% ammonium persulfate (APS), and 10 ul tetramethylethylenediamine (TEMED). Stacking gel solution consisted of 2.5 mL 4X stacking gel buffer, (0.5 M Tris-HCl, pH 6.8, 0.4% SDS) 1.3 mL 30% acrylamide, 6.33 mL non-sterile H2O, 50 ul 25% APS, and 15 ul TEMED. For both gel solutions, APS and TEMED were added right before pouring. After using 500 ul separating gel mixture as a plug, the gel was poured with the rest of the separating gel mixture and smoothed with 150 ul H2O-saturated isobutanol. After the separating gel polymerized, the stacking gel was poured and allowed to polymerize around the comb. Samples were run in 1X Tris-glycine SDS at 30 mA per gel. After running, the gel was transferred onto a 0.2 um reinforced nitrocellulose membrane using the tank transfer method. The gel and membrane were sandwiched between 3 pieces of Whatman transfer paper and pads, and transferred in transfer buffer + SDS (1 X Tris-glycine + SDS, 20% MeOH by volume) at 90 V for 2 hrs. Membranes were stained with Ponceau S., (0.1% Ponceau S. in 2% acetic acid) destained in 2% acetic acid for imaging, and completely destained in TBST (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.05% Tween 20) before western blotting.

### 2.10 Western blotting and protein detection by fluorescence
The destained membrane was blocked for 1 hr in 5% milk solution (5 g powdered milk, 100 mL TBST) then incubated in primary antibody at a 1:1000 dilution in 2% milk solution overnight at 4°C. The primary antibodies used were 8WG16 (manufactured by Covance) for detecting Rpb1 and TAF68 (made in house) for detecting TFIID subunit 12. TAF68 is a housekeeping gene and was used as a control for sample protein levels. 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) or anti-rabbit (Taf68) 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% 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.

2.11 Generating plots of western blot results

Fluorescence was quantified using ImageQuant software (Version 5.2, Molecular Dynamics). A volume report was generated by drawing rectangles around each band of interest, with rectangle dimensions consistent within each scanned blot. A background reading of blot area without fluorescent sample was taken. This background (back.) reading was subtracted from the “average” (avg.) reading of each rectangle. Plots were generated by setting the wild type value of (Rpb1 time 0 min level) / (Taf68 time 0 min level) equal to 100%, and then dividing every other (Rpb1 time x min level) / (Taf68 time x min level, where x = 30, 60, 90, or 120 min) by the wild type time 0 min value and multiplying by 100% to get the % of Rpb1 there is in a certain sample when compared to wild type levels at time 0 min, both corrected with the Taf68 level. These values were plotted for each different strain over the 120 min time course. Error bars are not shown because a representative experiment, rather than an average of experiments, is
shown. Averaging all the experimental findings would obscure general trends because of the variations in both protein levels and drug activity from experiment to experiment.

2.12 Generating strains expressing Not4 subunits containing a RING-domain deletion, I64A point mutation, and full functionality

2.12.1 Transformation of chemically-competent E.coli with plasmids containing variations of the Not4 subunit

1 ul of each plasmid was added to 200 ul DH5α cells and incubated on ice for 30 min (Table 2.4). The cells were placed at 42°C for 2 min and then removed from heat. 1 mL of Luria broth (LB) + 10 mM MgSO4 + 0.2% dextrose was added and then the tubes incubated at 37°C for 20-30 min. For each plasmid, 10 ul and 100ul of the cells were plated on LB + 100 ug/mL ampicillin and grown at 37°C.

2.12.2 Mid-scale plasmid preparation of plasmids coding for variations of Not4

100 mL LB + 100 ug/mL ampicillin was inoculated with a single colony 37°C overnight. The culture was then spun at 3,400 rpm for 10 min before being resuspended in 5 mL solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0). 10 mL solution II (0.2 M NaOH, 1% SDS) was mixed in and the cells kept on ice for 5 min. 7.5 mL solution III (3 M KOAc, 5 M OAc) was mixed in and the cells kept on ice for 5 min. Cells were spun at 3,300 rpm for 15 min and the supernatant poured through 4 layers of cheesecloth. 0.7-0.8 volume isopropanol was added and the mixture incubated at room temperature for 15 min before being spun at 3,300 rpm for 10 min. The pellet was resuspended in 2 mL TE (10 mM Tris, 1 mM EDTA, pH 8.0) and 2 mL 5 M NH4OAc and incubated on ice for 10-15 min before spinning at
3,200 rpm for 10 min. The supernatant was brought to 15 mL with 100% ice-cold EtOH and kept on ice 20-30 min before being spun at 3,200 rpm for 15 min. The pellet was resuspended in 70 uL TE and 25 ug/mL RNase A was added before incubation at 37°C for 20-30 min 30 ul 5 M NaCl and 27.5 ul 30% PEG 3350 (in 1.5 M NaCl) was added and the tube incubated on ice for 60 min before spinning at 15,000 rpm for 10 min. The pellet was resuspended in 1 mL ice-cold EtOH, spun for 3 min., and resuspended in 200 ul TE, 200 ul 2X PK buffer, (100 mM Tris-HCl pH 8.0, 5 mM EDTA pH 8.0, 300 mM NaCl, 1% SDS) before incubation at 55°C for 20 min. One volume phenol chloroform isoamyl alcohol (PCIAA) was added and the tube was vortexed for 20 sec, spun at 15,000 rpm for 5 min, and the aqueous layer recovered. This procedure was repeated with 1 volume CIAA, and then 2-2.5 volume 100% EtOH was added to the recovered aqueous layer. The tube was incubated on ice 15 min and spun at 15,000 rpm for 8 min, whereupon 1 mL ice cold 70% EtOH was added and the tube spun at 15,000 rpm for 3 min. The pellet was air- dried and resuspended in 40 ul TE buffer. Plasmid concentration was measured using a ND-1000 NanoDrop spectrophotometer and stored at -20°C after being verified by restriction digest with SacI and PacI.

2.12.3 Introducing plasmids containing Not4 subunit variations into wild type and Δnot4

Yeast transformation was performed as described in section 2.4. Each of the 4 above plasmids was transformed into JR1 1466 (wild type) and JR 1469 (Δnot4) yeast and selected on SC-LEU plates.

2.12.4 Growth and 4NQO treatment of Not4 subunit RING-domain mutants

The RING-domain mutants were grown and treated as in section 2.7.1, except they were grown in SC-LEU media instead of YPD and were seeded at initial OD600 values of 0.3-0.4 to
compensate for their slow growth rates, especially in SC-LEU media. Extracts were prepared and analyzed as detailed in sections 2.8-11.

2.13 Spot-plating yeast containing Not4 subunit variations to analyze growth rates

Yeast were transformed as described in 2.12.3, and after selection on SC-LEU plates they were prepared for spot-plating as described in 2.6, using SC-LEU liquid and solid media. Images were taken 24 and 48 hrs after spot-plating.
Table 2.1: Strains used in the study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>BY4741</td>
<td>MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0</td>
<td>Wild Type</td>
</tr>
<tr>
<td>BY4742</td>
<td>MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0</td>
<td>Wild Type</td>
</tr>
<tr>
<td>JR1538A</td>
<td>BY4741/4742 (MATa, met15?) GAL1-YLR454::kanMX</td>
<td>Wild Type</td>
</tr>
<tr>
<td>JR1539A</td>
<td>BY4741/4742 (MATα, met15?) GAL1-YLR454::kanMX</td>
<td>Wild Type</td>
</tr>
<tr>
<td>JR1540A</td>
<td>JR1538A with dst1::URA3</td>
<td>Δdst1</td>
</tr>
<tr>
<td>JR1541A</td>
<td>JR1539A with dst1::URA3</td>
<td>Δdst1</td>
</tr>
<tr>
<td>JR1542</td>
<td>JR1538A with dhh1::HIS3</td>
<td>Δdhh1</td>
</tr>
<tr>
<td>JR1543A</td>
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<td>Δdhh1</td>
</tr>
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<td>JR1538A with dst1::URA3 and dhh1::HIS3</td>
<td>Δdst1/Δdhh1</td>
</tr>
<tr>
<td>JR1545</td>
<td>JR1539A with dst1::URA3 and dhh1::HIS3</td>
<td>Δdst1/Δdhh1</td>
</tr>
<tr>
<td>JR1550A</td>
<td>JR1538A with not5::HIS3</td>
<td>Δnot5</td>
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<td>JR1551A</td>
<td>JR1539A with not5::HIS3</td>
<td>Δnot5</td>
</tr>
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<td>JR1538A with dst1::URA3 and not5::HIS3</td>
<td>Δdst1/Δnot5</td>
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<td>Δdst1/Δnot5</td>
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<td>JR1560A</td>
<td>JR1538A with ccr4::natMX</td>
<td>Δccr4</td>
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<td>JR1561A</td>
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</tr>
<tr>
<td>JR1563A</td>
<td>JR1539A with dst1::URA3 and ccr4::natMX</td>
<td>Δdst1/Δccr4</td>
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<td>JR1575A</td>
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<td>JR1576A</td>
<td>JR1539A with caf40::natMX</td>
<td>Δcaf40</td>
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<td>JR1577A</td>
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<td>JR1581A</td>
<td>JR1538A with not4::natMX</td>
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<td>JR1583A</td>
<td>JR1539A with not4::natMX</td>
<td>Δnot4</td>
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<td>JR1585A</td>
<td>JR1539A with dst1::URA3 and not4::natMX</td>
<td>Δdst1/Δnot4</td>
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<td>BY4741 with trp1Δ::URA3</td>
<td>Wild type</td>
</tr>
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<td>BY4741 with trp1Δ::URA3 and dhh1::kanMX</td>
<td>Δdhh1</td>
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<td>JR1468</td>
<td>BY4741 with trp1Δ::URA3 and ccr4::kanMX</td>
<td>Δccr4</td>
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<tr>
<td>JR1469</td>
<td>BY4741 with trp1Δ::URA3 and not4::kanMX</td>
<td>Δnot4</td>
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Table 2.2: Sequences of primers used to construct knockout cassettes

