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Elucidation of the overexpression of Taf2 in eukaryotic cells

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Abstract

Through several studies, Taf2 has been found to be upregulated in various cancer cells. However, the mechanism through which this increased expression of Taf2 occurs remains unknown. As evolutionarily conserved ubiquitin-proteasome system (UPS) maintains protein homeostasis for normal cellular function, we hypothesized that stability of Taf2 may be regulated by this UPS and this UPS may be dysregulated in cancer cells causing overexpression of Taf2. To test our hypothesis, we assessed the role of the UPS in the regulation of the stability of Taf2 by 26S proteasome-mediated degradation. To do so, we performed molecular experiments mainly through two steps: 1st step includes the analysis of the ubiquitination status of Taf2, and 2nd step includes the analysis to determine if Taf2 is regulated by the 26S proteasome. Similarly to UPS, Taf2 is evolutionarily conserved and found in both yeast and humans as we conducted these experiments in yeast (*Saccharomyces cerevisiae*). To perform the 1st step, we conducted molecular cloning to introduce the pUB221 plasmid, expressing the hexahistidine-tagged ubiquitin under the *CUP1* promoter, into the yeast strain containing TAP-tagged Taf2. Next, using this strain, we performed Ni^{2+}- NTA-based ubiquitination assay to see if Taf2 is regulated by ubiquitination or not. Notably, for the 1st time we found that Taf2 undergoes polyubiquitination. Generally, if a protein undergoes polyubiquitination, it is likely to be degraded by the 26S proteasome. To evaluate this possibility, we investigated the activity of the 26S proteasome in regulating Taf2 stability. Briefly, we performed MG132 based proteasomal inhibition assay (2nd step). MG132 inhibits the proteolytic function of the 26S proteasome and therefore, if Taf2 is regulated by the proteasome we would observe increased abundance of Taf2 following proteasomal inhibition by MG132. However, yeast cells contain the multidrug resistance gene, therefore, to perform the MG132 based proteasomal inhibition assay, we

knocked out of the multidrug resistance gene, *PDR5*, in the yeast strain expressing TAP-tagged Taf2, and treat these cells with MG132. Importantly, we found that polyubiquitylated Taf2 is targeted for degradation by the 26S proteasome. Our results showed for the first time that Taf2 abundance is regulated by the UPS. In addition, our bioinformatics data suggest that protein overexpression, as opposed to mRNA overexpression, plays a role in several cancers implicating a mismanaged UPS system. Thus, our results reveal novel UPS regulation of Taf2 with potentials for future therapeutic intervention.

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Chapter 1: Introduction

TATA-binding proteins (TBP) serve as a significant component within the eukaryotic transcription initiation machinery. Within eukaryotic cells, RNA Polymerase II (RNA Pol II) mostly facilitates the transcription of protein-coding genes as well as some non-coding genes. In many RNA Pol II promoters, TBP operates within transcription factor IID (TFIID), which consists of evolutionarily conserved RNA Pol II specific TATA-box binding protein associated factors (Tafs) (Akhtar et al., 2011). Through several studies, the upregulation, also referred to as overexpression by producing increased copies, of TBP has been found to contribute to oncogenesis (Johnson et al., 2003) (Ribeiro et al, 2014). Additionally, numerous studies have provided evidence indicating that Tafs regulate differentiation as well as proliferation states; Taf expression has been observed to be higher in pluripotent cells, and consequently reduced in differentiation (Ribeiro et al., 2014). Significantly, Taf2 has been found to be overexpressed in several cancers, however the mechanism in which this occurs is not known at this time (Reghupaty, 2017) (Ribeiro et al., 2014).

In this study, we will examine the cellular abundance of Taf2. Our hypothesis is that Taf2 turnover might be important to maintain optimal levels of Taf2 for normal cellular function. This protein turnover can be regulated by targeted degradation by the 26S proteasome via ubiquitination or non-targeted degradation by proteases. Ubiquitin-26S proteasome dependent proteolysis moderates the selective destruction of few significant proteins including cell cycle regulators, transcription factors, as well as tumor suppressors. Within diverse regulatory pathways, key protein concentrations are managed by post-translational ubiquitination and degradation by the 26S proteasome (Deng et al., 2007). To investigate the cause of upregulation

of Taf2 in cancer cells, it is essential to know if Taf2 is ubiquitinated and degraded by 26S proteasome.

1.1 Eukaryotic Transcription

The basis of all known life is dependent upon the central dogma of molecular biology, which includes DNA replication to transcription of DNA into RNA to translation of RNA into proteins that are then expressed to perform specific functions. Important players in eukaryotic transcription include RNA Pol II, core promoter elements, and a synthetic promoter integrating TATA-box, initiator, downstream promoter element, as well as motif element that supplies a high affinity binding site for transcription machinery. However, most Pol II promoters do not possess a TATA box, which is the typical binding site of TATA-box binding proteins (TBP), subunits of general transcription factor IID (TFIID). TBP are essential in the assemblage of transcription machinery on Pol II promoters that lack TATA boxes as detection of other core promoter motifs by TFIID grant the addition of TBP onto DNA (Greber et al., 2019) (Patel et al., 2020).

There are three phases of transcription, including initiation, elongation, and termination. After RNA polymerase and general transcription factors are recruited to the promoter, known as the pre-initiation complex (PIC), the transcription bubble expands, which allows the synthesis of the first phosphodiester bond within RNA polymerase's active site. In elongation, Pol II conquers the promoter-proximal pause. After the RNA product has been synthesized, transcription is terminated in the termination phase (Greber et al., 2019) (Patel et al., 2020).

Transcription initiation is an exceptionally regulated process. The initiation of transcription and the regulation of RNA processing, stability, as well as subsequent translation of mRNA into protein illustrates the significance of this process in gene expression control.

Misregulation of transcription has been associated with human pathologies, and increased transcription levels by RNA Pol II has also been observed in cancer (Greber et al., 2019) (Patel et al., 2020).

1.2 Ubiquitin-Proteasome System

The UPS is a critical regulator of gene expression and cellular homeostasis. Both ubiquitin molecules and 26S proteasome have been observed to participate in non-proteolytic tasks such as nucleotide excision repair, receptor internalization, and ribosome function (Muratani et al., 2003). However, we will only discuss the proteolytic functions of the UPS as it relates to this study.

After cellular proteins are tagged with ubiquitin molecules, proteins made up by 76 amino acids, they are targeted for degradation by the ATP-dependent 26S proteasome. Ubiquitin mediated delivery of the protein substrate to the 26S proteasome generally occurs through an enzyme cascade as follows: E1 activating enzyme serves as the activating enzyme that transfers the ubiquitin molecule to the E2 conjugating carrier enzyme that tags ubiquitin to the protein substrate of interest with the assistance of E3 enzymes (Nandi et al., 2006). More specifically, in this ATP-dependent process, E1 catalyzes the generation of a thiol-ester bond between its cysteine residue and the C-terminal glycine residue of ubiquitin. Through a trans-thio esterification reaction catalyzed by E2 conjugase, the activated ubiquitin E1 is then transported to the active cysteine site of E2. From E2 conjugase, E3 ligase transfers ubiquitin to the target protein substrate through binding to both substrate and E2 conjugase, and catalyzing the construction of an isopeptide bond between the ε-amino group of lysine (K) residue of the substrate and C-terminal glycine (G) of ubiquitin (Davis & Gack, 2015). Identical linkages are observed between the carboxy terminus of ubiquitin and the $\boldsymbol{\varepsilon}$-amino group of K in a second

ubiquitin molecule to construct polyubiquitin chains, i.e. polyubiquitination. Polyubiquitination has been observed to occur on seven K residues of ubiquitin including K6, K11, K27, K29, K33, K48, as well as K63 (Ferdoush et al., 2024). The polyubiquitination that occurs on a specific K residue of a protein substrate is important as it designates subsequent actions. For example, if polyubiquitination occurs on K29 or K48 of the target protein, this is a signal for 26S proteasome mediated degradation of the substrate. However, if polyubiquitination occurs on other K residues of the substrate, such as K63, this action may act as a signal for other cellular activities such as activation of transcription factors and DNA repair (Nandi et al., 2006).

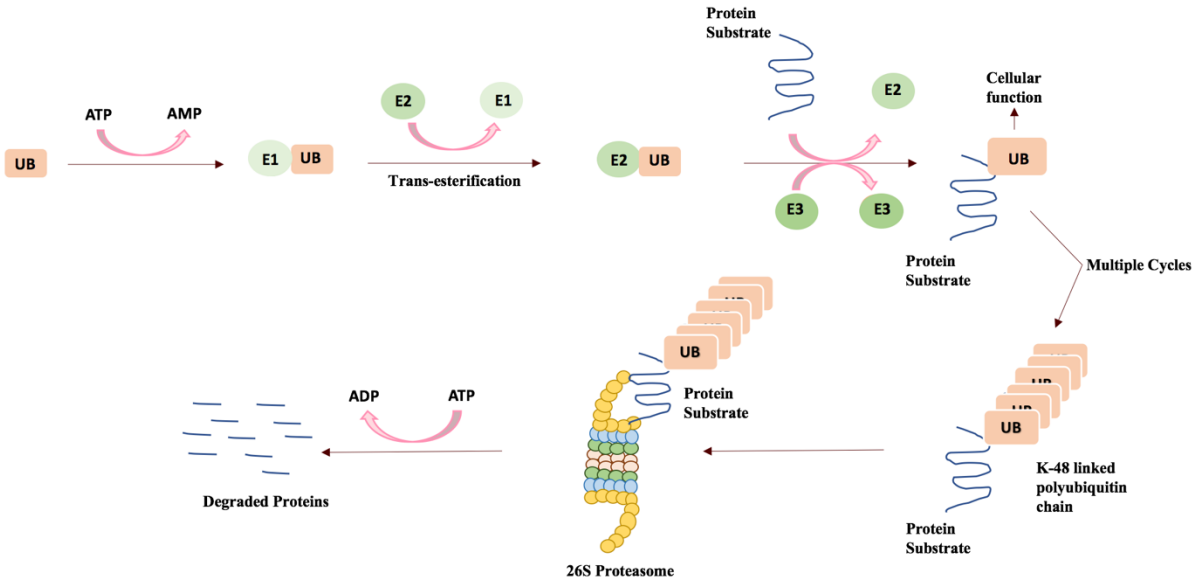

Figure 1.1: Regulation of normal cellular functions by ubiquitination pathway. Through this pathway, E1 catalyzes the formation of a thiol-ester bond between the cysteine residue and the C-terminal glycine residue of ubiquitin in an ATP-dependent reaction. The activated ubiquitin E1 is subsequently transferred to the active cysteine residue site of E2 by trans-thio esterification reaction, catalyzed by E2 conjugase. E3 ligase transfers the ubiquitin from E2 conjugase to the specific substrate by binding to both E2 conjugase and the substrate, and catalyzing the formation of an isopeptide bond between a K-residue of the protein substrate and C-terminal

glycine of ubiquitin. Polyubiquitin chains are formed by the one of the seven ubiquitin K residues. At least four K residue 48 (K48)-linked polyubiquitin serves as the major signal for degradation, while other linear ubiquitin chains participate in diverse processes including signal transduction as well as other regulatory processes. Following polyubiquitination, the polyubiquitinated substrate protein is degraded by the 26S proteasome complex (Ferdoush, 2019) (Ferdoush et al., 2024).

