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REGULATION OF BUBR1 BY ULTRAVIOLET RADIATION:
IMPLICATIONS IN SKIN CANCER

By

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A MASTER'S THESIS

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ABSTRACT

Non-melanoma skin cancer (NMSC) is the most common form of skin cancer. The primary risk factor of NMSC is prolonged exposure to ultraviolet (UV) radiation. UV radiation acts as both the tumor initiator and tumor promoter by causing molecular and cellular stress and DNA damage in cells within the skin, leading to mutations that promote the development of skin cancer. BubR1 is an essential mitotic checkpoint protein that plays a critical role in regulating chromosome segregation and maintaining genomic stability. BubR1 dysregulation can lead to an increase in chromosomal instability and aneuploidy, a common phenomenon associated with most cancers. Preliminary work from our lab have identified β -TRCP, an F-box containing protein, as an interacting factor for BubR1. β -TRCP is the substrate recognition subunit for the SCF (Skp, Cullin, F-box) complex, belonging to the Cullin RING E3 ubiquitin ligase (CRL) family of E3 ubiquitin ligases that regulates the stability of a wide range of cellular proteins by recognizing and binding specific substrates through a conserved degron motif and targeting them for ubiquitination and subsequent proteasome degradation. BubR1 contains a putative β -TRCP degron motif, indicating the possibility that β -TRCP may play a role in regulating BubR1 stability under various stimuli. Therefore, we hypothesized that UV radiation downregulates BubR1 protein levels through β -TRCP-dependent proteasome-mediated degradation. To test this hypothesis, short-term dose- and time-dependent UV experiments were carried out on human skin cell lines to assess the effect of UVC on BubR1 protein abundance. UV radiation led to the decrease of BubR1 protein and mRNA levels. In addition, β -TRCP protein abundance also showed a trend towards downregulation following UVC treatment, and a significant downregulation of both β -TRCP1 and β -TRCP2 transcripts was observed.

Global inhibition of the 26S proteasome, or specific inhibition of Cullin RING E3 ubiquitin ligases, blocked BubR1 downregulation in response to UVC treatment, indicating that UV-induced degradation of BubR1 was caused by both downregulation of BubR1 gene expression and protein degradation mediated by CRL-mediated ubiquitination. More specifically, we found that depletion of β -TRCP1 rescued BubR1 protein abundance and enhanced cell viability following UV exposure. Taken together, these results demonstrate that UV-induced BubR1 downregulation occurs in part through proteasome-dependent degradation regulated by β -TRCP which may function to promote cell death.

DEDICATION

I dedicate this thesis, first and foremost, to my parents, who constantly advised, supported, and prayed for me. To my brothers, who continually encouraged and motivated me. To my uncle and aunty who were always there for me in one capacity or the other. And to my friends that are friends indeed.

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ABBREVIATIONS

6-4-PP - pyrimidine-pyrimidone photoproducts

8-OXO-dG - 8-hydroxy-2-deoxyguanine

ANOVA - Analysis of Variance

APC/C - anaphase-promoting complex/cyclosome

BCC - Basal cell carcinoma

BubR1 - Budding uninhibited by benzimidazole 1

CDC20 - cell division cycle protein 20

CDK1 - cyclin-dependent kinase-1

CPD - cyclobutane pyrimidine dimers

CRLs - Cullin-RING ligases

DMEM - Dubecco's modified eagles medium

DMSO - Dimethyl sulfoxide

HBS - HEPES buffered saline

J/m² - Joules per square meter

MCC - Mitotic checkpoint complex

MSC - Melanoma skin cancer

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NER - Nucleotide excision repair

NMSC - Non-melanoma skin cancer

PCR - Polymerase chain reaction

ROS - Reactive oxygen species

rt-PCR - real-time Polymerase chain reaction

SAC - Spindle Assembly checkpoint

SCC - Squamous cell carcinoma

SCF - SKP1-CUL1-F-box protein

sh-RNA - short hairpin ribonucleic acid

UV - Ultraviolet

UVA - Ultraviolet A

UVB - Ultraviolet B

UVC - Ultraviolet C

UVR - Ultraviolet radiation

β -TRCP - Beta transducing protein

β -TRCP1 - Beta transducing protein 1

β -TRCP2 - Beta transducing protein 2

Chapter 1: Introduction and Background

Skin cancer prevalence

Globally, skin cancer is the most common type of cancer, and its incidence increases with age¹. In the United States (U.S.), the number of people diagnosed with skin cancer each year exceeds the diagnosis of all other cancers combined¹. Approximately 10,000 people in the U.S.) are diagnosed with skin cancer every day, an average of two people die from skin cancer every hour, and at least one in every five Americans is likely to develop skin cancer by the age of 70²⁻⁴. Skin cancers can be broadly divided into melanoma (MSC) and non-melanoma skin cancer (NMSC). Melanoma occurs when pigment-producing cells called melanocytes become cancerous. Although melanomas are less prevalent than NMSC, they have a higher mortality rate⁵. In the U.S., NMSC is the most common malignancy and is linked with significant morbidity and economic burden³. NMSC consists mainly of squamous cell carcinoma (SCC) and basal cell carcinoma (BCC); other less common forms of NMSC include Merkel cell carcinoma, Kaposi carcinoma, cutaneous skin lymphoma, and adnexal skin tumors.

BCC is a less aggressive form of skin cancer that originates from cells of the lower part of the epidermis known as the basal layer⁶. BCC is the most prevalent type of skin cancer in the U.S., where approximately 3.6 million cases of BCC are diagnosed annually^{3,7}. Although BCC has very low mortality and metastatic rates, it makes up around 60% of NMSC cases, while SCC accounts for 30-40% of NMSC cases⁸.

SCC is the second most common NMSC, with an estimated 1.8 million cases diagnosed annually in the U.S.⁹. Cutaneous SCC is a malignant tumor arising from

epidermal keratinocytes, which are the primary cell type within the epidermis. Lesions known as actinic keratosis (AK) usually precede the onset of SCC; however, some SCC may also develop *de novo*^{9,10}. Compared to normal skin cells, the gene expression profiles in both AK and SCC have been shown to have significant alterations. While AK and SCC have similar genetic profiles, SCC has a significantly higher mutation burden¹¹. Compared to BCC, SCC is more aggressive and has a higher probability of metastasizing.

Recent statistics have shown that more than 15,000 people die of cutaneous SCC yearly in the U.S., more than twice the deaths caused by melanoma^{1,2}. The estimated annual costs of skin cancer treatment in the U.S. increased from \$8.0 billion in 2012-2015 to \$8.9 billion in 2016-2018, roughly two-thirds of which is spent on treating NMSC. The remaining one-third is spent on MSC treatment^{8,12}.

If diagnosed early, SCC can be effectively treated; standard treatment options include excisional surgery, Mohs surgery, cryosurgery, curettage and electrodesiccation, laser surgery, radiation, photodynamic therapy, and topical medications using 5-fluorouracil (5-FU) and Imiquimod¹³. However, the increasing prevalence of SCC and the effect of SCCs, and SCC treatment, on quality of life pose a significant issue for public health and economic burden. Therefore, it is essential to identify innovative approaches for SCC prevention and treatment to improve patient quality of life and increase survival.

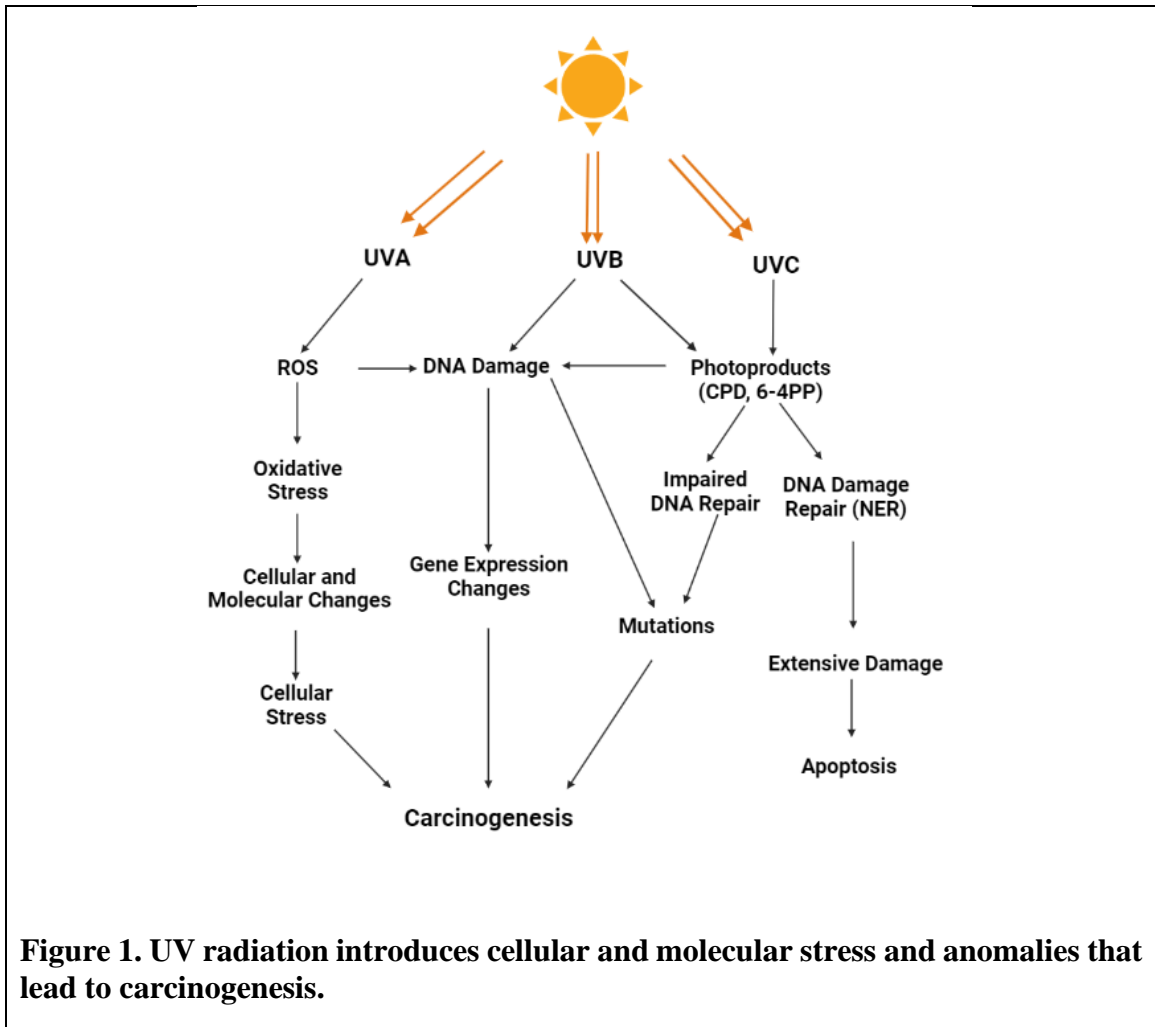
Ultraviolet radiation is a major cause of skin cancer

The risk factors of skin cancer include ultraviolet (UV) radiation exposure (e.g. sun exposure or indoor tanning), age, people with light complexions, exposure to certain chemicals, radiation exposure, previous incidence of skin cancer, precancerous skin

conditions such as AK or Bowen's disease (BD), inherited syndromes and certain disease states such as xeroderma pigmentosum, people with a deteriorated or repressed immune systems due to transplantation or diseases such as HIV/AIDS or leukemia, medications, and smoking. Of the listed risk factors, UV radiation is the primary cause of skin cancer development; roughly 90% of instances of NMSC can be attributed to exposure to UV emanating from the sun^{14,15}.