<table>
<thead>
<tr>
<th>Knockout pAG vector</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caf40</td>
<td>up: tgaataagtttaatgttttcgttcctcaaaagccatatatgccttcacGAGCCTGTTTTCGACACTGGAT</td>
</tr>
<tr>
<td></td>
<td>down: gttttctgtaatgtcttagcagacttctttgAGCTCGTTTTCGACACTGGAT</td>
</tr>
<tr>
<td>Ccr4</td>
<td>up: acgttatcctgcaaaactacgtaatgaaaggacctctttactTCAGGGGCATGATGTGACT</td>
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<tr>
<td></td>
<td>down: tgtgcggtttaactttactgtggttgtggttcataaatAGCTCGTTTTCGACACTGGAT</td>
</tr>
<tr>
<td>Not4</td>
<td>up: atccacaagttcaaaatttgaagaaaaacttcacagacacGGCATGATGTGACT</td>
</tr>
<tr>
<td></td>
<td>down: ttaatgcaaaaaagaaatatagtgctggatttacacgcAGCTCGTTTTCGACACTGGAT</td>
</tr>
<tr>
<td>Dst1</td>
<td>up: cataagagcttcatctgagtaagpgaagtaactgtgcgatTCAGGGGCATGATGTGACT</td>
</tr>
<tr>
<td></td>
<td>down: ctattctagagaaaaacttctcttggttaaccacatgtgccataAGCTCGTTTTCGACACTGGAT</td>
</tr>
</tbody>
</table>

Table 2.2: Sequences of primers used to construct knockout cassettes. The lowercase portions correspond to sequences upstream or downstream of the ORF of the gene to delete. The capitalized portions are the universal parts of the primer and are sequences homologous to pAG25.
Figure 2.1: The NatMX4 cassette is engineered into the pAG25 plasmid

(A) Map of the pAG25 plasmid including the location of the \textit{NAT1} gene.

(B) Diagram of the inserted \textit{NAT1} gene including restriction enzyme digest sites.

\textbf{Figure 2.1:} The NatMX4 cassette is engineered into the pAG25 plasmid. Taken from (24). (A) Map of the pAG25 plasmid including the location of the \textit{NAT1} gene. (B) Diagram of the inserted \textit{NAT1} gene including restriction enzyme digest sites.
Figure 2.2: Tetrad Dissection

Dissection plate. Each ascus is separated from the tetrad by dissection.

Figure 2.2: Tetrad Dissection. Taken from Reese lab Mating and Dissection protocol. (A) Example of a “stripe” of sporulation digest and dissected tetrads. Each horizontal row of colonies originated from spores in one tetrad. (B) View of dissection needle and tetrad to be dissected through the eyepiece of a compound light microscope.
### Table 2.3: Characteristics analyzed by replica plating

<table>
<thead>
<tr>
<th>Plate</th>
<th>Gene</th>
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</thead>
<tbody>
<tr>
<td>-URA</td>
<td>DST1</td>
</tr>
<tr>
<td>G418 resistance</td>
<td>GAL1-YLR454</td>
</tr>
<tr>
<td>a, α-tests</td>
<td>Mating type</td>
</tr>
<tr>
<td>-HIS</td>
<td>NOT5, DHH1</td>
</tr>
<tr>
<td>natMX resistance</td>
<td>NOT4, CCR4, CAF40</td>
</tr>
<tr>
<td>-LYS</td>
<td>Genotype (BY4741 or BY4742)</td>
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</table>

### Table 2.4: Plasmids coding for variations of the Not4 subunit transformed into *E.coli*

<table>
<thead>
<tr>
<th>Plasmid Name</th>
<th>Description</th>
<th>Notes</th>
<th>Reference/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMAC751</td>
<td>pNot4-Myc&lt;sub&gt;6&lt;/sub&gt;-Not4&lt;sub&gt;78-587&lt;/sub&gt;-LEU2</td>
<td>Not4 RING domain deletion</td>
<td>55</td>
</tr>
<tr>
<td>pMAC749</td>
<td>pNot4-Myc&lt;sub&gt;6&lt;/sub&gt;-Not4&lt;sub&gt;I64A&lt;/sub&gt;-LEU2</td>
<td>I64A Not4 RING domain mutant</td>
<td>55</td>
</tr>
<tr>
<td>pMAC684</td>
<td>pNot4-Myc&lt;sub&gt;6&lt;/sub&gt;-Not4-LEU2</td>
<td>Full Not4 subunit</td>
<td>55</td>
</tr>
<tr>
<td>pRS415</td>
<td>LEU2</td>
<td>Empty vector</td>
<td>65</td>
</tr>
</tbody>
</table>
CHAPTER 3

RESULTS PART I: GENETIC ANALYSIS OF ELONGATION PATHWAYS OF TFIIS AND CCR4-NOT SUBUNITS

3.1 Verification of single mutants

To begin studying the role of Ccr4-Not subunits in transcription elongation, single mutants in Ccr4-Not subunits were generated. Of the Ccr4-Not subunits, only Not1 is coded for by an essential gene. Knockout cassettes for NOT4, CCR4, CAF40, and DST1 (TFIIS) were constructed and used to create yeast strains with NOT4, CCR4, and CAF40 deletions (Table 2.1). To make these strains, the designer deletion method detailed by Brachmann, C.B., et. al. was used in combination with pAG25, a vector originally constructed by Goldstein, A.L. and McCusker, J.H. (12, 24). pAG25 contains the natMX4 cassette, which confers resistance to clonNAT-Nourseothricin (CLONAT) through the nourseothricin N-acetyl-transferase (NAT1) gene.

The general gene knockout scheme using pAG25 relies heavily on PCR (Figure 3.1). The first step involves generating a cassette to replace a target gene with the CLONAT resistance gene. Upstream and downstream 62 nucleotide (nt) PCR primers are used (Table 2.2). The 20 nts of sequence at the 3’ end of each primer is specific for the NAT1 cassette on pAG25, and the 42 nts of sequence at the 5’ ends is identical to the genomic sequence flanking the gene of interest, in this case either NOT4, CCR4, CAF40, or DST1. Using these primers, NAT1 is PCR-amplified from pAG25 and the product contains NAT1 along with 42 bp of genomic DNA sequence upstream and downstream of the desired gene deletion. The resulting PCR product was amplified using the appropriate gene-specific primer (CCR4, CAF40, NOT4, or DST1), and purified by
phenol- chloroform isoamyl (PCIAA) extraction and ethanol precipitation as detailed in section 2.2. Both the unpurified and purified products were separated by gel electrophoresis and stained with ethidium bromide (EtBr) to verify the proper incorporation of the NAT1 gene into the knockout cassettes and to visualize the effectiveness of the purification procedure (Figure 3.2). A 1.2 kb DNA fragment was generated by PCR since the NAT1 cassette is ~1.2 kb (Figure 2.1). The purified products exhibited brighter bands and thus higher DNA concentrations than their corresponding unpurified counterparts, except for the CAF40 knockout cassette in Figure 3.2a. This inconsistency may be explained by error during the purification of the CAF40 cassette, and the effect was likely exacerbated by the fact that there was little product to begin with. Figure 3.2a shows sufficient amplification of the CCR4 and DST1 knockout cassettes, but insufficient amplification of the NOT4 and CAF40 cassettes. This was probably a consequence of error during the experiment or PCR procedure, since a repeat of the PCR and purification procedure generated appropriate amounts of the NOT4 and CAF40 cassettes (Figure 3.2b). Thus, a sufficient amount of the CCR4, DST1, NOT4, and CAF40 knockout cassettes were generated to proceed.