1.3 Bioinformatic Analysis

Bioinformatics is a critical tool in the identification of biomarkers within various cancers through acquiring and interpreting biological data. Specifically, cancer bioinformatics provides meaningful data for clinical medicine and cancer research. Humans produce various genes, RNA, and proteins, all of which are programmed to function spatially and temporally in an intricate web. To provide a thorough representation of cellular activity, bioinformatics is utilized to organize, integrate, as well as interpret extensive data. Today, wet laboratory techniques and bioinformatic analyses are regularly utilized to work together (Nelakurthi et al., 2023). In this study, we utilize bioinformatic databases, i.e. UALCAN and cBioPortal, to assess the overexpression of Taf2 in various cancers. Similar bioinformatic analyses through UALCAN and cBioPortal has been conducted on the upregulation of Paf1, which is the protein that we utilize as our positive control in our research as it has been found to be fully regulated by the UPS (Ferdoush et al., 2017). Paf1 was found to be upregulated at the protein level, rather than the mRNA level in several cancers (Barman et al., 2024). In a likewise manner, we aim to evaluate whether Taf2 is upregulated at the protein or mRNA level in cancer patient samples as this would support our hypothesized association between Taf2 overexpression in cancer cells and regulation by UPS.

1.4 Research Project Summary

Through this research, we aspired to determine whether UPS plays a role in regulation of Taf2's abundance in yeast (*Saccharomyces cerevisiae*) through two sub-aims. Primarily, to investigate regulation of Taf2 by UPS, we evaluated whether Taf2 undergoes polyubiquitination. We assessed the status of Taf2's ubiquitylation using a Ni2+-NTA-based ubiquitination assay as described in the methods and in this paper (Ferdoush et al., 2017) to ascertain whether the abundance of Taf2 is controlled by the UPS. We first carried out a transformation/cloning experiment in which we inserted pUB221 plasmid expressing hexahistidine-tagged ubiquitin under the CUP1 promoter, which is induced in the presence of Cu^{2+} (Pickart, 2000) within the yeast strain expressing tandem affinity purification (TAP)-tagged Taf2. Second, we used this strain to carry out the ubiquitination assay as previously reported in (Ferdoush et al., 2017) and as illustrated in Figure 2.4. Ubiquitin and ubiquitylated proteins were precipitated from whole cell extract (WCE) by binding to the hexahistidine-tagged ubiquitin if UPS regulates the quantity of Taf2 in the cell. Using an anti-TAP antibody against TAP-tagged Taf2, the precipitate was examined for the presence of Taf2 using a western blot test. We carried out comparable tests using the yeast strain without the plasmid encoding hexahistidine-tagged ubiquitin as a loading control (but expressed TAP-tagged Taf2). The expected result consisted of Taf2 not being present in the precipitate; these findings conveyed that cellular Taf2 may be ubiquitylated, and as a result, Ni^{2+}- NTA agarose beads engaged with covalently bonded hexahistidine-tagged ubiquitin to pull down Taf2.

Through our second sub-aim, we assessed 26S proteasome's involvement in the degradation of TAP-tagged Taf2. To examine how the 26S proteasome regulates Taf2 abundance and stability to maintain effective transcription and other cellular processes, we examined Taf2

stability in the presence and lack of MG132 (carbobenzoxy-Leu-Leu-leucinal), a peptide aldehyde that inhibits the 26S proteasome from performing its proteolytic function, in order to gauge the frequency of Taf2 regulation by the proteasome. If polyubiquitinated Taf2 is regulated by 26S proteasomal degradation, the stability and abundance of Taf2 would be enhanced by the pharmacological inhibition of 26S proteasome's proteolytic activity. We deactivated the multidrug resistance gene (*PDR5*) within yeast cells in the strain expressing TAP-tagged Taf2, and then we compared the levels of Taf2 with and without MG132 therapies. Following MG132 treatment, the regulation of Taf2's abundance by the 26S proteasome would enhance Taf2's stability if Taf2's abundance is controlled by this enzyme. Since DMSO was used to make the MG132 solution, we did not anticipate any changes in Taf2's stability. Furthermore, actin served as the loading control and is not controlled by the 26S proteasome, so we did not expect any changes in actin levels in the presence of MG132. Therefore, if Taf2's abundance and stability was controlled by the 26S proteasome, pharmacological inhibitions of the 26S proteasome's proteolytic activity would affect Taf2 through its increased stability and abundance.

Chapter 2: Materials and Methods

2.1 Plasmids

For the ubiquitination assay, we used the pUB221 plasmid which expresses hexahistidine-tagged ubiquitin under the CUP1 promoter. First, we extract the pUB221 plasmid (by doing Plasmid miniprep) from the bacteria strain expressing pUB221 grown in LB and ampicillin. Then, this pUB221 plasmid was transformed into the haploid yeast strain BY4741 bearing TAP-tagged Taf2.

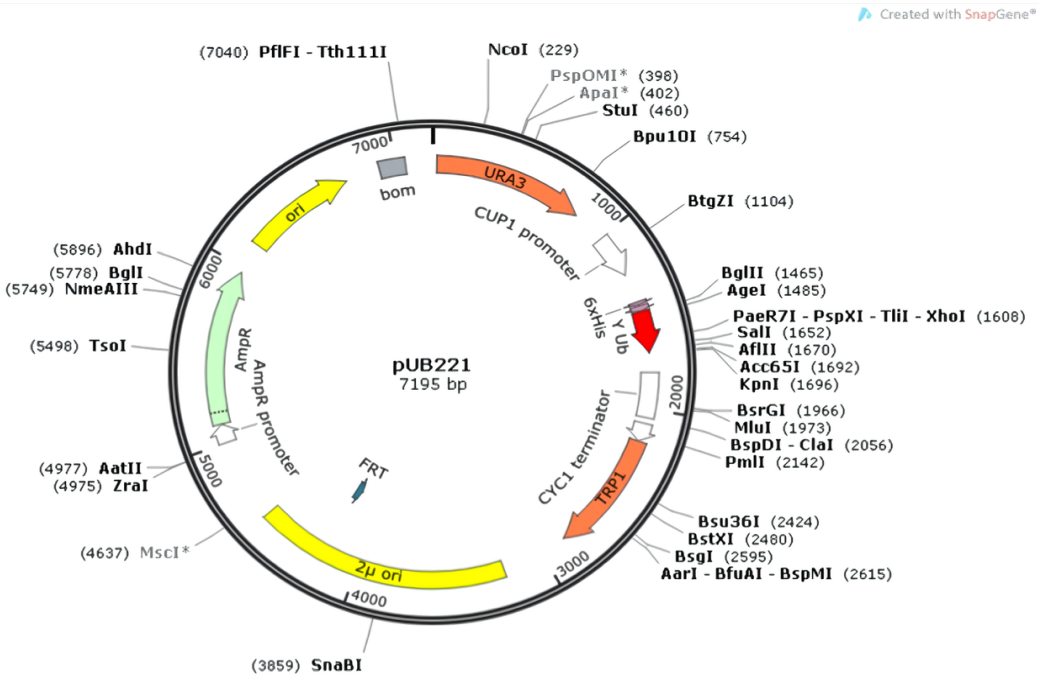

Figure 2.1: Vector map of pUB221 plasmid containing the *URA3* marker and hexahistidine tagged ubiquitin.

Next, for the PCR-mediated disruption of the *PDR5* gene, we used two plasmids: pRS406 for the TAP-tagged Taf13 yeast strain and pRS426 for the TAP-tagged Paf1 yeast strain. Because we had to amplify these two plasmids, first both plasmids were expressed in the *Escherichia coli* (*E. coli*) strain XL1B. Next, using plasmid miniprep protocol, we extracted these two plasmids from the XL1B strains.

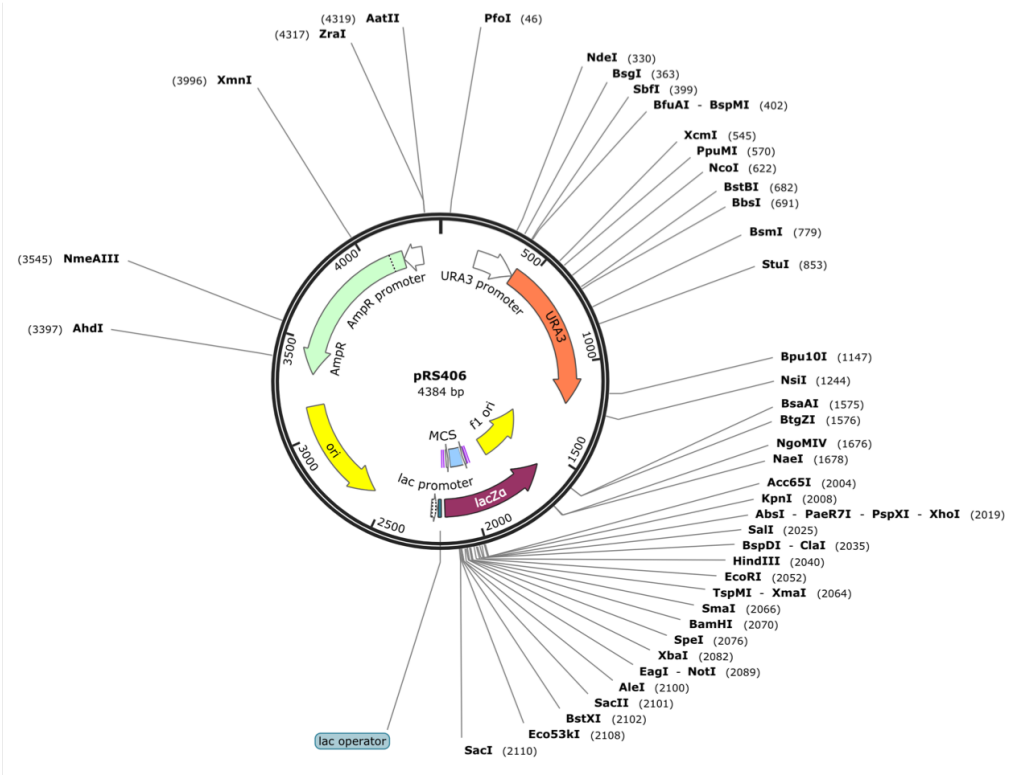

Figure 2.2: Vector map of pRS406 plasmid containing the *URA3* marker.

