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Understanding Taf13 (TATA box-binding protein-associated factor 13) upregulation in eukaryotic cells

Selin Kaplanoglu
University of Tennessee at Chattanooga, bnh413@mocs.utc.edu

Understanding Taf13 (TATA box-binding protein-associated factor 13) Upregulation in Eukaryotic Cells

Selin S. Kaplanoglu

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Department of Biology, Geology, and Environmental Science

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Dr. Jannatul Ferdoush

Assistant Professor of Biology

Thesis Director

Dr. Henry G. Spratt

Professor of Biology

Department Examiner

Dr. Jose F. Barbosa

Professor of Biology

Department Examiner

Abstract

TATA-binding protein (TBP) and TBP-associated factors (Tafs) comprise RNA Polymerase II (RNA Pol II) pre-initiation complex. This universal component carefully controls the transcriptional initiation process. One of the Tafs, Taf13, also plays an important role in the regulation of RNA Pol II transcription initiation which is evolutionarily conserved from yeast to humans. It is found that Taf13 is overexpressed in cancer cells, although the exact mechanism that is responsible for this overexpression is unclear. Our hypothesis suggests that targeted degradation by the 26S proteasome via ubiquitylation [Ubiquitin-Proteasome System (UPS)] may be the mechanism that regulates the stability of Taf13. To test this possibility, we evaluated the role of UPS on the stability of Taf13 in yeast (*Saccharomyces cerevisiae*). Importantly for the first time, we found that Taf13 undergoes polyubiquitylation but it is not regulated by the 26S proteasome. These findings suggest further oncologic research topics for the development of therapeutic interventions for future patients of cancer.

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Chapter 1: Introduction

The biological process of RNA polymerase II (RNA Pol II) gene expression is highly coordinated and controls the information drift from DNA to RNA to protein in eukaryotic cells. Transcription Factor IID (TFIID) is one of the universal components required for RNA Pol II to accurately and carefully control the transcriptional initiation process. TATA-binding protein (TBP) and TBP-associated factors (Tafs) make up TFIID. TFIID is instrumental in forming the pre-initiation complex (PIC) required for the initiation of transcription. One of the Tafs, Taf13, is involved in the initiation of RNA Pol II transcription and is evolutionarily conserved from yeast to humans (Tora, 2002). Various cellular diseases are linked to the dysregulation of these transcriptional initiation factors. It is found that Taf13 is overexpressed in cancer cells, including thyroid carcinoma cells (Zhang, 2022). However, the exact mechanism underlying Taf13's upregulation in cancer cells is unclear. The importance of maintaining the optimal level of Taf13 in the cell, which is necessary for healthy cellular activity, is the main question in our research. Targeted degradation by the 26S proteasome via ubiquitination or non-targeted degradation by proteases can both control this protein turnover. The 26S proteasome, a piece of non-lysosomal proteolytic machinery found in eukaryotes (Voges et al., 1999) (Coux, 2002), regulates several cellular processes including gene regulation, transcription, and DNA repair, cell cycle regulation, chemotaxis, angiogenesis, and apoptosis (Frankland-Searby, & Bhaumik, 2012).

We hypothesize that the stability of Taf13 may be regulated by the targeted degradation of Taf13 by the 26S proteasome via polyubiquitylation [Ubiquitin-Proteasome System (UPS)]. In the end, we found for the first time that Taf13 undergoes polyubiquitylation but is not targeted for degradation by the 26S proteasome.

1.1. Transcription in Eukaryotic Cells

The central dogma of molecular biology serves as a fundamental framework for understanding how genetic information flows within biological systems, particularly within eukaryotic cells. It outlines a sequential process wherein genetic information moves from DNA to RNA to proteins. In eukaryotes, transcription is a pivotal step where genetic information encoded in DNA is transcribed into various RNA molecules, primarily messenger RNA (mRNA), which act as templates for protein synthesis. This transcription process is regulated by RNA Pol II, which synthesizes not only mRNA but also other non-coding RNAs such as long non-coding RNAs, microRNAs, and small nuclear RNAs. Meanwhile, transfer RNAs and other short RNA molecules are synthesized by RNA Pol III (Girbig et al., 2022). RNA Pol II's gene expression is carefully coordinated to regulate the information transaction from DNA, to RNA, and then to protein. Transcription by the three polymerases can be divided into three steps: transcription initiation, transcription elongation, and transcription termination. Several general transcription factors (GTFs), including TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH, and RNA Pol II holoenzyme assemble at the promoter during transcriptional initiation, leading to the creation of PIC, which initiates transcription (Bhaumik et al. 2011). The regulation of gene expression at the transcriptional initiation level is crucial in maintaining cellular function, and its dysregulation is associated with numerous human diseases. That is why it is important to understand the regulatory mechanisms of transcription initiation. According to Conaway et al. (2002), there is evidence that ubiquitin regulates transcription through proteasome-dependent transcription factor degradation or proteasome-independent pathways. Here, we focus on the particular ubiquitin-proteasome-dependent degradation system by the 26S proteasome.

1.2. The Ubiquitination Proteasome System (UPS)

In eukaryotes, the UPS is the main proteolytic system that controls protein degradation to maintain protein homeostasis. Moreover, it regulates many cellular processes including transcription. In UPS, the process of substrate protein degradation initiates with ubiquitination of the substrate protein. Ubiquitination of the substrate protein depends on the usage of certain enzymes, which help the tagging of the substrate protein with multiple ubiquitin molecules. This polyubiquitylated substrate protein is then targeted for degradation by the 26S proteasome complex (Park et al., 2020) (Mata-Cantero et al., 2015).

Briefly, in eukaryotes, the process of ubiquitination initiates through a cascade of three enzymatic reactions with enzymes (E) E1, E2, and E3 that help to connect poly-ubiquitination chains to the protein substrate. E1 is also called the Ubiquitin-activation enzyme, it is responsible for the ATP-dependent thiol-ester bond synthesis in the ubiquitination pathway between its cysteine residue and ubiquitin's C-terminal glycine residue. Next, the trans-thioesterification step, which is catalyzed by the E2 ubiquitin-conjugase enzyme, transfers the activated ubiquitin E1 to the active cysteine site of E2. (Weissman, 2011). Then, by attaching to both the substrate and E2 conjugase, the E3 ubiquitin-ligase enzyme facilitates the transfer of ubiquitin from E2 to the designated substrate by accelerating the formation of an isopeptide bond between the C-terminal glycine of the substrate protein and a lysine residue of the ubiquitin (Davis & Gack, 2015). Ubiquitin has seven lysine (K) residues and an N-terminus that serves as points of ubiquitination. These K residues that make up ubiquitin can construct polyubiquitin chains. Among these seven K residues (K6, K11, K27, K29, K33, K48, and K63), the primary signal for degradation is a chain of at least four K48-linked polyubiquitins. The other linear ubiquitin chains instead are involved in a variety of processes, including signal transduction and other regulatory functions (Ferdoush

et al., 2024). Following the K48-linked polyubiquitination, the 26S proteasome complex recognizes and breaks down the polyubiquitylated substrate protein. Numerous illnesses, including cancer, have been linked to UPS dysregulation. A variety of cancers usually contain mutations or irregular expressions of UPS components. (Park et al.,2020)

