THE UNIVERSITY OF CHICAGO

AUTOPHAGY-INDEPENDENT REGULATION AND FUNCTION OF AUTOPHAGY ADAPTOR PROTEIN P62

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE DIVISION OF THE BIOLOGICAL SCIENCES
AND THE PRITZKER SCHOOL OF MEDICINE
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

COMMITTEE ON CANCER BIOLOGY

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CHICAGO, ILLINOIS
AUGUST 2017

To my grandmothers Sherry McClure and Vickie Sample.

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ACKNOWLEDGMENTS

First, I would like to acknowledge my incredibly supportive mentor, Dr. Yu-Ying He, who has been the best PI a grad student could hope for. Additionally, I would like to thank the He lab members who were key to competing my thesis work: Dr. Lei Qiang, who taught me so much about how to do science; the helpful and hilarious Palak Shah; Seungwon Yang, Yu Deng, Sixia Xiao, and Baozhong Zhao, who did the preliminary screening for p62 in UVA response. The other members of the Section of Dermatology, particularly Dr. Keyoumars Soltani and Dr. Deborah Lang, who have been unfailingly supportive. I would also like to acknowledge committee members Dr. Lev Becker who helpfully shared techniques with me, Dr. Xiaoyang Wu, and Dr. Seungmin Hwang.

Many thanks to the faculty and students of the Committee on Cancer Biology, CCB chairs Dr. Kay Macleod and Dr. Geoff Greene, and the many funding sources that have supported my work. Additional thanks to the administrators who work tirelessly behind the scenes in the Biomed Cluster office-Emily Traw, Nadja Otikor, Helen Polan, Lisa Abston-Leftridge and Liz Cartwright in Dermatology.

To the students and faculty of the Committee on Cancer Biology. My dear friends, classmates, and colleagues in suffering Maya Springer, Yuanyuan Li, John Cao, Lindsey Ludwig, Anna Chen, Hannah Brechka, and my beautiful tropical fish Kaitlin Perry.

To the many many people who have been essential in guiding me to this point: career advisors and my mentors Drs. Abby Stayart and Briana Konnick. To the people who taught me science in the first place- Drs. Xiaoting Wu, Chotirat Rattanasinchai, Chengfeng Yang and Zhishan Wang. To Dr. Kathy Gallo, who took a bet on me in the first place. Most of all, I would like to thank Eva Miller, who helped me more than anyone to shape me into the scientist I am today. Also, to the great teachers-Mr. Philip Loewen, Mrs. Regina Hicks, Mrs. Linda Wise, and Mrs. Linda Ford who were instrumental in getting me to this point.

I also need to thank my family, I could never have done it without them. To my parents, Kenny Sample and Tammy Wiser, my step families the Wisers and the Allisons, my little

brothers Isaac and Zach, my cats, and my dog brother Mr. Beefy. To the person who helped me through every day, Bryce Lanham. Finally, I would like to thank my grandparents. To Dave and Sherry McClure who worked so hard every day to ensure that I succeeded. To Kenny and Vickie Sample, who have been unfailingly loving and supportive my entire life.