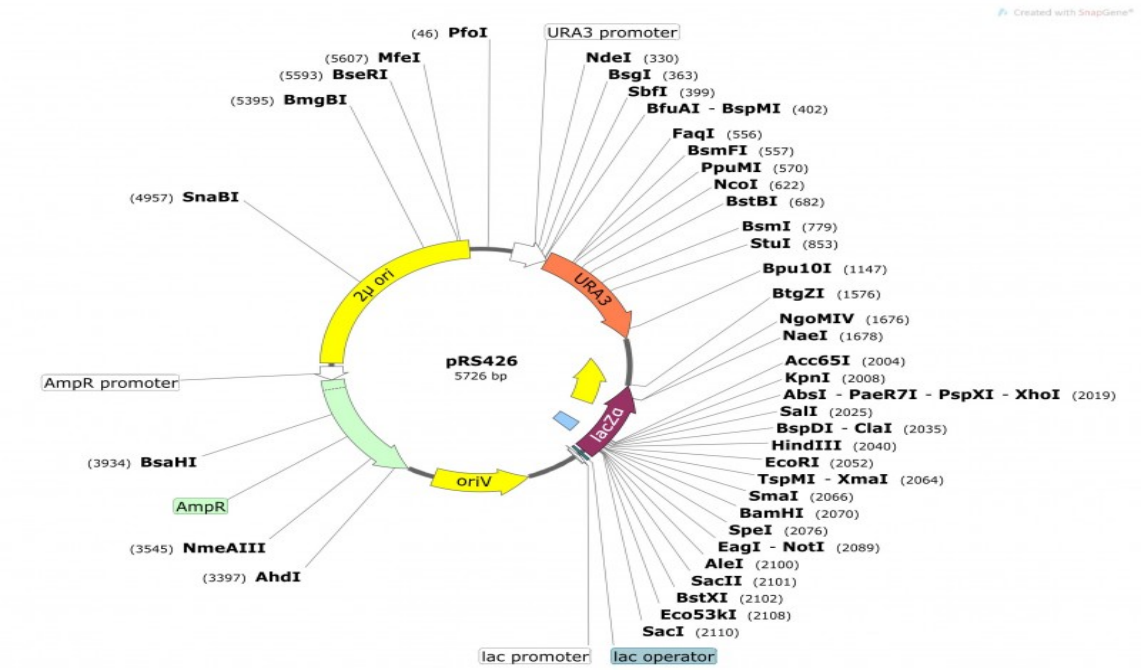

Figure 2.3: Vector map of pRS426 plasmid containing the *URA3* marker.

2.2 Strains

The following bacterial strains were made in Dr. Ferdoush's molecular biology lab and used in this study.

Name	Genotype/Description	Source
JSMBO1	pRS426 in XL1B *(URA3* marker) (Grown in LB + tet^r (*KAN*) (*AMP*))	In lab
JSMBO5a	pRS406 in XL1B (*URA3* marker) (Grown in LB + tet^r (*KAN*) (*AMP*))	In lab
JSMBO5b	pRS406 in XL1B (*URA3* marker) (Grown in LB + tet^r (*KAN*) (*AMP*))	In lab

The following yeast strains were made in Dr. Ferdoush's molecular biology lab and used in this study.

Name	Genotype/Description	Source
JSMO5a	pUB221 plasmid (*URA3* marker) introduced into Taf2-TAP (grown in YNB-ura/dex)	In lab
JSMO5b	pUB221 plasmid (*URA3* marker) introduced into Taf2-TAP (grown in YNB-ura/dex)	In lab
JSMO8a	pUB221 plasmid (*URA3* marker) introduced into Paf1-TAP (grown in YNB-ura/dex)	In lab
JSMO8b	pUB221 plasmid (*URA3* marker) introduced into Paf1-TAP (grown in YNB-ura/dex)	In lab
JSMO2a	Δ*pdr5* in Taf2-TAP tag (pRS406 (*URA3* marker)) (grown in YNB-ura/dex)	In lab
JSMO2b	Δ*pdr5* in Taf2-TAP tag (pRS406 (*URA3* marker)) (grown in YNB-ura/dex)	In lab

2.3. Growth Media

To amplify pRS406 and pRS426 plasmids, we initially expressed them in XLIB strains followed by extraction through plasmid miniprep. To grow the XLIB strain, we prepared luria broth + ampicillin agar (LB + amp) media as ampicillin serves as the selective antibiotic marker.

In the transformation of yeast strains expressing TAP-tagged Taf2 and TAP-tagged Paf1, cells were selected for by the *URA3* marker present in all plasmids utilized in our laboratory (pUB221, pRS406, and pRS426). Yeast Nitrogen Base – uracil/dextrose (YNB-URA/Dex) served as the selective media for yeast cells. Yeast Extract-Peptone-Dextrose (YPD) served as the growth media for yeast cells.

2.4 Plasmid Extraction via Qia-Miniprep

We isolated pUB221 plasmid DNA from transformed *E. coli* cells on a LB + amp plate, provided by Dr. Daniel Finley of Harvard University. Using the QIAprep Spin Miniprep Kit, we cultured transformed *E. coli* in liquid LB + amp media for 12 hours at 37 ℃ and 200 xg. After centrifuging, we serially resuspended the cells in resuspension buffer P1, lysis buffer P2, and neutralization buffer N3. After another centrifugation step, we collected the supernatant, loaded it into a spin column, and washed with washing buffers PB and PE. Lastly, we eluted the plasmid DNA with EB buffer and stored it at -20℃.

This protocol was also used to extract both pRS406 and pRS426 plasmids from XLIB bacterial cells.

2.5 Ubiquitination Assay

2.5.1. Part 1: Transformation of pUB221 Plasmid into the Yeast Strains

After isolating the pUB221 plasmid through miniprep, we introduced it into our haploid yeast strain, BY4741, expressing TAP-tagged Taf2. Yeast cells were cultured in YPD media at 30℃ and 200 xg for 12 hours. Once the optical density at 600 nanometers (nm) (OD_{600}) reached approximately 1.00 OD_{600}, the culture was centrifuged. The cell pellet was resuspended in cold 1X tris-buffered saline (TBS), mixed with polyethylene glycol (PEG) solution, and pUB221 plasmid DNA. Herring sperm DNA was prepared by boiling at 95℃ for 5 minutes and subsequently added, and the mixture was incubated at 42℃ for 15 minutes. After centrifugation and resuspension in YPD media, the cells were incubated for 1.5 hours at 30℃. Following centrifugation and resuspension in 1X tris-ethylenediaminetetraacetic acid (EDTA) (TE) buffer, the cells were plated onto YNB-URA/Dex agar and incubated at 30℃ for 24-48 hours.

2.5.2. Part 2: Whole Cell Extract (WCE) Preparation and Histidine-Ubiquitination Assay

To the collected pellets, we added buffer A (6 M guanidine-HCl, 100 mM Na_2HPO_4/NaH_2PO_4 at pH 8.0, 10 mM imidazole) and glass beads, which we vortexed for 30 minutes at 4°C. After 30 minutes, we punctured the bottom of the microcentrifuge tube to collect the lysate within a new microcentrifuge tube. We added Ni^{2+}-NTA agarose beads to the lysate and spun the mixture for 4 minutes at 3,000 xg. After removing the supernatant, we resuspended the beads in buffer A and incubated them on a rocker. After incubation, we washed the beads three times each with buffers A, A/TI (1 volume of buffer A and 3 volumes of buffer TI), and TI (25 mM Tris-Cl, 20mM imidazole at pH 6.8) and discarded the supernatant each time. We resuspended the pellet in 2X sodium-dodecyl sulfate (SDS) with 0.2 M imidazole, boiled the mixture for 10 minutes, and transferred the supernatant to a new microcentrifuge tube using a 22 gauge needle as this allowed us to accurately extract only the supernatant, instead of the agarose beads located at the bottom of the tube. This was stored at -80°C. Figure 2.4 represents the schematic diagram for Ni^{2+}-NTA-based ubiquitination assay.

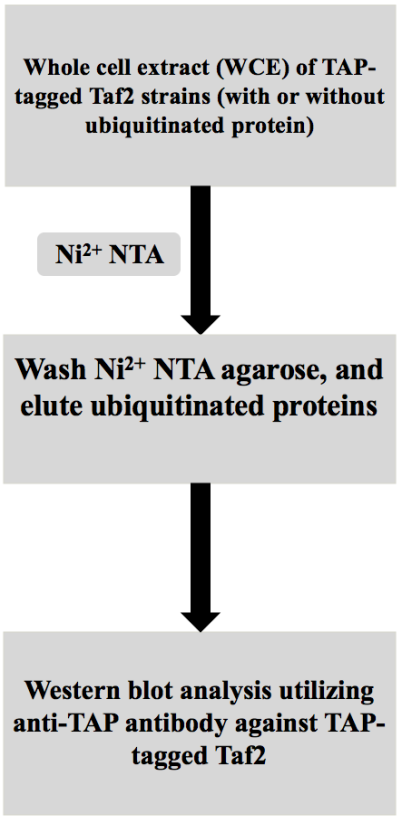

Figure 2.4: Schematic diagram for Ni^{2+}-NTA-based ubiquitination assay.

2.5.3. Part 3: Western Blot (WB) Analysis of Ubiquitinated Proteins

We prepared a 7.5% SDS-polyacrylamide (PAGE) gel. After loading the protein samples along with BLUeye Protein Ladder and running the gel in 1X running buffer at 120 Volts (V) and 35 ampere (A), we transferred proteins onto a polyvinylidene difluoride (PVDF) membrane in 1X transfer buffer at 100 V and 350 milliampere (mA). To develop the membrane, we briefly exposed the membrane to methanol to induce hydrophilicity, blocked it with blocking buffer, incubated with the primary anti-TAP antibody, followed by the secondary anti-rabbit-horseradish peroxidase (HRP) antibody. After washing with 1X Tris-buffered saline, 0.1% Tween20 (TBST), we applied chemiluminescent reagents, and visualized the blot using Dr. Benjamin Stein's ChemiDoc machine at the University of Tennessee at Chattanooga. We stored the blot at 4°C.