The knockout cassettes were transformed into wild type BY4742 yeast. Transformants were selected on medium containing 100ug/mL CLONAT. Two crossover events occurred in the transformants to replace the gene of interest with NAT1; one between each end of the 42 bp target sequences of the PCR product and the corresponding genomic DNA sequence. It is already known that Ccr4-Not subunit deletion mutants grow slowly. Therefore, it was expected that true transformants would be slow growing, and thus form small colonies, while transformants that gain CLONAT resistance in some fashion besides gene deletion would form larger colonies. Therefore, both large and small colonies were checked for the presence of the gene targeted for
deletion by priming within the ORF of the deleted gene. True knockouts should not generate a PCR product (Figure 3.3). Controls were knockout candidates in other subunits, which retain the open reading frame for the gene examined. For example, a candidate CAF40 knockout was used as a control for NOT4 candidate knockouts, as it still has the NOT4 gene. Predictably, small colonies tended to be true gene knockouts. None of the NOT4 deletion candidates from small colonies had a PCR product, while large colonies did. Similarly, only one CCR4 and CAF40 deletion candidate from a small colony generated a PCR product and only one from a large colony did not. In this manner, NOT4, CCR4, and CAF40 deletion candidates were isolated.

Deletion candidates were verified once again using PCR from genomic DNA with a NAT1 gene forward primer and gene-specific reverse primer. Controls were known unsuccessful transformants. Figure 2.2 shows that the NAT1 cassette on the pAG25 vector is about 1.2 kb long, consisting of a ~420 bp TEF gene promoter (p-TEF), the 573 bp long NAT1 gene, and ~250 bp long TEF gene terminator (t-TEF). Since the PCR product contains the whole 573 bp NAT1 gene along with the 250 bp t-TEF region along with 42 bp of the deleted gene, successful knockouts generate a ~850 bp PCR product. Each candidate displayed a PCR product. The products generated here appeared slightly longer than 800 bp after gel electrophoresis, which is a reasonable range for the expected product, thus the experiment supports that CCR4, NOT4, and CAF40 mutants were created (Figure 3.4). CAF40 PCR products were not as abundant as those from NOT4 or CCR4 knockouts, perhaps because of the efficiency of the CAF40 gene-specific primer or optimization of the PCR procedure for amplifying the sequence of the CAF40 knockout cassette in particular. Regardless, the single mutants in NOT4, CCR4, and CAF40 subunits that were generated and verified in this experiment were used to study the roles of each subunit in transcription elongation.
3.2 Ccr4-Not subunits and TFIIS have different functions in transcription elongation under a normal environment

Single and double mutants in Ccr4-Not subunits and TFIIS were characterized by spot-plating to characterize their roles in transcription elongation. Ccr4, Dhh1, and Not4 were previously identified as probable factors in RNAPII elongation, and Caf40 and Not5 were included in the study to see double mutants in these factors and TFIIS exhibited growth phenotypes that warranted further investigation (35). Double mutants in Ccr4-Not subunits and TFIIS were constructed by mating two parent haploid yeast cells to produce a diploid cell, which was forced to undergo sporulation to induce meiosis and produce four haploid spores per diploid cell. The four haploid spores were separated through dissection as detailed in section 2.5 of the Materials and Methods (Figure 2.2). A tetrad dissection plate already gives important phenotypic information about the double mutant to be isolated, as spores containing both mutations often exhibit a much slower growth rate than single mutants or wild type strains (Figure 3.5). The genotype of each spore with regards to the genes detailed in Table 2.3 was determined by replica plating the colonies on SC media lacking a specific amino acid, or YPD containing 100 ug/mL CLONAT as described in section 2.5.4. In particular, mutants in TFIIS and Ccr4-Not subunits and were identified by the ability of the spore to grow on –URA and –HIS or CLONAT-containing plates. Yeast with the DST1 deletion contain the URA3 gene, which is missing from the BY4741 background, and therefore can grow on –URA plates. Likewise, cells with NOT5 and DHH1 deletions contain the HIS3 gene, which is missing from BY4741, and therefore can grow on –HIS plates. Mutants in NOT4, CCR4, and CAF40 contain the NAT1 gene, which confers resistance to CLONAT, and therefore can grow on media that contains the drug. Double mutants in TFIIS and Ccr4-Not subunits are identified by their ability to grow on both –URA and
either –HIS or CLONAT-containing media (depending on the Ccr4-Not subunit). In this way, double mutants in DST1 and either DHH1, CCR4, NOT5, NOT4, or CAF40 were isolated.

The growth rates of these single and double mutants were examined under conditions stressful for elongation, including hydroxyurea (HU) and mycophenolic acid (MPA) treatment. HU is a ribonucleotide reductase inhibitor which starves the cell for deoxynucleotides (33). HU sensitivity is a known phenotype in Ccr4-Not mutants. Ccr4-Not mutants are also sensitive to MPA, which places stress on elongating RNAPII by inhibiting IMP dehydrogenase, which catalyzes an important step in the synthesis of guanine nucleotides (34). Spot plating assays reveal that under conditions stressful for elongation, a mutant in a possible elongation factor tends to grow more slowly than wild-type cells (Figure 3.6).

In this study, we first examined the growth phenotypes of single and double mutants in DST1 and genes encoding Ccr4-Not subunits, including NOT4, NOT5, CCR4, DHH1, and CAF40 compared to wild type yeast on SC media after 48 hrs (Figure 3.7a). DHH1, NOT5, and especially NOT4 single mutants grew more slowly than wild type cells. Likewise, each Ccr4-Not subunit/TFIIS double mutant except CAF40/DST1 grew more slowly than wild type cells. Upon comparing growth under untreated and 10ug/mL MPA treatment conditions at the 48 hr time point, it is evident that each strain is affected by the drug to different extents. Of the single mutants, the dst1Δ mutant displayed the most severe slow-growth phenotype on MPA plates. The Ccr4-Not single mutants each experienced similar growth rate reductions, all less severe than that seen in the dst1Δ mutant. At the 48 hr time point, it is evident that each Ccr4-Not subunit/TFIIS double mutant is impacted by the drug to a greater extent than either the dst1Δ mutant or its corresponding Ccr4-Not subunit single mutant, but the level to which each differs from its untreated counterpart is difficult to discern at 48 hrs because of the severe slow growth
of the treated double mutants. Examination of the growth of the double mutants at the 96 hr timepoint provides a clearer picture. By 96 hrs, the single mutants show a substantial amount of growth on MPA, even \textit{dst1A} cells. However, none of the double mutants have recovered to such an extent. This assay shows the synthetic lethality of Ccr4-Not subunit/TFIIS double mutants, but does not give a clear indication as to which particular Ccr4-Not subunit deletion; \textit{dhh1A}, \textit{ccr4A}, \textit{not5A}, \textit{not4A}, or \textit{caf40A}, displays the most severe phenotype when combined with a \textit{DST1} deletion. In sum, this assay shows that \textit{not4A} mutants exhibit the slowest growth in the absence of MPA treatment, the \textit{dst1A} mutant is affected to the greatest extent by MPA treatment, and double mutants in Ccr4-Not subunits/TFIIS exhibit synthetic lethality under MPA treatment. Synthetic lethality is related to epistasis, the concept of how two genes involved in the same phenotype interact (see section 5.1). In this case, compromised transcription elongation is causing the slow growth phenotype. If the Ccr4-Not subunits and TFIIS acted in the same transcription elongation pathway, the growth rates of each single mutant would be the same as the double mutant, because the same pathway would be compromised in all three strains. The fact that the double mutants exhibited a severe growth decrease compared to either single mutant indicates that TFIIS and Ccr4-Not are not acting in the same transcription elongation pathway.

