A BIOCHEMICAL ANALYSIS OF TRANSCRIPTION-COUPLED REPAIR PROTEINS IN YEAST AND THEIR ROLE IN TRANSCRIPTION elongation AND mRNA Degradation

ANNA WING
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Reviewed and approved* by the following:

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

Craig Cameron
Professor of Biochemistry and Molecular Biology
Honors Adviser

* Signatures are on file in the Schreyer Honors College.
ABSTRACT

Transcription-coupled repair (TCR) is an essential part of the cell’s DNA repair machinery. It is the most effective way to fix damage caused by UV radiation and oxidative stress before dangerous or cancerous mutations can form. This mechanism is associated in *S. cerevisiae* with the Ccr4-Not complex, a complex of nine proteins that are highly conserved in eukaryotes from yeast to humans. In addition to activities related to the cell stress response, the Ccr4-Not complex plays a role in key pathways of transcription elongation and mRNA degradation as demonstrated by elongation and degradation defects present in Ccr4-Not mutants. However, the precise proteins that link these two processes are currently unclear. In this project, several TCR-associated proteins were analyzed to determine their role in transcription elongation and mRNA degradation through the quantification of gene-length dependent elongation rates and mRNA degradation rates in mutant strains of yeast. While none of the proteins analyzed had an effect on transcription elongation, two of the five proteins studied were shown to significantly impact mRNA degradation rates within the cell—Not4, a subunit of the Ccr4-Not complex, and Def1. The other three TCR-associated proteins analyzed, Ubp3, Bre5, and Rad26, did not demonstrate an effect and therefore are not implicated in the mRNA degradation pathways. Further study of the Not4 protein and its domains will be necessary to determine which protein domains are essential for efficient RNA turnover and to better understand the role of Def1 in linking transcription-coupled repair to mRNA degradation.
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CHAPTER 1
INTRODUCTION

1.1 DNA Repair

Eukaryotic cells employ a variety of mechanisms to maintain the integrity of the DNA sequence. Mammalian DNA polymerase, for example, utilizes a proofreading domain during DNA synthesis to check for errors and recruit additional corrective factors. When damage from UV radiation or oxidative stress leads to modified bases or lesions, other methods are frequently required to repair the damage before it is copied incorrectly (1). Two such methods are base excision repair (BER) and nucleotide excision repair (NER), both of which are essential for the repair of different types of DNA damage (2).

BER is a conserved pathway responsible for the repair of non-helix-distorting base lesions and other small base modifications caused by oxidative damage. Eukaryotes utilize a set of DNA N-glycosylases, each responsible for recognizing and excising a specific type of damaged base—for example the Ogg1 protein in yeast binds to and removes 7,8-dihydro-8-oxoguanine to leave behind an apurinic/apyrimidinic (AP) site. In \textit{S. cerevisiae}, AP endonucleases Apn1p, Ntg1p, and Ntg2p then cleave the AP site and the 5’ terminus is processed by deoxyribose phosphodiesterase. The gap is filled by DNA polymerase and the nick is sealed by DNA ligase to complete the repair (1).

NER is a highly conserved pathway responsible primarily for correction of helix-distorting bulky lesions and other damage caused by UV radiation (2). In \textit{S. cerevisiae}, the damage is recognized by Rad14, which recruits the Rad4-Rad23 complex to the site. The helicase domains of TFIH unwind the DNA at the site of the damage, allowing RPA to selectively bind the single-stranded DNA and to specify the damaged strand. Endonucleases Rad1-Rad10 and Rad2 then cooperatively make single-stranded nicks on either side of the damaged sequence. The 25-30 bp fragment is removed, and DNA polymerase fills in the complimentary sequence to the non-damaged strand while DNA ligase seals the nicks (3). Mammalian homologues for each of these proteins have been identified as well (4).
1.2 Transcription-coupled Repair

Repair happens more quickly on the transcribed strand than the non-transcribed strand because damage on the transcribed strand stalls RNAPII, creating a signal that recruits repair machinery to the site of the damage in a pathway that is displayed in Figure 2 (4). This pathway is a rich ground for study due to its close ties to a number of different human diseases—failures of the transcription-coupled repair pathway have been linked to skin cancer, xeroderma pigmentosum, Cockayne’s syndrome, and other diseases (5, 6). While the mechanism of TCR has been studied extensively in prokaryotes, the eukaryotic pathway of repair is complex, less clearly understood and continues to be researched.

The initiation of transcription-coupled repair relies upon the arrest of RNAPII at a site of DNA damage (Figure 3). In the presence of a DNA lesion, stalled RNAPII cannot always be rescued via backtracking by TFIIS as it is during the normal process of transcription. Additionally, RNAPII does not utilize a lesion-bypass mechanism like many DNA polymerases. Instead, RNAPII must be ubiquitylated and degraded to prevent the irreversible blockage of later transcription while the lesion is repaired by TCR to allow later RNAPII to transcribe unhindered (8). In *S. cerevisiae*, this problem is primarily tackled by two proteins, Def1 and Rad26 that associate with each other and with stalled RNAPII. Def1 is an RNAPII-ubiquitylation promoting factor that marks RNAPII for degradation by the ubiquitylation machinery. Def1 communicates with structural components of the RNAPII complex, such as the Rpb9 subunit and the C-terminal repeat domain (CTD) on the largest subunit of RNAPII, Rpb1 (9). *DEF1* also plays a role in elongation in the absence of induced DNA damage and deletion of *DEF1* leads to increased sensitivity to transcription elongation inhibitors such as 6AU (7).

Meanwhile, Rad26 coordinates the repair of the transcription-stalling lesion along with a series of other TCR-linked proteins such as Rad28, Paf1, the THO complex, the Thp1-Scd3 complex, and Spt4/5 along with the assistance of transcription factors like TFIIH as seen structurally in Figure 4 (9). Rad26 is part of the SNF2 sub-family of DNA helicases which are often responsible for chromatin remodeling in the genome. The factor therefore may be responsible for unwinding the DNA near a damage site to
promote accessibility for other TCR repair factors and may also have a role in helping RNAPII bypass some types of lesions through association with TFIH(10, 11). Rad28 may be responsible for maintaining the integrity of the repair proteins as well as RNAPII by blocking the E3 ubiquitin ligase from marking these proteins for degradation. Rad26 and Rad28 may both act in creating time and space for TCR to occur, through they never form a stable association in the cell (11).

Crucially, some factors involved in the process of TCR are closely linked not just to transcription via elongation factors but also to mRNA metabolism and transport. Mutants in the THO and Thp1-Scd3 complexes, both of which play important roles in mRNP biogenesis and export from the nucleus, are deficient in TCR, possibly due to a failure to either rescue or degrade RNAPII from the transcript during repair (12). However, the overall links between transcription elongation, TCR, and mRNA/mRNP activity remain poorly understood.

1.3 mRNA degradation

mRNA degradation is a process in constant equilibrium with transcription. In eukaryotes, this process occurs through several different pathways based on the type of mRNA transcript targeted for degradation and specific to the structure of the eukaryotic mRNA (Figure 5). Rate of mRNA turnover can be an important regulatory tool in determining the rate of formation of the eventual protein product of the transcript and the proteins involved in regulation of these processes are therefore essential to maintaining proper levels of product under varying conditions (13).

The process of mRNA destruction can be broken down into two key categories: deadenylation-dependent decay and deadenylation-independent decay, which are depicted in Figure 6 (63). In the first category, degradation is initiated by the shortening of the poly-A tail, followed by either 3’ to 5’ decay or decapping and 5’ to 3’ decay (13). The deadenylation step is an important target for regulatory molecules such as miRNAs to control protein levels in the cell, as seen in Figure 7. Micro RNAs (miRNAs) act to
downregulate gene expression via a variety of mechanisms and are primarily associated with translation inhibition, but miRNA activity has also been linked to the initiation of degradation through the proteins GW182 and Argonaute 1 (AGO1). These miRNA complexes also associate with the decapping complexes Dcp1/Dcp2 and with 5- to 3’ exonucleases such as Xrn1 and 3’ to 5’ exonucleases called the exosome. It is likely that mRNA messages that are translationally inhibited by miRNAs are marked for mRNA degradation by these complexes, then deadenylated (14).

Proteins Ccr4 and Caf1 (two subunits of the Ccr4-Not complex discussed in Section 1.5) are members of the main cytoplasmic deadenylase in S. cerevisiae. These proteins both contain nuclease domains and are recruited to the mRNA message. Recruitment of ccr4-Not can occur through RNA binding proteins (e.g. Puf3, Puf4, and Puf5) (17). Caf1 also associates with Dhh1, which is part of a decapping complex in yeast. The Pan2p/Pan3p nuclease complex plays a role as an alternative deadenylase in mRNA degradation—it acts as a poly-A nuclease during nuclear mRNA processing to trim the poly-A tail to the proper length, but can act as a deadenylase in the cytoplasm to a limited extent as well. Each of these proteins is conserved in higher eukaryotes as well (15).

In deadenylation-independent decay, the process begins by either decapping the mRNA via a method that does not depend on poly-A shortening or by endonucleolytic cleavage in the middle of the mRNA transcript. Both possibilities are followed by a subsequent decay pattern that is mediated by exonucleases (13). Many transcripts degraded by a deadenylation-independent manner as part of a process called mRNA surveillance, utilized when a premature stop codon is incorporated into mRNAs or when mRNAs are misprocessed. The error is recognized by the translation machinery, then the transcript is decapped without deadenylation by Dcp1 and degraded by exoribonuclease Xrn1 in a 5’ to 3’ manner (13, 16). Though this pathway has been observed to degrade wild type transcripts (e.g. EDC1) as well, it is far less common than the adenylation-dependent pathway. Interestingly, the deadenylation-independent pathway has also been linked to subunits Not2, Not4, and Not5 of the Ccr4-Not Complex (18).
1.4 The Ccr4-Not Complex

1.4.1 General Structure and Function

The Ccr4-Not complex is an important regulatory complex in eukaryotes that plays a role in several gene expression and regulation pathways such as transcription, mRNA export and metabolism, translation, and protein ubiquitylation. It is made up of nine highly-conserved subunits: Not1, Not2, Not3, Not4/Mot2, Not5, Caf1/Pop2, Caf40, Caf130, and Ccr4 that make up a 0.9-1.2 MDa protein (Figure 8). The complex can also be purified in *S. cerevisiae* in a 1.9-2 MDa form that also contains a combination of other proteins such as Dhh1, Caf4, Caf16, Btt1, and Dbf2 (20, 21) which may bind less strongly with the complex but are nonetheless necessary for some associated functions of the complex with the core nine subunits.