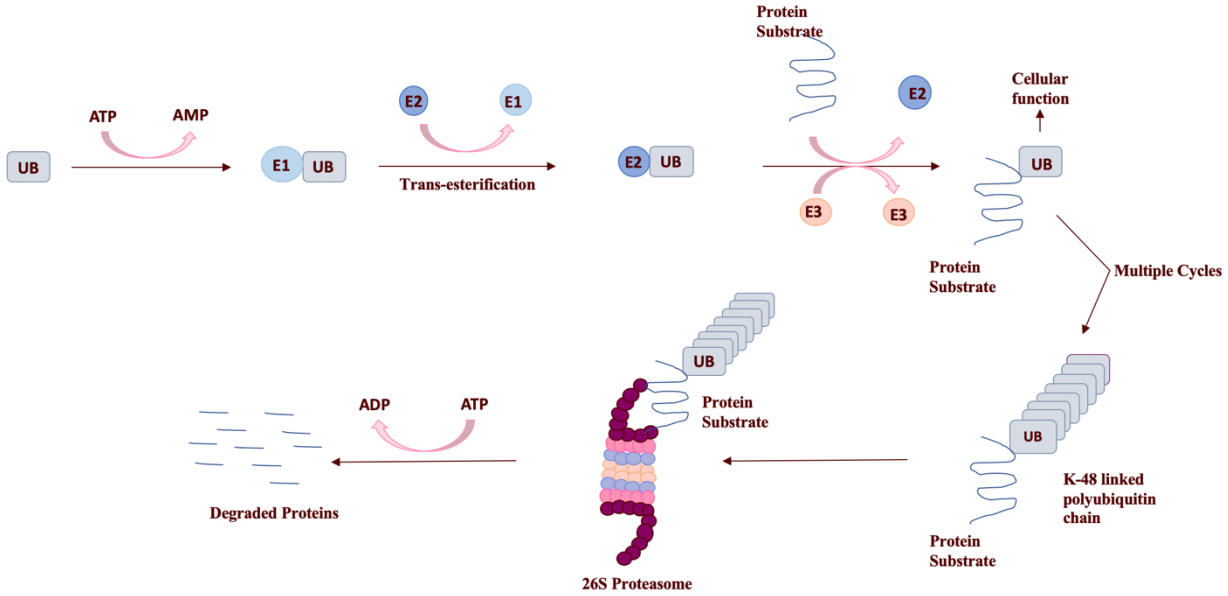


Figure 1.1. The ubiquitination pathway and its role in the regulation of normal cellular functions. In the ubiquitination pathway, E1 catalyzes a thiol-ester bond formation between its cysteine residue and the C-terminal glycine residue of ubiquitin in an ATP-dependent fashion. Then, the activated ubiquitin E1 is transferred to the active cysteine site of E2 by a trans-thio esterification reaction, catalyzed by E2 conjugase. Subsequently, 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. Ubiquitin has seven K residues, all of which can form polyubiquitin chains. A chain of at least four K48-linked polyubiquitin is the major signal for degradation, whereas other linear ubiquitin chains contribute to diverse processes such as signal transduction and other regulatory processes. After polyubiquitination, the polyubiquitylated substrate protein is documented and degraded by the 26S proteasome complex. (Ferdoush et al., 2024)

1.3. Taf13 and its Association with Cancers

TFIID can interact with activators and repressors that are particular to a gene and is involved in identifying nearby chromatin marks and core promoter regions. Protein-coding genes begin to transcribe when TFIID identifies key promoter sites on DNA and PIC consisting of Pol II, TFIIH, and mediator complexes is subsequently assembled. TFIID is a large assembly

consisting of 14 distinct Tafs and TBP (Patel et al., 2020). Among those Tafs, recruiting TBP and each other requires Taf11 and Taf13. Taf11 and Taf13 may offer the majority of functional connections with TBP during activator-mediated recruitment, according to research (Shen et al., 2003) Together with Taf11, Taf13 forms a heterodimer that resembles a histone structure, and this heterodimer is essential for recruiting into the general TFIID protein complex of RNA Pol II. (Gupta, 2017). Taf13's evolutionary conservation for the transcription pathway suggests that its misregulation might lead to cellular pathologies, given that genetic changes that inevitably result in dysregulated transcriptional processes are the root cause of cancer (Bradner & Young, 2017).

Importantly, Taf13 is found to be overexpressed in cancers including Head and Neck cancer (https://www.cbioportal.org/; (Cerami et al., 2012; de Bruijn et al., 2023; Gao et al., 2013), http://ualcan.path.uab.edu/; (Chandrashekar et al., 2017; Chandrashekar et al., 2022)) and thyroid cancer (Zhang et al., 2022). It was found that the inhibition of Taf13 by introducing Taf13-specific small interfering RNA (siRNA) via transfection significantly slowed down the growth and proliferation of thyroid carcinoma (TC) cells (Zhang et al., 2022). Understanding the basis of overexpression Taf13 in these cancers could potentially help develop therapeutics in the future.

1.4. Summary of Research

Our work aimed to address two sub-aims to determine the role of the UPS in controlling Taf13 expression in *Saccharomyces cerevisiae*. First, we wanted to know if the ubiquitination mechanism regulates the stability of Taf13. We carried out a Ni^{2+}-NTA-based ubiquitination experiment to achieve this. This entailed transforming and cloning of a pUB221 plasmid [plasmid with hexa-histidine tagged ubiquitin and controlled by the CUP1 promoter (activated with Cu^{2+} present; Pickart, 2000)] into the Taf13 yeast strain that had been tagged with Tandem Affinity Purification (TAP). This tampered strain was used as the basis for the ubiquitination experiment,

which allowed the presence of ubiquitinated Taf13 to be determined. We did the ubiquitination assay followed by the western blot (WB) analysis to see if hexahistidine-tagged ubiquitin had bound to proteins from whole cell extract (WCE). To do this, we applied an anti-TAP antibody to the precipitate and then employed it in the WB test against the TAP-tagged Taf13. An alternative yeast strain that lacked the pUB221 ubiquitin plasmid was used as a control; with this strain, no precipitate was expected. The results of these tests shed light on whether poly-ubiquitination of Taf13 occurred, which would indicate if the UPS had a role in controlling the turnover of this Taf13 protein.

We then pursued our other sub-aim, which was to determine if the 26S proteasome was involved in the degradation of Taf13. To study how the 26S proteasome controls Taf13 stability and abundance for effective transcription and other cellular processes, we assessed Taf13 stability in the presence and absence of MG132, a peptide aldehyde that is known to be a 26S proteasome inhibitor. MG132 was predicted to increase Taf13's stability by preventing the 26S proteasome from performing its proteolytic function, assuming poly-ubiquitylated Taf13 was the target of 26S proteasomal degradation. Because *PDR5* could interfere with MG132's activity, it was essential to knock out or delete the *PDR5* multidrug resistance gene from the yeast strain expressing TAP-tagged Taf13 before doing any experiments.

We sought to determine if the 26S proteasome regulated Taf13 abundance by monitoring the effects of MG132 on Taf13 stability and using DMSO as a control for the MG132 solution, which should not affect Taf13’s stability owing to its pharmacological features. As a loading control, actin levels were monitored as it was not impacted by the proteasome. If Taf13 levels had increased after MG132 treatment, it would have indicated that Taf13’s quantity and stability were regulated by the 26S proteasome. It was therefore expected that MG132, which pharmacologically

inhibits the 26S proteasome's proteolytic activity, would increase Taf13 stability and abundance, confirming its regulatory function over Taf13.

We have also conducted bioinformatics research to support our findings. In oncology, bioinformatics research is essential for comprehending the complex processes underlying carcinogenesis and developing potential therapeutic interventions.

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 extracted 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 yeast strain BY4741 bearing TAP-tagged Taf13.

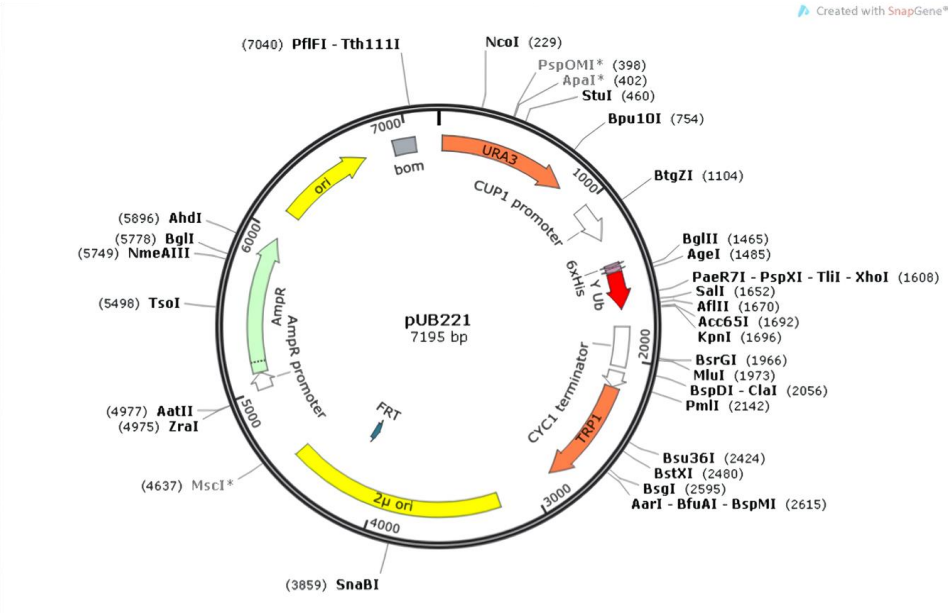


Figure 2.1.1. pUB221 plasmid containing hexahistidine tagged ubiquitin (under the CUP1 promoter) and URA3 marker

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 the plasmid miniprep protocol, we extracted these two plasmids from the XL1B strains.