UV is categorized as a "complete carcinogen" as it is a mutagen and a non-specific damaging agent that initiates and promotes tumorigenesis¹⁶. UV consists of three wavelengths, UVA (400-320 nm), UVB (320-280 nm), and UVC (280-100 nm). UVC has a short wavelength and is mainly absorbed by the ozone layer. UVA and UVB rays have longer wavelengths that reach the earth. UVA and UVB can penetrate the skin and cause permanent damage, resulting in sunburn and skin aging (also known as photoaging) as well as contribute to skin cancer development. However, these UV wavelengths have different effects on the skin and mechanisms leading to the development of skin cancers caused by UV exposure¹⁷.

UVA has the longest wavelength and therefore its energy level is lower compared to UVB and UVC¹⁶. UVA is able to penetrate the skin down to the dermis and induces long-term changes in epidermal keratinocytes and dermal fibroblasts¹⁸. UVA causes damage primarily by generating reactive oxygen species (ROS); the absorption of UVA energy mediates this by several endogenous chromophores, which include melanin (and its precursors and metabolites), and amino acids. ROS can react with DNA bases, leading to the formation of modified bases such as 8-oxo-7,8-dihydroguanine (8-oxoG), these modified bases can disrupt normal base pairing and lead to mutations (Figure 1)¹⁸⁻²⁰.



Oxidative stress resulting from UVA leads to a wide array of cellular and molecular changes, including lipid peroxidation, DNA lesions, protein carbonylation, and activation of intracellular signaling pathways, which induce cellular stress contributing to carcinogenesis (Figure 1)²⁰.

UVB penetrates cells in the upper layers of the epidermis, causing damage to these cells. UVB is responsible for sunburn, a significant risk factor for skin cancer. DNA absorbs UVB directly, leading to DNA damage (Figure 1).

UVC radiation leads to the induction of oxidative stress that leads to the downstream occurrence of cell death through the initiation of an apoptotic pathway; this apoptotic pathway is triggered by the activation of caspase-3 and poly-ADP-ribose polymerase-1 (PARP-1) ^{19,21}.

As a result of exposure to UV, covalent bonding between consecutive bases occurs near their double carbon bonds along the nucleotide chain leading to the production of photo-products known as cyclobutane pyrimidine dimers (CPD) and pyrimidine-pyrimidone photoproducts (6-4PP) (Figure 1)^{22,23}. In a phenomenon known as the “dark pathway”, melanin-containing melanocytes, exposed to UV radiation, have also been found to produce a high-energy triplet state molecule that creates delayed CPDs by energy transfer, which are chemically identical to the CPDs induced by direct UV exposure but are generated in the absence of light²⁴. Normally, cells have mechanisms in place to repair DNA damage caused by UV exposure, mainly by a process known as Nucleotide Excision Repair (NER), or undergo programmed cell death if the damage is severe. However, DNA repair can be inefficient or suppressed due to genetic factors such as inherited mutations, single nucleotide polymorphisms, and epigenetic alterations, or environmental factors such as smoking, contact with certain chemicals, and exposure to UV or ionizing radiation²⁵. If DNA damage goes unrepaired or the repair mechanisms are faulty or inadequate, it can lead to genetic mutations that can accumulate over time.

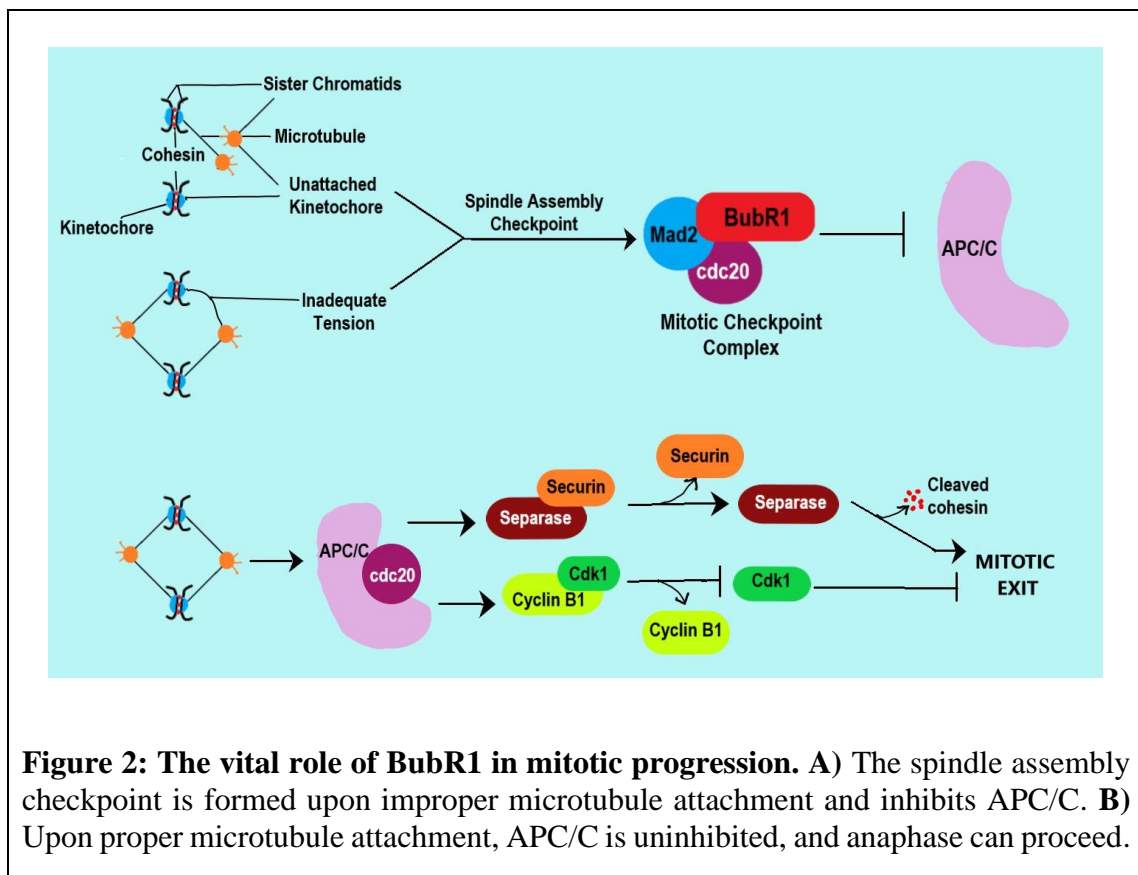
Additionally, DNA damage can also interfere with the ability of cells to undergo programmed cell death or apoptosis by inducing mutations in key regulators such as p53 by inducing the formation of pyrimidine dimers, specifically thymine-thymine (TT) dimers, in the DNA sequence²⁶. The downstream effects of UV-induced mutations in p53

can be significant, as p53 plays a critical role in regulating the cell cycle and preventing the division of cells with damaged DNA. Mutations in p53 can lead to a reduction in expression or to the production of a mutant form of the protein that does not function properly, both of which can impair the cell's ability to respond to DNA damage²⁷. This can result in increased susceptibility to cancer development and other diseases and disorders. Additionally, p53 mutations have been associated with increased UV sensitivity and decreased DNA repair capacity, further increasing the risk of DNA damage and mutation. DNA damage can therefore initiate a cascade of events that can ultimately lead to the development of cancer, and an accumulation of mutations is a key hallmark in the development of skin cancers²⁷.

BubR1 plays a vital role in the Spindle Assembly Checkpoint

Budding uninhibited by benzimidazole-related 1 (BubR1) is a serine-threonine kinase that plays a vital role in regulating the spindle assembly checkpoint (SAC). The SAC is a safety mechanism that monitors the connection between the spindle fibers and kinetochores, which are protein structures on mitotic chromosomes facilitating attachment with microtubules²⁸. Unattached, or improperly attached, kinetochores are sensed by the SAC which signals to prevent anaphase onset. For a cell to progress to anaphase, the attachment of microtubules to the kinetochore and the application of appropriate tension are necessary. This tension across kinetochores can only be achieved when sister chromatids are accurately aligned to the metaphase plate and attached to bipolar spindle fibers. If these criteria are not met, the signaling function in the SAC leads to the creation of the mitotic checkpoint complex (MCC), which comprises the proteins MAD2, Bub3, and BubR1, linked to the SAC target protein cell division cycle protein 20 (cdc20), which

functions as a cofactor for the anaphase-promoting complex/cyclosome (APC/C)^{29,30}. When activated, the MCC will inhibit the APC/C, a ubiquitin ligase required to separate sister chromatids and drive anaphase onset (Figure 2)³¹. If the criteria for microtubule attachment and tension are met then the SAC is silenced and the APC/C, in conjunction with Cdc20 (APC/C^{Cdc20}), polyubiquitinates a number of mitotic regulators including securin and cyclin B, activating a protease known as separase and inactivating cyclin-dependent kinase-1 (Cdk1), respectively. Activated separase mediates the cleavage of cohesion complexes responsible for holding the sister chromatids together, leading to their separation, whereas inactivation of Cdk1 allows for the dephosphorylation of its substrates by protein phosphatases. The SAC is rapidly silenced upon successful alignment, enabling anaphase initiation and mitotic exit (Figure 2)³².



During prophase, BubR1 is localized to kinetochores to monitor microtubule-kinetochore attachment, enabling chromosomes to migrate to the metaphase plate^{33,34}. Loss of BubR1 is associated with chromosome missegregation leading to genomic instability in the form of aneuploidy, which can cause infertility, congenital defects, and tumorigenesis^{35,36}.