ABBREVIATIONS

UV: ultraviolet; NMSC: non-melanoma skin cancer; BCC: basal cell carcinoma; SCC: squamous cell carcinoma; ROS: Reactive oxygen species; DNA: deoxyribonucleic acids; MITF: microphthalmia-associated transcription factor; TFEB: transcription factor EB; CLEAR: coordinated lysosomal expression and regulation; mTORC1: mammalian target of rapamycin complex 1; AMP: Adenosine monophosphate; AMPK: 5' AMP-activated protein kinase; LKB1: liver kinase B1; ATP: Adenosine triphosphate; TSC1/2: tuberous sclerosis complexes 1 and 2; GTP: guanosine triphosphate; PI3K: phosphatidylinositol-3 kinase; AKT: protein kinase B; PTEN: phosphatase and tensin homolog; PIP: phosphatidyl inositol phosphate; ATM: ataxia telangiectasia mutated; ATR: ataxia telangiectasia and Rad3-related; ULK1/2: Unc-like kinase 1/2; ATG: autophagy-related gene; FIP200: focal adhesion kinase family-interacting protein of 200 kD; UVRAG: UV radiation resistance-associated gene; VPS34: class III PI3-kinase; BCL: B-cell lymphoma 2; BH3: BCL-2 homology domain; LC3: Microtubule-associated protein 1A/1B-light chain 3; PE: phosphatidylethanolamine; LIR: LC3-interacting region; UBA: ubiquitin-associated domain; NF-κB: Nuclear factor κB; TNF: tumor necrosis factor; TRAF6: TNF receptor associated factor 6; RIP: receptor-interacting protein; PB1: phox and Bhem1 domain; ZZ: zinc finger domain; KIR: Keap1-interacting region; TBS: TRAF6-binding domain; ER: endoplasmic reticulum; MEK: mitogen-activated protein kinase kinase; LOH: Loss-of-heterozygosity; MCL-1: induced myeloid leukemia cell differentiation protein Mcl-1; CPD: cyclobutane pyrimidine dimers; 6-4PP: pyrimidine-(6-4)-pyrimidone photoproducts; TC-NER: transcription-coupled nucleotide excision repair; GG-NER: global genome nucleotide excision repair; BER: base-excision repair; RNA: ribonucleic acid; XP: Xeroderma Pigmentosum; DDB2: damage-specific DNA-binding protein 2; TFIIH: transcription IIH; RPA: replication protein A; PCNA: proliferating cell nuclear antigen; ssDNA: single-stranded DNA; Chk1: checkpoint kinase 1; DSB: double-stranded break; E2F4/RBL2: transcriptional repressor complex; PUVA: psoralen and UVA therapy; 8-oxo-dG: 8-oxo-2'deoxyguanosine; hOGG1: human 8-oxo-guanine glycosylase 1;

RNS: reactive nitrogen species; MC1R: melanocortin 1 receptor; H2O2: hydrogen peroxide; NRF2: nuclear factor (erythroid-derived 2)-like 2; KEAP1: Kelch-like ECH-associated protein 1; ARE: antioxidant response element; MMP: matrix metalloproteinase; NAC: N-acetylcysteine; RhoA: ras homolog gene family, member A; Ccr2: C-C motif chemokine receptor type 2; ATF2: activating transcription factor 2; CCL8: chemokine (C-C motif) ligand 8; IL: interleukin; γH2AX: histone H2A; IFN-γ: interferon gamma; PD1: programmed cell death 1; PD-L1: programmed cell death ligand 1; TIL: tumor-infiltrating lymphocytes; COX-2: cyclooxygenase-2; PGE_2: prostaglandin E2; TP53: tumor protein 53; RAC1: Ras-related C3 botulinum toxin substrate 1; alpha-MSH: alpha-melanocyte-stimulating hormone; ROCK: rho-associated protein kinase; AICAR: 5-aminoimidazole-4-carboxamide ribonucleotide; HIF1α: hypoxia-inducible factor 1α; EMT: epithelial-mesenchymal transition; ERK: extracellular signal-related kinase; SPF: sun protection factor; NHEK: normal human epidermal keratinocytes; NHEM: normal human epidermal melanocytes; MEF: mouse embryonic fibroblasts; siRNA: small interfering RNA; siNC/siCon: control siRNA; shRNA: small hairpin RNA; iBMK: immortalized mouse baby kidney cells; HRP: horseradish peroxidase; RNA-Seq: RNA sequencing; DAVID: Database for Annotation, Visualization and Integrated Discovery; qPCR: quantitative polymerase chain reaction; CT: threshold cycle number; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; ChIP: chromatin immunoprecipitation; GSH: glutathione; GSSG: glutathione disulfide; MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; SKH1: immunocompetent hairless mouse; BALB/c: immunocompetent albino mouse; NaN3: sodium azide; mito-CP: mitochondria-targeted cationic carboxyproxyl nitroxide; ERAD: ER-associated degradation