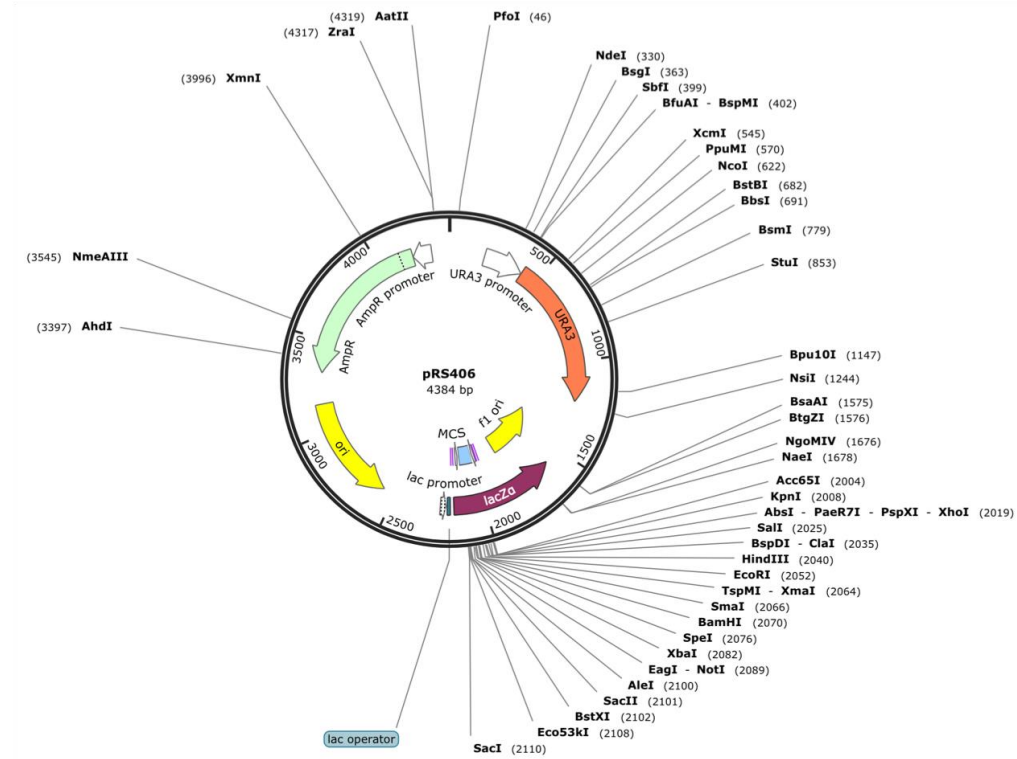


Figure 2.1.2. pRS406 plasmid

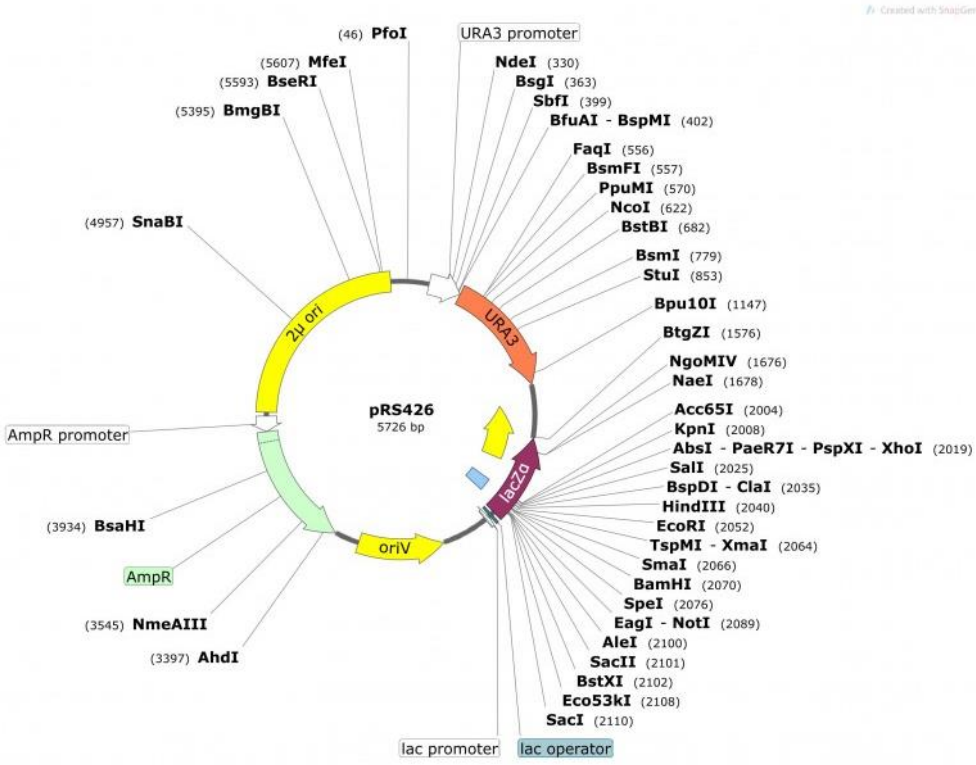


Figure 2.1.3. pRS426 plasmid

2.2 Strains

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

Type	Name	Genotype/Description	Source
Bacterial	JSMB01	pRS426 in XL1B strain *(URA3* marker) (Grown in LB + tet^r (*KAN*) (*AMP*))	In lab
Bacterial	JSMB05a	pRS406 in XL1B strain (*URA3* marker) (Grown in LB + tet^r (*KAN*) (*AMP*))	In lab
Bacterial	JSMB05b	pRS406 in XL1B strain (*URA3* marker) (Grown in LB + tet^r (*KAN*) (*AMP*))	In lab
Yeast	SKY01	pUB221 plasmid introduced into yeast strain containing TAP-tagged Taf13 (*URA3* marker) (Grown in YNB-Ura/dex)	In lab
Yeast	JFY01	pUB221 plasmid introduced into yeast strain containing TAP-tagged Paf1(*URA3* marker)	In lab

		(Grown in YNB-Ura/dex)	
Yeast	JSM03a	Δ*pdr5* in yeast strain containing TAP-tagged Taf13 (plasmid pRS406) (*URA3* marker) (Grown in YNB-Ura/dex)	In lab
Yeast	JSM04a	Δ*pdr5* introduced into yeast strain containing TAP-tagged Paf1 (plasmid pRS406) (*URA3* marker) (Grown in YNB-Ura/dex)	In lab

2.3. Growth Media

To amplify plasmids pRS406 and pRS426, first both plasmids were expressed in the XL1B strain. Next, using the plasmid miniprep protocol, these two plasmids were extracted from the XL1B strains. To grow the XLIB strain, Luria broth+ ampicillin (LB +amp) was prepared by adding 100µL of 100 mg/mL ampicillin to 100 mL of LB. Ampicillin is commonly used for selection against the cells that do not contain the desired plasmid as it is the antibiotic of selection.

For transformational cloning of plasmids into yeast strains bearing TAP-tagged Paf1 and TAP-tagged Taf13 was completed, the yeast cell was able to be selected by the Uracil (*URA*) marker in the plasmids used (All plasmids used in this experiment contain *URA3* marker). The growth media used to select transformed yeast cells was Yeast Nitrogen Base - uracil/dextrose (YNB-Ura/dex). All other inoculations for our genes of interest were done with Yeast extract–peptone–dextrose (YPD) Medium.

2.4. Plasmid extraction via Qia-Miniprep

All three plasmids (pUB221, pRS406, pRS426) were first expressed in bacteria cells. Then the plasmids were extracted from the bacteria using the QIAprep 2.0 Spin Miniprep Kit obtained from QiaGen. A single colony of bacteria was grown overnight in LB+amp at 37°C. After approximately 12 hours, the bacterial cells were centrifuged at 8,000 xg for 3 minutes, and the supernatant was discarded. The cell pellet was then resuspended in 250 µL of resuspension buffer

P1, followed by the addition of 250 µL of lysis buffer P2 to initiate cell lysis. After brief mixing, 350 µL of neutralization buffer N3 was added, and the mixture was centrifuged at 13,000 xg for 10 minutes to separate cellular debris and chromosomal DNA. The supernatant, containing the plasmid, was transferred to special Qiaprep 2.0 Spin Column tubes and centrifuged again. The column was washed with washing buffers PB and PE to remove proteins and salts, respectively. Finally, the plasmid DNA was eluted from the column using elution buffer EB and stored at -20°C.

2.5. Ubiquitination assay

2.5.1. Part 1: Transformation of pUB221 plasmid into the desired yeast strains

After isolating the pUB221 plasmid through miniprep, it was then introduced into the TAP-tagged cells of interest. Both TAP-tagged Taf13 and Paf1 were acquired from the Yeast TAP Tagged ORFs Collection by Horizon (Cat No: YSC1177) with the background strain of *MatA* (BY4741). It is important to keep the environment and materials sterile during this process, as the cells are still alive and growing. This also means mixing the solutions by finger-tapping or spinning at very low xg. In a controlled environment, TAP-tagged Taf13 and Paf1 were inoculated into YPD liquid media to grow overnight in the shaker incubator at 29°C. After approximately 12 hours, when the optical density (OD) of both yeast cultures at 600 nm wavelength (OD_{600}) reached around 1.00, the cell pellet was collected to be washed with 1 mL 1X cold tris-buffered saline (TBS). TBS is isotonic and non-toxic to cells; therefore, it is used in many protocols as a washing buffer to help the cell remain at a constant pH. After transferring the washed solution to a new microcentrifuge tube, in the meantime, the polyethylene glycol (PEG) solution was prepared. PEG solution was then added to the cells of interest, and mixed. Following this, the PEG and cell mixture were combined with the foreign pUB221 plasmid DNA. Preparation of the Herring Sperm DNA, the carrier DNA, was done by boiling it at 95°C for 5 minutes. After adding the Herring

Sperm DNA, the mixture was mixed carefully and placed in the rotor to be incubated at 30℃ for 30 minutes for growth. Following the first incubation period, dimethyl sulfoxide (DMSO) was added and mixed. The microcentrifuge tube was then placed in a water bath to undergo heat shock at 42℃ for 15 minutes. This heat shock marks the entry of DNA into cells. After heat shock, the cells were spun at 2,000 xg for 10 seconds at room temperature. The supernatant was removed, and the cell pellet was resuspended in sterile YPD liquid media. After resuspension, both tubes were placed in the rotor to be incubated at 30℃ for 1.5 hours. At room temperature, the cells were spun at 4,000 xg for 2 minutes after the incubation, and the supernatant, consisting of YPD liquid media, was discarded. The cell pellets were then dissolved in 1X TE buffer. In maintained sterile conditions, the cells were poured onto the selective YNB-Ura/dex plates, and sterile glass beads were used to spread the cells evenly throughout the media. The plate was placed in the 30℃ incubator to grow for the next 24 to 48 hours.