BubR1 and Cancer

BubR1 levels progressively reduce with age, and hypomorphic mice expressing approximately 10% of wild-type BubR1 protein levels show premature aging phenotypes due to an increase in cellular senescence³⁶. Homozygous knockout of BubR1 in mice is embryonically lethal whereas heterozygous knockout mice show an increase in bypass of the SAC in a process referred to as 'mitotic slippage'. Mitotic slippage results in higher levels of mitotic abnormalities due to aneuploidy, shortened lifespan, premature aging phenotypes, and increased age-related spontaneous or carcinogen-induced tumorigenesis^{35,37-39}. BubR1 deficiency also leads to a compromised response to DNA damage⁴⁰. Truncating and missense mutations in BubR1 have been discovered in individuals with mosaic-variegated aneuploidy (MVA). MVA is a rare autosomal recessive disorder characterized by inaccurate chromosome segregation and high rates of aneuploidy resulting from premature segregation of chromosomes during mitosis. MVA is characterized by microcephaly, cataracts, growth and mental retardation, a shortened lifespan, and increased susceptibility to cancer^{35,38}.

Aneuploidy, is a condition where cells have an abnormal number of chromosomes, is a hallmark of cancer; around 90% of cells within tumors typically display aneuploidy^{33,41,42}. It is physiologically linked to severe cellular stress while also favoring

tumor progression⁴³. Aneuploidy can enhance the ability of cancer cells to evolve and adapt to chemotherapy, thereby facilitating disease progression and potential relapse⁴³. Given the key role BubR1 plays in suppressing aneuploidy, the increase in cancer incidence with age may also be associated with the age-related decline in BubR1.

Genetic modifications such as mutations in *KRAS*, which is involved in the regulation of cell division, are potent drivers of tumorigenesis and are linked with an increase in aneuploidy. Overexpression of BubR1 in mice maintains genomic integrity and suppresses tumorigenesis, and reduces *KRAS*-driven aneuploidy and tumorigenesis³⁷. In conditions where the mitotic checkpoint and the attachment of kinetochore-microtubules are disrupted, increased levels of BubR1 facilitates the correction of this impairment of the mitotic checkpoint and defects in the attachment of microtubules to kinetochores⁴⁴. Furthermore, sustained BubR1 expression with age positively affects longevity and delays age-related decline in tissues and aneuploidy³⁷.

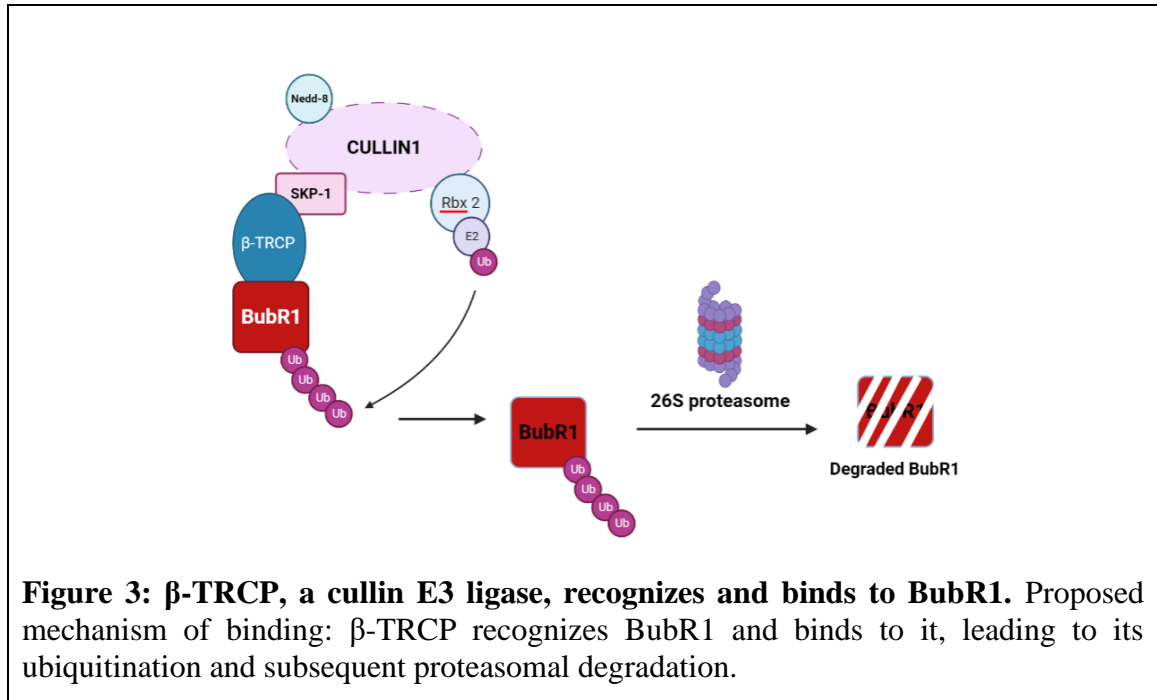
SCF^{β-TRCP} recognizes and ubiquitinates BubR1

The Cullin-RING ligases (CRLs) are a family of E3 ubiquitin ligases of which SKP1-CUL1-F-box protein (SCF) complexes are some of the most well characterized members. SCF complexes are comprised of a core complex including the S-phase Kinase-Associated Protein 1 (SKP1), which functions as an adaptor protein; CUL1, which functions as a scaffold protein, RBX1, which is a RING domain containing protein; and an interchangeable F-box protein, which serves as the substrate receptor⁴⁵. The human genome encodes 69 F-box proteins, each facilitating the recruitment of a variety of cellular proteins into the SCF complex for ubiquitination^{46,47}. F-box proteins are categorized into three groups: FBXW, which are F-box proteins with WD40 domains; FBXL, which are F-

box proteins with leucine-rich repeats; and FBXO, which are F-box proteins that have other domains. This classification is based on the nature of the substrate recognition and binding domain. FBXW comprises FBXW1 to FBXW5 and FBXW7 to FBXW11, making up ten members. FBXW1 and FBXW11 are also known as β -transducing repeat-containing protein 1 (β -TRCP1) and β -transducing repeat-containing protein 2 (β -TRCP2), respectively. β -TRCP plays a crucial role in recruiting substrates involved in regulation of cell division, cell migration, DNA damage response, and signal transduction, all of which play a pivotal role in cancer development^{48,49}.

Numerous oncoproteins and tumor suppressors are recognized as substrates of β -TRCP, and their dysregulation is observed in human cancers. The serine or threonine residues within the degron motif (DSGxxxS, or its variations) found in substrate proteins require phosphorylation by specific kinases in order to be recognized by β -TRCP^{48,49}. Upon β -TRCP recognition, RBX1 facilitates the transfer of a ubiquitin protein from a E2 ubiquitin conjugating enzyme to the substrate recruited into the SCF complex by β -TRCP⁴⁹⁻⁵¹. BubR1 contains a putative β -TRCP degron motif (SSGFSGS) that may mediate its recognition and polyubiquitination (Figure 3).

β -TRCP has been shown to primarily play an oncogenic role in human cancers by targeting the degradation of tumor suppressors, and β -TRCP1 is overexpressed in many human carcinomas⁵². High expression of β -TRCP1 mRNA and protein has also been reported in 56% of colorectal tissues associated with poor clinical prognosis and a high probability of tumor recurrence⁵⁰. Although previous studies suggest that β -TRCP1 and β -TRCP2 are functionally redundant with numerous overlapping substrates, recent findings have shown that both proteins also have unique functions^{48,53}. However, both play roles in



regulating the cellular response to genotoxic stress and furthermore may regulate each other's protein stability⁵³. β -TRCP1 levels have been shown to be elevated upon genotoxic stress induction, while β -TRCP2 levels are decreased⁵³. The levels and activity of β -TRCP1 also increase with advancing age,⁵⁴ indicating its potential involvement in the age-related deterioration of BubR1. There is ample evidence pointing to the participation of BubR1 in cell cycle progression⁴⁴, and both BubR1 and β -TRCP are implicated in the DNA damage response and carcinogenesis^{40,55}. Considering the highlighted background information, we hypothesize that UV radiation mediates the downregulation of BubR1 via β -TRCP-dependent ubiquitin-proteasome-mediated degradation. Understanding the role of β -TRCP in regulating BubR1 loss following UV exposure in skin cells may give insight into possible therapeutic targets for skin cancer (Figure 4). In this research, we used immortalized human keratinocyte cells (HaCaT)⁵⁶ and cutaneous SCC cells (SCC13)⁵⁷ to perform a series of short-term UV experiments. Our experiments revealed that UV leads

to the loss of BubR1 protein levels in a proteasome-dependent manner and reduces BubR1 mRNA levels. Inhibition of β -TRCP1 results in a rescue of BubR1 protein levels, indicating that β -TRCP plays a role in the UV-mediated degradation of BubR1.