ABSTRACT

Skin cancer is the most common form of cancer, representing 40-50% of all cancers diagnosed. Exposure to ultraviolet (UV) radiation, namely UVA (315-400nm) and UVB (280-315nm) is the major risk factor for skin cancer development. Of these, UVA is more abundant in both sunlight and tanning beds. UVA is significantly less effective in causing direct DNA damage than UVB, but UVA has been shown to increase skin cancer risk through producing reactive oxygen species (ROS). However, the mechanism by which UVA contributes to skin cancer progression remains unclear. Autophagy adaptor protein p62 is a known oncogene and signaling hub. We and others have shown that p62 is up-regulated in melanoma and squamous cell carcinoma (SCC) and that this up-regulation correlates with poor prognosis. Here, we show that UVA induces p62 in both melanocytes and keratinocytes by different mechanisms. In melanocytes and melanoma cells, p62 is up-regulated by UVA in an Nrf2- and ROS-dependent manner. We also identified a novel regulatory feedback loop between p62 and PTEN in melanoma cells. In keratinocytes, however, UVA activates the transcription factor EB (TFEB), a known regulator of autophagy and lysosomal gene expression, to induce p62 transcription. Next, we identified a novel link between p62 and cyclooxygenase-2 (COX-2) in SCC cells. COX-2 expression was up-regulated by UVA-induced p62 in SCC cells, suggesting that p62 plays a role in UVA-induced SCC. Moreover, we found that p62 stabilizes COX-2 protein through the p62 ubiquitin-associated domain and that p62 regulates prostaglandin E2 (PGE_2) production *in vitro*. In a syngeneic squamous cell carcinoma mouse model, p62 knockdown inhibited tumor growth and metastasis. Furthermore, p62-deficient tumors exhibited reduced immune cell infiltration and increased cell differentiation. As PGE_2 is known to promote pro-tumorigenic immune cell infiltration, increase proliferation, and inhibit keratinocyte differentiation *in vivo*, this work suggests that UVA-induced p62 acts through COX-2 to promote skin tumor growth and progression. These findings expand our understanding of UVA-induced skin tumorigenesis and tumor progression and suggest that targeting p62 can help prevent or treat UVA-associated skin cancer.

BACKGROUND AND SIGNIFICANCE

Ultraviolet (UV) Radiation Exposure Causes Skin Cancer

Skin cancer is the most common form of cancer, representing 40-50% of all cancers diagnosed in the US. Approximately 3.5 million cases of skin cancer are diagnosed each year in the US alone[1, 2]. Skin cancer causes more than 20,000 deaths each year in the United States, and treatment costs US $8.1 billion annually[3]. Worldwide, incidence of skin cancer is rapidly rising each year[1, 4], increasing the number of people for whom skin cancer will become a costly and potentially deadly disease.[1]

Skin cancers are broadly classified into two major types by the cell type of origin: melanoma and nonmelanoma skin cancer (NMSC). NMSC consists of two major types: in squamous cell carcinoma (SCC) and basal cell carcinoma (BCC). BCC is the most common type of skin cancer, representing 80% of skin cancer cases[5]. BCC accounts for approximately 3,000 deaths each year in the United States and can be disfiguring[5]. SCC represents accounts for approximately 16% of skin cancers[5] and has potential to metastasize[6]. An estimated 9,000 Americans die each year of SCC[6].

Melanoma is the least common, but most aggressive form of skin cancer. Despite accounting for only 4% of skin cancer cases, melanoma is responsible for nearly half of all skin cancer deaths[3, 7]. In 2017, it is expected that nearly 90,000 cases of melanoma will be diagnosed in the US, leading to nearly 10,000 melanoma-related deaths[8]. Recent advancements in targeted therapy (vemurafenib) and immunotherapy (pembrolizumab) for melanoma have offered improvements in survival to some patients, but most patients fail to have a sustained response[9]. Furthermore, estimates predict the death rate for melanoma to fall, but the number of deaths will continue to increase unless more effective therapies are developed[10].

Ultraviolet (UV) radiation exposure is the major risk factor for both NMSC and melanoma.

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An estimated 90% of NMSCs and 60-70% of cutaneous malignant melanomas are thought to be caused by UV radiation exposure[11, 12]. A number of factors drive UV response and consequently risk of skin cancer, however much remains to be understood about the effects of UV radiation, its roles in skin cancer, and its relationship with the other factors involved in skin damage.

Exposure to ultraviolet (UV) radiation through sunlight and indoor tanning beds is the major risk factor for skin cancer development and skin photoaging. UV radiation is divided into three major types by wavelength: UVA (315-400 nm), UVB (280-315 nm), and UVC (100-280 nm). Of these, UVA is the most abundant, as it accounts for about 95% of solar UV radiation, and indoor tanning beds emit UVA at doses up 12-fold higher than the sun[13]. UVB accounts for the remaining 5% of solar UV radiation, while UVC is filtered out by the ozone[14].