2.6 Proteasomal Degradation Assay

2.6.1. Part 1: PCR-Mediated Disruption of *PDR5*

To delete *PDR5* in yeast cells expressing TAP-tagged Taf2, we utilized PCR-based gene disruption which was schematically represented in Figure 2.5. To do this, pRS406 and pRS426 plasmids, both containing genes that encode auxotrophic markers, were amplified through PCR. We designed two primers that were 80 nucleotides (nt) in length. Each primer was designed in such a way that the 60 nt sequence at the 5' end of each primer overlapped with regions that flanked the gene of interest to be deleted. Additionally, the remaining 20 nt sequence of each primer is specific to the portion of the plasmid that was amplified. The following PCR program was utilized to amplify the plasmid DNA:

94°C – 2 min

94°C – 1 min
55°C – 1 min
72°C – 3 min
} 10 cycles

94°C – 1 min
65°C – 1 min
72°C – 3 min
} 20 cycles

First, we produced the PCR Master Mix cocktail for 24 plasmid DNA samples, pRS406 plasmid and pRS426 plasmid, made with 600 µL of Dream Taq (50%), 24 µL of forward primer (2%), and 24 µL of reverse primer (2%), and 550 µL of nuclease-free water (46%). To specifically delete *PDR5*, we designed both primers. The forward primer (DelIA) consists of a nucleotide sequence downstream of the start codon 60 kilobases (kb) of the *PDR5* open reading frame (ORF) and 20 kb of the universal sequence from the pRS406 plasmid and pRS426. The reverse primer (DelIB) consists of a nucleotide sequence of the stop codon 60 kb of the *PDR5* ORF and 20 kb of the universal sequence of pRS406 plasmid:

PDR5 del1A: 5'- AAGAAATTAAAGACCCTTTTAAGTTTTCGTATCCGCTCGTTCGAA AGACTTTAGACAAAA **CTGTGCGGTATTTCACACCG** -3'

PDR5 del1B: 5'- ATGTTTATTAAAAAAGTCCATCTTGGTAAGTTTCTTTTCTTAACCAAA TTCAAAATTCTA **AGATTGTACTGAGAGTGCAC** -3'

We added 1 µL of the template DNA (1%) into 24 thin-walled PCR tubes. Then, we added 99 µL of the Master Mix cocktail into each PCR tube. After placing the lids on the tubes, we carefully mixed the samples by tapping followed by quickly centrifuging all tubes. Then, we placed all samples in the thermocycler with lids on and selected the first (of two) PCR-mediated disruption program. After the program concluded, we stored all samples in 4°C. When observing our PCR product through agarose gel electrophoresis, the band was seen at 1145 base pairs (bp).

After transforming yeast cells expressing Taf2-TAP and Paf1-TAP with the first PCR program product, we observed the successful transformation that resulted in homologous recombination between the gene-specific 60 nt sequence of PCR amplified DNA as well as the corresponding genomic sequence of the endogenous gene. This integrated the auxotrophic marker, *URA3*, into the yeast genome by disrupting the endogenous gene from its chromosomal locus. We selected individual colonies of TAP-tagged Taf2 and TAP-tagged Paf1 on selective media, YNB-URA/Dex, according to their selective marker. During PCR screening of selected individual colonies, we designed the primers to target against a region within the *PRD5* open reading frame.

The PCR program occurred as follows:

94°C – 2 min

94°C – 2 min
55°C – 1 min
72°C – 2 min
} 30 cycles

72°C – 10 min

First, we produced the PCR Master Mix cocktail for 24 DNA samples made with 300 µL of Dream Taq (50%), 12 µL of forward primer (2%), and 12 µL of reverse primer (2%), and 276 µL of nuclease-free water (46%). To ensure that *PDR5* was successfully deleted, we designed both primers from the open reading frame of the *PDR5* gene in yeast cells; the forward primer was referred to as "ORF2A," and the reverse primer was referred to as "ORF2B:"

PDR5 ORF 2A: 5'- GGA TGC TAG AAG TTG TTG GTG CA - 3'

PDR5 ORF 2B: 5'- CTC AGC TGC AGT TAT CGA ACC TT -3'

We added 1 µL of the template DNA (1%) into 24 thin-walled PCR tubes. Then, we added 24 µL of the Master Mix cocktail into each PCR tube. After placing the lids on the tubes, we carefully mixed the samples by mixing followed by quickly centrifuging all tubes. Then, we placed all samples in the PCR machine with the lids on and selected the second PCR program to accomplish the PCR-mediated disruption of *PDR5*. After the program concluded, we stored all samples in 4°C.

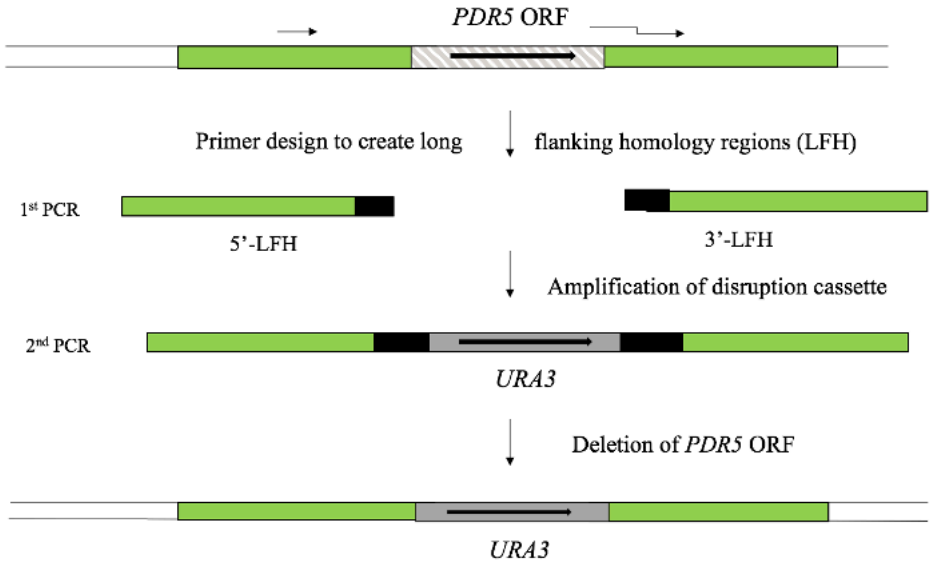

Figure 2.5: Schematic diagram for PCR-mediated disruption of *PDR5* in yeast strain expressing TAP-tagged Taf2 and TAP-tagged Paf1. During transformation, through homologous recombination yeast endogenous *PDR5* gene was replaced by the PCR product leaving *URA* marker in the place of *PDR5* gene. PRS406 plasmid and PRS426 plasmid were used which contain URA selectable marker.

2.6.2. Part 2: Positive Colony Screening by Agarose Gel Electrophoresis

In sterile conditions, we inoculated individual colonies in YPD media to grow for 12 hours at 30℃ and 200 RPM for positive colony screening. After 12 hours, we transferred the cultures, centrifuged them, and resuspended the cell pellets in 1X TBS. Following centrifugation, we resuspended the pellets in formic acid lysis buffer (FALB), and vortexed the samples for 30 minutes. After 30 minutes, we centrifuged the sample, collected the supernatants into new microcentrifuge tubes, and added phenol:chloroform:isoamyl alcohol. Following an additional centrifugation, we transferred the aqueous layer to new microcentrifuge tubes, added 100% EtOH, and placed the samples in -80℃ for at least 30 minutes to precipitate the DNA. After

precipitation, we centrifuged the samples, washed the residual salts with 70% ethanol, and dried the DNA. We resuspended the DNA in 1X TE buffer, centrifuged, and stored samples in -20℃.

To visualize the disruption of *PDR5* in yeast strains expressing TAP-tagged Taf2 and TAP-tagged Paf1, we prepared a 1% agarose gel by mixing agarose powder with 1X Tris-borate EDTA buffer (TBE) and ethidium bromide (EtBr). After the gel solidifies, we added 6X DNA dye to the PCR samples, mixed, and loaded them in addition to the 100 bp DNA ladder into the gel. After running the agarose gel at 100 volts, the PCR samples were ready to be observed under ultraviolet (UV) light using our laboratory's GelDoc machine. The successful deletion of *PDR5* resulted in the absence of a DNA band at approximately 175 bp.

2.6.3. Part 3: Proteasomal Degradation Assay via MG132 and DMSO treatment

After *PDR5* was successfully deleted in our yeast strain expressing TAP-tagged Taf2 and TAP-tagged Paf1, we treated the cells with MG132 and DMSO solutions. First, we inoculated the yeast strain in liquid YPD media and allowed the cells to grow for approximately 12 hours at 30℃ and 200 RPM. After OD_{600} reached approximately 0.7 OD_{600}, we transferred the culture into two separate tubes. In one tube, we added MG132 solution, and incubated for 2 hours before collecting the cells for western blot analysis. In the other tube, we added DMSO solution, and incubated for 2 hours. We stored the cells at -80℃ for further processing.

In the microcentrifuge tubes containing the cell pellets treated with MG132, we added FALB, protease inhibitor, and 1 mM phenylmethylsulfonyl fluoride (PMSF), which inhibits serine proteases, such as trypsin and chymotrypsin, from degrading our protein samples by sulfonylation of the serine residue in the active site of the enzyme while processing our samples (Nichols et al., 2020). It was critical to process our cells quickly as PMSF has a very small half-life. With glass beads, we vortexed the tube at 4℃ for 30 minutes to break the cell membrane

and extract our protein of interest(s). After 30 minutes, we extracted the lysate with a 22 gauge needle into new microcentrifuge tubes and centrifuged the samples. We transferred the supernatants into new microcentrifuge tubes and stored them at -80℃. Alternatively, if we were ready to perform a western blot assay, we would add 6X SDS dye and load the samples directly into the SDS-PAGE gel. Figure 2.6 represents the schematic diagram for MG132-based proteasomal degradation assay.

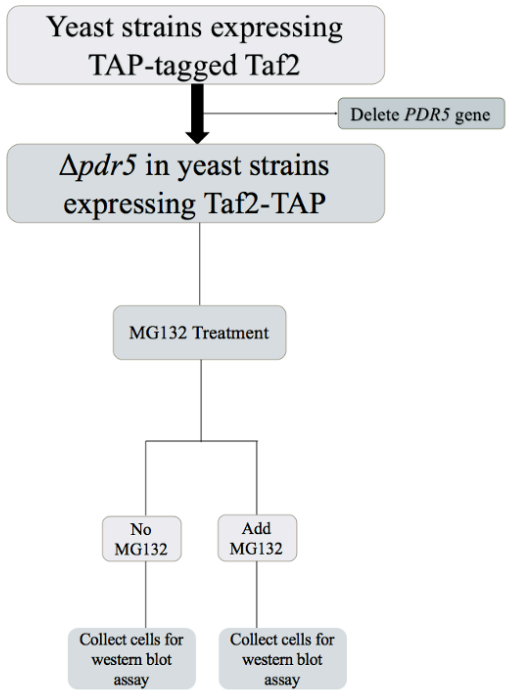

Figure 2.6: Schematic diagram for MG132-based proteasomal degradation assay.