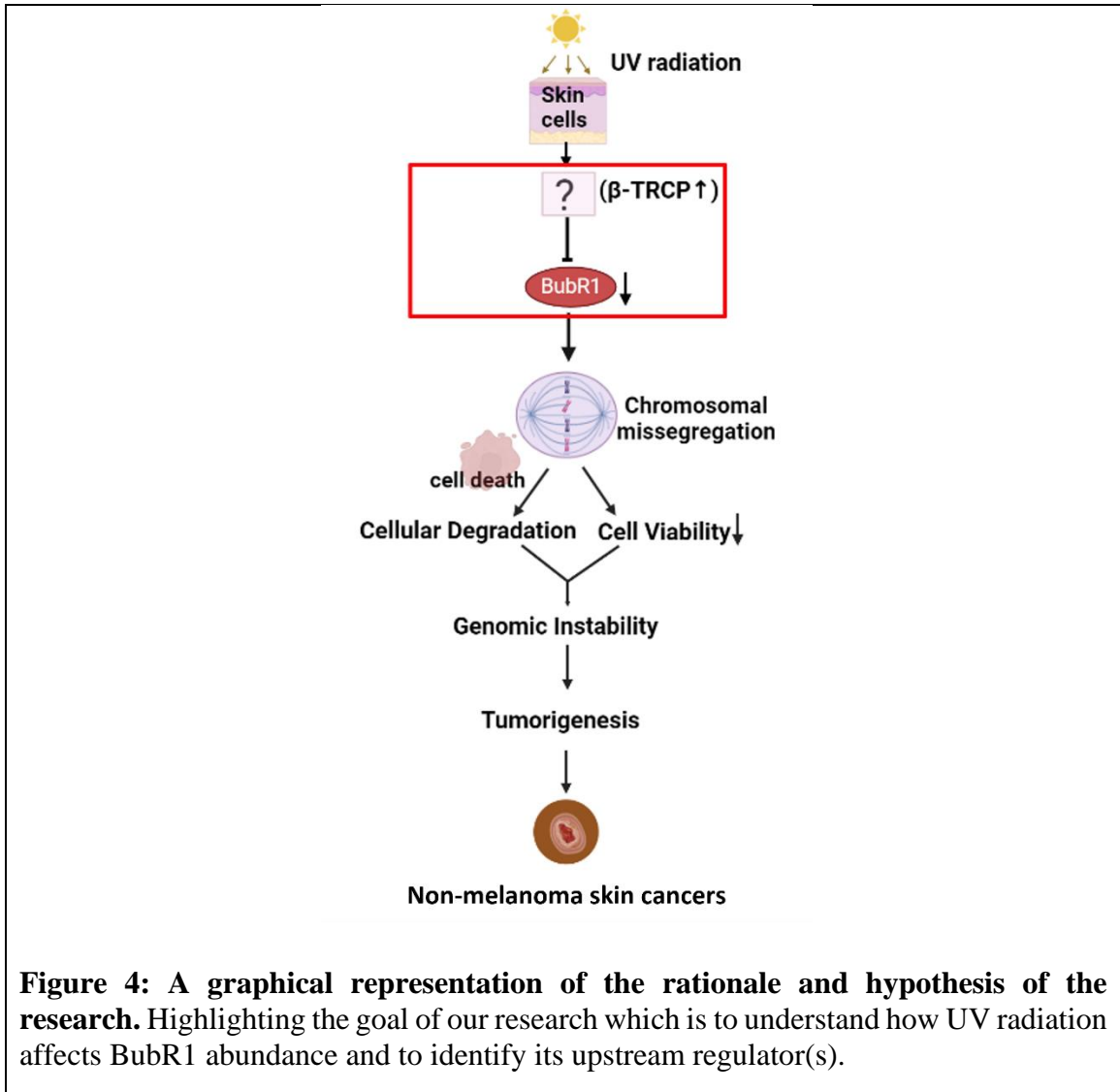


Figure 4: A graphical representation of the rationale and hypothesis of the research. Highlighting the goal of our research which is to understand how UV radiation affects BubR1 abundance and to identify its upstream regulator(s).

Chapter 2: Materials and Methods

Cell lines, culture, and reagents

293T cells were supplied by the American Type Culture Collection (ATCC), and HaCaT⁵⁶ and SCC13⁵⁷ cells were kind gifts from Dr. Laura Hansen (Creighton University School of Medicine, Omaha, NE). HaCaT, SCC13, and 293T cells were cultured in Dulbecco's modified Eagles' medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 100 units/ml penicillin and 100 µg/ml streptomycin (GIBCO). Cells were seeded into culture plates and incubated at 37°C in a humidified atmosphere with 5% CO₂. After 24 hours of incubation, the cells were subjected to indicated treatments.

UVC radiation

Cells were seeded in 60mm culture dishes, incubated, and allowed to reach 75% confluency. Media was aspirated from the culture plates, and cells were washed with Phosphate-Buffered Saline (PBS). PBS was removed, and the cells were covered with 500 µl PBS containing 0.1 mM CaCl₂ and exposed to UVC. The UVC dose for timed experiments was 600 J/m²; for the dose-response experiments, the doses were 0, 200, 600, and 1,200 J/m². Following UV exposure, PBS was aspirated, and fresh media was added to the plates. Cells were incubated for 8 hours for the dose-response experiments and 2, 4, 8, 16, and 24 hours post-UV exposure for the timed experiments. UVC radiation was performed using a UV Stratalinker model 1800 (Stratagene catalog #40071).

MG132 treatment

HaCaT and SCC13 cells were grown to approximately 75% confluency. MG132 was added to a final concentration of 10 μ M, and an equal volume of dimethyl sulfoxide (DMSO) was added to control plates for 1 hour prior to UV radiation.

MLN4924 treatment

HaCaT and SCC13 cells were grown to approximately 75% confluency. MLN4924 was added to a concentration of 10 μ g/ml, and an equal volume of DMSO was added to control plates and incubated for 16 hours prior to UV radiation.

Cell Viability Assay

Cells were seeded into 96 well plates at a density of 8,000 cells/well and incubated for 16 hrs. The media was removed, and cells were washed with PBS. PBS was aspirated, and cells were covered with 20 μ l of PBS containing 0.1 mM CaCl₂ and exposed to UV. PBS was removed and fresh media was added, and the cells were incubated for 24 hours. Following the incubation period, 20 μ l of a 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to each well, and the plate was incubated for 2 hours. Subsequently, the media was aspirated, and 100 μ l of DMSO was added to each well. The plate was then placed on a rocker for 10 minutes to dissolve the formazan crystals. After the crystals had dissolved, the absorbance was spectrophotometrically measured at 570 nm on a Biotek Cytation 5 imaging reader.

Packaging of shRNA-encoding lentivirus

293T cells were grown in 100 mm plates to 75% confluency. Cells were transfected using the calcium phosphate precipitation method. Briefly, 5 µg of the lentiviral transfer vector (shGFP and shβ-TRCP1), 3.5 µg of the packaging vector (psPAX2), and 1.5 µg of the envelope vector (pMD2.G) were combined, and water was added to yield a volume of 427.5 µl. Subsequently, 62.5 µl of 2 M CaCl₂ was added to the DNA mixture and vortexed, followed by the addition of 500 µl of 2X HEPES buffered saline (HBS), consisting of 140 mM NaCl, 1.5 mM Na₂HPO₄, 50 mM HEPES, pH 7.05, and vortexed. The resulting transfection solution was then added to the plates dropwise, the cells were incubated overnight, and the media was changed. After 24 hours following the media change, the media containing virus particles was collected in 50 ml tubes, and fresh media was added to the plates for another 24 hours and subsequently collected and combined with previously isolated media. The virus-containing media was centrifuged at 1,500 rpm for 5 minutes and subsequently filtered using a 0.45 µm syringe filter to remove any cells or cell debris. Aliquots of the viral media were made for either immediate use or stored at -80°C.

Reverse transduction of cells with shRNA-encoding lentivirus

To infect cells with shRNA-encoding lentivirus, 20 µl of 10 mg/ml polybrene was added to 20 ml of media, the lentiviral aliquots were rapidly thawed, and 1 ml of each viral solution was added to the plates (one well per sample), excluding the well that would serve as the 'no virus' control, For the 'no-virus' control plate, 1 mL of standard media was added. Cells were trypsinized and pelleted at 1,500 rpm for 5 minutes, the supernatant was aspirated, and the cells were resuspended in media + polybrene solution and mixed by pipetting. The cell suspensions were added 1mL per well, and the cells were incubated for

24 hours. After 24 hours, the virus-containing media was removed and replaced with fresh DMEM, and puromycin was added to a final concentration of 1 µg/ml of puromycin. The cells were monitored daily until all non-infected (control) cells died. After completion of the selection process, cells were utilized for further experiments.

Western blotting

Media was aspirated from the cells, and the plates were rinsed with room-temperature PBS twice. Lysis buffer (50 mM Tris HCl (pH 7.5), 0.5 mM EDTA, 0.5% NP-40, 150 mM NaCl and 1X protease and phosphatase inhibitor) was prepared and added to each well and cells were harvested on ice using cell lifters (Corning). The protein concentration was quantified using Bio-Rad Dc Protein Assay Kit, protein concentration was normalized, and the samples were boiled at 95°C for 10 minutes. Equal amounts of the samples were run on 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred to nitrocellulose membranes. Confirmation of even loading and transfer was done via Ponceau S staining and immunoblotting for α -tubulin. Membranes were blocked with 5% nonfat dry milk in Tris Buffered Saline and 0.1% Tween (TBS-T) buffer and incubated at 4°C overnight with primary antibodies. The membranes were washed three times in TBS-T buffer for 15 minutes each and incubated with the respective secondary antibodies at room temperature. The blots were washed again with TBS-T and developed using chemiluminescence HRP substrate and imaged using the BioRad Chemi Doc XRS+ with image lab software.

Table 1: Antibodies and reagents

Reagent/ Antibody	Dilution for Immunoblotting	Source	Catalog number
MG132	-	MedChem Express	<u>HY-13259</u>
MLN4924	-	MedChem Express	HY-70062
DMSO	-	ThermoFisher Scientific	D1391
MTT	-	Tocris Bioscience	296-93-1
Polybrene	-	Santa Cruz Biotechnology	SC-134220
HALT protease and phosphatase inhibitor	-	ThermoFisher Scientific	78442
Chemiluminescence HRP substrate	-	Millipore Immunobilon	WBKLS0500
5% nonfat dry milk	-	Great Value, Walmart Inc.	-
BubR1	1:5000	BD Transduction	612503
β -TRCP	3:5000	Cell Signaling Technology	4394S
α -tubulin	1:5000	Sigma	SC-23948

Quantitative RT-PCR

Based on the manufacturer's protocol, total RNA was isolated from cells using RNeasy Mini Kit (Qiagen, #74104) and RNA quality and quantity were assessed with NanoDrop spectrophotometer/fluorometer (Denovix V 3.51). Based on the manufacturer's protocol, cDNAs were reverse transcribed from 1 μ g of RNA using the qScript cDNA synthesis kit (Quantabio) on a BioRad C1000 Touch thermal cycler. Samples were prepared using Powerup SYBR green (Applied Biosystems), and quantitative real-time Polymerase Chain Reaction (qRT-PCR) was performed using the Applied biosystems 7500 Fast real-time PCR system.

Table 2: Primer sequences

Gene	Orientation	Primer sequence
BubR1	Forward	5'-CTCGTGGCAATACAGCTTCA-3'
	Reverse	5'-CTGGTCAATAGCTCGGCTTC-3'
β -Actin	Forward	5'-CCACGAAACTACCTTCAAC-3'
	Reverse	5'-GATCTTCATTGTGCTGGG-3'
β -TRCP1	Forward	5'-TGGACACAAACGAGGCATTG-3'
	Reverse	5'-TTCCATCATAGGCCCCACTG-3'
β -TRCP2	Forward	5'-TCTAACTGGCGGTGTGGACG-3'
	Reverse	5'-TGCAGACAGAGGACAGAGCC-3'

Statistical analysis

One-way ANOVA or two-way ANOVA tests with multiple comparisons were used to determine statistical significance. $p \leq 0.05$ was considered to be significant.

Chapter 3: Results

UVC radiation leads to a reduction of BubR1 protein levels

To investigate the effect of UVC radiation on BubR1 protein levels, immortalized human keratinocyte cells (HaCaT) and squamous cell carcinoma cells (SCC13) were exposed to 0, 200, 600 and 1,200 J/m² of UVC and harvested after 8 hours incubation. Western blot results revealed a dose-dependent decrease in BubR1 levels following UVC exposure (Figure 5A and C). Quantification of immunoblots probed with BubR1 from three independent experiments revealed a significant decrease in BubR1 protein following UVC exposure (Figure 5B and D). To further examine the dynamics of UVC radiation on BubR1 loss, HaCaT, and SCC13, cells were exposed to 600 J/m² of UVC and incubated for increasing time (2, 4, 8, 16, and 24 hours) post-exposure. Immunoblotting revealed a time-dependent reduction in BubR1 levels after UVC exposure (Figure 5E, G), and quantification of immunoblots probed with BubR1 from three independent experiments showed a significant time-dependent loss of BubR1 levels after exposure to UVC (Figure 5F and H). These results indicate that UV exposure reduces BubR1 levels in both a dose and time-dependent manner.