In a similar experiment, the same strains were grown on YPD treated with 50 mM HU in order to compare their growth rates. Once again, the \textit{DHH1}, \textit{NOT5}, and especially \textit{NOT4} single mutants exhibited a slower growth rate than wild type cells on untreated YPD after 48 hrs (Figure 3.7b). Each double mutant except \textit{CAF40/DST1} grew more slowly than wild type cells under the same conditions. Upon comparing untreated cells with cells treated with 50 mM HU at 48 hrs, it is evident that each strain is affected by the drug to a different extent. In contrast to 10ug/mL MPA treatment, single mutants in Ccr4-Not subunits (except perhaps \textit{CAF40}) show a
more severe decreased growth rate phenotype with HU treatment than $dst1\Delta$ cells. Comparing the treated and untreated double mutants at 48 hrs shows that the double mutants in $DHH1$, $NOT5$, $CCR4$, and $NOT4$ and $DST1$ grow more slowly than their untreated counterparts. Further examination of these double mutant phenotypes after 72 hrs on HU plates revealed that even as the single mutants grow more substantially, the double mutants do not recover to a similar extent. At this point, it is evident that deletions in $DHH1$, $NOT5$, $CCR4$, or $NOT4$ exhibit synthetic lethality with the $DST1$ deletion when grown on YPD treated with 50 mM HU. Arguably, double mutants in $DHH1$, $CCR4$, $NOT4$ and $DST1$ exhibit the most severe phenotype. In sum, this assay showed that $NOT4$ is the slowest growing Ccr4-Not subunit deletion, Ccr4-Not subunits as well as TFIIS play a role in transcription elongation, and that double mutants in $DST1$ and $DHH1$, $NOT5$, $CCR4$, or $NOT4$ genes exhibit synthetic lethality. Clearly, treatment with HU elicited slightly different growth rate phenotype results than MPA treatment.

As a whole, these results suggest that of the Ccr4-Not subunits tested, Not4 plays the most significant role in cell vitality. The $dst1\Delta$ mutant was more severely affected by MPA treatment than single mutants in Ccr4-Not, while the opposite was true under HU treatment. This shows the importance of analyzing growth phenotypes under treatment with two different drugs that produce conditions stressful for transcription elongation in order to gain an accurate picture of the relative importance of the elongation factors studied. This observation shows that both TFIIS and Ccr4-Not subunits likely play substantial roles in elongation. Lastly, the synthetic lethal double mutant phenotypes seen under HU treatment were more specific than those seen under MPA treatment, and show that Dhh1, Not5, Ccr4, and Not4 subunits in particular may play a role in elongation independently of TFIIS.
Figure 3.1: Deleting genes in yeast using PCR and transformation

Primers containing a 20 nucleotides (nt) of a universal pAG25 sequence in conjunction with 42 nt of gene-specific sequences are used to create a knockout cassette to replace your favorite gene (YFG) with the nourseothricin N-acetyl-transferase (NAT) gene to confer clonNAT-Nourseothricin (CLONAT) resistance to transformants. The pAG25 plasmid was used in lieu of a pRS plasmid in this case but the method is the same.
Figure 3.2: Knockout Cassettes for Ccr4-Not Subunits and TFIIS
Figure 3.2: Knockouts in Ccr4-Not subunits and TFIIIS. PCR was used to generate knockout cassettes with the pAG25 vector. The desired cassettes confer resistance to CLONAT and contain the NAT1 gene flanked upstream and downstream by regions of the NOT4, CCR4, DST1, or CAF40 genes. The PCR product was visualized both before and after purification by phenol-chloroform isoamyl (PCIAA) extraction and ethanol precipitation. When analyzed by gel electrophoresis on a 1.5% agarose gel, successful knockout cassettes generate a 1.2 kbp product. CCR4 and DST1 (A) and NOT4 and CCR4 (B) knockout cassettes were successfully generated.
Figure 3.3: Verification of Ccr4-Not subunit knockouts with PCR.

A.

True NOT4 knockout

42 nt of NOT4

NAT1 cassette

42 nt of NOT4

upstream NOT4 gene
diagnostic primer

primers do not bind!

downstream NOT4
gene diagnostic primer

3’

5’

No PCR product

Not a true NOT4 knockout

42 nt of NOT4

NOT4 gene

42 nt of NOT4

upstream NOT4 gene
diagnostic primer

primers bind!

downstream NOT4
gene diagnostic primer

3’

5’

PCR product
**Figure 3.3: Verification of Ccr4-Not subunit knockouts with PCR.** Genomic DNA from possible *not4Δ, ccr4Δ*, and *caf40Δ* mutants was isolated from 4 small and 4 large colonies after transformation with the appropriate knockout cassette and selection on SC + CLONAT plates. (A) Schematic of the process using the *NOT4* gene as an example. PCR was performed within the deleted ORF. Successful knockouts produce no product upon analysis by gel electrophoresis. Alternate mutants were used as controls for each mutant, i.e. the *CAF40* knockout was used to generate a product from priming within the *NOT4* ORF. (B) *not4Δ* mutant candidate colonies 1-4 are successful knockouts, as well as *ccr4Δ* mutant candidate colonies 1, 2, 4, 5, 6, 9 (C). The control for the *ccr4Δ* mutant candidates was a known negative result from a previous experiment. (D) *caf40Δ* mutant candidate colonies 1, 2, 3, 4, 6 surfaced as successful knockouts.
Figure 3.4: Verifying Ccr4-Not subunit mutants by checking for the presence of the \textit{NAT1} gene

A.

\textbf{True NOT4 knockout}

\textbf{Not a true NOT4 knockout}
Figure 3.4: Verifying Ccr4-Not subunit mutants by checking for the presence of the \textit{NAT1} gene. The putative successful knockouts visualized in Figure 3.4 were re-confirmed by PCR, this time using a \textit{NAT1} gene-specific primer. (A) Schematic of the process, using the \textit{NOT4} gene as an example. Successful knockouts generate a \textasciitilde850 bp product, and controls were samples shown unsuccessful for gene knockout in Figure 3.4. Colony numbers are kept consistent with Figure 3.4. (B) \textit{not4Δ} mutant candidate colonies 1-4 and \textit{ccr4Δ} mutant candidates colonies 1-6 produced \textit{NAT1} products. (C) An inverted image of the gel showing samples analyzed from \textit{caf40Δ} mutant candidates shows bands from colonies 2-6 and a very faint band from colony 1.
Figure 3.5: Tetrad dissection plate

Figure 3.5: Tetrad dissection plate. This is an example of a dissection conducted to isolate double mutants in NOT5 and DST1. Complete tetrads exhibit two large and two small colonies, indicating that two of the spores contain the NOT5 deletion (which results in slow growth) and thus true tetrads were selected.

Figure 3.6: Spot Plating Assays Identify Important Elongation Factors

Figure 3.6: Spot Plating Assays Identify Important Elongation Factors. Under conditions stressful for transcription, strains deficient in important elongation factors exhibit reduced growth, while wild type cells are able to sustain a normal growth phenotype.
Figure 3.7: Dhh1, Ccr4, and Not4 function independently of TFIIS in transcription elongation.

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<tr>
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<td>0.1</td>
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<tr>
<td>caf40Δ/dst1Δ</td>
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</table>
Figure 3.7: Dhh1, Ccr4, and Not4 function independently of Dst1 in transcription elongation. These two spot plating assays exhibit the growth patterns of different mutants in TFIIS and Ccr4-Not subunits. (A) Under treatment with 10 μg/mL mycophenolic acid (MPA), growth of all strains suffered but the double mutants in DST1 and DHH1, NOT5, CCR4, NOT4, or CAF40 exhibited poorer growth than either single mutant, and poorer growth than its untreated counterpart. (B) Under treatment with 50 mM hydroxyurea (HU), double mutants in DST1 and DHH1, NOT5, CCR4, or NOT4 exhibited poorer growth than either single mutant, and poorer growth than its untreated counterpart.
CHAPTER 4

RESULTS PART II: TURNOVER OF RNA POLYMERASE II LARGE SUBUNIT RPB1 UNDER DNA-DAMAGING CONDITIONS REQUIRES NOT4

4.1 Rpb1 turnover is reduced in Ccr4-Not mutants

Previous studies have shown that Rpb1, but not other RNAPII subunits, is degraded in response to DNA damage (42). The purpose of Rpb1 degradation may be to remove the rest of the RNAPII complex from the DNA so that repair factors can access the damaged site. The fact that Rpb1 is the only subunit degraded enables the cell to both access the DNA and recycle other RNAPII subunits. Experiments have shown that DNA-damage-dependent Rpb1 degradation is mediated by the ubiquitin-proteosome pathway (69, 74). Several pathways implicating the ubiquitin degradation pathway in Rpb1 turnover have already been discovered, such as that involving the E3 ubiquitin ligases Rsp5 and the Elongin-Cullin complex (75). It is quite possible that there are additional pathways acting in a parallel manner. Since Not4 is an E3 ubiquitin ligase and is associated with transcription elongation, and Ccr4-Not is required for TCR, there are grounds to investigate whether it modulates Rpb1 degradation under DNA damaging conditions.