2.6.4. Part 4: Western Blot Analysis of MG132 and DMSO Treated Cells

In a 7.5% SDS-PAGE gel, we loaded samples and BLUeye Protein Ladder to run at 120 V and 35 A in 1X running buffer. In 1X transfer buffer, we transferred the proteins onto a PVDF membrane at 100 V and 350 mA. To develop the membrane, we briefly exposed the membrane to methanol to induce hydrophilicity. After blocking buffer incubation, we treated the membrane with primary anti-TAP antibody for one half of the membrane, primary anti-Actin antibody for

the other half of the membrane, and washed each with 1X TBST. We treated both halves of the membrane with the same secondary anti-rabbit-HRP antibody, and washed with 1X TBST. We applied the chemiluminescent reagents, and visualized the blot using Dr. Stein's ChemiDoc machine. Lastly, we stored the blot at 4°C.

2.7 Analysis of Cancer Patient Samples

To analyze Taf2 amplification and deletion in several cancers, we used the cBioPortal database (https://www.cbioportal.org/) (Cerami et al., 2012) (de Bruijn et al., 2023) (Gao et al., 2013) selecting 32 TCGA Studies. A total of 10967 samples (or 10,953 cancer patients) from these studies were used for the Taf2 analysis in various cancers with a minimum sample number of 3 and frequency of 0.1%. In addition, we used the UALCAN (The University of Alabama at Birmingham cancer data analysis) database (http://ualcan.path.uab.edu/) (Chandrashekar et al., 2017) (Chandrashekar et al., 2022) to analyze Taf2 protein and mRNA levels in various cancers. Similarly, Taf2 mutations in various cancer patient samples were identified through the cBioPortal database (with 10967 samples/10,953 patients) with a minimum sample number of 3 and frequency of 0.1%. The DNA methylation of the Taf2 promoter DNA and liver hepatocellular carcinoma (LIHC) patient survival probability was analyzed using the UALCAN portal. Based on these results, box and whisker plots were obtained. The red boxes represent tumor data whereas the blue represents normal cell lines. In this diagram, 50% of the data fall between the boxes with the median represented by a line. Significant change is shown generally by analyzing the position of the boxes.

Chapter 3: Results

3.1 Background

Within this study, we investigated the role of the UPS in regulating the overexpression of Taf2 in yeast cells through two sub-aims. Primarily, we performed a Ni^{2+}-NTA based ubiquitination assay to determine if TAP-tagged Taf2 expressing the pUB221 plasmid with hexahistidine tagged ubiquitin underwent polyubiquitination. Secondly, we evaluated the stability of TAP-tagged Taf2 in the presence and absence of MG132, which inhibits the 26S proteasome from performing its proteolytic function, to assess Taf2 regulation by the 26S proteasome. From our results, we have determined that Taf2 undergoes polyubiquitination and is subsequently degraded by the 26S proteasome.

3.2 Sub-Aim I Results

3.2.1. Ubiquitination assay demonstrates that TAP-tagged Taf2 undergoes polyubiquitination

Within our ubiquitination assay, we performed a western blot to determine if TAP-tagged Taf2 expressing the pUB221 plasmid with hexahistidine tagged ubiquitin experienced polyubiquitination. Figure 3.1 contains this blot demonstrating that Taf2 undergoes polyubiquitination. Using the protein ladder, we can observe this by identifying the molecular weight of Taf2, 161 kilodaltons (kDa), in addition to the TAP tag, 21 kDa, attached to the protein. However, we clearly see that the bands for both wells containing TAP-tagged Taf2 is well above 183 kDa (161 kDa + 21 kDa = 183 kDa); this indicates that polyubiquitin molecules (approximately 100 kDa) are indeed covalently attached to our protein of interest. Additionally, TAP-tagged Paf1 is utilized as our positive control in the ubiquitination assay as it has been found to be fully regulated by the UPS (Ferdoush et al., 2017).

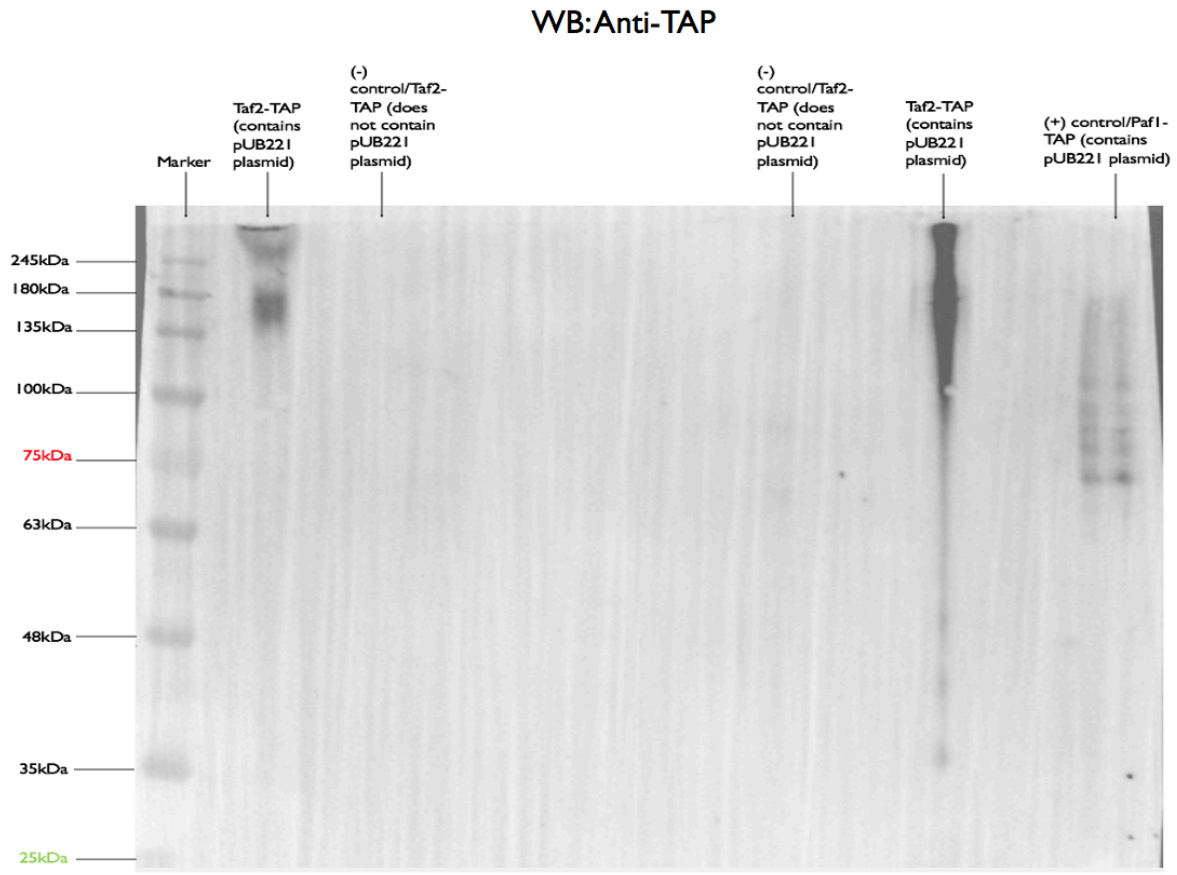

Figure 3.1: **Taf2 undergoes polyubiquitination.** Western blot analysis containing (left-to-right) BLUeye protein ladder, TAP-tagged Taf2 protein expressing the pUB221 plasmid containing hexahistidine-tagged ubiquitin, two samples of TAP-tagged Taf2 without the pUB221 plasmid (serving as our negative control), TAP-tagged Taf2 protein expressing the pUB221 plasmid containing hexahistidine-tagged ubiquitin, as well as TAP-tagged Paf1 expressing the pUB221 plasmid containing hexahistidine-tagged ubiquitin, serving as our positive control due to its regulation by UPS (Ferdoush et al., 2017). Both TAP-tagged Taf2 and TAP-tagged Paf1 were precipitated through Ni^{2+}- NTA agarose beads. From both wells containing TAP-tagged Taf2 expressing the pUB221 plasmid containing hexahistidine-tagged ubiquitin, we determined that Taf2 undergoes polyubiquitination.

3.3 Sub-Aim II Results

3.3.1. PCR-mediated disruption of *PDR5*: positive colony screening

Figure 3.2 presents individual colonies re-streaked after transformation of yeast strain expressing TAP-tagged Taf2 containing PCR product. Solid growth plate containing deletion of *PDR5* in TAP tagged Taf2 for colony screening.

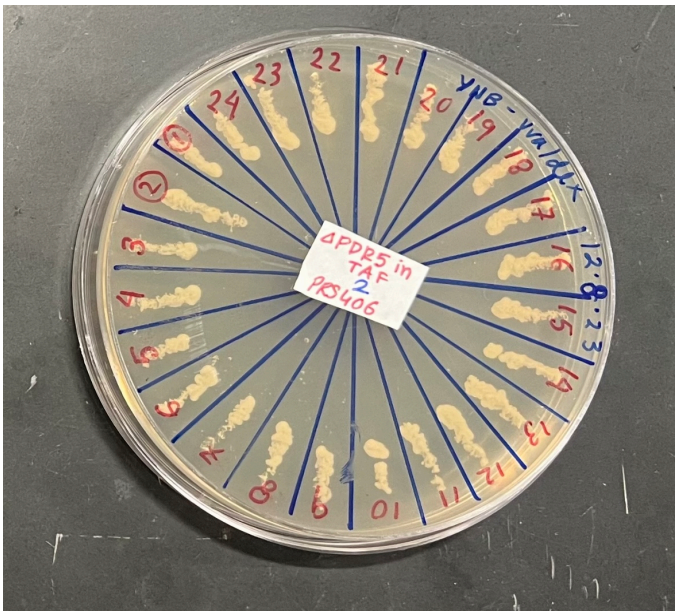

Figure 3.2: Individual colonies re-streaked after transformation of yeast strain expressing TAP-tagged Taf2 containing PCR product. Solid growth plate containing deletion of *PDR5* in TAP tagged Taf2 for colony screening.

Figure 3.3 presents the product size of the first PCR program to disrupt *PDR5*. The PCR product consists of the selectable marker gene, *URA*, which flanks 60 bp of the start codon and 60 bp of the stop codon of the *PDR5* ORF. The size of the PCR product is approximately 1,145 bp.