UV-induced BubR1 degradation occurs in a proteasome-dependent manner

In eukaryotic cells, the 26S proteasome plays an essential role in protein degradation in response to polyubiquitin conjugates, to recycle ubiquitin tags and destroy substrates irreversibly⁵⁸. Our previous finding that UVC radiation decreased BubR1 levels led us to assess whether the observed BubR1 decrease was proteasome dependent.

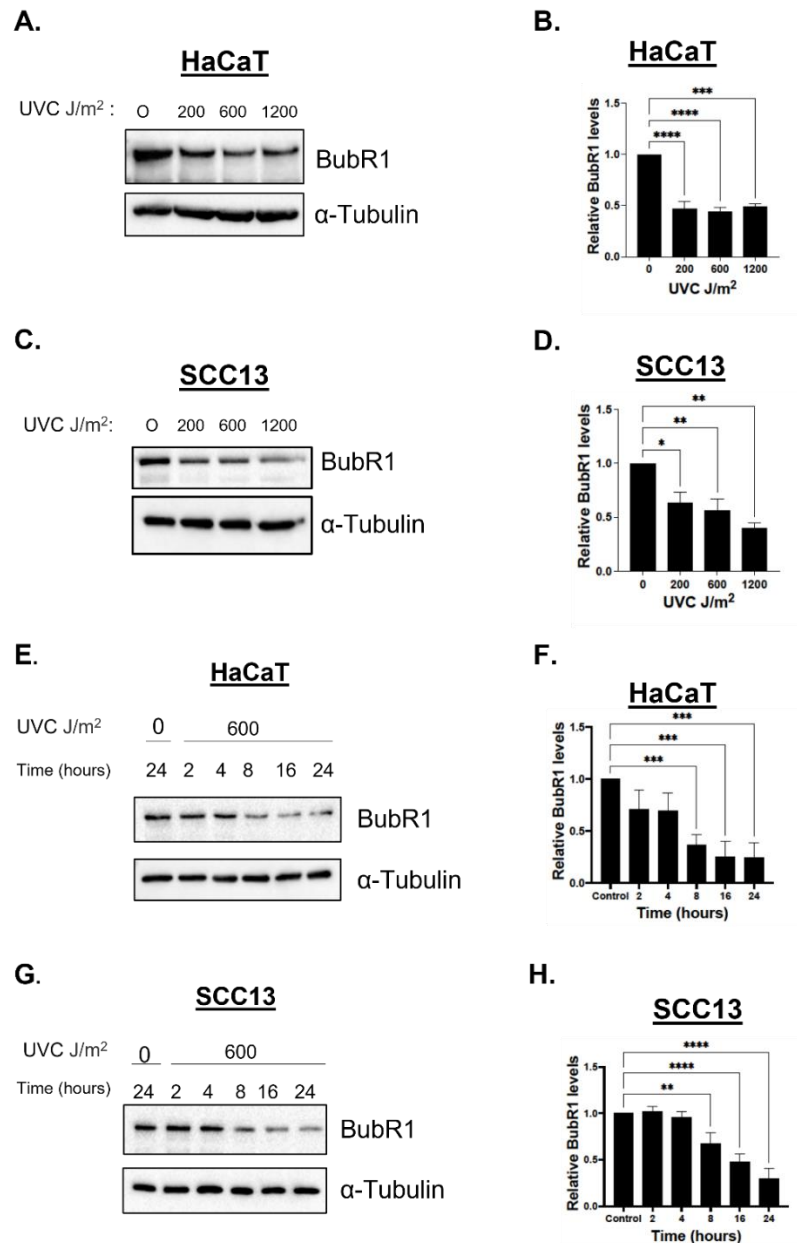
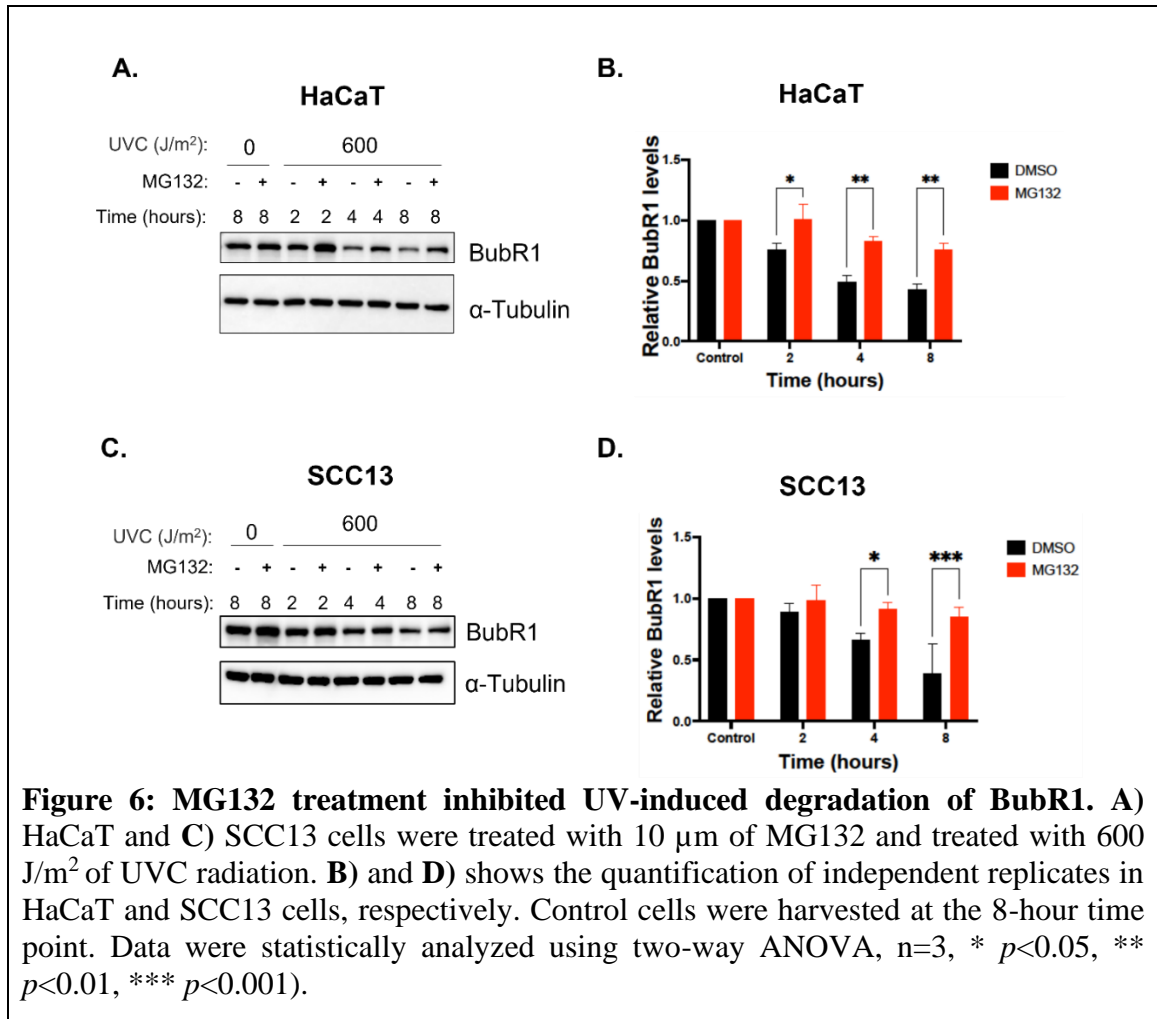


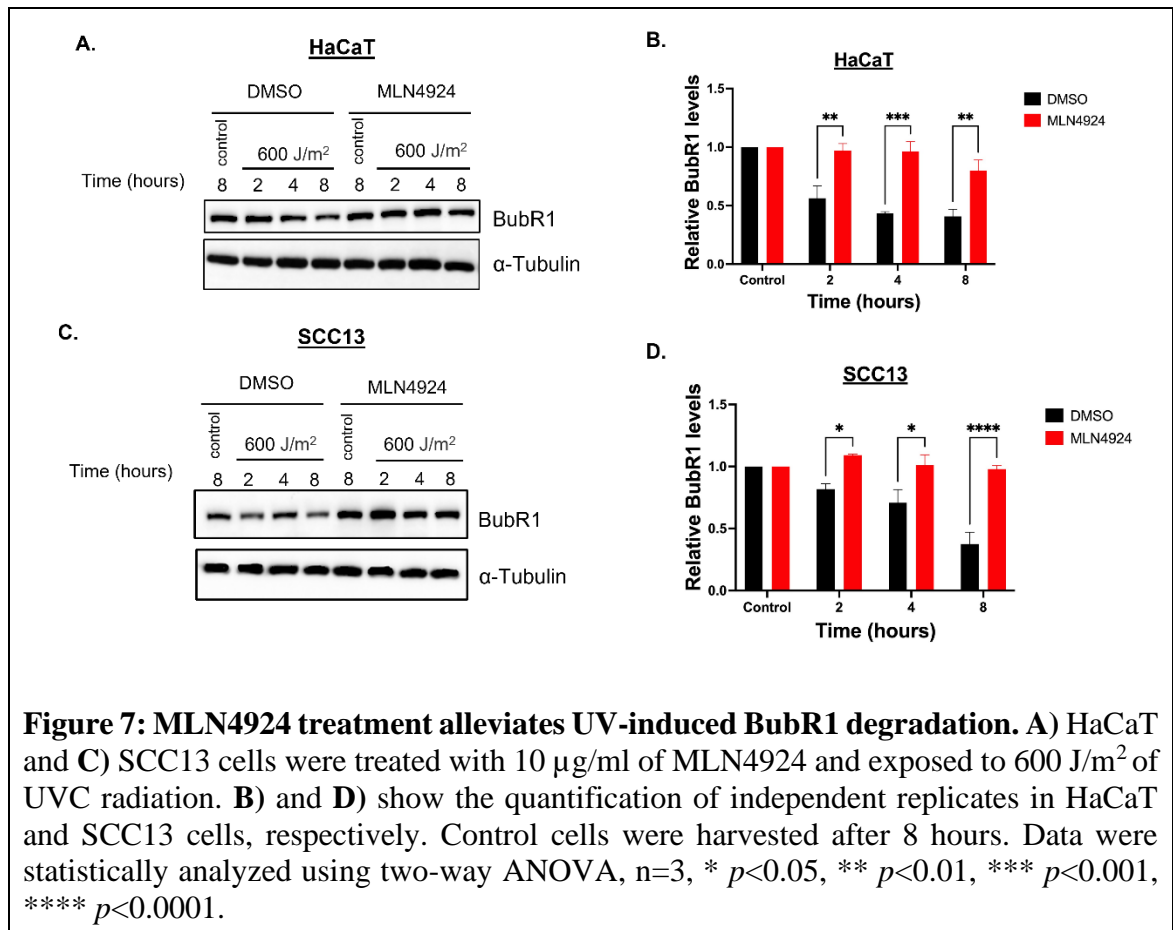
Figure 5: UV radiation induces BubR1 degradation. A) HaCaT and C) SCC13 cells were treated with UV at varying doses and incubated for 8 hours after UV exposure. Quantification of levels of BubR1 based on three independent replicates in B) HaCaT and D) SCC13 cells as seen in Figures 5A and 5C. E-H) BubR1 levels reduce in a time-dependent manner following exposure to 600 J/m² of UVC in both E) HaCaT and G) SCC13 cells. F) and H) show the quantification of independent replicates of Figure 5E and 5G in both HaCaT and SCC13 cells, respectively. Control cells were harvested at the 24 hour time point. Data were statistically analyzed using one-way ANOVA, n=3, (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