In this study, the role of Not4, Ccr4, and Dhh1 subunits of the Ccr4-Not complex in Rpb1 turnover was analyzed. Not4 is the Ccr4-Not subunit associated with the ubiquitylation function of Ccr4-Not, while the deadenylase Ccr4 and RNA helicase Dhh1 are major players in its deadenylation function. Figure 1.11 shows that these two functions are spatially separated on the Not1 scaffold of the Ccr4-Not complex, with Ccr4 and Dhh1 associated with the N-terminus, and Not4 the C-terminus. This spatial separation partially explains the modular nature of Ccr4-Not, namely, that few functions of the complex depend on more than one subunit (5). This
characteristic of Ccr4-Not explains why the possible roles of Ccr4 and Dhh1 in Rpb1 turnover were examined, namely, to determine which “module” of Ccr4-Not might act in TCR. The effect of CCR4 and DHH1 deletions on Rpb1 turnover was investigated to isolate the specificity of the role of Ccr4-Not in Rpb1 turnover to its E3 ubiquitin ligase activity versus other functions in the complex such as mRNA decay. If Ccr4 and/or Dhh1 play a role in Rpb1 turnover, then Ccr4-Not’s role in Rpb1 turnover may be indirect via its regulation of mRNA levels. Since Not4’s role in ubiquitylating other substrates such as the NAC was shown to be dependent on other Ccr4-Not subunits, another plausible explanation in this case is that Ccr4 or Dhh1 are structurally involved in Not4’s role as an E3 ubiquitin ligase (54).

This study was carried out over a two-hour time course under DNA-damaging conditions induced by the UV mimetic 4-Nitroquinoline 1-oxide (4NQO). 4NQO forms bulky lesions in DNA that are repaired similarly to UV-induced pyrimidine dimers (74). At the time of 4NQO treatment, the cells were also treated with cycloheximide (CHX), which prevents the synthesis of newly translated Rpb1. This prevents any newly synthesized Rpb1 from obscuring the extent of degradation occurring as a result of DNA damage. Data points for wild type cells and single mutants in CCR4, DHH1, and NOT4 were collected every 30 min and the amount of Rpb1 as well as Taf68, a housekeeping protein used as a control to normalize for protein recovery, in each sample was quantified. As explained in section 2.11, error bars are not shown on the plot because a representative experiment, rather than an average of experiments, is shown. Averaging all the experimental findings would obscure general trends because of the variations in both protein levels and drug activity from experiment to experiment.

Figure 4.1 shows that wild type cells had the greatest Rpb1 degradation by the end of the time course, closely followed by the ccr4Δ and dhh1Δ mutants. These cells exhibited
approximately two-fold reductions in Rpb1 over the time course, with slightly greater Rpb1 reduction in wild type cells. The inconsistent drop in Rpb1 signal at the 60 min time point in \textit{dhh1A} cells and slightly high level at 120 min are specific to this experiment and not normally seen. They are likely a result of inconsistencies during sample preparation or cellular responses. Therefore, correction of Rpb1 levels with Taf68 levels did not completely eliminate these fluctuations. On the whole, \textit{dhh1A} and \textit{ccr4A} cells exhibit Rpb1 turnover rates similar to wild type cells.

On the other hand, the \textit{not4A} mutant exhibited less Rpb1 degradation than wild type cells. The 90 min time point of \textit{not4A} cells shows a small increase in Rpb1 levels, again, this is probably caused by a small inconsistency in the TCA preparation as detailed above. Since Ccr4 is the enzyme responsible for initiating decay and this mutant impairs mRNA decay equally as the NOT4 mutation, these results indicate that Ccr4-Not’s role in mRNA turnover is not responsible for its actions in Rpb1 degradation. It is still possible that Dhh1 and Ccr4 play minor structural roles in the process, but this is unlikely as these subunits are located on the N-terminal of Not1, the major scaffold of Ccr4-Not (Figure 1.11). The ubiquitylation machinery, including Not4, is located on the C-terminus of Not1. This experiment suggests that the ubiquitin ligase activity of Not4 might be responsible for Rpb1 turnover under DNA damaging conditions, therefore, the role of Not4 in this process was investigated further.

\textbf{4.2 Slow growth is not responsible for the decreased Rpb1 turnover in \textit{not4A} cells}

Though \textit{CCR4} and \textit{DHH1} deletion strains have growth rates similar to wild type, \textit{not4A} cells grow about half as fast. Therefore, the reduced rate of Rpb1 turnover in the \textit{not4A} mutant compared to that of wild type cells might be a result of their slow growth, not as a result of the
function of Not4 in Rpb1 turnover. Deleting *LSM1*, a gene that codes for a protein involved in cytoplasmic mRNA decapping and decay, also results in slow growth (68). Therefore, the level of Rpb1 in 4NQO-treated *lsm1Δ* cells was analyzed every 30 min over a 2 hr time course and compared with Rpb1 levels in wild type and *not4Δ* cells. This experiment was conducted prior to the optimization of experimental conditions and the cells were treated with 4 ug/mL rather than 6 ug/mL 4NQO, and CHX was not added. Therefore, Rpb1 degradation in WT cells is not as prominent as in Figure 4.1, but is still observable. Rpb3 levels were used as a control, as it was shown that levels of RNAPII subunits besides Rpb1 did not decrease under DNA damage (42). In each strain, Rpb3 levels remained stable or increased slightly over the time course.

Figure 4.2 shows that Rpb1 levels in WT and *lsm1Δ* cells decreased with 4NQO treatment. The Rpb1 level in *not4Δ* cells fell, but not to the same extent as the other strains. There is an inconsistent drop of Rpb1 signal in the *lsm1Δ* mutant at the 60 min time point, which returns to expected levels. The fact that *lsm1Δ* cells, which grow just as slowly as *not4Δ* cells, exhibit Rpb1 degradation under 4NQO treatment in a manner similar to wild type cells supports the fact that the slow growth rate of cells with the NOT4 deletion is not responsible for the reduced rate of Rpb1 turnover in these cells. Furthermore, since Lsm1 also plays a role in mRNA decay, this result reinforces the idea that impaired mRNA turnover does not result in Rpb1 degradation defects. In other experiments, conditions were optimized to better convey that Rpb1 turnover is reduced in *not4Δ* cells even as wild type cells experience significant Rpb1 turnover. In particular, growth media was supplemented with cycloheximide (CHX) at the time of 4NQO treatment in accordance with the Verma, et. al, and Malik, et. al. studies (69, 74). CHX prevents the synthesis of newly translated proteins, which prevents any newly synthesized Rpb1 from obscuring the degradation occurring as a result of DNA damage. In addition, cells in future
experiments were treated with a slightly higher concentration of 4NQO, 6 mg/mL. Keeping these conditions in mind, Not4 remains a candidate for directly or indirectly modulating Rpb1 turnover under DNA damaging conditions, independently of its slow growth rate or role in mRNA decay.

4.3 Rpb1 turnover is reduced upon complete deletion of the Not4 RING domain

The C4C4 RING domain of Not4 is important for its function in ubiquitylation, but not for its association with other Ccr4-Not subunits (27, 55). The RING domain of Not4 is also important for its association with E2 ligases Ubc4/5 (3). To support the role of Not4 in Rpb1 degradation by ubiquitylation, analysis of Rpb1 turnover in Not4 RING domain mutants was conducted. The \textit{not4}Δ mutant was transformed with plasmids coding for wild type Not4, Not4 with an I64A point mutation in its RING domain, and Not4 with a RING domain deletion (55). The I64A point mutation reportedly disrupts the interaction between the RING finger and E2 ubiquitin-conjugating enzyme (3).