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

The ubiquitination assay was performed to analyze the ubiquitination status of both TAP-tagged Paf1 and TAP-tagged Taf13. Yeast strains JFY01 and SKY01 harboring pUB221 plasmid were inoculated to OD_{600} of 0.700 in liquid YPD media. Copper sulfate ($CuSO_4$) at a final concentration of 250 mM was added to plasmid pUB221 to promote the production of hexahistidine-tagged ubiquitin. Collected cells were suspended in buffer A (6 M guanidine-HCl, 100 mM Na_2HPO_4/NaH_2PO_4 at pH 8.0, 10 mM imidazole) and lysed by glass beads by vortexing it in the highest speed. In the meantime, Ni^{2+}-NTA beads were washed equilibrated with buffer A after centrifuging it for 4 minutes at 3000xg to discard the old supernatant. Buffer A and the beads were mixed by putting them in the rotor for 10 minutes. This establishes a constant environment for the Ni^{2+}-NTA beads to express the hexahistidine tag when added to the lysate.

After the vortex of the cell, glass beads and buffer A, the lysate was collected by poking a hole at the bottom of the microcentrifuge tube using a needle. The lysate was added to the Ni^{2+}-NTA equilibrated beads, and incubated first at 4°C for 1 hour, and then at room temperature for 45 minutes. Following the incubation, the solution was spun at 3000 xg for 5 minutes to pellet them. Subsequently, the pellet was washed three times by buffer A, three times by buffer A/TI (1 volume of buffer A and 3 volumes of buffer TI), and once by buffer TI (25 mM Tris-Cl, 20mM imidazole at pH 6.8). For a fully efficient washing, each wash was done by putting the sample through the rotor for 3 minutes before centrifuging it at 3000 xg for 4 minutes and discarding the supernatant. Finally, hexahistidine-tagged ubiquitin/ubiquitylated proteins were eluted with 2X SDS loading buffer containing 200 mM imidazole, and boiled at 95°C for 5 minutes. The supernatant was collected with a syringe into a new microcentrifuge tube to store at -80°C until gel loading.

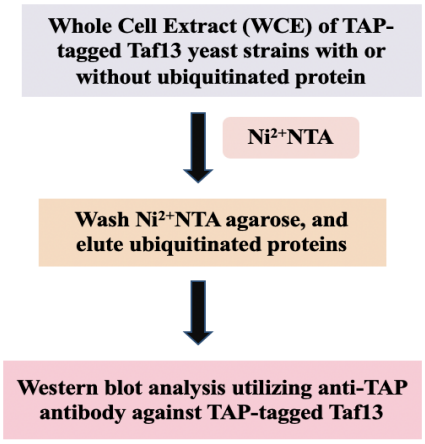


Figure 2.2. Schematic diagram of Ni^{2+}-NTA based Ubiquitin Assay

2.5.3. Part 3: Western Blot (WB) Analysis of ubiquitylated proteins

WB analysis was conducted to assess the protein levels. After the WCEs prepared before were heated with an SDS-gel loading buffer at 95°C for 5 minutes, they were subsequently loaded

onto an SDS-polyacrylamide gel for electrophoresis. Using a dry transfer device, the separated proteins were subsequently transferred onto a polyvinylidene difluoride (PVDF) membrane, a process that took one to two hours. During WB analysis, particular primary antibodies were chosen and used to probe the PVDF membrane to identify anything. To specifically look at the possible ubiquitination of TAP-tagged Taf13 and TAP-tagged Paf1 proteins, an anti-TAP antibody was used. Following antibody incubation, the membrane underwent a series of washing steps with Tris-Buffered Saline, 0.1% Tween 20 Detergent (TBST) buffer to remove the nonspecific binding. Subsequently, the membrane was probed with an anti-rabbit-HRP secondary antibody to enhance the signal. After another round of washing, the membrane was subjected to chemiluminescent reagents for signal development, followed by visualization using a ChemiDoc machine. This facilitated the determination of the ubiquitination status of the TAP-tagged Taf13 and TAP-tagged Paf1 proteins.

2.6. Proteasomal Degradation Assay:

2.6.1. Part 1: PCR-Mediated Disruption of PDR5

To test whether 26S Proteasome regulates the stability of Taf13, we performed an MG132-based proteasomal degradation assay. MG132 inhibits the activity of 26S proteasome, therefore, we could determine the role of the proteasome on the stability of Taf13 in the presence and absence of MG132. However, the *PDR5* multidrug resistance gene is a gene expressed in yeast that inhibits the activity of MG132. Therefore, to see whether MG132 inhibits the activity of 26S proteasome, proving its role in the stability of Taf13, *PDR5* needs to be knocked out. In deleting the gene *PDR5,* a part of pRS406 and pRS426 containing the gene that encodes marker URA was amplified by a pair of primers (*PDR5* del1A and *PDR5* del1B). The 80 base pairs (bp) primers were designed such that the 60 bp from the downstream of either the start (*PDR5* del1A) or stop (*PDR5* del1B)

codon of the *PDR5* ORF were selected to overlap with regions, flanking the gene of interest to be deleted. The remaining 20 additional bp from the universal sequence of the plasmid being amplified:

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

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

The 1st program was run after preparing a Master Mix cocktail for plasmid DNA samples. pRS406 plasmid was used for *PDR5* deletion in yeast strain bearing Taf13-TAP and pRS426 plasmid was used for *PDR5* deletion in yeast strain containing Paf1-TAP. The following program was used for the disruption of *PDR5*:

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

The DNA isolation process from yeast cells transformed with PCR-amplified DNA commenced by pooling the PCR product into designated microcentrifuge tubes. Subsequently, 10% of the total volume of 3 M sodium acetate was added to each tube, followed by centrifugation to separate the aqueous phase. Phenol:chloroform:isoamyl alcohol was then introduced to facilitate DNA extraction, with mechanical vortexing enhancing the process. Following centrifugation, the aqueous phase containing DNA was carefully collected and transferred to new microcentrifuge tubes. Next, the DNA was precipitated by adding 2.5 times the volume of ice-cold 100% ethanol along with glycogen. After incubation in a freezer, centrifugation at maximum speed separated the

precipitated DNA, which was then washed with 70% ethanol to remove residual salts. Subsequent centrifugation and drying steps ensured the DNA was thoroughly purified.

For confirmation of successful transformation, yeast colonies were grown and subjected to further processing. Upon harvest, the cells were lysed using Tris-buffered saline (TBS) and glass beads, allowing for efficient DNA extraction. The resulting supernatants containing DNA were collected, followed by the addition of phenol:chloroform:isoamyl alcohol for further purification. After centrifugation, the DNA-containing aqueous phase was isolated and subjected to ethanol precipitation. Following centrifugation and removal of the supernatant, the DNA pellets were washed with 70% ethanol, dried, and resuspended in TE buffer. The resultant DNA samples were stored at -20℃ for future use.