The complex was discovered in screens designed to find proteins associated with gene expression and transcriptional regulators. *NOT1* and *NOT2* were the first two genes defined, identified as CDC genes in a screen for G1 cell cycle arrest genes (19). Later, the first four *NOT* genes were found in a screen for mutations that increased *HIS3* expression. The genes appeared to globally repress transcription, and they specifically acted upon the TATA-less core promoter of *HIS3*. This screen is the origin of the most common name for these proteins: NOT comes from Negative On TATA-less (23). The other components of the Ccr4-Not complex were identified through genetics and their physical association with known components of the complex (24).

Not1 is the only protein essential for viability in yeast and acts as a scaffold for the remaining subunits. It is necessary for the overall association of the complex, therefore indicating that the formation of the complex is essential for viability as well. However, cells lacking Not2p, Not5p, or Not4p have increased doubling times as compared to wild type cells (19). Most other subunits associate primarily with the C-terminal region of Not1, as demonstrated by the fact that a truncated 800 amino acid form of Not1 is sufficient for yeast viability. Caf1 and Ccr4 associate with the non-essential N-terminal region,
and are generally considered to be functionally and sometimes physically separate, though their interactions are important for structural integrity of the complex (25). As such, mutants missing two or more of the nonessential subunits are sometimes nonviable, possibly due to a loss of structure or to the combined loss of multiple regulatory functions (19).

Due to the screens through which it was discovered, Ccr4-Not was initially studied as a regulator of transcription initiation (22). The complex associates with several initiation factors, such as TFIID and the TATA-binding protein (TBP), providing an explanation for the TATA-dependent activity in the screen that gave some of its subunits its name, Negative On TATA. It is particularly important in transcription of genes regulated by stress-regulated promoters, indicating that the Ccr4-Not complex differentially regulates the expression of genes under stressed conditions (26). The complex has been implicated most strongly in the regulation of TATA-containing stress response genes through its connection to the SAGA co-activator complex, but has also been identified as a factor with some control over TATA-less housekeeping genes through TFIID. The precise mechanism for this regulation remains unclear and may act through indirect effects (27). However, the Not5p subunit has been shown to associate directly with promoters in a TAF1-dependent manner, providing evidence for its function in regulation of transcription at promoters (29). It can act as either a positive or negative regulator of transcription based on the gene and the environmental conditions (22). However, while the Ccr4-Not complex is most closely associated with regulation of the initiation of transcription, recent work has illuminated its role in several other processes. Its role in the three crucial processes in eukaryotes described earlier will be explored here: transcription elongation, TCR and mRNA decay.
1.4.2 Role in Transcription Elongation

The Ccr4-Not complex works with a variety of transcription elongation factors to ensure efficient elongation in *S. cerevisiae* (Figure 9). Deletion of several subunits (e.g. *not5*) results in a dramatic decrease in transcription efficiency in yeast that is multiplied by additional mutations in elongation factors (9). Several Ccr4-Not mutants also show synthetic lethality with transcription elongation factors such as Paf1c and TFIIS (22, 28). Recent studies indicate that loss of complex subunits such as CCR4 and NOT4 led to decreased relative RNAPII density at the 5’ end of the gene due to the accumulation of RNAPII at the 3’ end of the gene, which is caused by defects in RNAPII elongation through the gene. This indicates that rather than playing a role in maintaining RNAPII contact to the DNA, the Ccr4-Not complex might aid the RNAPII in passing nucleosomes and other blocks in transcription. Like TFIIS, it helps stalled and backtracked RNAPII resume elongation, likely through direct interaction with RNAPII, though it acts through a distinct method to TFIIS (30). Synthetic lethality between Ccr4-Not and TFIIS mutants may be explained by their cooperation to ensure the rescue of stalled RNAPII. One or more subunits of the Ccr4-Not complex also interacts with the transcript, an association which may be responsible for its activity in transcription elongation (22).

1.4.3 Role in Transcription-coupled Repair

Its role in stress regulation of transcription suits the Ccr4-Not complex to a role in repair of DNA damage, which is frequently caused by UV and oxidative stress. Indeed, the complex has been linked to TCR in eukaryotes along with another transcription elongation complex, PAF, based on its phenotype upon exposure to UV radiation. Double mutants in *RAD7* along with a Ccr4-Not subunit (*NOT3, NOT4, NOT5, CAF1*, or *CCR4*) are more sensitive to UV radiation, indicating that the DNA damage repair pathways that would correct UV-caused damage are deficient under these conditions (9).
Another important possibility for its activity lies in its ability to ubiquitylate proteins. This activity is performed by the Not4 subunit of the complex, a protein that acts as a RING E3 ubiquitin ligase in a fashion highly conserved among eukaryotes. Ubiquitylation of a protein is important for both targeted degradation pathways and localization. An E1 ubiquitin activating enzyme attaches to a ubiquitin molecule and is activated by an E2 activating enzyme. A RING domain E3 ubiquitin ligase such as Not4 then acts in a substrate targeting to allow the E2 enzyme to transfer the ubiquitin to the target substrate. For Not4, the primary E2 partners are Ubc4 and Ubc5 (22). This ubiquitylation activity allows the Ccr4-Not complex to play a role in protein quality control. For example, the complex has been linked to the degradation of arrested translation products based on a $NOT4$ mutant that stabilized these products (32). Only the $NOT4$ mutant caused this phenotype—mutations in other Ccr4-Not subunits do not have an affect. This further supports the role of complex in a direct and specific ubiquitylation activity catalyzed by Not4. Another major cytoplasmic target of Not4 is the nascent-associated polypeptide complex (NAC or EGD), which protects nascent polypeptides bound to the ribosome. Its role is somewhat unclear, but Not4 as well as several other Ccr4-Not subunits have been found to physically associate with the NAC subunits and Not4 ubiquitylates NAC under stressed conditions (33).

Not4 also targets nuclear proteins for destruction. Its nuclear targets include histone demethylase Jhd2, an important transcription regulator via H3K4me3 control; Cdc17, a DNAP subunit; and Yap1, a transcription activator (22). The nuclear activity of Not4 relates to another possible role in transcription-coupled repair. Previous research in the Reese Lab shows that the subunit may play a role in the ubiquitylation and degradation of stalled RNAPII to ensure transcription continues as necessary during conditions of DNA damage (73).

Interestingly, the ubiquitylation activity of Not4 does not necessarily appear to act in concert with the rest of the complex in the normal transcription elongation activity of the Ccr4-Not complex. Expression of ribonucleotide reductase genes (RNR), which is highly Ccr4-Not dependent, is reduced by a $NOT4$ mutant genotype (30).
1.4.4 Role in mRNA degradation

As described briefly above and in Figure 7, the Ccr4-Not complex also plays a major role in mRNA degradation—both through the deadenylase subunits Ccr4 and Caf1 and through its strong association in *S. cerevisiae* with Dhh1, a member of the decapping complex. The complex is most associated with the deadenylation-dependent pathway of degradation, which begins with the degradation of the poly-A tail. This is initiated by the Ccr4/Caf1 deadenylase in conjunction with Pan2/Pan3, a minor deadenylase in *S. cerevisiae* (15, 17). It is likely that Pan2/Pan3 is involved in the initiation of decay, then the Ccr4/Caf1 portion of the Ccr4-Not complex completes the deadenylation process. This biphasic process allows for another checkpoint in the regulation of decay—a message that has only been degraded by the Pan2/Pan3 complex may be able to be salvaged by polyA polymerase if necessary (22, 34). *CCR4* and *CAF1* mutants both display decreased mRNA degradation capacity and may act on different substrates within the cell (15).

Ccr4-Not is recruited to a particular mRNA message via a network of RNA binding proteins, the most carefully studied of which are the pumilio fem binding proteins *PUF1*-5. *PUF* proteins associate with Caf1 and Ccr4 and are can induce Ccr4-Not dependent deadenylation of HO mRNA (35).

While the initiation of deadenylation is the crucial Ccr4-Not dependent step in mRNA degradation, the complex is also strongly associated with Dhh1, a member of the decapping complex. As described in the Ccr4-Not structure section above, Dhh1 is an RNA helicase that strongly associates with the complex in its larger 2 MDa form. It associates with the nonessential N-terminus of the Not1 subunit, physically near the Caf1 and Ccr4 subunits (21). Additionally, *CAFI* and *CCR4* mutants show a Ccr4-Not complex phenotype with greatly decreased binding to Dhh1 (21). Dhh1 likely plays a role in the mRNA transcription and decay balance mediated by the Ccr4-Not complex. The Ccr4-Not complex is particularly crucial in mRNA degradation patterns under stressed conditions. Ccr4-Not subunit mutants are more sensitive to stress, as are *PUF* mutants (22). The RNA-binding proteins responsible for mRNA targeting and Ccr4-Not likely create a network of regulation under stressed conditions.
1.5 The Ubp3/Bre5 Complex

The regulation of ubiquitylation requires two key pieces. The ubiquitylation of proteins, as described above, is performed by E1, E2, and E3 enzymes. Equally important, however, is the de-ubiquitylation activity of ubiquitin proteases to more precisely control targeted degradation and localization pathways. Ubp3 is a ubiquitin protease in *S. cerevisiae* that is part of the UBP (ubiquitin-specific processing protease) class of proteins which cleaves ubiquitin from a conjugated protein and is the most widespread and well-studied class of deubiquitylating proteases. UBP proteins share a 350 residue catalytic core domain while variable N and C-terminal portion of the protein are responsible for selection of UBP substrates (36). While these proteins are necessary for wild type functioning of de-ubiquitylation pathways, they are very rarely necessary and sufficient for the de-ubiquitylation of any specific substrate. Instead, these ubiquitin proteases must interact with a variety of other protein cofactors to carry out their full functions (37).