Interestingly, the growth rates of the strains examined fell along a spectrum, with the \textit{NOT4} deletion the slowest growing, followed by \textit{not4}Δ cells expressing \textit{NOT4}_{\Delta \text{RING}}, \textit{NOT4}_{\text{I64A}}, \textit{NOT4}_{\text{WT}}, respectively (Figure 4.4). Wild type yeast grew faster than all of these strains. Despite utilizing the same background strain, BY4742, Halter, D., et. al., did not report these substantial growth rate differences between these variations of Not4 (27). This may be due to the fact that the plasmids containing the \textit{NOT4} variations were transformed into the cells using a plasmid containing the \textit{URA3} gene, an experiment that generates more false positives than using \textit{LEU}-containing plasmids. Once again, Taf68 levels were used as a control for protein level at each time point and remained stable over the time course for each strain.
Upon analyzing the Rpb1 levels under 4NQO treatment for 2 hours, it was found that not4Δ cells expressing NOT4WT exhibited Rpb1 turnover similar to wild type yeast (Figure 4.3a). Both not4Δ cells expressing NOT4ARING and not4Δ cells showed less Rpb1 turnover over the time course than wild type cells (Figure 4.3b and c). The Not4 point mutant (I64A) exhibited an intermediate phenotype with regards to Rpb1 turnover, a result with two possible explanations. The point mutation may not completely disrupt the interaction between the Not4 RING domain and its E2s, allowing some Rpb1 turnover. It has been shown that some RING finger point mutations abolish this E2-RING interaction while others have a lesser effect (3). Alternatively, the Not4 RING domain is important for some other interaction that results in the degradation of Rpb1, which is disrupted by the RING domain deletion, but not the point mutation. Either way, these results indicate that the RING domain of Not4 is required for Rpb1 turnover. Since RING domains are classically associated with ubiquitylation, the possibility that Not4 directly ubiquitylates Rpb1 or another protein required for Rpb1 turnover needs to be explored.
Figure 4.1: *not4Δ* cells show the most pronounced lack of Rpb1 degradation upon 4NQO treatment.

A.

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B.

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</table>

C.

Rpb1 Levels in 4NQO-treated Yeast Cells
Figure 4.1: not4Δ cells show the most pronounced lack of Rpb1 degradation upon 4NQO treatment. 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. (A) Levels of Rpb1 in wild type cells dropped over time course when compared to the Taf68 control while Rpb1 levels in not4Δ cells remained relatively stable over the same time period, except for a rise at the 90 min. time point which was likely caused by inconsistencies in TCA preparation. (B) dhh1Δ cells exhibited an unexpected drop in Rpb1 at 60 min and an unexpected rise at 120 min, probably due to inconsistencies in the TCA preparation procedure. On the whole, dhh1Δ and ccr4Δ cells exhibited similar Rpb1 degradation to wild type cells. (C) Line graphs of Rpb1 levels in each strain upon 4NQO treatment. Protein levels for each timepoint were plotted based on a % of Rpb1 detected at the 0 min. timepoint for each strain divided by the corresponding a % of Taf68 detected at the 0 min timepoint for each strain.
Figure 4.2: *not4Δ* cells exhibit decreased RPB1 turnover compared to wild type and *lsm1Δ* cells under 4NQO treatment.

**A.**

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**B.**

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<td>4 ug/mL 4NQO</td>
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<td>(+)</td>
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Figure 4.2: *not4Δ* cells exhibit decreased Rpb1 turnover compared to WT and *lsm1Δ* cells under 4NQO treatment. Cells were grown in YPD and treated in log phase growth. At time 0 min., indicated samples were treated with 4 ug/mL 4NQO. WT (A) and *lsm1Δ* (B) cells exhibited greater Rpb1 degradation after 120 min of treatment with 4NQO when compared to *not4Δ* cells (C). Rpb3 was used as a loading control, and levels remained stable or increased slightly over the time course in each strain. (D) Visualization of Rpb1 levels in each strain over the time course. Rpb1 levels fell in WT and *lsm1Δ* cells over the time course. Rpb1 levels in *not4Δ* cells fell, but not as severely as in WT and *lsm1Δ* cells.
Figure 4.3: Spot-plating assay showing growth differences between Not4 mutants (work of Jonathan Willis). Species following the “+” sign were transformed into the indicated cell, transformants were selected on –LEU plates and then seeded for spot-plating. This image was taken 48 hrs after spot-plating. See Table 2.3 for a description of the plasmids used. From fastest to slowest growing: wild type, not4Δ + NOT4WT, not4Δ + NOT4I64A, not4Δ + NOT4ΔRING, and not4Δ cells. not4Δ + NOT4ΔRING cells had a very similar growth phenotype to the not4Δ mutant. Also, not4Δ + NOT4WT cells had a similar growth phenotype to wild type cells. However, not4Δ + NOT4I64A cells, which contain Not4 with a point mutation in the RING domain which affects its association with E2s, had an intermediate growth rate. The fact that not4Δ + NOT4I64A has a faster growth rate than the not4Δ and not4Δ + NOT4ΔRING cells indicates that the I64A point mutation may not completely inactivate the function of the Not4 RING domain in the cell.
Figure 4.4: Rpb1 turnover is reduced upon complete deletion of the Not4 RING domain.

A.

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B.

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Taf68 Control
Figure 4.4: Rpb1 turnover is reduced upon complete deletion of the Not4 RING domain. Cells were grown in minimal media with all amino acids except leucine, and were treated with 100 μg/mL CHX at time 0. Indicated cells were also treated with 6 μg/mL 4NQO at this time. (A) WT and not4Δ + NOT4 WT cells exhibited the greatest levels of Rpb1 degradation over the time course. (B) not4Δ + NOT4I64A cells exhibited some degradation of Rpb1, while both not4Δ + NOT4ΔRING and (C) not4Δ exhibited almost no Rpb1 degradation. (D) Rpb1 amounts in each strain as a % of 0 min protein levels, corrected by dividing each Rpb1 % value by the Taf68 % value from 0 min at the same time.
CHAPTER 5

DISCUSSION

5.1 Known roles of Ccr4-Not in removing RNA Polymerase for DNA damage repair

Growth rate analysis of single and double mutants in TFIIS and Ccr4-Not subunits by spot plating provided evidence to support the theory that Ccr4-Not acts differently in transcription elongation than TFIIS. This conclusion can be explained by epistasis, which is a broad term encompassing the ways in which two genes affecting the same phenotype can interact (46). Two epistatic genes can be identified by comparing the effects of each single mutant with the double mutant. If the phenotype of the double mutant is not merely the additive of both single mutant phenotypes, the genes are epistatic. This concept can be applied to examine whether two separate gene deletions that produce the same phenotype act in the same pathway to produce that phenotype. For example, if a double mutant exhibits a slow growth phenotype similar to that of each separate single mutant, the two genes may act in the same pathway. On the other hand, if the double mutant displays a slower growth rate, then the genes might act in different pathways.

Woudstra, et. al., applied this logic to determine that both the Def1 protein and TFIIS act in transcription elongation, but proceed through different mechanisms (77). DEFI and DSTI deletions separately caused a slow growth phenotype, but the double mutant grew even more slowly than either single mutant. Further investigation revealed that Def1 promotes transcription elongation by acting in ubiquitin-dependent Rpb1 degradation, a mechanism distinct from TFIIS’s role in stimulating the nuclease activity of RNAPII (35, 75, 77). Several elongation factors, such as Rad26 and Paf1c, have been shown to act in TCR in addition to transcription,
however, mutants in TFIIS show no TCR phenotype (21, 48, 72). Similarly to the \textit{def1Δ/dst1Δ} double mutant (77), deletions in \textit{DHH1}, \textit{CCR4}, \textit{NOT5}, and \textit{NOT4} have a synthetic lethal phenotype when combined with a \textit{DST1} deletion.

Since Def1’s role in RNAPII turnover implicates it in both transcription elongation and TCR, we investigated the possibility that Ccr4-Not subunits also have implications in the steps required for TCR, such as RNAPII turnover. There is precedent for the activity of Ccr4-Not in DNA repair, for example, mutants in several subunits were found to be sensitive to DNA damaging agents and mutants in Ccr4-Not subunits show reduced TCR (21, 70). Moreover, Traven, A., et. al. provide grounds that Ccr4 might play a role in directing DNA damage repair processes by showing that the \textit{CCR4} deletion exacerbates the effects of the \textit{DUN1} deletion.