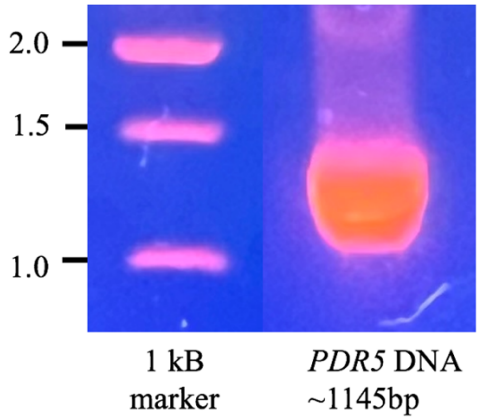

Figure 3.3: Agarose gel exhibiting PCR product containing *PDR5* DNA at approximately 1,145 base pairs (between 1,000 bp and 2,000 bp).

Figures 3.4 and 3.5 display agarose gels after the second PCR program. The two designed primers, containing *PDR5* ORF, are approximately 175 bp. When successfully disrupted, the ORFs were not expressed, which can be observed through the absence of a band around 175 bp. In both figures, the Taf2-TAP and Paf1-TAP controls contain bands around 175 bp, indicating that *PDR5* was not successfully deleted in these colonies.

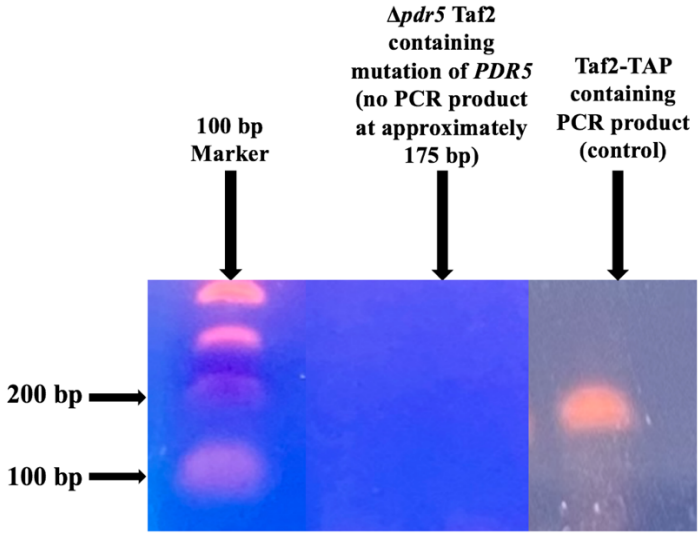

Figure 3.4: Deletion of *PDR5* in Taf2-TAP through the absence of the band at 175 bp. Δ*pdr5* screening in Taf2-TAP individual colonies through agarose gel electrophoresis, which is loaded with

(left-to-right) 100 bp DNA ladder, Taf2-TAP containing mutation of *PDR5* (no PCR product around 175 bp) that was utilized through Sub-Aim II procedures, and Taf2-TAP containing PCR product (control). We can confirm the deletion of *PDR5* through the absence of the band at 175 bp (between 100 bp and 200 bp).

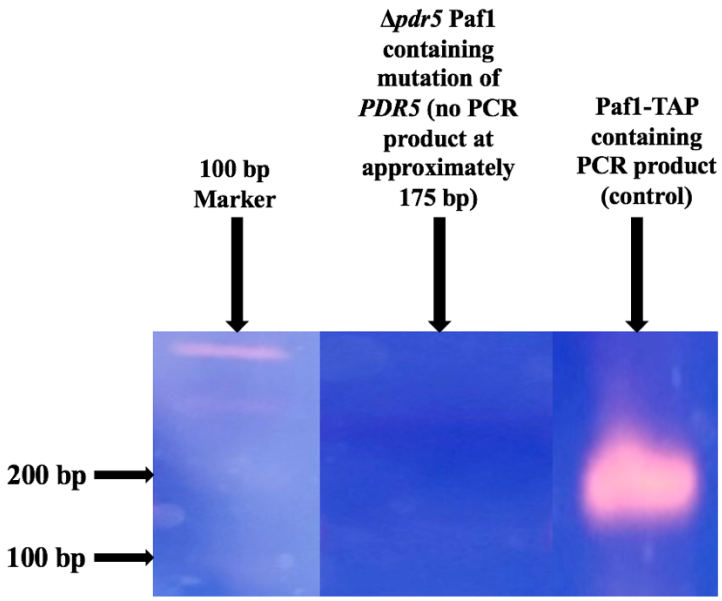

Figure 3.5: Deletion of *PDR5* in Paf1-TAP through the absence of the band at 175 bp. Δ*pdr5* screening in Paf1-TAP individual colonies through agarose gel electrophoresis, which is loaded with (left-to-right) 100 bp DNA ladder, Paf1-TAP containing mutation of *PDR5* (no PCR product around 175 bp) that was utilized through Sub-Aim II procedures, and Paf1-TAP containing PCR product (control). We can confirm the deletion of *PDR5* through the absence of the band at 175 bp (between 100 bp and 200 bp).

3.3.2. Western blot demonstrates that TAP-tagged Taf2 is regulated by 26S proteasomal degradation

In our assessment of the 26S proteasome regulating the stability of TAP-tagged Taf2, we performed a western blot to observe TAP-tagged Taf2 proteins treated with and without MG132 proteasome inhibitor. Figure 3.6 contains two blots developed with two different antibodies, anti-

TAP (A), acting against the TAP tag, and anti-Actin (B), acting against the actin present in TAP-tagged Taf2 collected from yeast cells; actin is constitutively expressed as it is involved in structure and movement of the yeast cytoskeleton (Mulholland et al., 1994). In the blot treated with the anti-TAP antibody (A), we observed that the band representing TAP-tagged Taf2 collected from yeast cells treated with MG132 at 183 kDa (Taf2 + TAP tag) was more saturated than the protein collected from cells treated with DMSO. Significantly, this indicates that upon the knockout of *PDR5*, MG132 was successful in inhibiting the 26S proteasome from degrading TAP-tagged Taf2, thus leading to the band appearing more saturated compared to the less saturated band representing TAP-tagged Taf2 collected from cells treated with DMSO, instead of MG132. This can also be observed in our positive control, TAP-tagged Paf1. Similarly, in the blot treated with the anti-Actin antibody (B), we observed the band representing actin expressed in TAP-tagged Taf2 collected from yeast cells treated with MG132 at 43 kDa (molecular weight of actin) was more saturated than the protein collected from cells treated with DMSO. This finding is confirmed with our positive control, Paf1.

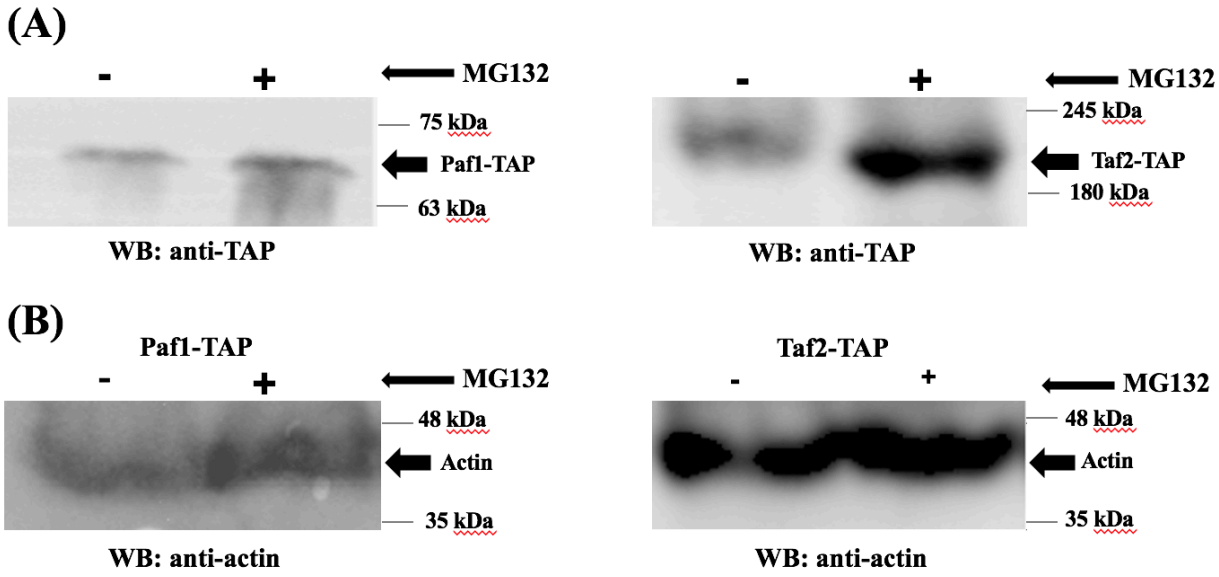

Figure 3.6: Polyubiquitylated Taf2 undergoes 26S Proteasomal degradation. Western blot analysis of TAP-tagged Taf2 abundance in the presence and absence of MG132, a proteasome inhibitor that suppresses the proteolytic activity of the 26S proteasome. To develop the PVDF membrane, we utilized (A) anti-TAP and (B) anti-Actin antibodies on blots containing TAP-tagged Paf1, serving as our positive control due to its regulation by the UPS (Ferdoush et al., 2017) (left) and TAP-tagged Taf2 with a null mutation of *PDR5* (right). In (B), we utilized actin as our loading control to assess the activity of the 26S proteasome in the presence and absence of MG132. From these observations, we determined that TAP-tagged Taf2 abundance and stability was impacted in the presence of MG132, suggesting that Taf2 undergoes regulation by 26S proteasome mediated degradation.

3.4 Bioinformatic data suggests that protein overexpression, rather than mRNA overexpression, plays a role in several cancers suggesting a mismanaged UPS system

Through cBioPortal, we acquired an analysis of cancer patient samples revealing that Taf2 is upregulated via gene amplification in numerous cancers (Fig. 3.7A). In other cancers, such as thyroid cancer and pleural mesothelioma, Taf2 was deleted, but at a lower frequency (Fig. 3.7B). Through an examination of UALCAN cancer patient samples using Z-tests, we confirmed that Taf2 protein expression was heightened throughout multiple samples (Fig. 3.7C), which was accompanied by differential mRNA expression levels of Taf2 (Fig. 3.7D).