Ubp3 associates with protein cofactor Bre5 to form a deubiquitylating complex. Bre5 has a conserved N-terminal domain related to nuclear transport factor 2 (NTF2) that is necessary and sufficient for binding to Ubp3 as well as a C-terminal RNA-binding domain. This interaction was first discovered by a high-throughput two-hybrid screen for interacting proteins in the *S. cerevisiae* genome and subsequently confirmed by co-immunoprecipitation assays (37, 38). The Bre5/Ubp3 complex was first linked to a COPII protein substrate, Sec23 and later to the β'-COP protein, two subunits in the COPII and COPI complexes responsible for regulation of transport between the ER and Golgi apparatus (36).

More recently, the Ubp3/Bre5 complex has also been linked to the yeast response to DNA damage. In this role, the deubiquitylation of proteins may be less related to the prevention of a degradation signal and more a regulatory covalent modification to control enzyme activity. The complex is essential for the facilitation of normal levels of non-homologous end joining—a DNA repair process crucial for the correction of double-stranded breaks (39). Double-stranded breaks are some of the most dangerous due to the many possibilities for incorrect recombination or replication, as demonstrated by the
negative synthetic interaction of BRE5 mutants with other DNA repair genes (40). The deubiquitylation activity of Ubp3 also plays a role in nucleotide excision repair by acting in opposition to the ubiquitylating factors of Rpb1, delaying ubiquitylation and degradation of RNAPII until strictly necessary, as seen in Figure 10. This is referred to as the “Ub-clock,” which can be extended by Ubp3 function and shortened dramatically by its failure. Ubp3 is thought to deubiquitylate an RNAPII that has been marked for destruction by a ubiquitin ligase like Not4, rescuing it from degradation (41). The far-reaching and not entirely understood breadth of Ubp3/Bre5 activity makes it an excellent target for study.

1.6 Scope and Significance

Many human diseases are closely related to the regulation of genes under stress and the mechanism of gene repair in the cell. As previously described, xeroderma pigmentosa and Cockayne’s syndrome are both linked to faulty regulation of transcription-coupled repair within the cell (5, 6). Xeroderma pigmentosa, depicted in Figure 11, is a rare disorder characterized by extreme UV sensitivity, abnormal pigmentation, and neurological degeneration that is linked to failure in the nucleotide excision repair pathway and the therefore dramatically increased DNA mutation rate upon UV exposure (42). For similar reasons, transcription-coupled repair failures are closely associated with increased rates of skin cancer, a disease that affects approximately 20% of Americans at some point in their lives (5).

Cockayne’s syndrome is a genetic disorder with similar symptoms of UV sensitivity but with more severe neurological degeneration and slow growth. The disorder is caused by a specific defect in selective repair based on helicase activity during the process of TCR, particularly in one protein that is studied closely in this project, Rad26, which has a human homolog CSB that is mutated in many Cockayne’s syndrome patients (43). While skin cancer rates are not increased, patients are unable to transcribe effectively following UV radiation and the assumed initiation of the repair pathway (42).
In the recent history of biochemistry study, many pathways that were previously thought to be independent have been found to share enzymes and protein factors. One multifunctional enzyme complex highly involved in the crosstalk between regulatory mechanisms in the cell is the Ccr4-Not complex, which has been found to impact processes from “birth to death” of the mRNA (22). Additionally, the Ccr4-Not complex has been found to associate with several other proteins that have been implicated in one of the regulatory processes; however, their association with the multifunctional complex may indicate they are multifunctional themselves and participate in additional regulatory pathways. This project examines proteins that are involved in transcription-coupled repair with the Ccr4-Not complex to determine their possible roles in transcription elongation and mRNA degradation.

This project studied five of these TCR proteins. Rad26 and Def1 were studied as crucial transcription-coupled repair factors that may be involved in the regulation process, and Ubp3 and Bre5 were studied as proteins with roles in both DNA repair and stress signaling pathways. Not4, the E3 ubiquitin ligase subunit of the Ccr4-Not complex, was also studied to determine if the ubiquitylation or structural roles of Not4 were involved in alternate processes. This project provides evidence of a role for Not4 and Def1 in mRNA degradation, uncovering an interesting link between the nuclear activities of the Ccr4-Not complex and its cytoplasmic degradation activities. By understanding the proteins involved in these processes, regulation-related diseases can be better understood and new treatment approaches can be developed.
Figure 1: Role of transcription factors in transcription initiation and elongation

Role of transcription factors in transcription initiation and elongation. Taken from (66). Transcription begins with the formation of the pre-initiation complex at the site of the promoter, followed by promoter clearance with the help of initiation factor TFIIH. The RNA is capped as elongation continues with cotranscriptional processing and elongation factors depicted in (4) above. Phosphorylation of RNAPII and transcription factors is represented by the red circles.
Figure 2: Transcription-coupled Repair in Mammalian Cells

Transcription-coupled repair in mammalian cells. Taken from (8). A stepwise representation of the steps in repair. First, damage is detected via transcriptional arrest and TCR factors are recruited to the damage site. Incisions are made on the 5’ and 3’ sides of the damaged strand to remove the lesion and DNA polymerase is recruited to fill in the removed nucleotides. DNA ligase seals the gap and transcription can continue normally.
Nuclear Response to Stalled or Arrested RNAPII. Taken from (7). Upon DNA damage, RNAPII is stalled or arrested at the site. Elongation factors and transcription-coupled repair factors (e.g. Rad26) are recruited to the site. If possible, these factors rescue RNAPII without causing its removal either through lesion bypass or RNAPII retrograde translocation. If rescue does not occur, RNAPII ubiquitylation occurs which either triggers a ubiquitylation-dependent rescue mechanism or leads to the degradation of the RNAPII and the later transcription by a different RNAPII protein.
Transcription-coupled repair factor recruitment. Adapted from (65). Protein complexes including THO, PAF, and Ccr4-Not are crucial in the interaction between transcription elongation and transcription-coupled repair. Rad26 recruits other essential TCR proteins such as Def1, which is necessary for RNAPII removal. The Ubp3/Bre5 complex is also recruited to the site. These complexes mediate the rescue or removal of RNAPII and the reinitiation of transcription.
Figure 5: Structural organization of eukaryotic mRNA

Structural organization of eukaryotic mRNA. Taken from (68). The structure of mRNA includes a 5’ UTR region with a 7-methylguanylate cap and translation factors such as eIF-4G and eIF-4E. Following the coding region, eukaryotic mRNA also includes a 3’ UTR important for regulatory factor binding and a poly(A) tail that helps maintain mRNA integrity. IREs in the 5’ UTR are found only in a small percentage of eukaryotic mRNAs.
Figure 6: Pathways of mRNA degradation

Pathways of mRNA degradation. Taken from (63). mRNA degradation can occur via multiple pathways. Deadenylation-dependent mRNA decay occurs most frequently and is initiated by deadenylase activity, followed by either 5’ decapping with the DCP complex and 5’ to 3’ decay or by direct 3’ to 5’ decay. Deadenylation-independent pathways also exist—in one, the decapping complex acts first followed by Xrn1 exonuclease activity and in the other, an endonuclease creates a degradation site with a break in the central region of the mRNA.
Figure 7: The miRNA RISC complex in mRNA degradation

The miRNA RISC complex in mRNA degradation. Taken from (71). mRNA degradation is an important regulatory tool for controlling protein expression in the cell. In addition to translation repression, one mechanism of miRNA gene silencing involves the recruitment of the Ccr4-Not deadenylase to the site of miRNA-targeted genes.
Figure 8: The structure and functions of the Ccr4-Not complex

The structure and functions of the Ccr4-Not complex. Adapted from (19). The nine subunits of the Ccr4-Not complex with some known protein interactions and complex functions.
Figure 9: The Ccr4-Not complex in transcription elongation

Arrest and misalignment of transcript in the active site

Forward transient excursions by RNAPII realign the transcript and cause the threading of RNA through the exit channel

Ccr4-Not engages the transcript, "locking" RNAPII into an elongation competent form and rescuing the arrested complex

The Ccr4-Not complex in transcription elongation. Taken from (30). The Ccr4-Not complex plays a role in rescue of arrested RNAPII during the normal process of transcription elongation.
Deubiquitylation activity of the Ubp3/Bre5 Complex. Taken from (41). The Ubp3/Bre5 complex acts antagonistically to the ubiquitylating factor Def1 to balance the less-energetically costly RNAPII rescue with RNAPII degradation when rescue is unsuccessful.
Symptoms of Xeroderma Pigmentosum. Taken from (67). Symptoms of xeroderma pigmentosa include sun hypersensitivity and skin hypopigmentation. It also leads to neurologic abnormalities such as delayed motor skills and microcephaly. It is caused by a defective transcription-coupled repair mechanism.
CHAPTER 2
MATERIALS AND METHODS

2.1. Materials

Reagents and media materials came from Sigma-Aldrich, American Bioanalytical, and BD Biosciences. Synthetic dropout (SD) media is composed of 6.8 g/L yeast nitrogen base with ammonium sulfate, 10% 10X amino acid mix, and 2% dextrose. SD plates contain an additional 22 g yeast bacto-agar. Yeast Peptone (YP) media is composed of 10 g/L yeast extract and 20 g/L bacto-peptone. YPAD media contains 2% adenine sulfate and 2% dextrose and YPAG media contains 2% galactose. A Thermo Scientific Genesys 20 was used for all optical density (OD) measurements.