Dun1 is a checkpoint kinase important for activating transcription of DNA damage-inducible genes and regulating repair pathways. The fact that Ccr4 appears to act in a similar pathway as Dun1 indicates that it may act in global DNA repair, separately from TCR. The results presented here indicate that Not4, on the other hand, appears to play an important role in TCR (Figures 4.1, 4.3). These findings indicate that Ccr4-Not can affect DNA damage repair pathways on two levels; either providing nucleotides for global DNA repair (through Ccr4) or promoting Rpb1 degradation to facilitate TCR (though Not4). Interestingly, Ccr4-Not’s role in global DNA repair seems to require the mRNA decay function of the complex, while its actions in TCR do not as evidenced by the fact that \textit{ccr4Δ} and \textit{dhh1Δ} mutants have similar Rpb1 degradation as wild type cells under DNA damaging conditions (Section 4.1).

The E3 ubiquitin ligase Not4 has already been implicated in TCR by studies in \textit{not4Δ} cells (21). Without global genome-NER (GG-NER), which is not dependent on transcription, deficiencies in TC-NER lead to increased UV sensitivity. \textit{RAD7} is a gene that is essential for
GG-NER, and the viability of the not4Δ/rad7Δ double mutant dropped far below that of either single mutant under UV treatment. In my study, strains lacking the Ccr4, Dhh1, and Not4 subunits were analyzed to see if deletions in these subunits affected Rpb1 turnover under DNA damaging conditions and by extension TCR. The potential role of the Ccr4 deadenylase and Dhh1 RNA helicase in Rpb1 turnover was investigated to determine whether the Ccr4-Not complex played a role in Rpb1 turnover indirectly through regulating mRNA levels. Results in my study indicate that Not4 should be further investigated as a possible player in TCR rather than Ccr4 or Dhh1, since deletions of the latter two exert less of an effect on Rpb1 turnover (Figure 4.1). This shows that Ccr4 and Dhh1 are not important for Rpb1 degradation under DNA damaging conditions, therefore, the deadenylase function of Ccr4-Not and by extension its role in mRNA turnover probably do not play a role in this process.

On the other hand, the NOT4 deletion caused a noticeable reduction in Rpb1 turnover. Not4 might very well directly ubiquitylate Rpb1, targeting it for degradation and thus facilitating TCR. Not4 requires its RING domain to ubiquitylate the NAC, the first substrate of Not4 discovered (54). Results presented here show that a Not4 RING domain deletion mutant decreased Rpb1 turnover to a similar level observed in a NOT4 deletion mutant (Figure 4.3). Therefore, the ability of Not4 to interact with its E2 protein, and thus, ubiquitylate proteins, is essential for its regulation of Rpb1 turnover. Though the point mutant did not show as drastic a reduction in turnover, this could be due to a reduction, rather than complete elimination, of the interaction between Not4 and its E2s Ubc4/5. It is reasonable to suppose that the RING domain of Not4 directly ubiquitylates Rpb1, but more concrete evidence must be provided to prove the direct nature of this interaction. Rsp5 and the Elongin-Cullin complexes are major E3 ubiquitin ligases responsible for Rpb1 mono-and poly-ubiquitylation, respectively (detailed in section 5.2).
Therefore, perhaps the first step in further examining the role of Not4 in this process is to compare the effect of double and single mutants in NOT4 and RSP5 and the Elongin-Cullin complex to see if Not4 acts independently of this pathway.

5.2 Known Pathways of RNA Polymerase II Large Subunit Rpb1 Ubiquitylation

The cell must expend much energy and material to manufacture RNAPII, so it is likely that attempts to restart stalled RNAPII precede Rpb1 degradation and complex dissolution in solving problems of transcriptional arrest. However, if RNAPII remains arrested, the cell turns to Rpb1 degradation pathways to remove RNAPII and expose the site of DNA damage so that repair factors can fix the problem. One DNA-damage-induced pathway for Rpb1 degradation, involving the Rsp5 and Elongin-Cullin complex E3 ubiquitin ligases, has been elucidated in reasonable detail (74, 75).

First, arrested RNAPII is recognized by Rsp5 in conjunction with the E2 enzyme Ubc5. Rsp5 adds a single ubiquitin molecule to lysine residues on Rpb1. At the same time, a separate pool of cytoplasmic Rsp5 ubiquitylates Def1, allowing it to undergo processing to enter the nucleus. By binding the Elongin-Cullin complex by means of its ubiquitin-homology domain, Def1 helps recruit this E3 ligase to arrested Rpb1 molecules so it can extend the ubiquitin chains begun by Rsp5. Cul3 is the catalytic subunit of the Elongin-Cullin complex. Once polyubiquitylation has occurred, Cdc48, a ubiquitin-dependent ATPase, helps deliver the 26S proteasome to Rpb1 for degradation (Figure 5.1).

Since RNAPII must be removed from the DNA damaged site in order for TCR to occur, it is likely that Rpb1 ubiquitylation and degradation is a recurrent theme and parallel pathways act to preserve it. These experiments indicate that Not4 is involved in this process via its RING
domain. Interestingly, Rsp5 performs at least two ubiquitylation events in the Rpb1 degradation pathway; one to facilitate the nuclear localization of Def1 and one on the Rpb1 subunit itself. This information, along with the observation of the many adaptor proteins implicated in Rpb1 degradation beyond the basic E1-E2-E3 ubiquitylation system, indicate that the role Not4 plays in this process may not be simply to directly ubiquitylate Rpb1, but perhaps to ubiquitylate another protein involved in targeting Rpb1 for degradation.

My attempts to show that Not4 is required for Rpb1 ubiquitylation were not successful due to technical reasons. I tried to isolate ubiquitylated Rpb1 by affinity chromatography and immunopurification. First, GST-Dsk2, which contains a ubiquitin binding domain, was constructed used to bind ubiquitylated species from cell extracts so to compare levels of ubiquitylated Rpb1 between wild-type cells and Ccr4-Not mutants. Signal from Rpb1 was unreliable under these conditions, so a myc-tagged version of ubiquitin, so that antibodies to myc can be used to enrich ubiquitylated proteins from extracts. However, Rpb1 still could not be detected under these conditions. It is possible that Rpb1 is ubiquitylated in a manner rendering it difficult to bind to beads, the experimental conditions need to be changed so that ubiquitylated Rpb1 can be analyzed or the ubiquitylated Rpb1 is too short-lived to accumulate to detectable levels. Whether by optimized GST-pull down conditions or another means, investigating Rpb1 ubiquitylation in single and double mutants in NOT4 and either RSP5 and or CUL3 will indicate if Not4 acts independently of Rsp5 in the ubiquitylation of Rpb1.

5.3 Model for the Not4 subunit in Transcription-coupled repair

Figure 5.2 presents four possibilities for the role of Not4 in Rpb1 degradation upon DNA-damage induced RNAPII arrest. The first is that Not4 directly ubiquitylates Rpb1 when
RNAPII is stalled on a lesion. This is the simplest scenario that correlates with the experimental results garnered thus far. The second scenario is that the Not4 subunit present on an upstream RNAPII ubiquitylates the Rpb1 subunit of a stalled downstream RNAPII when the two molecules collide. This way, the downstream RNAPII is removed from the damaged site and the upstream RNAPII can continue transcribing through. In a similar scenario, the Not4 subunit on an upstream RNAPII ubiquitylates part of the Ccr4-Not complex on a downstream stalled RNAPII molecule. This may prompt the Not4 subunit of the stalled RNAPII molecule to ubiquitylate Rpb1 so that the RNAPII molecule is removed from the damaged site. The fact that there is evidence that Not4 might ubiquitylate other Ccr4-Not subunits supports this third scenario (16, 27, 59). A fourth possible scenario is that Not4 on RNAPII ubiquitylates an adaptor protein, which proceeds to trigger Rpb1 degradation. The last three models are consistent with the theory that Rpb1 degradation is the pathway of “last resort,” that is, it only occurs when a pileup of RNAPII begins to form because of DNA damage. Clarifying whether the ubiquitin ligase activity of Not4 acts directly or indirectly on Rpb1 under DNA damaging conditions will give direction as to which model is the best representation.