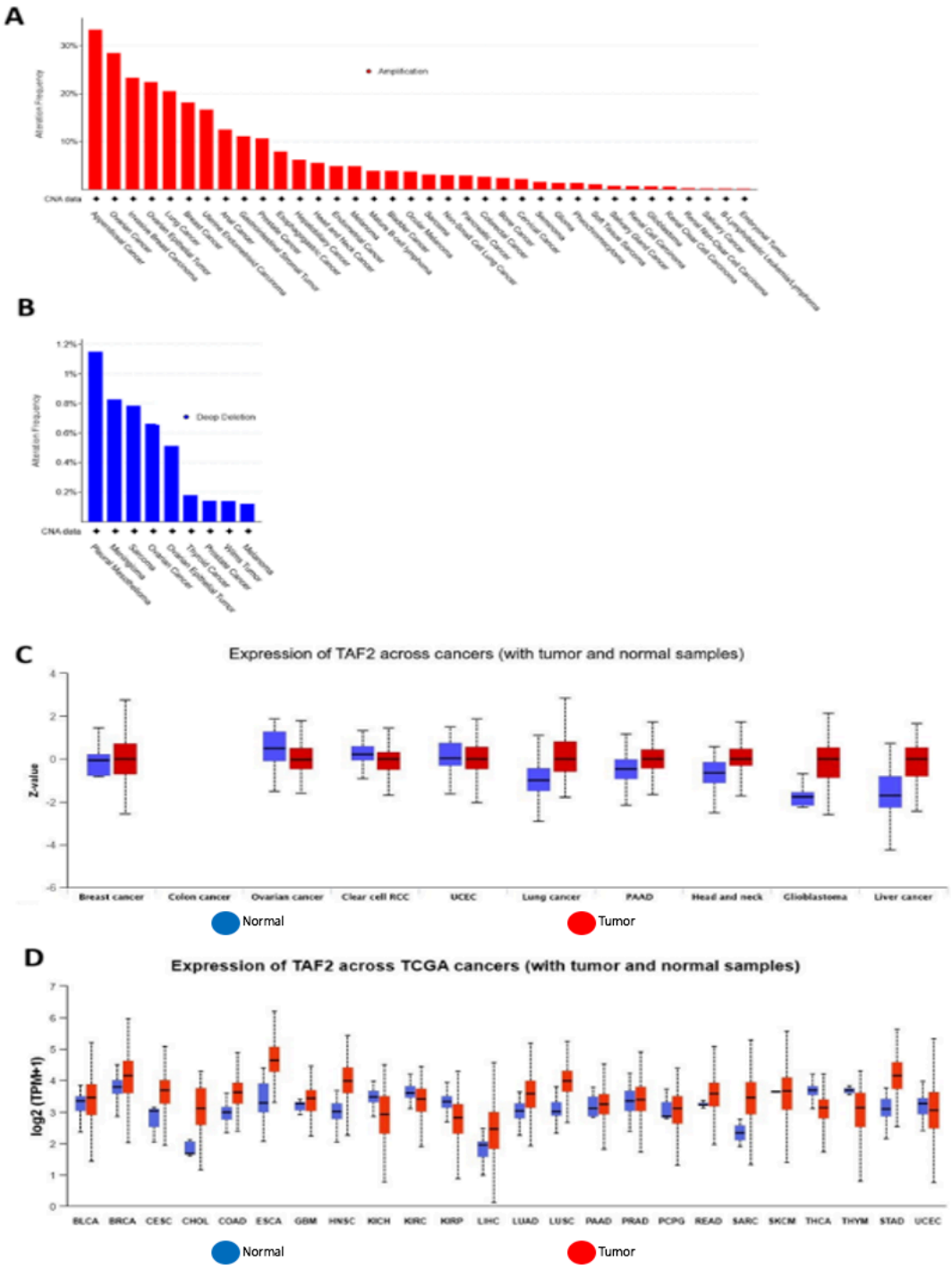

Figure 3.7: Taf2 expression in different cancer patient samples. (A & B) Cross-cancer analysis of copy number alterations (CNA) in Taf2 based on cancer patient samples derived from cBioPortal database [217 no overlapping samples]. The frequencies of 0.1 % and above were plotted with a minimum sample number of 3. (C) Taf2 protein levels in various cancer patient samples based on CPTAC (Clinical

proteomic tumor analysis consortium) in the UALCAN. Z value represents standard deviation from the median across samples for the given cancer type. UCEC, uterine corpus endometrial carcinoma; PAAD, pancreatic adenocarcinoma; and RCC, renal cell carcinoma. (D) Taf2 mRNA levels in various cancer patient samples based on TCGA in the UALCAN. TPM (Transcripts per million) is a normalized number, and is read as the number of RNA of a particular gene of interest out of 1 million RNA molecules. KIRC, kidney renal clear cell carcinoma; LUAD, lung adenocarcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRP, kidney renal papillary cell carcinoma; LIHC, liver hepatocellular carcinoma; LUSC, lung squamous cell carcinoma; PRAD, prostate adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; READ, rectal carcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; THCA, thyroid carcinoma; THYM, thymoma; and STAD, stomach adenocarcinoma.

We examined both Taf2 mRNA and protein levels in many cancer patient samples including glioblastoma multiforme (GBM) and kidney renal cell carcinoma (KIRC) through the UALCAN database (Fig. 3.8A-D). Taf2 protein and mRNA levels in GBM and KIRC patient samples exhibited increased upregulation at the protein level (Fig. 3.8B and 3.8D) as opposed to the mRNA level (Fig. 3.8A and 3.8C) suggesting a mismanaged UPS.

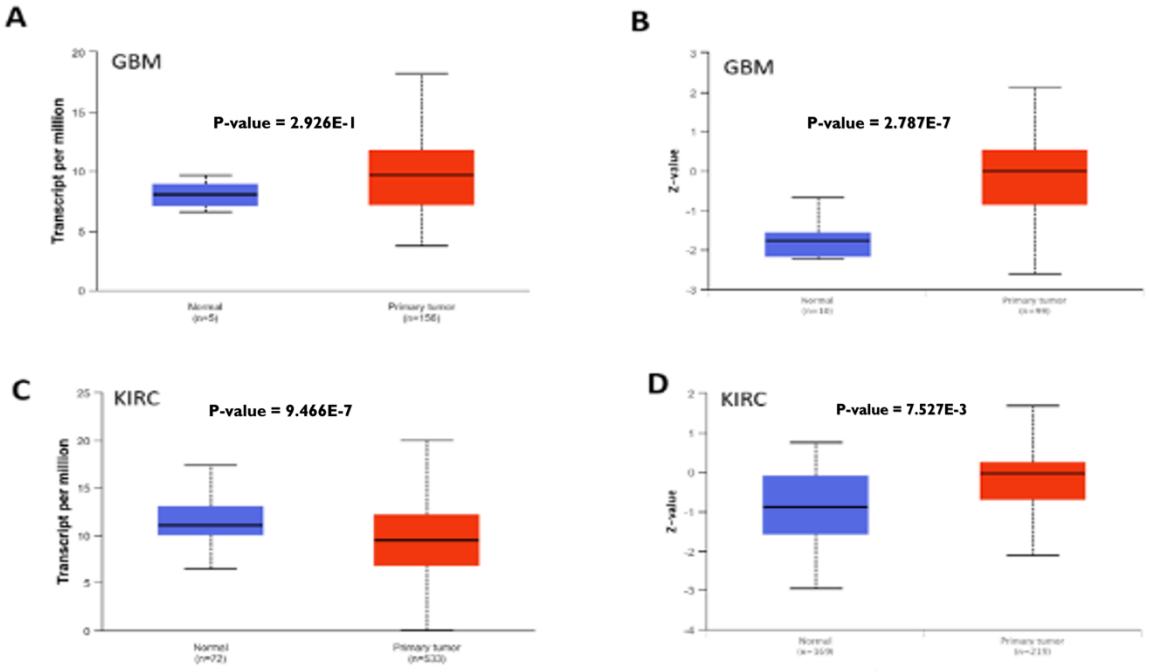

Figure 3.8: Z-test analysis of Taf2 mRNA and protein levels in GBM and KIRC based on CPTAC and TCGA cancer patient samples in the UALCAN. (A and B) Taf2 mRNA (A) and Protein (B) levels in GBM. n, number of samples; and p, p value for the analysis of the statistical significance of the change between normal and tumor/cancer cells. (C and D) Taf2 mRNA (C) and Protein (D) levels in KIRC.

As previously discussed in section 1.2, K residues within ubiquitin molecules are utilized to construct polyubiquitin chains, and therefore serve an important role in the polyubiquitination of substrate proteins. As ubiquitination commonly occurs on K residues of proteins, this suggests that K residue mutations may lead to decreased proteasomal degradation. However, we observed many cancer patient samples exhibiting K mutations in Taf2 within various tissues (Fig. 3.9A). At least one of these K residues may be involved in ubiquitination and subsequent proteasomal degradation of Taf2, providing a potential explanation for the observed increase in Taf2's abundance. Furthermore, several cancer patient samples exhibited mutations at several K residues (Fig. 3.9B and 3.9C). It is possible that at least one of these K residues may be involved

in ubiquitination and proteasomal degradation of the Taf2 protein, suggesting that UPS may be dysregulated in the cancer cell causing overexpression of Taf2 in those cancer cell.

A

K Mutations	Location
E172K	Breast
K603N	Colon
E205K	Colorectal
K1002T	Colorectal
K475R	Endometrial
K720T	Esophagus
R101K	Head & Neck
R343K	Larynx
K565E	Liver
K1157R	Liver
K156Q	Lung
K1001R	Lung
K1002T	Rectal
K1110N	Skin
E1106K	Skin
E770K	Skin
S731K	Skin
K1110N	Uterine
K14N	Uterine
K932N	Uterine
K601Q	Uterine

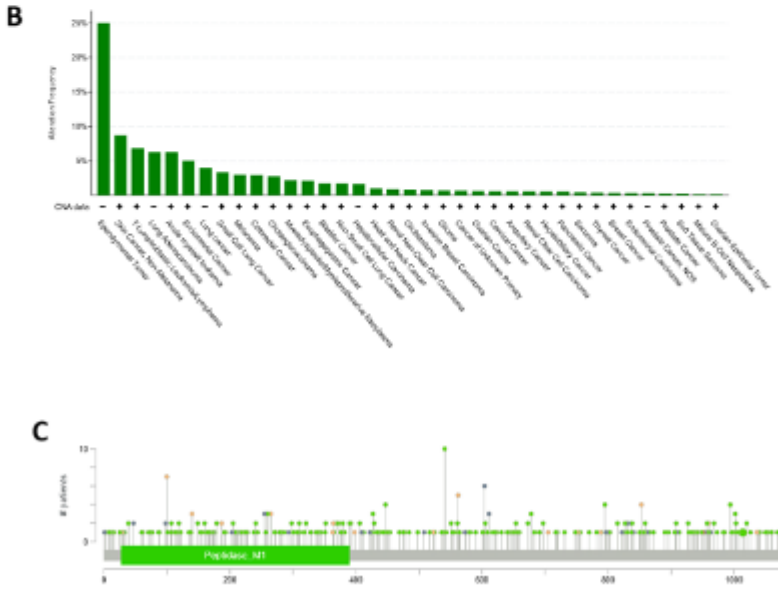

Figure 3.9: Analysis of Taf2 mutations in various cancer patient samples within cBioPortal. (A) K (K) mutation of Taf2 in different cancer patient samples. (B) Cross- cancer analysis of mutations in Taf2 based on the patient samples in the cBioPortal database [217 studies]. The frequencies of 0.1 % and above

were plotted with a minimum sample number of 3. (C) Mutations at different residues/amino acids (aa) of Taf2 in various cancer patient samples.