2.1.1. Strains Used

Yeast strains used include wild type strains BY4742 and BY4741 originally designed by Brachman, et. al. (45) as well as mutant knockout strains derived from the wild type strains listed in the table below.
Table 1. Yeast Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>BY4742</td>
<td>MATα; his3Δ 1; leu2Δ 0; met15Δ 0; ura3Δ 0</td>
<td>Wild type</td>
</tr>
<tr>
<td>BY4741</td>
<td>MATα; his3Δ 1; leu2Δ 0; lys2Δ 0; ura3Δ 0</td>
<td>Wild type</td>
</tr>
<tr>
<td>JR1531</td>
<td>BY4742 with not4::kanMX</td>
<td>ΔNot4</td>
</tr>
<tr>
<td>JR1696</td>
<td>BY4742 with def1::kanMX</td>
<td>ΔDef1</td>
</tr>
<tr>
<td>JR1697</td>
<td>BY4741 with def1::kanMX</td>
<td>ΔDef1</td>
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<tr>
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<td>BY4742 with rad26::kanMX</td>
<td>ΔRad26</td>
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<tr>
<td>JR1700</td>
<td>BY4742 with ubp3::kanMX</td>
<td>ΔUbp3</td>
</tr>
<tr>
<td>JR1714</td>
<td>BY4742 with bre5::kanMX</td>
<td>ΔBre5</td>
</tr>
</tbody>
</table>

2.2. Gene-length Dependent Accumulation of mRNAs Assay

2.2.1 Transformation and GAL1 Promoter Induction

Plasmids pSCH202 or pSCH212 containing, respectively, reporter genes $P_{GAL1}$-PHO5 and $P_{GAL1}$-PHO5::lacZ developed by Morillo-Huesca et. al. (46) were transformed into the investigated yeast strains for a gene-length dependent accumulation of mRNAs (GLAM) Assay. The plasmids described are found in Figure 12. A blank plasmid, pRS416, not containing the PHO5 acid phosphatase gene was also transformed into cells of each yeast strain. The cells were plated on –URA SD dextrose plates and incubated at 30°C for 2-4 days. One colony from each plate was restruck on a –URA SD plate in four quadrants for four replicates.
Four individual transformants were seeded in SD-URA Galactose medium. Samples were incubated for 20 hours at 30°C while rolling. Cells from this culture were diluted in SD-URA Galactose medium as to achieve an OD600 of 0.6-1.0 after incubation for 16-20 hours at 30°C.

### 2.2.2. Acid Phosphatase Activity Assay

One milliliter of culture was centrifuged at 14,000 rpm for 2 min and the supernatant was discarded. Cells were resuspended in 1 mL of 0.1 M Sodium Acetate, pH 3.8 and OD600 values were measured and recorded for each sample. 5-100 µL of cells (based on expected activity) and buffer to reach a total of 100 µL were mixed with 400 µL p-nitrophenol phosphate and incubated 20 min at 30°C. 500 µL of 1M sodium carbonate was added to stop the reaction, and centrifuged at 14,000 rpm for 5 min. OD400 of the supernatant was measured to determine acid phosphatase activity in Miller Units.

\[
Miller \text{ Units} = \frac{A_{400} \times 1000}{OD_{600} \times Vol (mL) \times rxn \ time (min)}
\]

### 2.3. Halting Transcription with thiolutin or 1,10-phenanthroline

Strains were seeded in 5 mL YPAD medium and incubated at 30°C for 24 hours until saturated. Cells were then reseeded in 50 mL YPAD as to achieve an OD600 of 0.6-1.0 after 16 hours of incubation at 30°C while shaking. Once the OD600 reached 0.6-1.0, cultures were treated with 150 µL of 1 mg/mL thiolutin to achieve a final concentration of 3 µg/mL or 55 µL of 100 mg/mL 1,10-phenanthroline to achieve a final concentration of 110 µg/mL. 5 mL samples of each culture were collected at the time points 0, 10, 20, 30 and 40 minutes after inhibitor was added and centrifuged at 10,000 rpm for 1 min. The supernatant was discarded and cells were frozen on dry ice. Samples were stored at -80°C. Between time points, the cultures were kept shaking at 30°C.
2.4. Halting transcription of *GAL1* with glucose shutoff

Strains were grown overnight in YPA medium with 2% raffinose and 2% galactose and incubated at 30°C for 24 hours until saturated. Cells were then reseeded in 50 mL YPAG with 2% raffinose as to achieve an OD600 of 0.6-1.0 after 16 hours of incubation at 30°C while shaking. Once an OD600 of 0.6-1.0 was reached, the culture was centrifuged at 3,200 rpm for 5 min. The cells were resuspended in 45 mL YPAD and mixed to initiate *GAL1* shutoff. 5 mL samples were collected at time points 0, 5, 10, 15, 20, and 40 minutes and centrifuged at 10,000 rpm for 1 min. The supernatant was discarded and cells were frozen on dry ice. Samples were stored at -80°C. Between time points, the cultures were kept shaking at 30°C.

2.5. Acid Phenol RNA Isolation

The samples collected in the transcription shutoff experiments were thawed and resuspended in 600 µL TES Buffer (10 mM Tris-HCl pH 7.4, 7.5 mM EDTA, and 0.5% SDS) on ice. 600 µL acid phenol (at 65°C) was added to each sample, vortexed, and incubated at 65°C. Samples were vortexed at intervals of every 10 min for 1 hour to aid extraction, then placed on ice for 5 min. Samples were then centrifuged at 14,000 rpm for 5 min at 4°C. The aqueous phase was transferred into a fresh tube and mixed with 550 µL acid phenol chloroform (APCIAA). APCIAA is composed of 1 part acid phenol and 1 part chloroform isoamyl alcohol, which is in turn made up of a 24:1 ratio of chloroform to isoamyl alcohol. The samples were vortexed and incubated at 65°C for 5 min, followed by a repetition of the ice and centrifuge steps.

The aqueous phase was transferred into a fresh tube and mixed with 25 µL 1 M Tris pH8 solution, 30 µL 5M NaCl, and 500 µL buffered PCIAA. Samples were vigorously vortexed, then the ice and centrifugation steps were repeated a final time. The aqueous phase was transferred into a fresh tube and mixed with 1.1 mL ethanol, then precipitated at -20°C for 12 hours. Samples were then centrifuged at high speed for 10 min at room temperature, rinsed with 1 mL 75% cold ethanol, and centrifuged at high
speed again for 5 min. The ethanol was discarded and the samples were resuspended in 25 µL DEPC-treated water (0.2% Diethyl pyrocarbonate, autoclaved).

RNA concentration measurements were made with the Nanodrop machine using 1.5 µL of the desired sample to measure an OD260 value. The values were then used to normalize the amount of RNA loaded on to the agarose gel.

2.6. Northern blotting

Formaldehyde gel-loading dye (95% formamide, 5 mM EDTA, 0.1% Bromophenol Blue, 0.1% Xylene Cyanolff) was used to prepare a gel-loading buffer (20 µL 10X MOPS buffer, 220 µL formaldehyde gel-loading dye, 70 µL formaldehyde) that was added to 10 µg RNA sample (approximately 5-6 µL). Samples were incubated for 15 min at 65°C and chilled on ice before loading.

1.2% agarose gels were made using 300 mL total solution composed of 255 mL DEPC-treated water, 30 mL 10X MOPS Buffer (50 mM sodium acetate, 0.2 M MOPS, 10 mM EDTA), and 20 mL of 12.2 M formaldehyde. The MOPS Buffer and formaldehyde were added once the solution had cooled to 70°C. Gel was run in 1X MOPS Buffer at 170 volts for two hours. After running, the gel was transferred to Hybond XL nylon membrane between 3 pieces of Whatman transfer paper on a transfer apparatus composed of a floating Styrofoam base with a Whatman transfer paper wick in 20X SSC (3M sodium chloride, 300 mM sodium citrate). The gel and transfer paper was topped by a stack of 10-12 cm paper towels and a 500 mg weight on a board. The transfer lasted 20-24 hours. The membrane was then washed, shaking in 1X SSC for 2 min and crosslinked using the UV Strateinker 2400 on the autocrosslink setting.
2.7. Labeling Probe

Agilent Technologies Prime-It II Random Primer Labeling Kit was used for random nucleotide and primer stocks. 30 ng of the desired DNA probe (GAL1, RPL25, or SCR1) was combined with 19 µL ddH₂O and 10 µL random oligonucleotide primers, boiled at 100°C for 5 min, spun briefly, and incubated at room temperature for 5 min then at 37°C for 20 min. 10 µL of 5X Primer buffer was added with 10 µL of α³²P-dATP and 1 µL Exo(-) Klenow and mixed gently. The probe was incubated at 37°C for 10 min, then 2 µL 0.5 M EDTA and 20 µL STE Buffer was added. A G50 column (made up of G-50 resin in STE buffer) was used to purify the probe away from free labeled nucleotides by spinning at 2000 rpm for 2 min. The probe was counted in a scintillation counter using 1 µL probe in scintillation fluid.

2.8. Hybridization, Washing, and Blot Exposure

Blot was washed two times for 30 min in wash buffer (1X SSC, 0.1% SDS) at 65°C. Blot was then incubated, with shaking, in 50 mL per blot of prehybridization solution (6X SSC, 5X Denhardt’s reagent, 0.5% SDS, 100 µg/mL boiled salmon sperm DNA). The prehybridization lasted four hours at 65°C. The probe was boiled for 5 min and quick chilled on ice before adding it to the solution. The probe was added to a concentration of 300,000 cpm/mL hybridization solution. Blot was incubated with hybridization solution overnight at 65°C, shaking.

Blot was removed from hybridization solution and placed in wash buffer for 5 min at room temperature. This was repeated a total of three times, transferring the blot into a fresh container and solution each time. The blot was then washed in wash buffer at 65°C for 30 minutes; this step was repeated once. Finally, the blot was washed twice in wash buffer for 3 minutes at room temperature and secured on a plastic screen.

The blot was exposed on a phosphor screen for a time period selected based on the abundance of the mRNA message probed (6-24) hours. The screen was then visualized using a Typhoon 8600 to
perform a phosphor scan at 200 microns to detect radiation and brightness/contrast values were adjusted using ImageJ 1.48v.