5.4 Summary and Perspective

Previous studies have implicated the Ccr4-Not complex in both global DNA repair and TCR (21, 70). TCR is an important process for maintaining the fidelity of the protein-coding portion of the genome since by definition it is targeted to actively transcribed genes. The Ccr4-Not complex acts in many gene regulatory processes through its deadenylation and ubiquitylation functions. Therefore, it makes sense that it may act to preserve TCR in the cell, perhaps by playing a role in the removal of arrested RNAPII from damaged DNA via Rpb1.
degradation, in parallel with other known processes. It was known that Ccr4-Not is necessary for efficient TCR, however, until this study there was no indication as to which subunit of Ccr4-Not plays a key role in TCR (21). Ccr4-Not is modular in structure; each of its subunits acts in either deadenylation or ubiquitylation of species targeted by the complex. It was therefore necessary to determine whether Ccr4-Not plays a role in TCR through either of these functions. I determined that the E3 ubiquitin ligase Not4, its RING domain specifically, is important for Rpb1 degradation, a prerequisite for TCR. This indicates that Not4 may be directly involved in Rpb1 decay under DNA-damaging conditions, but further experiments will investigate whether Not4 acts directly or indirectly on Rpb1 in this degradation pathway.

Since TCR is key to maintaining the fidelity of frequently transcribed genes, it follows logically that problems with TCR might well result in accumulation of DNA damage that leads to cancers or other phenotypes such as premature aging. Deficiencies in TCR proteins have already been shown to result in pathological conditions such as Cockayne’s syndrome. The Ccr4-Not complex is conserved from yeast to humans, much like Rad26 is the yeast homolog of CSB, the major species deficient in patients with Cockayne’s syndrome. Though Cockayne’s syndrome results in sunlight sensitivity and premature aging rather than cancers, the fact that DNA damage leads to cancers in general implies that mutations in other TCR factors may contribute to cancer. Using yeast as a tool to better understand the role of the Ccr4-Not complex in TCR will provide a knowledge base that will facilitate comprehension of this process in humans. In turn, this mechanistic understanding might eventually provide knowledge that will prove useful to relating misregulation of TCR to pathological conditions.
Figure 5.1: Model of Rpb1 degradation via the Rsp5 and Elongin-Cullin E3 ligases

(A) When RNAPII is stalled on a lesion, Rsp5 in conjunction with the E2 Ubc5 monoubiquitylates Rpb1 and ubiquitylates Def1, so that part of Def1 is degraded and it is processed for nuclear transition. Next, Def1 binds RNAPII via its ubiquitin-binding domain and recruits the Elongin-Cullin complex, which elongates the ubiquitin chain Rsp5 began on Rpb1. (B) Cul3 is part of the Elongin-Cullin complex. After the Elongin-Cullin complex elongates the ubiquitin chain on Rpb1, the ATP-dependent adaptor protein Cdc48 is recruited to Rpb1 via Ubc5, and helps poly-Ub Rpb1 transition into the 26S proteasome for processing.
Figure 5.2: Models for possible roles of Not4 in Rpb1 degradation
Figure 5.2: Models for possible roles of Not4 in Rpb1 degradation. Upon a DNA-damaging event, one of four scenarios might arise. (A) Not4 could directly ubiquitylate Rpb1 of the stalled RNAPII, promoting the dissolution of the complex and its release from the site of DNA damage. (B) After encountering a stalled RNAPII molecule, the Not4 subunit of the upstream RNAPII could ubiquitylate Rpb1 of the original stalled RNAPII molecule. (C) The same situation, but instead of ubiquitylating Rpb1, the upstream Not4 ubiquitylates another Ccr4-Not subunit, prompting that Not4 to ubiquitylate Rpb1. (D) Again the same situation, but Not4 ubiquitylates some adaptor protein which leads to the downstream ubiquitylation of Rpb1, perhaps via the Rsp5 pathway.
BIBLIOGRAPHY


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EDUCATION
The Pennsylvania State University, University Park, PA
B.Sc. In Biochemistry and Molecular Biology
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RESEARCH EXPERIENCE

Honors Thesis Research January 2011-Present
Penn State University, University Park, PA: Dept. of Biochemistry and Molecular Biology (Principal Investigator: Prof. Joseph Reese)
Title: Genetic and biochemical analysis of elongation mechanisms of RNA polymerase II.
Description: Use of molecular biology and genetics to study the interactions between elongation factors in budding yeast S. cerevisiae. Construction of mutants in elongation factors, and analysis of their phenotypes to determine how elongation factors regulate transcription.
• Deleted genes in yeast and verified mutants using transformation PCR, and complementation
• Dissected spores to isolate double mutants
• Analyzing mutant phenotypes by spot plating and western blotting

Summer Research Intern Summer 2013
Lausanne, Switzerland: Ecole Polytechnique Federale de Lausanne (Principal Investigator: Prof. Viesturs Simanis)
Title: Studying cell division in fission yeast.
Description: Use of genetics and confocal microscopy to generate statistics on the resetting of Septation Initiation Network (SIN) regulatory proteins in S. pombe. Evaluation of cell cycle events that may determine SIN resetting and realization that multiple control points modulate the process. Manuscript in preparation.
• Constructed yeast strains by means of tetrad dissection
• Used confocal microscopy to generate images and films of dividing cells
• Quantified levels of the tagged protein through ImageJ analysis

Summer Research Intern Summer 2012
Harvard University, Cambridge, MA: FAS Center for Systems Biology (Principal Investigator: Dr. John Calarco, worked with post-doctoral fellow Dr. Adam Norris)
Title: Exploring RNA diversity in the nervous system.
Description: Use of biochemistry and genetics to study novel alternative splicing regulators in the nervous system of C. elegans. Identification of new splicing regulators by forward genetic screening of organisms with fluorescent reporters of specific splicing events, and analysis of the resultant mutants to elucidate the mechanism of these factors.
• Conducted snip-SNP mapping to locate mutation sites
• Carried out forward genetic screen (EMS mutagenesis) for alternative splicing regulator mutants
• Verified activity of two splicing regulators using RT-PCR

RELEVANT PROJECTS/COURSEWORK
Analysis of Proteins Using Aldolase as a Model

Spring 2013

• Verified the mechanism of aldolase using coupling Assays I & II and graphical determination of kinetic parameters
• Extracted and purified aldolase using salt fractionation, dialysis, and column chromatography
• Analyzed molecular weight, N terminus, and isoelectric point of aldolase with HPLC, crystallization, and isoelectric focusing

RNAi in Drosophila Class Project

Fall 2012

• Conducted RNAi screen for ddaE neuronal phenotypes in protein localization and dendrite structure
• Took pictures of fluorescent neurons and finalized them with Adobe Photoshop
• Carried out statistical t-test to determine phenotype significance

LEADERSHIP/TEACHING

Teaching Assistant Penn State University, State College, PA

• General Biochemistry II (Spring 2013) and Fundamental Of Organic Chemistry (Fall 2012)
• Held exam review sessions (~200 students enrolled, 6-8 hrs/wk)

Penn State Biochemistry Society President 2012-2013

• In charge of undergraduate society with 38 members, organized academic, volunteering, and social events

Penn State Biochemistry Society THON Co-Chair 2011-2012

• Helped coordinate the Society’s involvement in the world’s largest student-run fundraiser. Funds are for pediatric cancer treatment and research

ACTIVITIES/WORK EXPERIENCE

• Emergency Department Volunteer, Mt. Nittany Medical Center, State College, PA  Fa. 2013-Present
• Penn State Club Swimming Member 2010-Present
• Phi Beta Kappa Society, General Member Sp. 2013-Present
• National Health Preprofessional Honor Society (Alpha Epsilon Delta), General Member 2012-2013
• Penn State Concert Band, Flute Fall 2011
• Volunteer, Friends Retirement Home, Kennett Square, PA, and Jenner’s Pond Retirement Community, West Grove, PA Summer 2011
• Committee Member, Rules and Regulations, PSU Pan-Hellenic Dance Marathon (THON) 2010-2011
• Café Employee/Prep Cook, Waitress, Ashworth by the Sea, Hampton, NH Summer 2010-11

COMPUTER SKILLS: C++, ImageJ, Adobe Illustrator, ImageQuant

HONORS/AWARDS

• Penn State Eberly College of Science Braddock Scholarship (Full Tuition/Room/Board) 2010-2014
• Schreyer Honors College Academic Excellence Scholarship 2010-2014
• Penn State Evan Pugh Scholar Award, President Sparks Award, Freshman Award 2011-2013
• Morrow Award Scholarship (Biochemistry Dept. Award, 1 of 2 given) 2011