Absorption of UV radiation, and consequently the extent of skin damage, is dependent on a number of factors, including skin color/type, time of exposure, latitude, altitude, season, and wavelength[15]. UVA penetrates deep into the dermis[16], while UVB reaches only the epidermis[17]. However, UVA is much less efficient in causing direct DNA damage than UVB, instead inducing oxidative DNA damage through reactive oxygen species (ROS)[18]. Both UVA and UVB have been shown to cause cell proliferation, growth arrest, and apoptosis, although these responses can be highly context-dependent. The known effects of UVA and UVB on skin will be discussed here. UVC, although the most mutagenic type of UV radiation[19], will not be discussed here, as it is not a major relevant source of skin-damaging radiation.

Genetics and Skin Color Affect UV Response

Genetics Affect UV Response

Skin cancer risk is associated with familial and somatic mutations. Xeroderma pigmen-

tosum (XP) is an autosomal recessive disorder caused by mutations in XP genes involved in DNA damage repair. XP patients have a 10,000-fold increased susceptibility to UV-induced NMSC and 2,000-fold increased risk of melanoma[20]. XP patients develop skin cancer 50 years younger than the general population.

Melanoma has one of the highest rates of mutation of any cancer[21]. Approximately 3-15% of melanomas arise due to familial genetic predisposition, in which UV-independent mutations play a significant role[22]. Germline mutations in Cyclin Dependent Kinase Inhibitor 2A (CDKN2A, also p16-INK4A-ARF), while rare, correlate significantly with the development of melanoma[23]. Other key somatic mutations in melanoma are UV-independent, including the $BRAF^{V600E}$ mutation found in 60% of melanomas and NRAS mutations found in 15-20% of melanomas[24]. While these mutations are not UV-signature mutations[25], they are more common in sun-exposed skin[25, 26, 27, 28].

$BRAF^{V600E}$ mutation alone is often insufficient to drive malignant transformation of melanocytes[29]. Acquired mutations due to UV exposure can synergize with mutant BRAF to drive transformation. In mice with melanocyte-specific $BRAF^{V600E}$ mutations, UV exposure accelerates melanomagenesis[30]. 40% of the resulting tumors developed UV-signature p53 mutations, which further accelerated UV-induced melanomagenesis[30]. Similar UV-induced p53 mutations are seen in approximately 20% of human $BRAF^{V600E}$ mutant melanomas[30, 31, 32]. $BRAF^{V600E}$ mutation in melanocytes can also synergize with Arf deletion *in vivo* to accelerate UV-induced melanoma development[33].

Recent work indicates that UV-induced mutations accumulate as melanocytic nevi transform into melanoma, including driver mutations in CDKN2A, TP53, neurofibromatosis type 1 (NF1), Ras-related C3 botulinum toxin substrate 1 (RAC1), and phosphatase and tensin homolog (PTEN)[30, 34]. One study linked UV-induced DNA damage signatures to approximately 46% of driver mutations[31]. Melanomas from UV-exposed areas exhibited higher mutation load than melanomas developing in protected areas[32]. Whole-genome sequencing of melanoma patients has identified a number of additional mutations that are significant to

melanoma development, many of which can be linked to UV-induced DNA damage.

Skin Color Affects UV Response

Melanocytes produce two types of melanin: eumelanin and pheomelanin. Eumelanin is the most common type in dark skin and dark hair, and is synthesized upon binding of α-melanocyte-stimulating hormone (αMSH) to melanocortin-1 receptor (MC1R)[35]. In individuals with red hair and freckles, a loss-of-function mutation in MC1R prevents eumelanin production, leading to a higher proportion of pheomelanin[36]. Eumelanin reduces the accumulation of UV-induced photoproducts[37], while pheomelanin may actually contribute to UV-induced DNA damage by inducing free radical formation after UV[37]. Total melanin levels dictate UV response in melanocytes, independent of MC1R signaling. Higher melanin levels correlate with reduced UV-induced photoproduct formation, proliferation, and apoptosis independent of MC1R function in melanocytes[38, 39].

Signaling through αMSH and MC1R suppresses melanomagenesis by modulating UV radiation response. In melanoma patients, loss-of-function mutations in MC1R are linked to enhanced sensitivity to UV-induced cytotoxicity and increased incidence of melanoma, largely independent of skin type or hair color[40]. A recent meta-analysis of MC1R variants and melanoma risk showed that most variants increased risk and were associated with red hair and fair skin, but two were associated with melanoma risk independent of red hair or fair skin[37, 41].