2.9. mRNA degradation plots and results

Quantification of RNA on Northern blots was performed using ImageQuant software (Version 5.2, Molecular Dynamics) which generated a volume report of each lane. Background was subtracted using a “rolling ball” method that generated an average level of background for the blot. Values were then normalized to a control by dividing the volume of the mRNA transcript of interest, RPL25 or GAL1, by the SCR1 control volume at the same time point. Finally, these values were normalized to the zero time point of their respective mutant in order to compare percent degradation over time most accurately over the 40 minute time course. Each graph represents the wild type and two or three mutant strains that were processed simultaneously.
Gene-length dependent Accumulation of mRNAs (GLAM) Assay Plasmid Composition. Plasmid pSCh202 is 1.5 kB in length and plasmid pSCh212 is 4.5 kB in length due to the addition of the lacZ domain, which does not otherwise play a role in acid phosphatase activity. Both utilize a GAL1 promoter, therefore growth in galactose medium is indicated. Figure is adapted from Morillo-Huesca 2006.
CHAPTER 3

RESULTS PART I: THE ROLE OF TCR PROTEINS IN TRANSCRIPTION ELONGATION

3.1 Transcription-coupled Repair Proteins do not play a role in transcription elongation under Ideal Growth Conditions

The Ccr4-Not complex has been linked to both transcription-coupled repair and transcription elongation within the cell. Its role in transcription elongation may be similar to its role in transcription-coupled repair, in which it is involved in the rescue or removal of stalled RNAPII at damage sites (30). The Ccr4-Not complex physically associates with several other TCR proteins, including Def1, Ubp3, and Bre5, indicating that these proteins may also play a role in transcription elongation and were therefore good targets for this study. Def1 and Rad26 are both crucial to the process of transcription-coupled repair—Def1 has a role in the degradation of stalled RNAPII and Rad26 is an ATP-dependent protein with roles in protein recruitment, although previous studies have shown that its role may be more expansive in the process of RNAPII removal or in interactions with transcription elongation factors to halt their progress during repair (43).

The two other studied proteins that associate with the Ccr4-Not complex, Ubp3 and Bre5, form a deubiquitylation complex within the cell that has been loosely linked to various mechanisms of DNA repair. Its role also likely lies in the control of RNAPII degradation, possibly slowing the process of RNAPII removal to allow a longer window of opportunity for rescue (44). Not4, the E3 ubiquitin ligase subunit of the Ccr4-Not complex, was also studied due to its potential related role in the removal of RNAPII during TCR. The Ccr4-Not complex as a whole has been linked to transcription elongation, but the role of the Not4 subunit is unknown (28). These relationships between Def1, Rad26, Ubp3, Bre5, and Not4 make them good targets for transcription elongation study.
Analysis of these proteins was performed through a GLAM Assay (see Section 2.2), a process which measures differential gene-length dependent transcription efficiency at the elongation stage of transcription. The reporter genes $P_{GAL1-PHO5}$ (1.5 kB) and $P_{GAL1-PHO5::lacZ}$ (4.5 kB) were transformed into deletion strains for each of the investigated yeast genes and the resulting acid phosphatase activity was measured using the spectroscopic properties of the enzyme product (46). The acid phosphatase sequence and promoter were identical for both reporter genes—the difference between the plasmids is the 3 kB lacZ gene found in the longer reporter gene. The lacZ gene does not affect acid phosphatase activity but does increase the length of the mRNA required to produce the protein and the importance of transcription elongation factors in transcription efficiency. Mutants with dramatically lower transcription efficiency in the longer gene compared to the shorter gene may therefore have a transcription elongation defect that can be detected based on the activity of the acid phosphatase protein product.

These comparisons are made using a GLAM ratio, the ratio between the acid phosphatase activity resulting from the longer $P_{GAL1-PHO5::lacZ}$ transcript and the acid phosphatase activity resulting from the shorter $P_{GAL1-PHO5}$ transcript normalized to the activity level found in a control colony transformed with the blank plasmid pRS416. In wild type cells, this ratio is generally around 0.5-0.6, as longer genes are less efficiently transcribed regardless of additional gene mutations due to the greater chance of stalled RNAPII, inaccurate transcription, and other processes. However, a GLAM ratio dramatically lower than that of the wild type strain would suggest the mutant yeast strain has a elongation defect, as the relative contribution of transcription elongation factors is greater in longer genes than in shorter ones (47).

Each experiment consisted of four replicates for each transformation averaged together to account for random variations in acid phosphatase activity and spectrophotometer measurement. The data shown is the result of one representative experiment, showing the means and standard deviation of these four replicates. Figure 13 shows there is no statistically significant difference between the GLAM ratio of the wild type $S. cerevisiae$ strain and the mutant strains def1Δ, not4Δ, rad26Δ, and ubp3Δ. The average ratios did vary between the five strains. The $ubp3Δ$ strain had an average ratio of nearly 0.6 while the wild type
had an average ratio of just under 0.4, seeming to indicate a more efficient gene-length dependent transcription process, for example, and the not4Δ strain had an average ratio of slightly less than the wild type strain. However, a Student’s t-test indicates that none of the differences between these values are statistically significant. The P value for each sample as compared to the wild type is as follows: ubp3Δ has a p = 0.349, rad26Δ has a p = 0.6196, not4Δ has a p = 0.795, and def1Δ has a p = 0.7203. All of these P values fall well outside the conventional criteria for statistical significance that requires a p < 0.05. The large range of these error bars indicates the vast margin of error for acid phosphatase activity measurement. This error is due both to variation in acid phosphatase enzyme activity from one strain isolate to another as well as differences in plasmid copy number among individual transformation isolate.

This experiment demonstrates that none of the four tested TCR-related proteins are involved in the gene-length dependent processes of transcription elongation. If their contribution was crucial at the later stages of elongation, transcription efficiency of the longer reporter gene would decrease and lower the GLAM ratio below that of the wild type gene. In the absence of this observation, there is no evidence that Def1, Not4, Rad26, and Ubp3 act in transcription elongation under normal growth conditions.
TCR protein-related mutants do not show a transcription elongation defect in gene-length dependent transcription efficiency. Cells were grown in YPAD and transformed with reporter or control plasmids; transformed cells were then grown in SC–URA galactose medium to log phase. p-nitrophenol phosphate was added to 800 µg/mL to each sample, and acid phosphatase activity (in µmol/min) was determined based on appearance of the product measured using a spectrophotometer. GLAM ratios are equal to the relative activity of acid phosphatase expressed with the longer transcription unit with respect to the activity expressed with the shorter transcription unit, correcting for background using the control. All four tested strains (def1Δ, not4Δ, rad26Δ, ubp3Δ) show very similar GLAM ratios to the wild type strain and there is no statistical significance between strains. Values given with mean ± standard deviation with n = 4 in all cases: WT 0.377 ± 0.190, ubp3Δ 0.549 ± 0.281, rad26Δ 0.443 ± 0.168, not4Δ 0.347 ± 0.106, def1Δ 0.420 ± 0.127; p > 0.05)
CHAPTER 4

RESULTS PART II: THE ROLE OF TCR PROTEINS IN mRNA DEGRADATION

4.1 TCR proteins associated with Ccr4-Not may play a role in mRNA degradation

While the GLAM assay did not reveal a role for the four studied TCR proteins in transcription elongation, the physical and functional association of these proteins with the Ccr4-Not complex, as found in previous studies, still suggested the possibility that these proteins play a role in Ccr4-Not-regulated processes. mRNA degradation is a critical counterpoint to mRNA transcription, as it is the simultaneous regulation of these two processes which determines the quantity of any given mRNA transcript within the cell. The Ccr4-Not complex is involved in both processes, particularly under stressed conditions, to regulate final protein levels in the cell (22). The Ccr4-Not complex has a clear role in mRNA degradation through the Ccr4/Caf1 deadenylase (15, 21). This opens up the possibility that other TCR proteins, previously thought to be involved only in transcription-coupled repair, may also play a role in mRNA degradation.

Once again, four crucial TCR-related proteins were selected for study based on their close association with the Ccr4-Not complex. Ubp3 was studied based on its deubiquitylation function as well as its complex partner Bre5, which is thought to act in counterpoint to the RING family of ubiquitin ligases, including Ccr4-Not complex subunit Not4, and there is some possibility that ubiquitylation of proteins is involved in regulation of decay as the Ccr4-Not complex has ubiquitylation activity (48). Additionally, Bre5 has an RNA binding domain and Ubp3 was isolated in a screen to identify uncharacterized RNA binding proteins, raising the possibility that the Ubp3/Bre5 complex binds mRNAs in the cytoplasm to regulate decay (74). Rad26 and Def1 are TCR-related proteins that may also play a role. Double caf1Δ/rad26Δ mutants show markedly increased hydroxyurea sensitivity compared to single mutants during studies of mRNA decay in *Saccharomyces pombe*, a different yeast species in the same genus as *S. cerevisiae*, indicating possible redundancy between the roles of these two proteins (49).
4.2 Rationale for the Northern blot as a measurement of mRNA degradation

While Northern blotting was once the most common method of RNA quantification, the development and refinement of quantitative reverse transcription-PCR (RT-PCR) have brought these new techniques into greater prominence. These techniques are faster and allow for increased sample throughput in large-scale experiments (51). In studies testing gene expression, northern blot and RT-PCR data is highly correlated when normalized to an internal control (52). However, mRNA degradation is more difficult to accurately quantify using RT-PCR methods. Many mRNA transcripts have stabilized decay intermediates that may result in inaccurate quantification using the non-visual medium of RT-PCR (53). Reese lab data has indicated RT-PCR produces artifactual data in mRNA degradation experiments (unpublished). A Northern blot allows for the quantification of full length mRNA transcripts without interference from accumulated decay intermediates (Figure 14).

4.3 The use of thiolutin as a global transcription inhibitor for mRNA degradation studies

In order to measure degradation, transcription of the mRNA of interest must be inhibited to prevent interference with the newly produced transcripts. One method to do so involves using global transcription inhibitors, which halt cellular transcription. Several transcription inhibitors have been isolated and utilized for this purpose in previous studies, including thiolutin, 1,10-phenanthroline, and 6-azauracil (54).