A 2nd PCR program is performed to analyze whether the 1st PCR program and transformation worked, resulting in cells that have the *PDR5* deletion (Δ*pdr5*) gene. To perform the PCR, two ORF primers (*PDR5* ORF 2A and *PDR5* ORF 2B) were designed in order to initiate *PDR5* expression in the cell. In the case of a positive result, *PDR5* wasn't going to be expressed in the cell. The two ORF primers are as follows:

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'

The 2nd program was run with the template DNA acquired after the transformation. The PCR program used for colony screening is as follows:

94°C – 2 min

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

72°C – 10 min

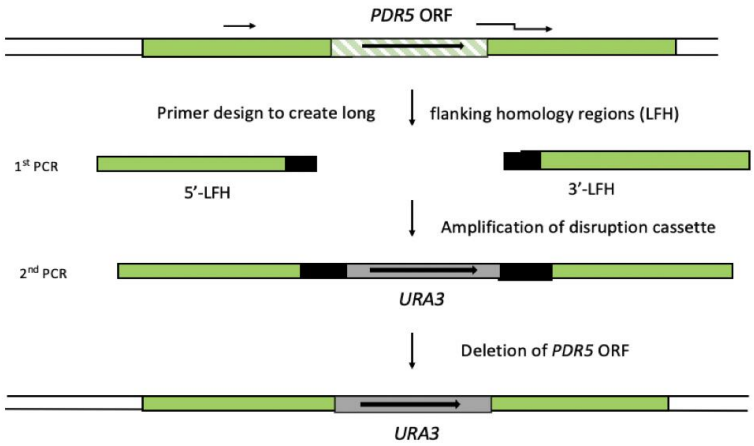


Figure 2.3: Schematic diagram for PCR-mediated disruption of PDR5 in yeast strain expressing TAP-tagged Taf13 and TAP-tagged Paf1

2.6.2. Part 2: Transformant (Positive Colony screening) by Agarose gel Electrophoresis

The preparation of a 1% agarose gel facilitated the visualization of bands under UV light. After the sample was run in the agarose gel, the *PDR5* deletion was verified by observing the gel under UV light and imaging it with the UVP GelSolo machine. A positive result of the Δ*pdr5* procedure indicated the presence of the selectable marker URA instead of the *PDR5* gene. Initially, the PCR product following the 1st program (2.6.1) was assessed, confirming a product size of ~1200 bp. In the 2nd PCR program (2.6.1), the product size of the *PDR5* ORFs were approximately 175 bp, which should not be expressed in the cell. Consequently, the band around ~175bp in the agarose gel would not be visible under successful mutation of *PDR5*. Following screenings, two strains, JSM03a and JSM04a, were found to harbor the Δ*pdr5* gene, validating the success of the mutation.

2.6.3. Part 3: Proteasomal Degradation Assay via MG132 & DMSO Treatment

Strains JSM03a and JSM04a were cultured in YPD media overnight at 30℃ with agitation at 200 xg. When the OD_{600} reached 0.7, the culture was divided into three tubes for further treatment: one tube treated with MG132 (75μM), one with DMSO, and one none. All three falcon tubes were then incubated for 2 hours in a shaker incubator at 30℃ and 200 xg. After incubation, the cells were collected and processed.

For processing, the cell pellets were thawed from -80℃ and transferred to microcentrifuge tubes. To each tube, formic acid lysis buffer (FALB) and protease inhibitors were added. Glass beads were added to aid in cell membrane disruption, and the tubes were vortexed mechanically at 4℃ for 30 minutes. After vortexing, a sterile needle was used to puncture the bottom of each tube, which was then placed into another labeled tube. Centrifugation at 6,000 xg for 10 seconds at 4℃ allowed the liquid to transfer to the new tube, which was subsequently spun at 13,000 xg for 1 minute. The supernatant was collected and transferred to properly labeled microcentrifuge tubes, which were stored at -80℃. If a WB assay was to be conducted, 2X SDS-gel loading buffer was added to the sample, followed by boiling for 5 minutes at 95℃ before loading onto SDS-PAGE gels.

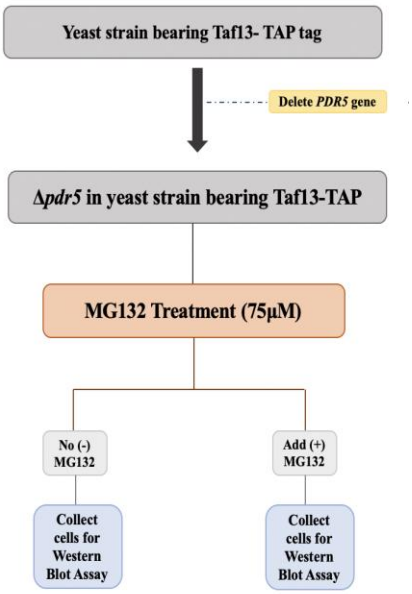


Figure 2.4. Schematic diagram for MG132-based proteasomal degradation assay.

2.6.4. Part 4: WB Analysis of MG132 & DMSO Treated Cells

Similar to the method displayed in 2.5.3 was used to run 10% SDS-PAGE gel. While developing the PVDF membrane, two different antibodies were used as the primary antibody. One of them was the Anti-TAP Antibody that bound onto the TAP tag on the strains, while the other antibody was the Anti-Actin Antibody produced in rabbits. Actin (*ACT1)* is a housekeeping gene that is a key component of the cytoskeleton of yeast. Actin levels don't change in the presence of MG132. In this experiment, the use of the Anti-Actin antibody provided loading control over yeast strains as Actin is constitutively expressed.

2.7. Bioinformatics Analysis

To analyze Taf13 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 Taf13 analysis in various cancers with a minimum sample number

of 1 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 Taf13 protein and mRNA levels in various cancers. Similarly, Taf13 mutations in various cancer patient samples were identified through the cBioPortal database (with 10967 samples/10,953 patients) with a minimum sample number of 1 and frequency of 0.1%. The DNA methylation of the Taf13 promoter DNA and Thyroid carcinoma patient survival probability was analyzed using the UALCAN portal. Based on these results, box and whisker plots were obtained.

Chapter 3: Results

3.1. Overview

Through two sub-aims, our main goal was to determine how the UPS controls the expression of Taf13 in yeast. First, we used a Ni^{2+}-NTA-based ubiquitination test to see if Taf13 underwent ubiquitination. In order to identify polyubiquitinated Taf13, a hexahistidine-tagged ubiquitin plasmid was transformed into a TAP-tagged Taf13 yeast strain. This was then evaluated using WB. Furthermore, by comparing the abundance of 26S proteasome in the presence and absence of the proteasome inhibitor MG132, we sought to determine if proteasome influences Taf13's stability. However, the *PDR5* multidrug resistance gene expressed in yeast inhibits the activity of MG132. Therefore, to see whether MG132 inhibits the activity of 26S proteasome, therefore proving its role in the stability of Taf13, the *PDR5* gene was knocked out. The overall goal of these investigations was to clarify UPS's function in Taf13 regulation, offering insights into molecular mechanisms essential for normal cellular function. Importantly, our results indicate that Taf13 is polyubiquitylated; however, it is not regulated by the 26S proteasome.

3.2. Taf13 Undergoes Polyubiquitination

Figure 3.1 shows the WB analysis results of the ubiquitination analysis of Taf13. Using an anti-TAP antibody against TAP-tagged Taf13, the precipitate was subjected to a WB experiment to detect the presence of Taf13. Taf13 has been shown to undergo ubiquitination. This is because we see the smear of Taf13 much above its molecular weight (19kDa + 21 kDa TAP-tag), which is the molecular weight of the ubiquitin proteins added together. Here, Paf1 is used as positive control which is already known to be regulated by UPS (Ferdoush et al., 2017).

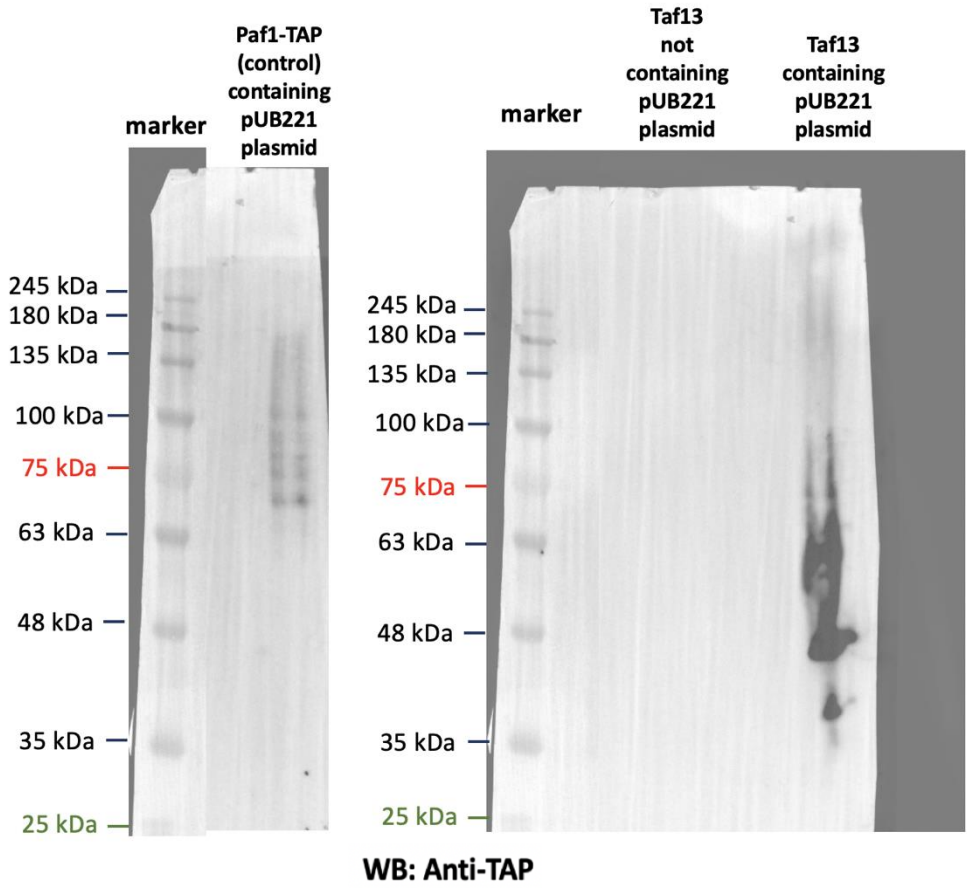


Figure. 3.1. Taf13 undergoes polyubiquitination. WB analysis results of ubiquitination analysis of Taf13 (left-to-right) with positive control (TAP-tagged Paf1) (Ferdoush et al.2017), negative control (Taf13 TAP tag without His-ubiquitin plasmid), polyubiquitylated TAP-tagged Taf13.