UVB radiation is capable of regulating MC1R signaling and pigmentation in melanocytes (Figure 1). UVB induces expression of a number of pigmentation-related genes in melanocytes [42], including αMSH and MC1R expression [35]. UVB activates expression of oxidative and ER stress response genes downstream of MC1R, although this is lost in cells expressing non-functional mutant MC1R [43]. UVB also induces the interaction of wild-type MC1R with PTEN, stabilizing PTEN and inhibiting phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT) signaling. In MC1R mutant cell lines, this interaction is lost and increased

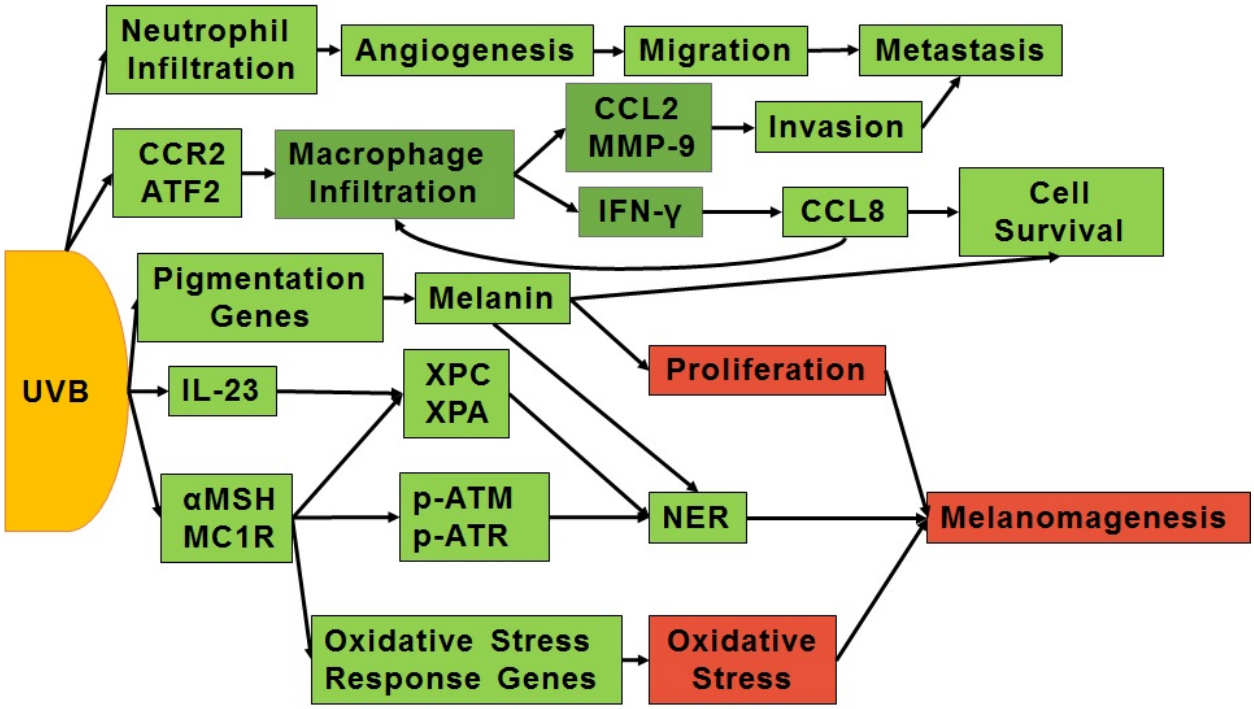


Figure 1: **Mechanisms of UVB Response in Melanoma.**
UVB exposure triggers macrophage and neutrophil infiltration into the skin. Up-regulation of CCR2 and ATF2 in melanocytes promotes recruitment of macrophages into the skin, which in turn stimulates production of CCL2, MMP-9, and IFN-γ in macrophages. IFN-γ signaling from macrophages promotes a positive feedback loop between melanocytes and macrophages, in which melanocytes up-regulate CCL8, a CCR2 ligand, and promote further recruitment of macrophages. The inflammatory response created by macrophage and neutrophil recruitment promotes angiogenesis, as well as melanoma cell invasion, survival, and metastasis. UVB also independently regulates melanin production and MC1R signaling. Induction of pigmentation genes and subsequent increase in melanin production following UVB increases cell survival and NER, but decreases proliferation and ultimately, melanomagenesis. Signaling through MC1R is induced by UVB and activates DNA damage response. Signaling through MSH and MC1R promotes phosphorylation of ATM and ATR, upregulates XPC, and promotes XPA recruitment to stimulate NER. αMSH also activates oxidative stress response genes to reduce oxidative stress in melanocytes/melanoma. UVB-induced expression of IL-23 also activates XPC and XPA to induce NER. IL-23 signaling, melanin production, and MC1R signaling can all inhibit melanomagenesis induced by UVB.