In this study, thiolutin was initially used to halt transcription activity in yeast. Previous studies have shown that thiolutin is one of the most effective transcription inhibitors in S. cerevisiae (54). Thiolutin is a metabolite produced by the bacterium Streptomyces luteoreticuli that was initially discovered to inhibit the growth of yeast. It interacts with all three RNA polymerases in yeast, though its specific mechanism of action is not entirely understood (55). Cells of each tested strain (wild type, ubp3Δ,
rad26Δ, not4Δ, def1Δ, and bre5Δ) were grown in YPAD medium, treated in log phase with 3 μg/mL thiolutin and samples were collected at 0, 10, 20, 30 and 40 minutes. RNA isolated from the cells were analyzed by Northern blotting using a probe of RPL25.

Despite its prior use in mRNA degradation studies, thiolutin proved to be an inconsistent transcription inhibitor in my hands, with wildly variable effectiveness in different experiments. A number of different modifications of the treatment procedure were attempted, such as keeping the cells shaking vigorously at 30° C between sample collections, different amounts of drug and temperatures, but none solved the inconsistent results. Figure 15 shows the results of one experiment using thiolutin as a transcription inhibitor. The ubp3Δ and rad26Δ strains appear to have an unexpected increased rate of mRNA degradation, a result which several future experiments refuted, while the not4Δ, def1Δ, and bre5Δ strains resulted in mRNA levels that increased or stayed constant over the time course. Of particular note are the bre5Δ and def1Δ strains. bre5Δ shows an increase of almost four times from the 0 minute time point to the 10 minute time point, a very unexpected result if transcription was completely inhibited by the thiolutin. The def1Δ mRNA levels at 40 minutes also increased above the def1Δ mRNA levels at the zero time point in the experiment.

There are a few possible explanations for this inconsistency. First, it is possible that thiolutin is simply not a consistent transcription inhibitor with the yeast strains used. This could be due to cell uptake problems/variability, or temperature changes by the exposure to cooler air during sample collection. To test the potency of the drug, a cell growth assay was performed to determine if the concentration being used in experiments, 3 μg/mL, was sufficient for the activity of thiolutin as a transcriptional inhibitor and suspend cell growth. The growth of a wild type yeast strain was tested over 5 hours under treatment conditions varying from 1 μg/mL to 10 μg/mL. As seen in Figure 16, concentrations of 3 μg/mL and 10 μg/mL were effective in halting yeast growth and therefore the concentration used in experiments should have been adequate for halting transcription. One possibility for this effect is that thiolutin’s inhibitory effect on transcription was counteracted by an inhibitory effect on the mRNA degradation machinery.
One study reports that high concentrations of thiolutin, significantly higher than that used here, reduce mRNA degradation and can also cause a stress response that changes the regulatory pattern of the cell (56). These factors, and the inconsistent results obtained experimentally made thiolutin a poor inhibitor choice for the mRNA degradation experiments in this project. However, patterns observed over several experiments indicated interesting possibilities for study using an alternate transcription inhibition technique.

Figure 17 shows an experiment with more consistent thiolutin data that indicates a possible degradation defect in not4Δ and in def1Δ. Both strains showed a markedly slower degradation rate over the 40 minute time course than the wild type cells. Due to the aforementioned issues with thiolutin and the very low mRNA levels present in the not4Δ strain, however, these results were not conclusive. I therefore turn my attention to different protocols to measure transcription inhibition that do not rely on thiolutin.

4.4 Def1 and Not4 mutants show mRNA degradation defect upon dextrose GAL1 shutoff treatment

One method of transcription inhibition that does not involve use of thiolutin is a promoter-based shutoff system. One of the most well characterized and versatile promoter systems is the GAL promoter, which is turned on by presence of galactose as the primary sugar source and turned off by the introduction of dextrose (57). To utilize this system, cells were cultured in YPA + 2% galactose + 2% raffinose medium to log phase to stabilize levels of GAL1 transcription, then spun down and transferred into YPAD (YPA + 2% dextrose) medium to initiate transcription shutoff of the GAL1 gene. mRNA degradation of GAL1 was then measured on a Northern blot delineated in Section 4.3, above. Due to the shorter half-life of GAL1 mRNA, time points were taken at shorter intervals for a more accurate analysis of degradation: 0, 5, 10, 15, 20, and 40 minutes.
Figure 18 shows the Northern blot and line graph quantification results of this experiment. While ubp3Δ and rad26Δ have a degradation rate very similar to wild type with an estimated half life of 5-7 minutes, both the not4Δ and def1Δ strains had a reduced decay rate of GAL1 mRNA, suggesting these strains have a mRNA degradation defect. The GAL1 half-life is approximately 12 minutes for the not4Δ strain and 15 minutes for the def1Δ strain. The GAL1 message is degraded more slowly in these strains than in the wild type strain, which indicates that the Not4 and Def1 proteins may play a role in mRNA degradation in the cell. The results shown are a representative of three experiments.

4.5 The Discovery of a Growth Suppressor Mutation

The results were tested for confirmation using alternate isolates of the mutants in question (Table 2.1). The result for the ubp3Δ, rad26Δ, bre5Δ, and not4Δ strains was consistent in each isolate, showing no degradation defect for the ubp3Δ, rad26Δ, and bre5Δ strains and a degradation defect in the not4Δ strain. The results from the two different def1Δ mutant strains, however, were not. Figure 19 shows these results—while not4Δ has a clear degradation defect, the def1Δ B strain has an almost identical degradation pattern to the wild type cells. Additionally, the alternate Δdef1-B strain demonstrated a faster growth rate compared to the Δdef1 A strain, which was observed on solid agarose medium and in liquid medium (not shown). Since it was previously reported and observed that a def1Δ mutant grows more slowly than wild type cells, this suggests that the def1Δ-B strain picked up an extragenic suppressor mutation that suppressed both the slow growth and the mRNA degradation phenotypes.

Extragenic suppressor mutations occur when a mutation somewhere in the genome reverses or reduces the phenotypic effect of another mutation. This is especially common in slow-growing strains, in which mutations that suppress the phenotype have a clear growth advantage over the cells without the suppressor and can over generations of growth become dominant in the strain. The def1Δ strain may have an additional layer of vulnerability because the Def1 protein normally plays a role in DNA repair. The
loss of its activity may increase the mutation rate in the cell and increase the likelihood of an extragenic suppressor.

To confirm this hypothesis, an additional test was performed using DEF1 knockout strains derived from an alternate wild type strain (see Table 1, BY4741) to determine whether the degradation defect was reproducible in strains without a growth suppressor mutation. Figure 4.6 C and D show that both alternate strains have a degradation defect, which confirms that Def1 plays a role in mRNA degradation in *S. cerevisiae* based on dextrose shutoff experiments. Because three out of the four def1Δ strains show the phenotype of a degradation defect, the def1Δ-B strain is most likely to have an extragenic suppressor that reverses some of the effects of the mutated Def1 protein and its phenotypes should not be considered standard for def1Δ strains.

### 4.6 Def1 and Not4 mutants show mRNA degradation upon drug treatment with 1,10-phenanthroline

Because different RNA messages may go through different mechanisms of mRNA degradation, it is desirable to test the degradation defect with other messages. The dextrose shutoff procedure relies upon the *GAL1* promoter system, and therefore is useful for measuring very few genes in the *S. cerevisiae* genome under the control of galactose. The use of a transcription inhibitor allows for the observation of a wider variety of mRNA messages.

1,10-phenanthroline is a chemical transcription inhibitor that inhibits RNAPII by chelating Mg^{2+}, a cofactor required for RNAPII activity (54). Much like thiolutin, 1,10-phenanthroline globally inhibits transcription within the cell, allowing mRNA degradation activity to be observed. Cells of each tested strain (wild type, *ubp3Δ, rad26Δ, not4Δ, def1Δ*, and *bre5Δ*) were grown in YPAD medium, treated in log phase with 110 µg/mL 1,10-phenanthroline and samples were collected at 0, 10, 20, 30 and 40 minutes. RNA isolated from the cells was analyzed by Northern blotting using a probe to *RPL25*. 
Figure 21 shows the Northern blot and line graph results of this experiment. Figures B and D show that upon global transcription shutoff with 1,10-phenanthroline. The *ubp3Δ*, *rad26Δ*, and *bre5Δ* strains show similar half-lives for *RPL25* mRNA to the wild type strain—about 20 minutes under these conditions, confirming that the proteins Ubp3, Rad26, and Bre5 are not involved in mRNA degradation. In the *not4Δ* strain and *def1Δ* strains, however, *RPL25* mRNA half-life was greater than the 40 minutes, the last time point measured in the experiment. This repeats the result found in the dextrose shutoff experiment with a different method of shutoff and a different mRNA message (Figure 18), confirming that the Not4 and Def1 proteins play a role in mRNA degradation under the normal growth conditions tested in this study.
Figure 14: Partially Degraded *GAL1* Transcript Observed in a Northern Blot

Partially Degraded *GAL1* Transcript Observed in a Northern Blot: Degradation intermediates are shorter and travel more quickly in an agarose gel, resulting in a characteristic “smearing” pattern consisting of these intermediates at various lengths. This image shows a blot of *GAL1* mRNA. The Northern blot was probed using a P<sup>32</sup>-labeled DNA probe, exposed for 24 hours on a phosphor screen, and visualized on a Typhoon Scanner.
Figure 15: Thiolutin shows inconsistent mRNA degradation trends in *S. cerevisiae* strains

(A)

(B)

(C)

(D)
**Thiolutin shows inconsistent mRNA degradation trends in *S. cerevisiae* strains.** Cells were treated in log phase with 3 µg/mL thiolutin at the zero time point. Northern blot used P32-dATP **RPL25** probe and a 24 hour exposure. **SCR1** was used as a loading control. (A) ∆ubp3 strain and ∆rad26 strain appear to show increased mRNA degradation compared to the wild type strain, possibly due to the inconsistent band intensity of various **SCR1** lanes (WT-0, ∆ubp3-20). (B) The three tested mutant strains each have at least one timepoint in which an increased level of **RPL25** is observed compared to the zero time point—the most obvious example being the comparison between the ∆bre5-0 timepoint and the following ∆bre5 timepoints. The inhibitory effect of thiolutin on mRNA degradation likely affects the results of this experiment. (C) Line graph representations of **RPL25** transcript levels over time after treatment with thiolutin. Values are normalized to the zero time point of each strain and to the **SCR1** control in each lane. The inconsistency in thiolutin effect results in mRNA levels that vary wildly over the time course.