HaCaT and SCC13 cells were treated with 600 J/m² of UVC and incubated at different time points (2, 4, and 8 hours) in the presence or absence of MG132, a 26S proteasome inhibitor (Figure 6A). Immunoblotting results showed that the UVC-induced decline in BubR1 was blocked following the inhibition of the proteasome by MG132 (Figure 6B and D), quantification of multiple western blots showed a significant rescue of BubR1 levels in the 4 and 8-hour MG132 treated time groups compared to their time-matched controls (Figure 6C and E). These findings suggest that proteasome-dependent degradation of BubR1 is induced following UV radiation.



Cullin RING E3 ubiquitin ligases play a role in UV-induced BubR1 degradation

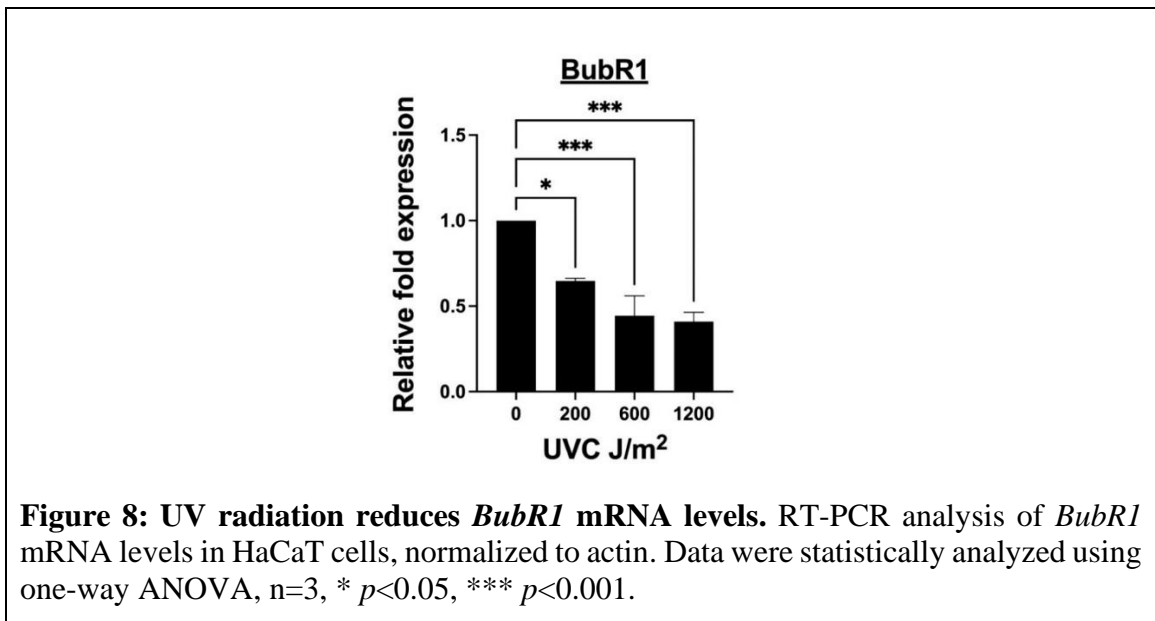
To investigate the role of Cullin RING E3 ubiquitin ligases (CRLs) in the degradation of BubR1 induced by UV radiation, we assessed whether MLN4924 treatment would block BubR1 protein loss following exposure to UVC. MLN4924 is a potent inhibitor of Cullin-RING E3 ubiquitin ligases, exerting its inhibitory effects by blocking CUL1 neddylation, a post-translational protein modification that required for CRL E3 ubiquitin ligase activity⁵⁹. HaCaT and SCC13 cells were pretreated with MLN4924 for 16 hrs, followed by exposure to 600 J/m² of UVC, and subsequently harvested at various time points. As expected, MLN4924 treatment prevented UV-induced loss of BubR1 compared to the time-matched DMSO controls (Figure 7A and C). Quantification of experimental



replicates showed a significant increase in BubR1 levels within the MLN4924-treated group compared to the control group (Figure 7B and D). Together, these results suggest that Cullin-RING E3 ubiquitin ligases regulate the UV-induced reduction of BubR1.

UV exposure results in the reduction of BubR1 mRNA levels

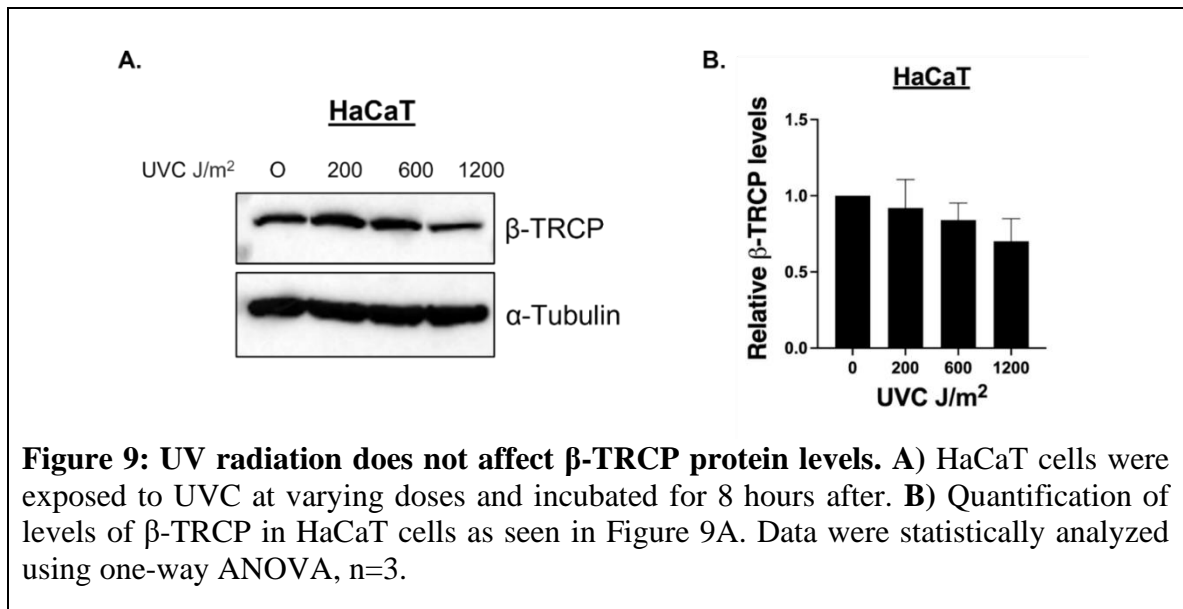
To determine whether UV exposure affected BubR1 at the transcript level, we monitored the mRNA levels of BubR1 following UV radiation. RT-PCR was carried out on HaCaT cells subjected to increasing doses of UVC (200, 600 and 1,200 J/m²) and incubated for 8 hours after UV treatment. RT-PCR results showed a dose-dependent decrease in BubR1 mRNA levels (Figure 8). This data suggests that BubR1 downregulation following UV exposure is regulated at both the transcriptional and post-translational levels.



UV exposure does not significantly decrease β -TRCP protein levels

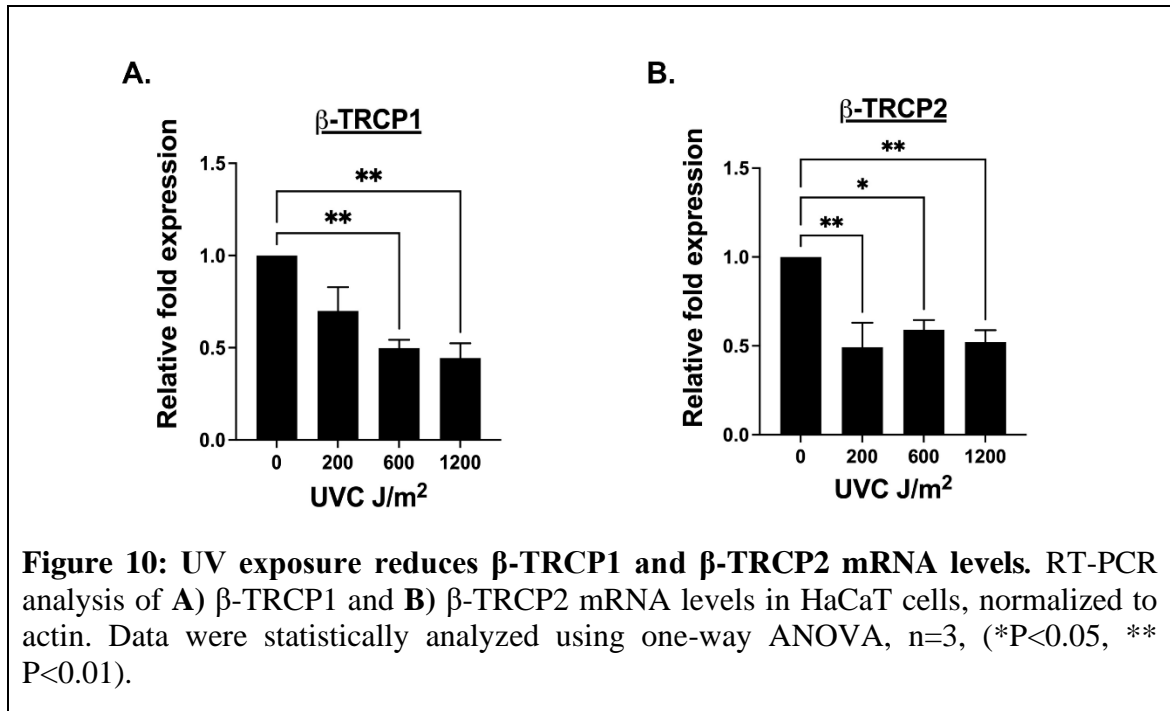
Our discovery that UV radiation diminished BubR1 levels led us to consider the possibility of UV also eliciting an effect on β -TRCP protein levels. HaCaT cells were

subjected to varying doses of UVC (200, 600, and 1,200 J/m²) and incubated for 8 hours after exposure. Immunoblotting revealed a slight reduction in β -TRCP levels following UVC exposure (Figure 9A). However, this reduction did not reach statistical significance when multiple replicates were assessed (Figure 9B), indicating that UV radiation may not substantially affect β -TRCP protein abundance.