3.3. PCR-mediated Disruption of *PDR5:* PCR Product and Transformant (Positive Colony Screening)

Figure 3.2 shows the visualization of the product size of the 1st PCR program. The PCR product contains a selectable marker gene (*URA*) flanking the 60 bp start codon and 60 bp stop codon of *PDR5* ORF. The size is around 1200 bp.

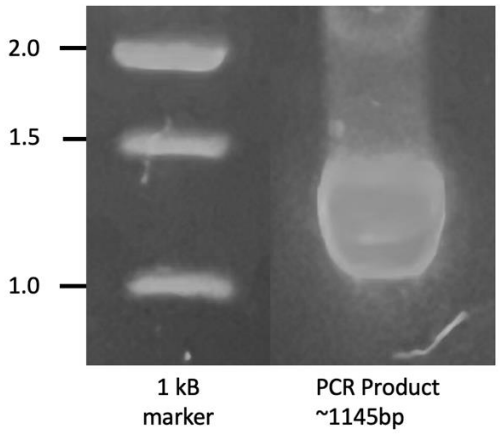


Figure 3.2. Size of PCR Product

Figure 3.3 shows the results of agarose gel electrophoresis after the 2nd PCR program. The two designed ORFs have a size of about 175 bp. This means that in the case of successful *PDR5* deletion, these ORFs wouldn't be expressed. This would lead to the lack of the band around 175 bp. In both A and B, a result that contains the 175bp band is shown as a control.

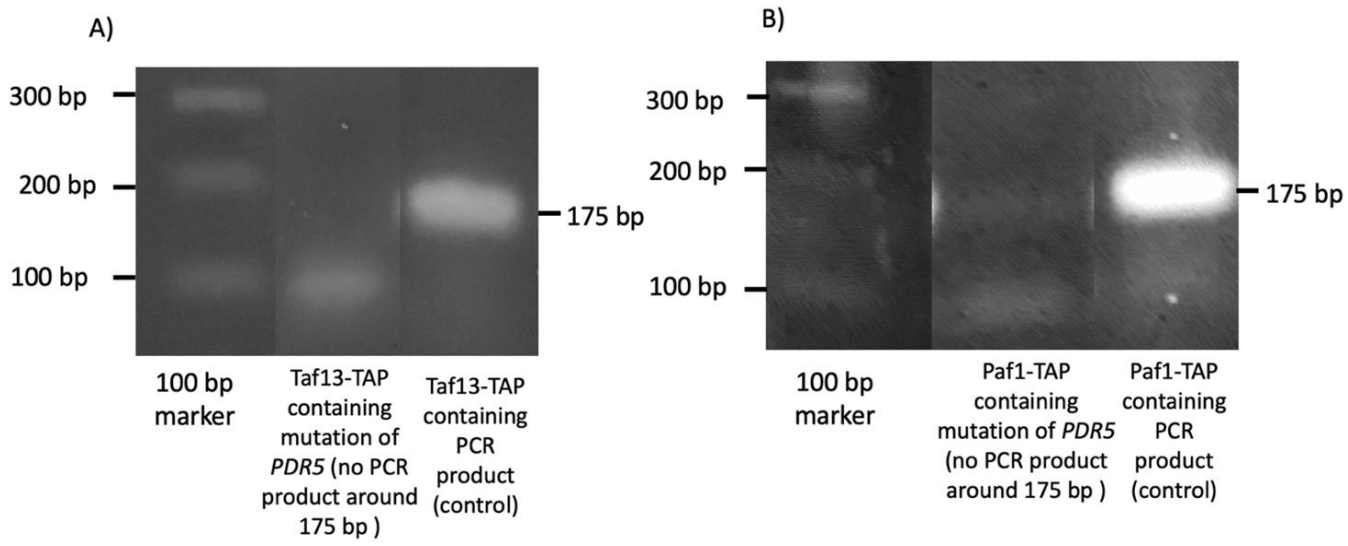


Figure. 3.3. Agarose gel showing deletion of PDR5 into the yeast strain bearing TAP-tagged Taf13 and TAP-tagged Paf1 A) Yeast strain bearing Taf13-TAP containing mutation of PDR5 (no PCR product around 175 bp) B) Yeast strain bearing Paf1-TAP containing mutation of PDR5 (no PCR product around 175 bp)

3.4. Taf13 is not Degraded by 26S Proteasome

Figure 3.4. shows the WB analysis results of MG132 & DMSO treatment of TAP-tagged Taf13. Here, Paf1 is used as a positive control as it is already known to be degraded by the 26S proteasome (Ferdoush et al., 2017). The results show that Taf13 is not degraded by 26S proteasome. This is because the smear depicting MG132-treated cells should be more saturated than their negative counterparts, which would suggest that MG132 has inhibited the degradation process led by the 26S proteasome. In this case, the saturation didn't have much change. These results indicate that polyubiquitylated Taf13 is not targeted for degradation by the 26S Proteasome.

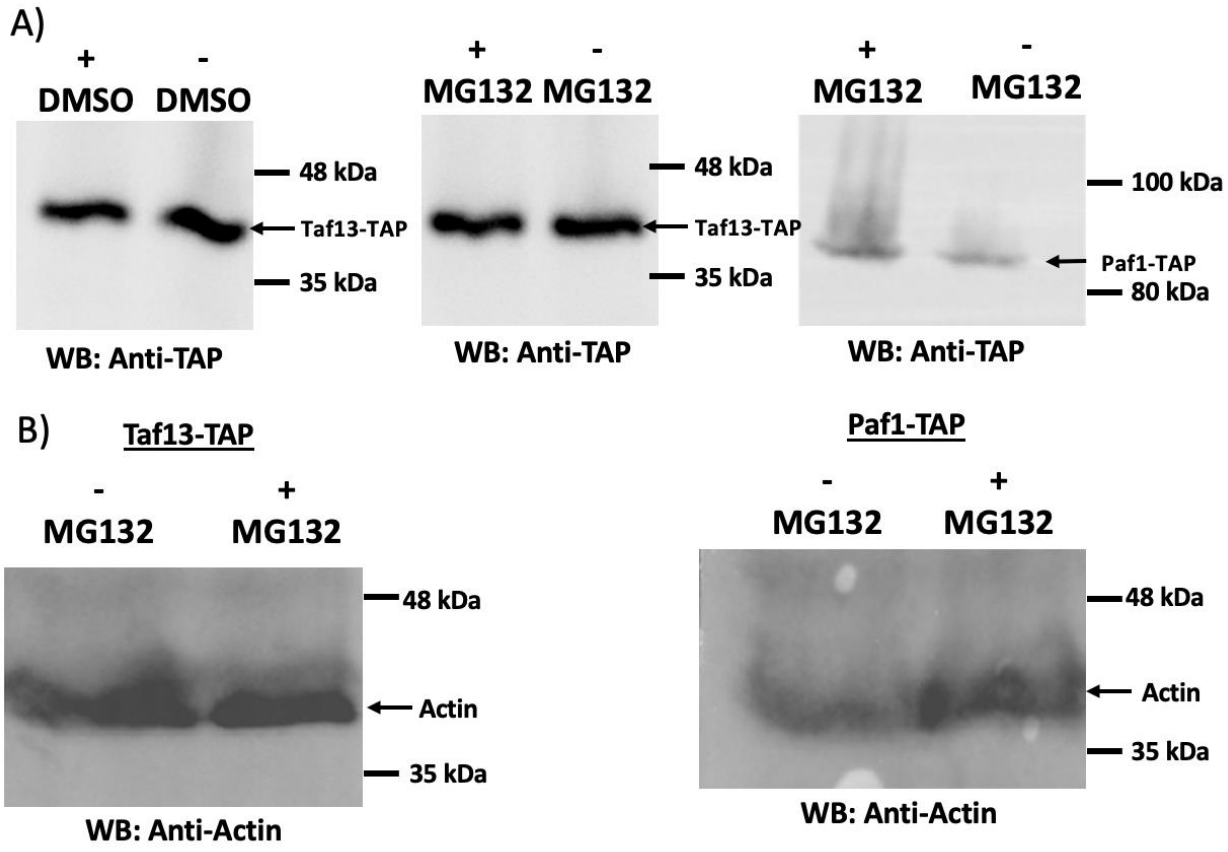


Figure. 3.4. Poly-ubiquitylated-Taf13 is not targeted for degradation by the 26S proteasome. (A) WB analysis of the abundance of Taf13 (with a null mutation of PDR5) in the presence and absence of DMSO or MG132. (-), absence of DMSO or MG132; (+), presence of DMSO or MG132. Paf1 with a null mutation of PDR5 was used as a control (Ferdoush et. al., 2017) (B) WB analysis of Actin as a loading control of the experiment

3.5. Bioinformatics Data Showing Cancer Cells Exhibiting Overexpression of mRNA Suggesting Mismanaged Ubiquitin-Mediated Transcription

An analysis of cancer patient samples presented in cBioPortal revealed that Taf13 is upregulated in some cancers and downregulated in others (Fig. 3.5A and 3.5B). Likewise, UALCAN patient samples show changed Taf13 protein and mRNA levels in different cancers (Fig. 3.5C and 3.5D). Importantly, we found that Taf13 mRNA (Fig. 3.5D) but not protein (Fig. 3.5C) is overexpressed in Head and Neck cancer (HNSC).