**Figure 16: The Concentration-dependent Effect of Thiolutin on Yeast Growth Rate**

![Effect of Thiolutin on Growth Rate](image1)

**The Concentration-dependent Effect of Thiolutin on Yeast Growth Rate.** WT BY4742 yeast strain was grown in YP media with 2% dextrose and treated at 0 hours with concentrations of thiolutin ranging from 1 µg/mL to 10 µg/mL. At concentrations of 3 µg/mL and above, yeast growth was dramatically hindered over the course of 5 hours compared to the untreated control. This indicates that these concentrations of thiolutin are adequate to kill *S. cerevisiae* cells through its role as a global transcriptional inhibitor.
Figure 17: $\Delta$not4 and $\Delta$def1 strains may be involved in mRNA degradation

(A)
\( \Delta \text{not4} \) and \( \Delta \text{def1} \) strains may be involved in mRNA degradation. All cells were treated at 0 minutes with 3 µg/mL thiolutin. Northern blot used P\(^{32}\)-dATP RPL25 probe and a 24 hour exposure. \( \text{SCR1} \) was used as a loading control. (A) \( \Delta \text{ubp3} \) and \( \Delta \text{rad26} \) strains show similar degradation to wild type cells. (B) \( \Delta \text{not4} \) and \( \Delta \text{def1} \) appear to have a slower degradation rate than the wild type or the wild type like \( \Delta \text{bre5} \) strain. The very low mRNA levels in the \( \Delta \text{not4} \) strain make quantification less reliable, however, and this result is therefore not conclusive. (C) and (D) Line graph representations of mRNA levels after treatment with thiolutin. \( \Delta \text{not4} \) and \( \Delta \text{def1} \) may have a degradation defect.
Figure 18: $\Delta$not4 and $\Delta$def1 strains show degradation defect with dextrose shutoff

(A) 

(B)
\( \Delta \text{not4} \) and \( \Delta \text{def1} \) strains show degradation defect with dextrose shutoff. Samples were grown in YPAG media + 2% raffinose to log phase, then cells were resuspended in YPAD medium to initiate \( \text{GAL} \) shutoff at 0 minutes. Northern blot used \( \text{P}^{32}\)-dATP \( \text{GAL1} \) probe and a 24 hour exposure. \( \text{SCR1} \) was used as a loading control. (A) \( \Delta \text{ubp3} \) and \( \Delta \text{rad26} \) strains show similar degradation to the wild type strain. No defect is observed. (B) \( \Delta \text{not4} \) and \( \Delta \text{def1} \) strains appear to have slower degradation than the comparative WT strain. The blue line indicates that the 0, 5, and 10 minute timepoints for the 2\(^{nd}\) wild type experiment were run on two separate gels, but the loading control allowed for normalization between these blots. The results indicate a degradation defect in \( \Delta \text{not4} \) and \( \Delta \text{def1} \) strains. \( \Delta \text{bre5} \) (data not pictured) was shown to have a degradation pattern very similar to the wild type cells. (C) and (D) Line graph representations of the mRNA levels after dextrose shutoff normalized to the \( \text{SCR1} \) control and mRNA level at 0 minutes. \( \Delta \text{not4} \) and \( \Delta \text{def1} \) strains have a degradation defect.
**Figure 19:** \(\Delta def1\)-B strain lacks degradation defect observed in \(\Delta def1\)-A strain

(A)

\(\Delta def1\)-B strain lacks degradation defect observed in \(\Delta def1\)-A strain. Samples were grown in YPAG media + 2% raffinose to log phase, then cells were resuspended in YPAD medium to initiate \(GAL\) shutoff at 0 minutes. Northern blot used \(P^{32}\)-dATP \(GAL1\) probe and a 24 hour exposure. \(SCR1\) was used as a loading control. (A) \(\Delta not4\)-B strain shows slower \(GAL1\) degradation over time course than WT strain, while \(\Delta def1\)-B and \(\Delta bre5\)-B strains show degradation rates and patterns very similar to WT strain. (B) Line graph representation of Northern blot after dextrose shutoff at 0 minute time point. Values are normalized to \(SCR1\) control and to 0 time point level of mRNA. Only \(\Delta not4\)-B appears to have a degradation defect, a change from the prior observation that both \(\Delta not4\)-A and \(\Delta def1\)-A have a degradation defect in Figure 18.
Figure 20: $\Delta$def1-B has a growth suppressor mutation that prevents the observation of a degradation defect.

(A) 

(B) 

(C)
\( \text{Δdef1-B has a growth suppressor mutation that prevents the observation of a degradation defect.} \) Samples were grown in YPAG media + 2% raffinose to log phase, then cells were resuspended in YPAD medium to initiate \( GAL \) shutoff at 0 minutes. Northern blot used \( \text{P}^{32}\)-dATP \( GAL1 \) probe and a 24 hour exposure. \( SCR1 \) was used as a loading control. (A) The \( \text{Δdef1-B} \) strain has wild type-like degradation while the \( \text{Δdef1-A} \) strain has a degradation defect (also observed in Figure 18). This discrepancy along with physical observation of the faster \( \text{Δdef1-B} \) growth rate points to a growth suppressor mutation in the B strain that changes the expression and degradation regulation of the cells. (B) Line graph representation of Northern blot after dextrose shutoff at 0 minute time point. Values are normalized to \( SCR1 \) control and to the level of mRNA at the initial time point. (C) \( DEF1 \) knockout strains made from a BY4741 wild type (see Table 1) were tested to confirm the growth suppressor mutation. Both BY4741-derived \( \text{Δdef1} \) strains have an observable degradation defect. (D) Line graph representation of Northern blot after dextrose shutoff at 0 minute time point. Values are normalized to \( SCR1 \) control and to the level of mRNA at the initial time point.
Figure 21: \( \Delta \text{not4} \) and \( \Delta \text{def1} \) have degradation defects confirmed by 1,10-phenanthroline-induced global transcription shutoff

(A)

(B)

(C)
Δnot4 and Δdef1 have degradation defects confirmed by 1,10-phenanthroline-induced global transcription shutoff. All cells were treated at 0 minutes with 110 µg/mL 1,10-phenanthroline. Northern blot used P32-dATP RPL25 probe and a 24 hour exposure. SCR1 was used as a loading control. (A) Δubp3 and Δrad26 strains show similar degradation to wild type cells. (B) Δnot4 and Δdef1 appear to have a slower degradation rate than the wild type or the wild type like Δbre5 strain. (C) and (D) Line graph representations of mRNA levels after treatment with thiolutin. Δnot4 and Δdef1 show a degradation defect compared to the WT strain.
CHAPTER 5
DISCUSSION

5.1 The Interconnected Metabolic and Regulatory Network

A pattern has emerged in biochemical research in which pathways once thought to be entirely separate are found to share regulatory pathways and proteins, indicating a more interconnected metabolic network than previously thought. One protein complex that has been found to span these various pathways is Ccr4-Not. Its regulatory activities have been linked to pathways as varied as transcription initiation and elongation, transcription-coupled repair, mRNA degradation, and more. The many activities of the Ccr4-Not complex opens the possibility that the proteins it associates with are also involved in multiple processes throughout the cell. This study particularly focuses on TCR proteins that are also associated with the Ccr4-Not complex and their role in transcription elongation and mRNA degradation. In the course of this study, it was discovered that a subset of these TCR proteins are important in mRNA degradation in S. cerevisiae cells.

5.2 Pathways of Transcription Elongation

Transcription elongation rates are affected proportionally more in longer genes, and thus can be measured and compared using a gene-length dependent assay. Mutants with a significantly impaired rate of mRNA accumulation in longer genes can be determined to have an elongation defect. Based on the GLAM Assay results described in Chapter 3, the TCR-associated proteins tested do not play a role in gene-length dependent processes of transcription elongation under normal growth conditions.
This result points to somewhat of a separation in activity between the proteins involved in stalled RNAPII processing in transcription elongation vs. those involved in the same process in transcription coupled repair. In TCR, Ubp3, Bre5, Rad26, and Def1 as well as the Not4 subunit of the Ccr4-Not complex are all linked to continuation of transcription through the site of a stalled RNAPII, whether by helping to restart transcription of the stalled polymerase or by marking it for degradation. Meanwhile, RNAPII density studies along transcribed genes have presented a similar role for various subunits of the Ccr4-Not complex in transcription elongation. There is evidence to support the fact that the Ccr4-Not complex acts in RNAPII rescue when the RNAPII is stalled or backtracked on a nucleosome or other block in transcription (30). This connection created the possibility of a link between the associated proteins involved in these pathways as well, but the results demonstrate that the TCR-related proteins are not involved in the similar RNAPII rescue during transcription elongation under normal growth conditions.

The larger “roadblocks” to transcription caused by the bulky lesions that initiate transcription-coupled repair therefore must necessitate a different pathway to handle the stalled RNAPII, possibly due to the greater likelihood that protein degradation will be required rather than the relatively more simple process of backtracking and restarting transcription. There may be other proteins that are involved in the transcription elongation RNAPII processing through nucleosomes and other natural roadblocks, such as the previously studied transcription factors TFIIS and Paf1c, whose mutants show synthetic lethality with several Ccr4-Not subunit mutants (22, 28).

Interestingly, this separation of roles also occurs within the Ccr4-Not complex itself. The Not5 and Ccr4 subunits of the complex have both been previously linked to transcription elongation and the rescue of RNAPII activity through the gene, but the results of the GLAM assay experiment show no indication of a role in transcription elongation for Not4 (9). One possibility for this discontinuity is due to the normal growth conditions under which the GLAM Assay was performed. The Ccr4-Not complex is experimentally linked to regulation of transcription and cellular processes under stressed conditions, and a
similar condition may apply to other TCR proteins such as those tested here. It is possible that under oxidative stress or UV light, the TCR proteins would play a much greater role in efficiency of transcription elongation.