PI3K/AKT signaling drives transformation of $BRAF^{V600E}$ mutant melanoma cells[44]. *In vivo*, however, UVB exposure accelerates melanomagenesis independent of MC1R mutation status and pigmentation [45], suggesting that UVB-induced melanomagenesis does not require the pigmentation and MC1R signaling response. Conversely, UVA does not induce a pigmentation response[42], but may require pigmentation to induce melanomagenesis[46].

Downstream of MC1R, cyclic AMP (cAMP) signaling activates melanoma-associated transcription factor (MITF)[35]. MITF is a master regulator of melanocyte differentiation required for melanocyte survival[47, 48, 49]. MITF controls UVB-induced expression of pigmentation genes[35] and DNA repair and proliferation genes in melanoma cells[50, 51]. Deletion of MITF in melanoma cells is sufficient to increase metastasis, concurrent with increases in mesenchymal markers[52] and ROCK-mediated invasion[53]. MITF overexpression promotes proliferation *in vitro*[47] and *in vivo*[52, 54], in addition to reducing metastasis[52]. Amplification of MITF occurs in up to 20% of all melanomas, with a higher incidence in metastatic melanoma and in BRAF mutant melanomas[47]. MITF protein expression is suppressed by $BRAF^{V600E}$ in melanocytes and melanomas, however, which allows cell proliferation[47, 55]. It is postulated that MITF amplification serves to maintain minimal MITF levels even in the presence of BRAF-mediated suppression for cells to survive the stresses of disease progression.

Autophagy Regulates UV Radiation Response

Macroautophagy (hereafter autophagy) is an essential, homeostatic cellular process of self-eating. Through this process, cells clear unwanted or damaged proteins, lipids and other cellular components, and in doing so regulate the availability of a number of cell signaling factors. Furthermore, autophagy-mediated recycling of cytoplasmic contents facilitates cell survival and adaptation during starvation, genotoxic stress and oxidative stress in normal cells[56]. Autophagy can also provide nutrients to sustain high rates of proliferation in times of growth. Dysregulation of autophagy can therefore contribute to the development of a

number of skin diseases, including skin cancer. UVA, UVB and UVC have all been reported to induce autophagosome formation and upregulation of autophagy markers[57, 58, 59]. However, given the varying effects of UV radiation, it is likely autophagy plays a number of context-dependent roles in UV response.

Mechanisms of Autophagy

Genotoxic stress can be induced by a number of pharmacological and environmental factors, including UV radiation. Genotoxic stress induces autophagy to mitigate the effects of DNA damage[60]. Defects in autophagy are associated with increased DNA damage, gene amplification and aneuploidy[61]. These effects are likely due to insufficient metabolic precursors in the absence of autophagy[61]. Genotoxic stress regulates autophagy at least in part through the stabilization of p53, which regulates the transcription of various autophagy and lysosomal genes[62].

One autophagy regulator is the MITF family member transcription factor EB (TFEB)[63, 64, 65]. TFEB is a basic helixloophelix leucine zipper transcription factor, which binds to the Coordinated Lysosomal Expression and Regulation (CLEAR) binding site found in the promoter of many autophagy and lysosomal genes[65]. TFEB overexpression enhances transcription of an autophagy and lysosomal gene program[63], as well as the degradation of autophagy substrates, mitochondria and lipid droplets[66]. TFEB has been reported to activate the transcription of an autophagy program in response to starvation[63].