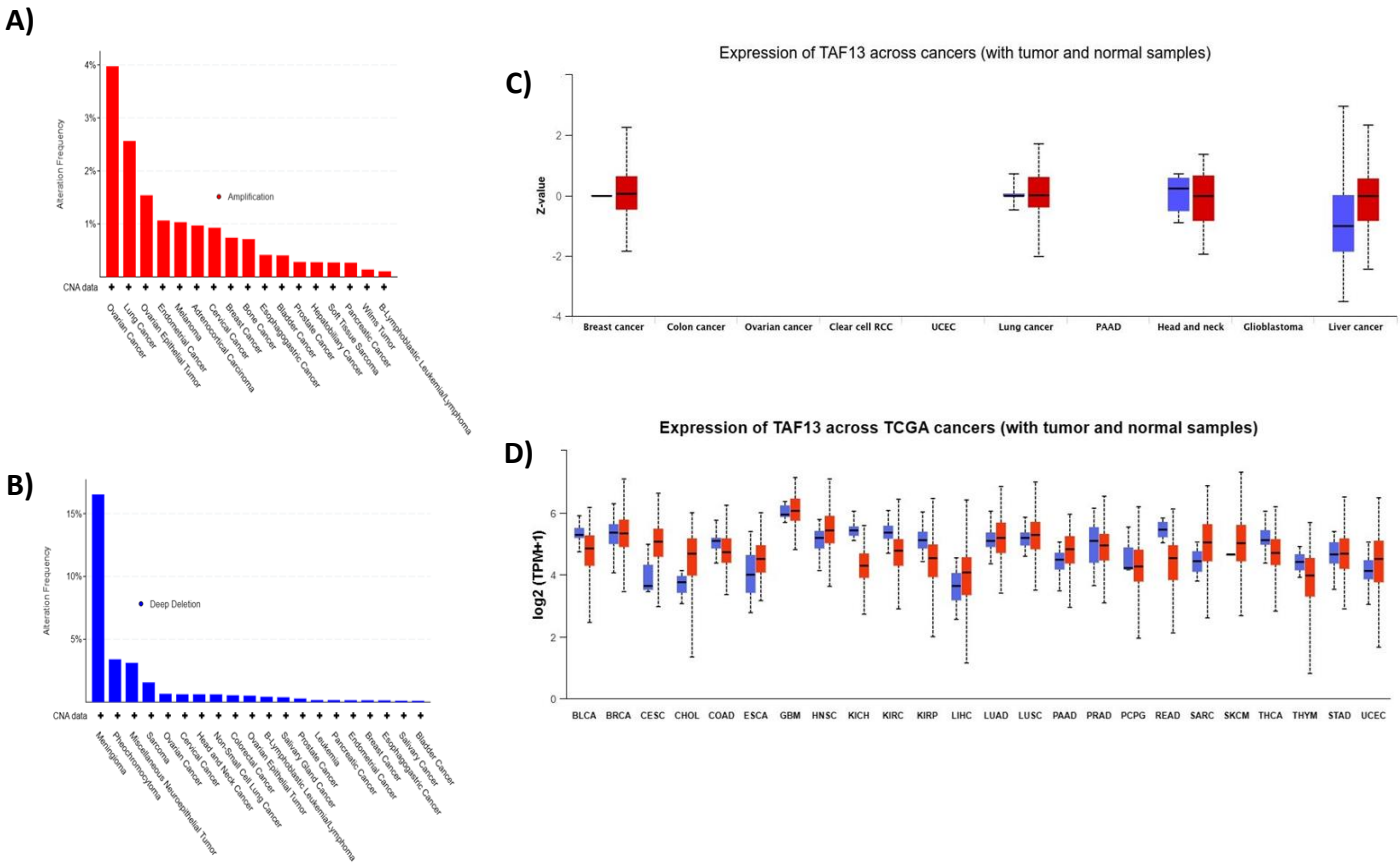


Figure. 3.5. Taf13 expression in different cancer patient samples. (A & B) Cross-cancer analysis of copy number alterations (CNA) in Taf13 based on the patient samples in the cBioPortal database [217 no overlapping samples]. (C) Taf13 protein levels in various cancer patient samples based on CPTAC in the UALCAN. (D) Taf13 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.

Taf13 protein and mRNA levels in Head and Neck cancer (HNSC) showed a much greater upregulation at the mRNA as opposed to the protein level suggesting a mismanaged ubiquitin-mediated transcription (Figure 3.6A and 3.6B). Unfortunately, we could not compare Taf13 protein and mRNA levels in THCA (thyroid carcinoma) since the protein expression data is not available.

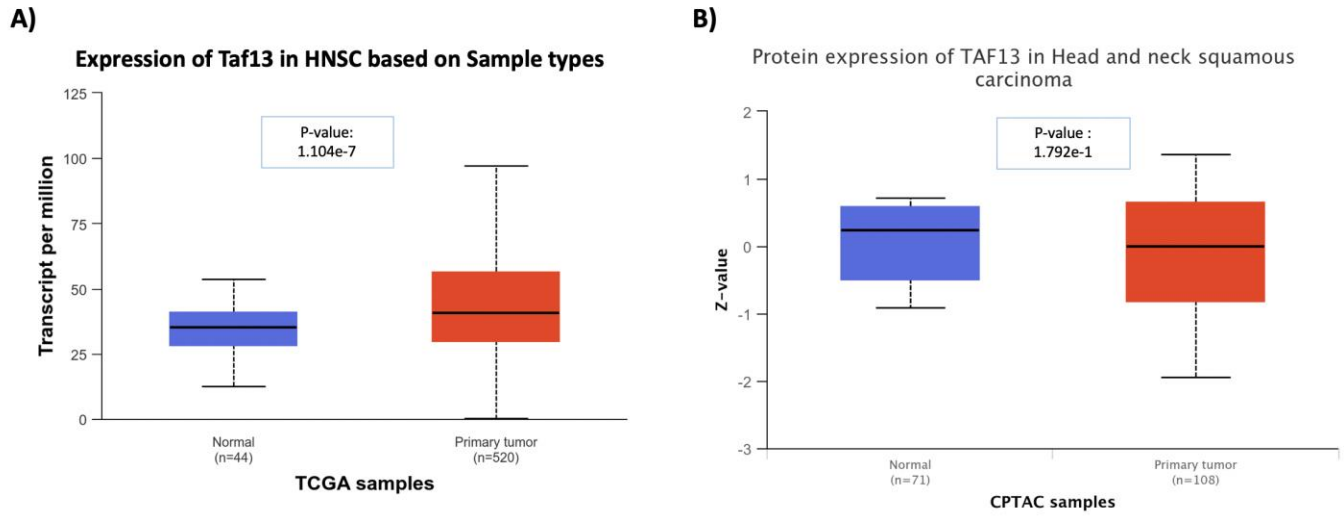
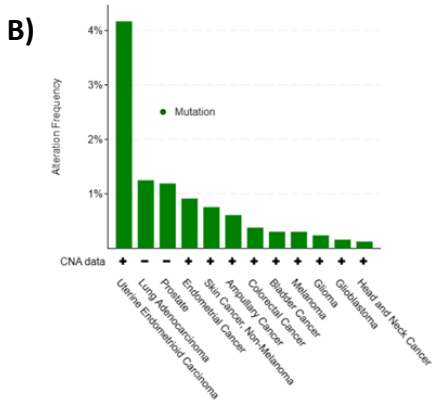


Figure. 3.6. Analysis of Taf13 mRNA and protein levels in HNSC based on CPTAC and TCGA cancer patient samples in the UALCAN. (A and B) Taf13 mRNA (A) and protein (B) expression levels in HNSC.