This model may indicate that the Not4 subunit plays a more important role in stress-regulated pathways. This separation of activities for the Not4 subunit is supported by other, prior, studies of the expression of Ccr4-Not dependent transcripts. For example, it has been demonstrated that mutations in the RING domain of Not4 have no effect on RNR expression or heat shock protein expression, two classes of proteins which are highly regulated by the Ccr4-Not complex as a whole (60). The null mutation not4Δ strain tested in the GLAM Assay experiment would certainly have different effects than the point mutants used in that experiment, but both of these studies raise the possibility that Not4 ubiquitylation activity, which is mediated through the RING domain and crucial for TCR, has different functions than the rest of the complex under normal growth conditions and is not involved in that normal transcription-coupled repair. A further study on transcription elongation utilizing UV radiation or induced oxidative stress may help elucidate the role of these TCR proteins in elongation under stressed conditions.

5.3 Pathways of mRNA degradation

While transcription elongation represents the “birth” of an mRNA transcript, mRNA degradation represents its “death,” also an essential regulatory point for the translation and therefore expression of proteins. In addition to its roles in transcription, the Ccr4-Not complex has been implicated in mRNA degradation, most obviously through its Ccr4/Caf1 deadenylase subunits. The Northern blot results presented in this thesis show a role in mRNA degradation for one of the other Ccr4-Not subunits, Not4, as well as for the TCR protein Def1.

The Ccr4 and Caf1 proteins, which serve as the main deadenylase in mRNA degradation in yeast, is linked to the rest of the complex by the non-essential N-terminus of Not1 which serves as the scaffold
for the overall complex. While this N-terminus is non-essential to the survival of the cell, it is essential for
decay activity and the association of Ccr4/Caf1 with the complex (25). It is currently suggested that this
interaction allows Not1 or other subunits to recruit the deadenylase complex to mRNAs in the cytoplasm.
In addition to Ccr4/Caf1, other subunits of the Ccr4-Not complex have been shown to affect mRNA
degradation in other ways. Previous research shows that NOT2, NOT4, and NOT5 mutants may exhibit a
decapping defect that also slows mRNA degradation (18). The discovery of a degradation defect in this
experiment suggests a further role for the Not4 subunit in this process, though it does not confirm the
mechanism through which it acts. It is possible that the Not4 subunit plays a role in recruitment or
interaction with the decapping complex, Dhh1, in the steps immediately following deadenylation by the
Ccr4/Caf1 complex. Another possibility is that the Not4 subunit, like Not1, is structurally essential as a
scaffold that allows the Ccr4/Caf1 proteins to interact with the mRNAs it degrades.

The degradation defect in DEF1 mutants is more puzzling. Def1 has not previously been linked to
a process of mRNA degradation, its only known role is the recruitment of ubiquitin ligase factors to
RNAPII during TCR. In contrast, Rad26, a protein which is closely linked to the TCR activity of Def1,
showed no degradation defect. The Reese lab found that Def1 co-purifies with the Ccr4-Not complex
through the Not4 subunit, which could indicate a connection to the mRNA degradation activity of Not4
discussed above (unpublished). However, the Ubp3/Bre5 complex also co-purified with Not4 and neither
of those proteins showed an mRNA degradation effect. Thus, the ability to bind to Not4 does not, in
itself, specify a role in mRNA decay. Recent research shows that a cytoplasmic form of Def1 is processed
by a proteasome in response to transcription stress and targeted to the nucleus where it acts in TCR (72).
The existence of two different cytoplasmic and nuclear forms points to the possibility of different
cytoplasmic and nuclear activities—for example, mRNA degradation in the cytoplasm and transcription-
coupled repair in the nucleus. It also seems likely that Def1 is involved through its physical association
with the Ccr4-Not complex, as discussed earlier. Within the nucleus, Def1 has a role in the recruitment of
an E3 ubiquitin ligase to the RNAPII; perhaps its cytoplasmic role is similar: recruiting the Ccr4-Not
complex to mRNAs. Alternately, Def1 may play a structurally important role in its association to the Ccr4-Not complex that corresponds to Ccr4-Not activity without directly binding the mRNA targeted for degradation. Perhaps Dhh1 association requires the presence of other associated proteins such as Def1 to effectively perform its decapping activity.

Another important question is the evolutionary reason for linking the pathways of transcription-coupled repair and mRNA degradation. One intriguing possibility is that both mechanisms serve as a way to clear damage— one by repairing damaged DNA through TCR and the other by destroying mRNAs damaged by the same conditions. Often, the same conditions that result in damaged DNA also result in damaged mRNA in the cytoplasm, rendering it useless for accurate protein translation. Perhaps the activation of Def1 under stressed conditions leads to its mediation of Ccr4-Not mRNA degradation activity to clear away the damaged mRNAs.

5.4 Significance and Further Questions

Transcription-coupled repair and its associated pathways are an important area for research, as the failure of repair pathways can lead to a wide variety of dangerous human diseases--namely, skin cancers, xeroderma pigmentosa, and Cockayne’s syndrome. While TCR was once thought to be independent, the multifunctional Ccr4-Not complex has been linked both to TCR and other processes throughout the mRNA cycle, such as transcription elongation and mRNA degradation, opening the possibility for other bifunctional or multifunctional proteins involved in TCR.

Not4, a Ccr4-Not complex subunit, and Def1, a TCR protein, were previously known to have both physical associations with the Ccr4-Not complex and roles in TCR. In this study, they were also shown to have a new effect on mRNA degradation. In addition to broadening the understanding of possible roles of TCR proteins, this also illuminates more possible pathways of mRNA degradation, once thought to be an entirely linear deadenylation-dependent pathway. The connection between TCR and
mRNA degradation creates the possibility that oxidative and UV stress are linked to mRNA degradation in addition to TCR. Further study into the role mRNA degradation may have in diseases associated with poor stress-regulatory activity like xeroderma pigmentosa and Cockayne’s syndrome would further aid in the understanding of these diseases and new therapeutic possibilities. Yeast can act as a useful model organism to study the highly conserved regulation patterns in eukaryotes and broaden the knowledge base associated with each mRNA transcript throughout its life in the cell.
BIBLIOGRAPHY


ACADEMIC VITA

Anna Wing
(814) 409-8275
annakaitwing@gmail.com

EDUCATION:

PENNSYLVANIA STATE UNIVERSITY, University Park, PA
Schreyer Honors College
Bachelor of Science in Biochemistry and Molecular Biology; English Minor

STATE COLLEGE AREA HIGH SCHOOL, State College, PA

ACADEMIC HONORS AND RESEARCH:

Lab of Dr. Joseph Reese | Penn State University 2013-present
• Biochemistry and Molecular Biology research, 10-15 hrs/week
• Earned Undergraduate Research Grant in Fall 2014
• Presented at Undergraduate Research Exhibition at Penn State University
• Earned Erickson Discovery Grant for full-time work in Summer 2015

DAAD-Rise Scholarship Recipient | University of Freiburg, Germany Summer 2014
• Full-time research intern in the Immunology lab of Dr. Kristina Schachtrup in Freiburg, Germany
• Presented at Science Undergraduate Experiences Poster Session
• Wrote article published in the Penn State Science Journal

Research Assistant | Biology of Eco-Health May-June, 2013
• Three-week research trip to Tanzania to study recovery of an ecosystem after overgrazing by cattle through vegetation surveys both inside and outside protected areas, visit to Mandela lab in Arusha.

Eberly College of Science Student Marshal Spring 2016
• Recognizes a student in the PSU College of Science with an excellent academic record

Herko Family Scholarship in Biochemistry and Molecular Biology 2015/16
• Recognizes a student in BMB who show promise of outstanding academic success

Ronald Venezie Scholarship in Science for Honors Education 2014/15
• Recognizes an outstanding student in Biochemistry and Molecular Biology

Bayard D. Kunkle Scholarship 2014/15
• Recognizes a student exhibiting good citizenship and leadership

WISER Scholarship 2013/14
• Grant for research in the lab of Dr. Joseph Reese

Presidential Leadership Academy Award and Travel Grants 2013-present
• Three-year critical thinking program with the president of Penn State, Rodney Erickson, and the Dean of the Schreyer Honors College, Christian Brady.

Braddock Scholarship 2012-present
• Four year scholarship that recognizes ~12 students in Eberly College of Science

**TEACHING AND WORK EXPERIENCE:**

**Organic Chemistry Tutor | PSUKnowHow**

Jan 2015-present

• Privately tutored college students through PSUKnowHow tutoring company in Organic Chemistry 1 and 2, 10-12 hours/week.

**Course Assistant and Tutor | CHEM 202**

Fall 2014

• Official tutor for students in *Fundamentals of Organic Chemistry* at Penn State University, held tutoring office hours and led review sessions for ~150 students before course exams and quizzes

**Employee | Kiwi Yogurt**

April 2012-Sept 2014

• Promoted to Team Leader in July 2013, where I worked in a Shift Manager capacity
• Trained new employees in customer service, register management, and food preparation

**SERVICE:**

**Admin Chair | Springfield**

2012-present

• Admin Chair March 2015-present, Member 2012-present. Organization dedicated to fundraising to Penn State THON, the largest student-run philanthropy in the world to help fight pediatric cancer. Springfield raised more than $270,000 in 2015.

**Volunteer | Mt. Nittany Medical Center**

2013-present

• Assisted medical staff through transport of patients, supplies, medical records, etc. to various locations in the hospital.

**Team Captain | Relay for Life**

2014

• Organized volunteers and donations for Penn State Relay for Life team

**PASS Leader | Penn State THON**

2012-2013

• Responsible for detailed knowledge of the system through which several thousand people received passes to visit dancers during Penn State THON, which raised over $13 million dollars for pediatric cancer treatment and research.