UV exposure reduces mRNA levels of β -TRCP1 and β -TRCP2

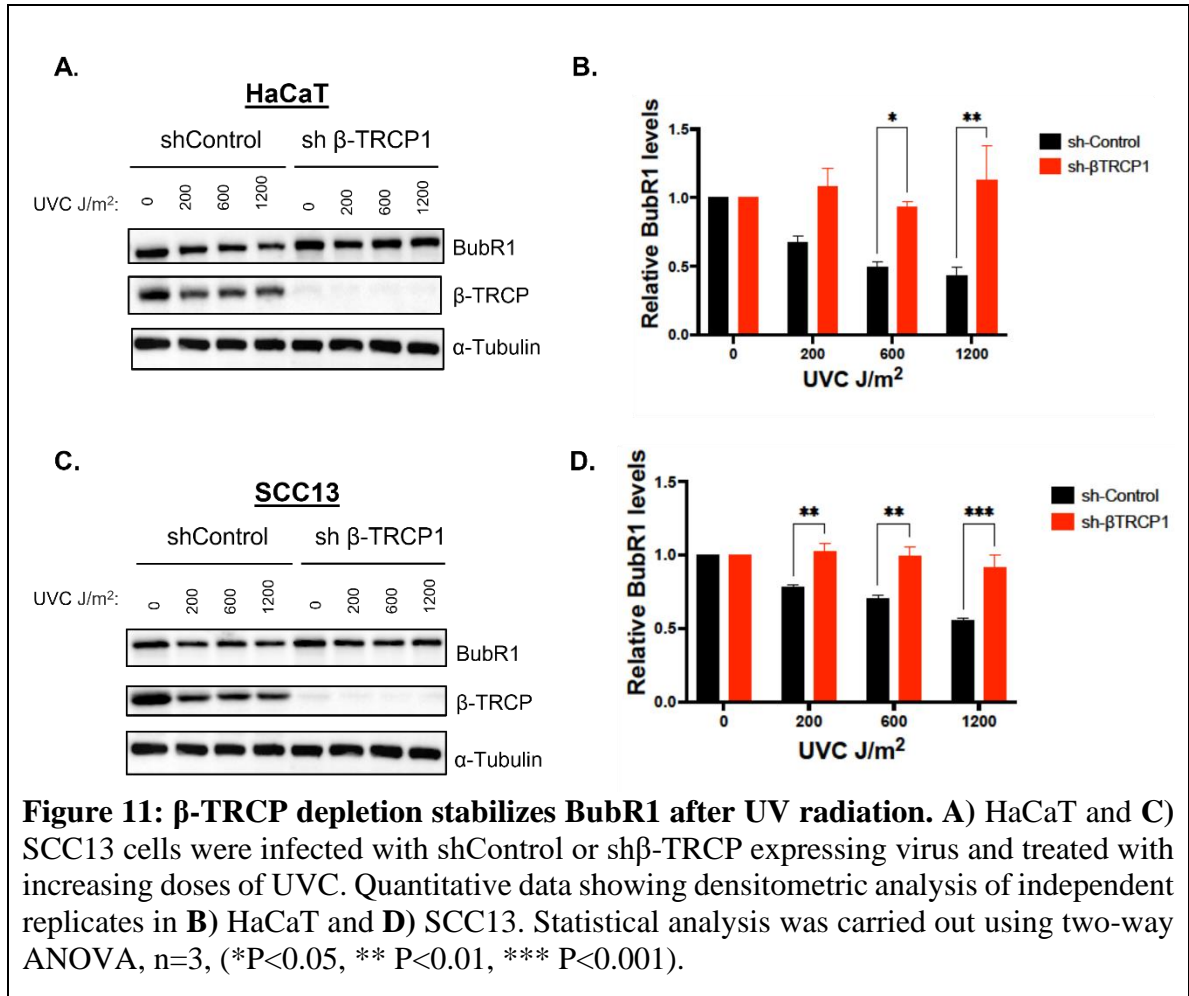
Next, we sought to understand the effect of UV exposure on β -TRCP mRNA levels. HaCaT cells were subjected to increasing doses of UVC (200, 600 and 1,200J/m²) and incubated for 8 hours after UVC treatment. The qPCR analysis showed that mRNA levels of β -TRCP1 and β -TRCP2 were significantly reduced with increasing doses of UV radiation (Figure 10A and B).



β -TRCP1 knockdown stabilizes BubR1 levels in response to UV radiation

Because our previous findings have shown that UV-induced BubR1 downregulation was regulated by Cullin RING E3 ligases, indicating the possibility of SCF (β -TRCP) involvement, we sought to test if β -TRCP1 was involved in controlling BubR1 degradation in response to UVC exposure. We generated HaCaT and SCC13 cells stably expressing shControl and sh β -TRCP1, in which we were able to achieve an approximate 90% depletion of β -TRCP1. Subsequently, we exposed these cells to increasing doses of UVC (200, 600, and 1,200 J/m²). Western blot analysis showed stabilization of BubR1 levels in the cells where β -TRCP1 is depleted compared to the control shRNA expressing cells (Figure 11A and C). Quantification of densitometric analysis of western blots from multiple experiments showed that BubR1 levels were maintained within the sh β -TRCP1

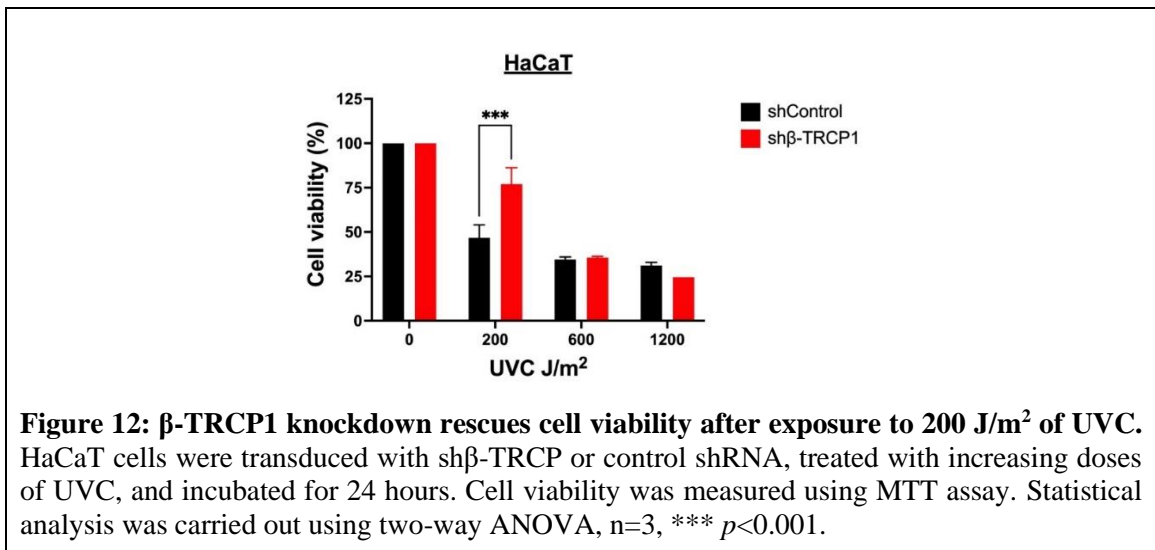
group after UV radiation (Figure 11B and D). Collectively, these results suggest that β -TRCP1 plays a specific role in regulating the UV-induced reduction of BubR1 levels.



β -TRCP1 depletion mediates cell viability stabilization after UV radiation in a dose-dependent manner

UV radiation is well established to induce cell death⁶⁰. Therefore, to understand whether loss of β -TRCP1 will influence cell viability in response to UV exposure, HaCaT cells depleted of β -TRCP1 were subjected to varying doses of UVC, incubated for 24 hours and followed by assessing for cell viability using an MTT assay. Cell viability was

significantly increased in the sh β -TRCP1 group after 200 J/m² of UVC compared to the dose matched cells expressing control shRNA. However, no significant changes in cell viability were seen within the sh β -TRCP1 group compared to the shControl group in the higher dose treatment groups (Figure 12). These results suggested that β -TRCP1 might play a role in promoting cell death following UV radiation at lower doses of UVC.



Chapter 4: Discussion

UV radiation is a major contributor to the generation of non-melanoma skin cancer, the most common type of skin cancer with a rising incidence worldwide. The high recurrence rates, potential for metastasis, and inadequate treatment options highlight the need for continued research on the mechanisms of NMSC tumorigenesis and development of novel treatment strategies to improve patient outcomes. The critical role of BubR1 in maintaining genomic stability, and its natural loss with age, underscores the importance of investigating its potential involvement in skin cancer development and progression, particularly in the context of the damaging effects of UV radiation.

BubR1 is an essential mitotic checkpoint protein that is critical in regulating chromosome segregation and maintaining genomic stability. Reduced BubR1 abundance caused by mutations has been shown to promote aneuploidy, a shortened lifespan, and the development of premature aging phenotypes, and cancer^{36,39,40}. On the other hand, BubR1 overexpression corrects mitotic defects, delays age-related deterioration in several tissues, and protects against³⁷cancer. Our studies demonstrate that UV radiation induces a decline in BubR1 protein which is attributed to a combined influence of β -TRCP-mediated proteasome-dependent BubR1 protein degradation and a reduction in mRNA levels.

Our first aim was to investigate how BubR1 levels are affected by UV exposure. Our preliminary findings show that following dose- and time-dependent exposure of SCC13 and HaCaT cells to UVC BubR1 protein levels are significantly downregulated. One potential implication is that the downregulation of BubR1 by UV radiation may contribute to the development of NMSC by increasing chromosomal instability and

aneuploidy⁶¹. This is consistent with previous studies that have linked BubR1 dysregulation to various cancers.