As discussed previously, K residues that make up ubiquitin can construct polyubiquitin chains, thus playing an important role in the polyubiquitination of the substrate protein. Hence, it is important to analyze the K mutations in Taf13 in many cancer patients. Notably, K mutations in Taf13 in various cancer patient samples were discovered in cancers located in many different tissues (Fig. 3.7A), suggesting that polyubiquitylation of Taf13 may be required for normal cellular activity such as regulation of transcription. In addition, Taf13 is also found to be mutated at other residues in various cancer patient samples (Fig. 3.7B and 3.7C)

A)

K Mutations	Location
E13K	Brain
R30K	Colon
E11K	Skin



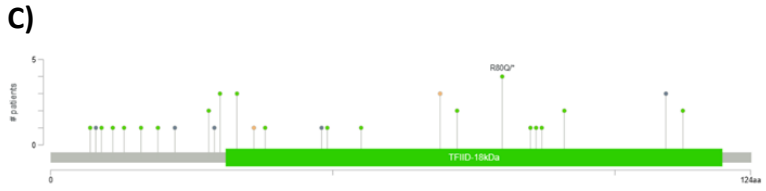


Figure. 3.7. Analysis of Taf13 mutations in various cancer patient samples in the cBioPortal. (A) Lysine (K) mutation of Taf13 in different cancer patient samples. (B) Cross-cancer analysis of mutations in Taf13 based on the patient samples in the cBioPortal database [217 studies]. (C) Mutations at different aa of Taf13 in various cancer patient samples.

Although we could not compare Taf13 protein and mRNA levels in THCA since the protein expression data is not available. However, we were able to analyze if the regulation of transcription via DNA methylation has a role in regulating the expression of Taf13 mRNA in thyroid cancers. Importantly, we found that Taf13 has decreased mRNA levels (Fig. 3.8.A). However, DNA methylation does not play a role in this since mRNA is lowered whereas no significant change in methylation is observed and is hypomethylated in both cases (Fig. 3.8.B). This suggests that DNA methylation, typically associated with inhibiting gene transcription, may not be playing a significant role in this context. Instead, the dysregulation of ubiquitination-

dependent transcription of the Taf13 pathway might play a role in influencing mRNA expression levels in thyroid cancer cells.

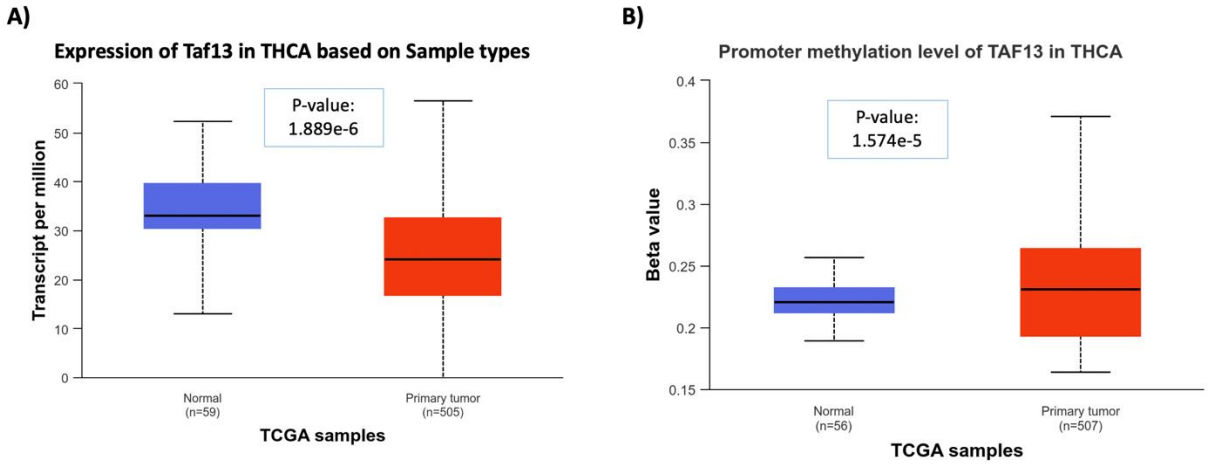


Figure 3.8. Analysis of Ta1f3 mRNA levels with promoter DNA methylation and survival probability in the thyroid carcinoma cells (THCA) in the UALCAN. (A) Taf13 mRNA levels in THCA. (B) Analysis of Taf13 promoter DNA methylation in THCA

Chapter 4: Discussion

4.1. Discussion

In our thesis work, we hypothesized that the stability of Taf13 may be regulated by the targeted degradation of Taf13 by the 26S proteasome via ubiquitylation (UPS) in *Saccharomyces cerevisiae*. We show that the TAP-tagged Taf13 strain precipitation that contained a hexahistidine-tagged plasmid was pulled down due to its interaction with the Ni^{2+}-NTA agarose beads. Above ~35 kDa, we have seen a ubiquitylated-Taf13 smear. A smear like this shows that Taf13 is polyubiquitylated rather than mono-ubiquitylated since each ubiquitin protein has a molecular weight of about 8 kDa and poly-ubiquitination adds around 8 proteins each. Overall, this result shows that Taf13 is polyubiquitylated. Next, in eukaryotes, if a protein undergoes polyubiquitylation, it is likely to be degraded by the 26S proteasome. To test it, we have examined the stability of Taf13 in the presence and absence of MG132, which suppresses the proteolytic

function of the proteasome, to determine if the poly-ubiquitylated Taf13 protein is a target for 26S proteasomal degradation. Taf13 would be more abundant if poly-ubiquitylated Taf13 is the target of the 26S proteasomal degradation, which may be achieved by pharmacologically inhibiting the proteasome's ability to catalyze proteolysis using MG132. However, we show that the abundance didn’t change after the MG132 treatment, meaning that the 26S proteasome does not degrade Taf13. Taken together, our results showed that Taf13 undergoes polyubiquitylation, however, is not degraded by the 26S proteasome. As discussed above in the introduction section, ubiquitin has seven K residues that serve as points of polyubiquitination. Among these seven K residues (K6, K11, K27, K29, K33, K48, and K63), the primary signal for degradation is a chain of at least four K48-linked polyubiquitin. The other linear ubiquitin chains instead are involved in a variety of processes, including signal transduction, transcription, etc. (Ferdoush et al., 2024). Therefore, it is possible that Taf13 polyubiquitylation is associated with other K residues, but not K48, which needs to be addressed in the future.

Moreover, to gain more mechanistic insight, we conducted a bioinformatics analysis and found that Taf13 is overexpressed in many cancer cells (Figure 3.5). As our control, it is proven that Paf1 is shown to be upregulated at the protein level but not at the mRNA level in pancreatic cancer, which is a result of its impaired 26S proteasomal degradation in cancer cells (Barman et al.,2024). However, this is not the case with Taf13. Taf13 protein and mRNA levels in Head and Neck cancer (HNSC) showed a much greater upregulation at the mRNA as opposed to the protein level suggesting a mismanaged ubiquitin-mediated transcription (Figure 3.6A and 3.6B). Unfortunately, we could not compare Taf13 protein and mRNA levels in THCA, thyroid carcinoma since the protein expression data is not available. We predict that protein upregulation in this case may be the primary consequence of mRNA regulation.

Further, since K residues are strongly associated with UPS, we looked at the K mutations in different cancers using the bioinformatics tool. Importantly we found that there is no mutation researched in K48, which is directly correlated to the 26S proteasomal degradation (Figure 3.7). This matches with the data found after our experiment, showing that 26S Proteasome is not regulating the stability of Taf13. In thyroid cancer, there is a notable underexpression of mRNA, while DNA methylation seems not to be a contributing factor, as both normal and cancerous cells exhibit hypomethylation. This suggests that DNA methylation, typically associated with inhibiting gene transcription, may not be playing a significant role in this context. Instead, the dysregulation of other transcription pathways, possibly due to mutations, appears to be more pertinent in influencing mRNA expression levels in thyroid cancer cells.

Research done by Kaiser et al. (2000) does explore the possibility of regulation of transcription by ubiquitination. Their research is conducted on the Met4 protein. Met4 is a transcription factor found in yeast cells, specifically *Saccharomyces cerevisiae*. It plays a crucial role in regulating the expression of genes involved in sulfur metabolism. Met4's ubiquitination prevents the Centromere Binding Factor 1 (Cbf1) association with the promoter regions at levels sufficient to promote transcription. We assume that ubiquitination of Taf13 might be required for normal transcription. However, dysregulation of ubiquitination of Taf13 might inhibit or overexpress transcription factors which could lead to altered expression of Taf13 in cancer cells. However, future research is needed to understand the full molecular mechanism.

4.2. Future Research

The next steps to be taken in the future would be to decipher the complex molecular mechanisms behind Ubiquitination-mediated Taf13 regulation, such as the enzymes (E1, E2, E3, ubiquitin protease, etc.) involved in the regulation of Taf13's ubiquitylation, which K residue it is connected to. Moreover, it is important to understand if ubiquitination of Taf13 might be required for normal transcription. If so, how the dysregulation of ubiquitination of Taf13 might inhibit or overexpress transcription factors which could lead to altered expression of Taf13 in cancer cells. After gaining more knowledge on the mechanisms of Taf13 protein turnover, these studies could be extended. In particular, our research could be extended to human cancer cell lines so that we can evaluate the effect of the polyubiquitination on Taf13 in a setting that is relevant to cancer.

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