TFEB activation is thought to be regulated primarily through phosphorylation. Under nutrient-rich conditions, TFEB is primarily cytosolic and inactive[67]. Upon nutrient deprivation, TFEB rapidly translocates to the nucleus and is activated to induce transcription of autophagy genes[67]. Phosphorylation at two sites, Ser211 and Ser142, determines the localization and activity of TFEB[68]. Ser211, when phosphorylated, is a docking site for chaperone 14-3-3, and this interaction retains TFEB in the cytoplasm[67, 69]. Furthermore, to maintain its cytosolic localization and inactivation, TFEB is phosphorylated at Ser211

by mammalian target of rapamycin complex 1 (mTORC1), a known negative regulator of autophagy[69].

mTORC1 is a key regulator of cell growth, proliferation, protein synthesis and autophagy [70]. mTORC1 consists of the core components mLST8, serine/threonine kinase mTOR and adaptor protein Raptor. mTORC1 is regulated in response to cellular stress by 5-AMP-activated protein kinase (AMPK)[70]. AMPK and its activator LKB1 sense reductions in cellular ATP and induce autophagy to replenish ATP stores. AMPK activation leads to the phosphorylation and activation of tuberous sclerosis complexes 1 and 2 (TSC1/2). TSC2 is a GTPase-activating protein which acts on small G-protein Rheb to inhibit mTORC1, and mTORC1 inhibition by TSC2 induces autophagy[70].

mTORC1 is also regulated by the PI3K/AKT pathway, and its negative regulator PTEN. PI3K phosphorylates PIP2 to form signaling intermediate PIP3, while PTEN dephosphorylates PIP3. PIP3 activates AKT, which in turn activates mTOR and phosphorylates Beclin1 to inhibit autophagy[71, 72]. PTEN has been shown to negatively regulate mTOR to induce autophagy in a variety of cell types[73, 74] and is activated by ATM in response to DNA damage to induce autophagy[75].

The mTORC1/AMPK pathway regulates the initiation of autophagy (Figure 2). In nutrient-rich conditions, mTORC1 binds, phosphorylates and inactivates Unc-51-like kinase 1 (ULK1) and Atg13[76]. AMPK can also bind and phosphorylate ULK1[77], blocking inhibition by mTOR and activating ULK1[78]. ULK1 activation by AMPK or by mTOR inhibition allows ULK1 to phosphorylate Atg13 and focal adhesion kinase family-interacting protein of 200 kD (FIP200)[76]. ULK1, Atg13 and FIP200 form a scaffold (called the ULK1 complex)[76], which localizes to the burgeoning phagophore and promotes the recruitment of other proteins essential for autophagy.

The ULK1 complex activates essential autophagy gene Beclin1[79, 80], via ULK1-mediated phosphorylation[81] (Figure 2). Beclin1, which is bound to anti-apoptotic protein Bcl-2 in normal conditions, dissociates[82] and binds to UVRAG[80]. UVRAG promotes the forma-