Given that the abundance of BubR1 significantly reduces after UV exposure, it was essential to determine the potential role of the ubiquitin-proteasome system in the observed BubR1 loss. Contrary to the findings of *Fang et al.*, which showed that MG132 did not prevent BubR1 degradation in HeLa cells treated with doxorubicin, which is known to induce DNA damage, we observed that MG132 caused a partial, but not complete recovery of BubR1 levels in HaCaT and SCC13 cells⁴⁰. These findings suggest that the MG132-mediated rescue of BubR1 levels may depend on the type or source of DNA damage or cell type. The partial rescue of BubR1 levels by MG132 treatment may also indicate the involvement of multiple mechanisms in regulating BubR1 levels following UV-induced damage, such as the activation of autophagy or the inhibition of protein synthesis. Additional studies are needed to more clearly define the mechanisms involved in the loss of BubR1 caused by UV exposure and other forms of DNA damage-inducing methods.

Cullin RING E3 ubiquitin ligases (CRLs) facilitate the transfer of ubiquitin molecules to target proteins, resulting in either altered protein function or protein degradation by the 26S proteasome. Previous studies have shown that inhibition of neddylation, a post-translational protein modification, by MLN4924, led to the accumulation of CRL substrates due to the inhibition of CRL activation⁶². Therefore, we conducted timed experiments to understand whether Cullin RING E3 ubiquitin ligases are involved in the downregulation of BubR1 after UVC treatment. Our findings revealed that MLN4924 led to a rescue of BubR1 levels following UVC exposure, confirming our hypothesis that CRLs played a role in mediating the degradation of BubR1 by the 26S

proteasome. Given that the inhibition of neddylation by MLN4924 has been investigated as a possible treatment strategy for cancer and other conditions involving CRL dysregulation, additional studies are necessary to determine whether the observed rescue of BubR1 by MLN4924 could be a contributing factor to its effectiveness in cancer treatment⁶³.

While our results implicate the ubiquitin-proteasome pathway in the UV-induced downregulation of BubR1, we further investigated if BubR1 downregulation is also controlled at the transcriptional level. To this end, we monitored mRNA levels of BubR1 in HaCaT cells subjected to increasing doses of UVC. RT-PCR analysis revealed a clear decrease in BubR1 mRNA levels in a dose-dependent manner. These findings suggest that UV-induced BubR1 decrease is likely regulated at both the transcript as well as post-translational levels, which likely involves the coordinated action of multiple pathways. These findings may also hint at the reason for the partial rescue of BubR1 protein levels after MG132 treatment. Future studies could focus on identifying these mechanisms and their respective contributions to BubR1 downregulation following UV treatment. The reduction of BubR1 mRNA levels may be due to different mechanisms including downregulation of transcription, increased mRNA degradation, degradation or inhibition of transcription factors, and DNA damage response that lead to changes in gene expression and alteration of mRNA stability. Further studies are needed to understand the exact mechanism of UV-induced downregulation of BubR1 mRNA.

β -TRCP has been previously shown to function as a substrate recognition subunit for the SCF subclass of CRLs, which targets proteins for ubiquitination and proteasomal degradation⁶⁴. β -TRCP recognizes and binds to a phospho-degron motif present in its

substrates⁴⁸. Unpublished data from our lab show that this motif has also present in BubR1. The observation of reduced BubR1 levels upon exposure to UV radiation and the rescue of its abundance following CRL inhibition with MLN4924 prompted us to investigate whether there was any potential impact of UV exposure on the abundance of β -TRCP protein and whether β -TRCP is involved in the UV-induced loss of BubR1. Similar to BubR1, we observed a trend towards a decrease in β -TRCP protein levels following UV exposure. However, this trend did not reach statistical significance. Since β -TRCP plays a crucial function in controlling substrate protein stability through its role as a substrate recognition subunit of the SCF complex to facilitate ubiquitination and subsequent degradation⁴⁹, we hypothesized that β -TRCP may be involved in the UV-induced BubR1 downregulation. We generated stable cell lines expressing shRNA targeting GFP or β -TRCP1 which allowed for the examination of the effects of β -TRCP1 depletion on BubR1 levels following UV radiation. Our results showed that the decrease in BubR1 levels induced by UV radiation was prevented when β -TRCP1 was depleted, indicating a critical role of β -TRCP1 in regulating the response to UV-induced stress on BubR1 protein levels.

Given that β -TRCP protein levels also show a trend towards decreasing following UV exposure, this raises the conundrum as to how β -TRCP is regulating BubR1 under these conditions. One possibility is that even if β -TRCP protein levels are falling, the recognition of BubR1 by β -TRCP increases in response to UV exposure. While our data suggest that β -TRCP may act as the substrate recognition subunit in the proteasome-mediated degradation of BubR1, further studies are necessary to define whether there is an increase in phosphorylation of the putative β -TRCP degron domain in BubR1 following UV exposure to increase binding and ubiquitination of BubR1 by β -TRCP.

β -TRCP has two distinct paralogs expressed in mammals, β -TRCP1 and β -TRCP2. Previous studies showed that a deletion of β -TRCP1 in male and female mice had little to no effect on their development, but a deletion of β -TRCP2 in mice was embryonically lethal, suggesting that the continued expression of β -TRCP2 may compensate for the lack of β -TRCP1, leading to the conclusion that β -TRCP1 and β -TRCP2 were partially functionally redundant^{49,65,66}. Recent studies have shown an intricate interplay between β -TRCP1 and β -TRCP2 in various cellular processes, including cell cycle progression and DNA damage response, where β -TRCP1 and β -TRCP2 target each other for degradation^{48,53,65}. This mutual regulation between the two isoforms suggests a complex regulatory mechanism that maintains the balance of β -TRCP activity within cells. Our real-time RT-PCR analysis of β -TRCP1 and β -TRCP2 mRNA levels after UV exposure revealed a significant reduction in the gene expression of both paralogs, contrary to the findings of *Islam et al.*, which showed that β -TRCP1 and β -TRCP2 mRNA levels were not altered following genotoxic stress induced by ionizing radiation⁵³. Several factors could account for this discrepancy. The response of cells to different types of stress could vary depending on the type and intensity of the stress, as well as the cellular context. Additionally, differences in the experimental conditions, such as the duration and intensity of the stress or the cell line used, could also contribute to this variation in results. Moreover, it is crucial to consider that the regulation of β -TRCP1 and β -TRCP2 expression could be complex and involve multiple signaling pathways. Therefore, the effect of genotoxic stress on β -TRCP1 and β -TRCP2 expression could be context-dependent and influenced by various factors.

We also observed that while β -TRCP mRNA levels decrease upon UV exposure, that the proteins abundance does not change to the same degree. One explanation for the discrepancy is that β -TRCP mRNA destabilization may be increased by UV radiation, whereas the protein may remain stabilized due to post-translational regulation. Another possibility is that there may be a delay between the decrease in mRNA levels and the decline in protein levels, as the half-life of β -TRCP protein may be longer or controlled differently in various cellular environments. In this context, β -TRCP protein levels may be governed by other mechanisms that are not influenced by UV radiation, such as protein-protein interactions or post-translational modifications. Additional research is needed to fully comprehend the mechanisms controlling the expression of β -TRCP1 and β -TRCP2 in response to various stress and the underlying mechanisms regulating β -TRCP following UV radiation exposure.

These results provide new insights into the cellular mechanisms involved in response to UV radiation and highlight the importance of β -TRCP1 in this process. Given prior studies assessing the anti-cancer effects of increased BubR1 levels in the skin³⁷, our studies suggest that the ability to regulate BubR1 levels through β -TRCP1 inhibition could potentially have significant implications for developing new therapies for conditions of the skin resulting from UV radiation exposure.

Despite the observed rescue of BubR1 protein levels following depletion of β -TRCP1 after UV exposure, a complete restoration was not achieved, suggesting two potential explanations. First, the presence of approximately 10% of β -TRCP1 protein remaining may still be adequate to mediate BubR1 degradation after UV treatment.

Alternatively, other mechanisms, such as regulation of BubR1 transcript levels, may be involved in controlling BubR1 degradation in response to UV exposure.

Identifying β -TRCP1 as a regulator of BubR1 stability suggests that targeting β -TRCP could be a potential therapeutic strategy for preventing or treating non-melanoma skin cancer. However, further research is required to fully understand the mechanisms underlying the regulation of BubR1 by β -TRCP1 and its potential as a therapeutic target. It is also essential to understand if β -TRCP2 also plays a role in regulating BubR1 after UV-induced damage, in addition defining other possible mechanisms involved in regulating BubR1 abundance after UV exposure.

UV radiation has been shown to negatively affect cell viability, and the proper regulation of BubR1 expression and function is essential for maintaining cell viability and genomic stability^{60,67}. Given our prior results showing depletion of β -TRCP1 led to a rescue in BubR1 levels following UV exposure, we reasoned that β -TRCP1 depletion would improve cell viability after UV treatment, potentially by increasing the levels of BubR1. Results from our cell viability assay showed an increase in cell survival in the sh β -TRCP1 group when subjected to 200 J/m² of UVC. However, there were no significant changes in cell viability between the sh β -TRCP1 and shControl groups in the higher dose treatment groups. We speculate that the lack of rescue in cell viability within the treatment groups subjected to higher doses of UVC was due to the irreversibility of the cell death caused by higher doses at the longer incubation time. This is supported by the observation that increasing the dose from 600 to 1200 J/m² did not result in a further decrease in cell viability indicating that these higher doses were likely inducing maximal cell death for the incubation time post UV exposure. However, the observed increase in cell viability in the

sh β -TRCP1 group following exposure to a lower dose of UVC suggests that β -TRCP1 may play a role in cell fate decisions under intermediate UV exposure. It would be important to determine whether this effect of β -TRCP1 depletion on cell viability is dose-dependent. Our results also suggest that assessing this UVC dose-response at shorter time course could enhance our understanding of the interrelationship between β -TRCP activity and cell viability in relation to genotoxic stress. Identifying other proteins that β -TRCP targets in response to UV exposure in addition to BubR1 will also be necessary to provide further insight into its role in regulating the cellular response to DNA damage caused by UV exposure.

This study focused on short-term dose- and time-dependent UV experiments on human skin cell lines; validating these findings in mouse and human primary keratinocytes as well as animal models will be necessary to enhance our understanding of the role of β -TRCP and BubR1 in regulating cellular response to UV exposure. Additionally, it will be important to investigate the downstream effects of BubR1 downregulation and β -TRCP inhibition on cell proliferation, apoptosis, and DNA damage response in the context of UV-induced skin cancer.

In conclusion, the downregulation of BubR1 following UV exposure is mediated by both transcriptional and post-translational mechanisms involving β -TRCP and the 26S proteasome. This study provides important insights into the molecular mechanisms underlying the development of non-melanoma skin cancer and identifies potential targets for therapeutic intervention.

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