Unlike GBM and KIRC, in some cancers, Taf2 is upregulated at both mRNA and protein levels (Fig. 3.10A and 3.10B), although protein expression is found to more overexpressed compared to mRNA expression (Fig. 3.10A and 3.10B). However, this overexpression of mRNA/transcriptional upregulation of Taf2 is not mediated via hypomethylation of its promoter DNA due to Taf2 promoter DNA being hypomethylated in normal as well as primary tumor samples (Fig. 3.10C). While Taf2 upregulation is associated with LIHC, its abundance does not significantly impact patient survival probability (Fig. 3.10D).This suggests that DNA methylation, which is closely associated with gene transcription inhibition, may not play an important role in the upregulation of Taf2 at mRNA and protein levels, as transcription and subsequent translation is not inhibited. Rather, this further emphasizes the role of a mismanaged UPS in the upregulation of Taf2 at protein level in LIHC patient samples (Fig. 3.10A and 3.10B).

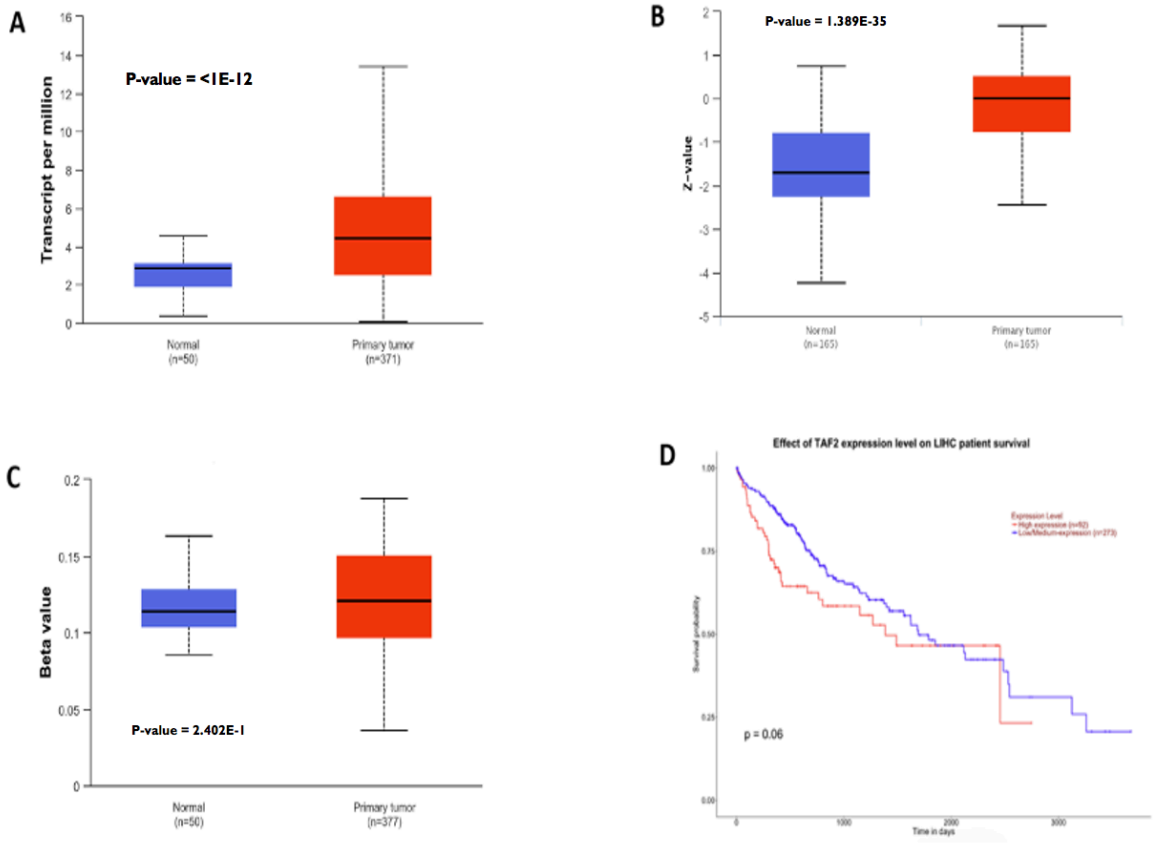

Figure 3.10: Z-test analysis of Taf2 mRNA and protein levels with promoter DNA methylation and survival probability in the liver hepatocellular carcinoma (LIHC) in the UALCAN. (A) Taf2 mRNA levels in LIHC. (B) Taf2 protein levels in LIHC. (C) Analysis of Taf2 promoter DNA methylation in LIHC. Beta value indicates the level of DNA methylation. It ranges from 0 (unmethylated) to 1 (fully methylated). Beta value of 0.7–0.5 represents hypermethylation, while hypomethylation is represented by the beta value between 0.3 and 0.25. (D) LIHC patient survival probability with high expression of Taf2.

Chapter 4: Discussion

4.1 Discussion

While previous work has identified the overexpression of Taf2 in various cancers (Reghupaty, 2017) (Ribeiro et al., 2014), we aspired to provide an explanation for this upregulation by investigating the role of UPS within the upregulation of Taf2. We hypothesized

that Taf2 turnover may be important to maintain optimal levels of Taf2 for normal cellular function as this protein turnover can be regulated by targeted degradation by the 26S proteasome via ubiquitination or non-targeted degradation by proteases. Through our work, we determined that Taf2 is polyubiquitinated and targeted for degradation by the 26S proteasome. Through the ubiquitination assay, we present that precipitated TAP-tagged Taf2 containing pUB221 plasmid expressing hexahistidine-tagged ubiquitin was pulled down through its covalent interaction with Ni^{2+}- NTA agarose beads. From this, we observed a smear surpassing the expected 183 kDa (Taf2 + TAP tag) above 183 kDa demonstrating that Taf2 undergoes polyubiquitination as polyubiquitin adds around 100 kDa and is covalently attached to Taf2-TAP (Fig. 3.1). Furthermore, if a protein is polyubiquitinated, this polyubiquitylated protein is likely to be regulated by 26S Proteasome. To evaluate the regulation of Taf2-TAP by 26S proteasome, we evaluated the stability and abundance of Taf2 in the presence and absence of the proteasome inhibitor, MG132. In MG132 treated yeast cells expressing TAP-tagged Taf2, we present enhanced stability and abundance through the pharmacological inhibition of 26S proteasome's proteolytic activity demonstrating that polyubiquitylated Taf2-TAP undergoes degradation by the 26S proteasome (Fig. 3.6).

Taf2 has been identified as a potential oncogene due to its upregulation in various cancers (Reghupaty, 2017) (Ribeiro et al., 2014). As oncogenes participate in cell growth, proliferation, and survival, their overexpression can lead to uncontrolled cell growth, a hallmark of cancer (Lockwood et al., 2008). As examined in section 3.4, we observed Taf2 upregulation via gene amplification in many cancers (Fig. 3.7A), which supports its standing as an oncogene (Cerami et al., 2012) (de Brujin et al., 2023) (Gao et al., 2013). Significantly, gene amplification is often

associated with increased protein levels. As Taf2 undergoes gene amplification, we would anticipate Taf2 protein expression to also increase.

Increased levels of protein are commonly associated with cancer as opposed to increased levels of mRNA. From our bioinformatic analyses, we determined that Taf2 is upregulated at the protein level in various cancers (Fig. 3.7C, 3.8B, 3.8D, and 3.10B), which supports the regulation of Taf2 by UPS as this pathway regulates proteins, instead of mRNA. This was also observed in Paf1, our positive control throughout this study that has been found to be regulated by the UPS (Barman et al., 2024) (Ferdoush et al., 2017). In Figure 3.7C, we observed greater expression of Taf2 at the protein level in breast cancer, lung cancer, pancreatic adenocarcinoma, head and neck cancer, and glioblastoma, which strongly indicates mismanaged regulation by UPS. The two most prominent cancers through which Taf2 is upregulated at the protein level is GBM and KIRC (Fig. 3.8B and 3.8D). In both cancer patient samples, we observed increased protein expression (Fig. 3.8B and 3.8D), however we observed their decreased mRNA expression (Fig. 3.8A and 3.8C). Notably, this finding confirms that Taf2 upregulation occurs at protein levels, rather than mRNA levels. These findings may be explained by a deficient proteasomal degradation pathway, i.e. 26S proteasomal degradation by UPS.

As previously discussed in section 1.2., ubiquitin molecules have been found to possess seven K residues, but K29 and K48 are the main residues through which polyubiquitin chains provide most potent signals for 26S proteasome mediated degradation (Park et al., 2020). As K residues are closely associated with the UPS, we investigated K residue mutations within Taf2, which was observed in various cancers through our bioinformatic analyses (Fig. 3.9A). Significantly, mutations within K residues can disrupt polyubiquitination and subsequently protein degradation. As a result, target proteins will not be marked for degradation by the 26S

proteasome, leading to that protein to aggregate within cells. This unregulated aggregation can intervene in normal cellular processes, which may contribute to the development of cancerous cells (Park et al., 2020). As supported by our bioinformatic analyses, this may be the case for the mutated K residues of Taf2 which might lead to mismanaged UPS and subsequent overexpression of Taf2 in cancer cells. Future research is necessary to confirm which K residue is involved in polyubiquitination of Taf2 as this could provide better molecular insight into the upregulation of Taf2 in cancer cells.

Overall, our wet lab results support that the UPS regulates the stability of Taf2. Our bioinformatics data showed that Taf2 upregulation occurs at protein levels, rather than mRNA levels. Thus, these findings may indicate a dysregulated UPS regulation Taf2 in cancer cells which could lead to overexpression of Taf2 in those cancer cells. Taken together, these findings suggest Taf2 protein as a particular target of interest for the development of therapeutic interventions against cancer patients.

4.2 Future Work

Here, we report for the first time that Taf2 undergoes polyubiquitination and is subsequently degraded by the 26S proteasome, and thus regulated by the UPS. The next phase of the work will include performing similar experiments in human cancer cell lines to confirm that this regulation is conserved from yeast to humans. Moreover, Future research is needed to identify and characterize the E1, E2 and E3 enzymes involved in the ubiquitination of Taf2 along with ubiquitylation sites towards offering insights into the UPS regulation of Taf2 in regulating gene expression. This would help developing future potential therapeutics against cancers.

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