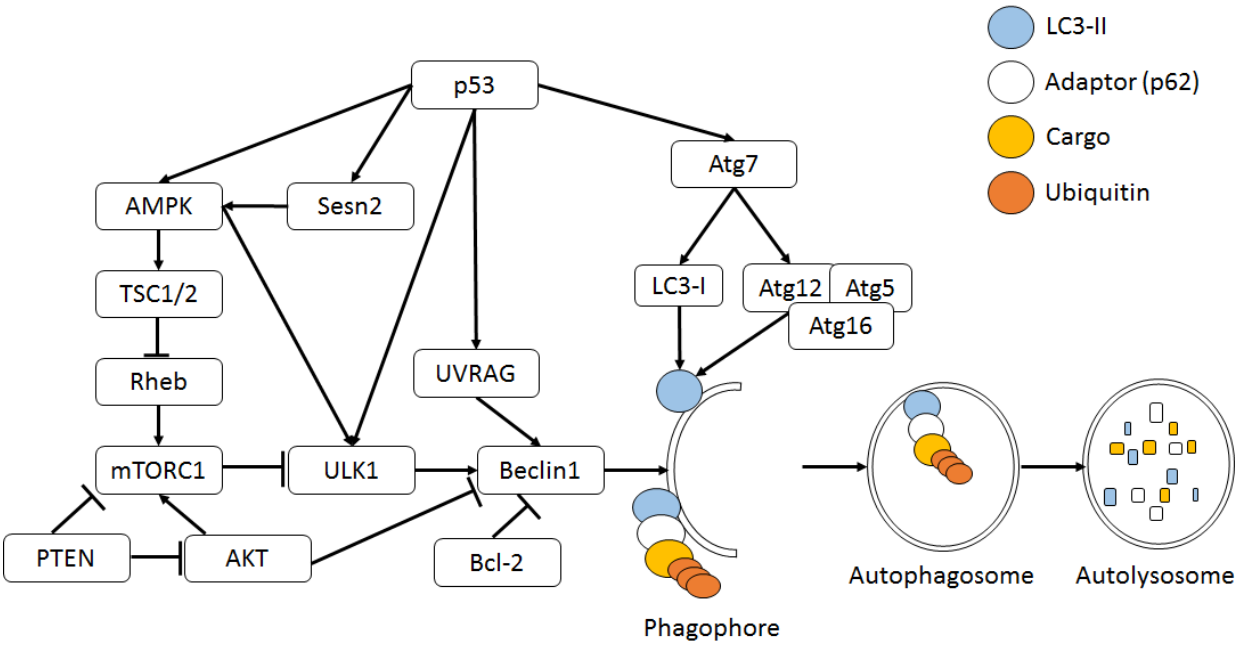


Figure 2: **Mechanisms of Autophagy.**
Mechanisms of Autophagy. Autophagy is initiated upon activation of the AMPK signaling pathway. AMPK can be activated by p53 and Sesn2 in response to cellular stress. AMPK then activates TSC1/2 to inhibit mTORC1. mTORC1 is further negatively regulated by PTEN to activate autophagy. ULK1 is activated by inhibition of mTORC1, direct phosphorylation by AMPK or p53-mediated transcriptional upregulation. ULK1 then forms a complex which activates Beclin1. Beclin1, which binds to Bcl-2 in normal conditions, dissociates from Bcl-2 and binds to UVRAG. UVRAG, a p53 target gene, promotes the formation of the Beclin1 complex to initiate phagophore nucleation. Two ubiquitin-like conjugation systems facilitate the lipidation of LC3-I to form LC3-II and elongation of the burgeoning phagophore. E1-like activating enzyme Atg7 leads to the activation of E2-like enzyme Atg3 (System 1) or Atg10 (System 2), subsequently the activation of the E3-link enzyme complex Atg5-Atg12 conjugate with Atg16 and subsequently the generation of LC3-II. LC3-II binds to an adaptor protein or autophagy receptor, such as p62, which in turn binds to cargo with modifications such as polyubiquitination. The autophagosome encloses the cargo and subsequently fuses with a lysosome to form an autolysosome. Adaptor and cargo are degraded at the autolysosome, and the resulting molecules are recycled to the cytoplasm.

tion of the Beclin1 complex, consisting of core components Beclin1, Vps34 and p150[83]. The Beclin1 complex can interact with other proteins to induce phagophore nucleation in response to a variety of stressors[84].

Upon initiation of phagophore nucleation by Beclin1 complex formation, two sequential ubiquitin-like conjugation systems are induced to facilitate phagophore elongation[85] (Figure 2). In the first, ubiquitin-like Atg12 is activated by the E1-like activating enzyme Atg7. Atg7 transfers Atg12 to the E2-conjugating enzyme Atg10, and Atg12 is irreversibly attached to Atg5. The Atg12Atg5 conjugate binds to Atg16 and attaches to the phagophore[85]. In the second system, LC3 is then processed by cysteine protease Atg4 to allow activation by Atg7. Atg7 transfers the activated LC3 (LC3-I) to E2 enzyme Atg3. Finally, a phosphatidylethanolamine (PE) lipid is attached to LC3-I by Atg12Atg5 conjugate to form LC3-II. LC3-II is linked to the phagophore membrane by the attached PE. The amount of LC3-II ultimately controls the size of the autophagosome and the amount of cargo included for degradation[85].

LC3-II facilitates selective autophagy of long-lived proteins, protein aggregates or damaged organelles by forming interactions with adaptor proteins (Figure 2). Selective autophagy adaptors, such as p62/SQSTM1, bind to cargo for degradation and to LC3-II on the autophagosome membrane through the LC3-interacting region (LIR)[86]. Upon autophagosomelysosome fusion, both the adaptor and cargo are degraded. The products of autophagy are recycled to the cytosol to maintain essential cellular processes after starvation or stress.

Autophagy in UV-Induced Disease

UVA-induced ROS generation leads to the oxidation of phospholipids and subsequent formation of oxidized phospholipidprotein adducts[58]. Autophagy induced by UVA degrades these adducts[58] to prevent damage caused by aggregation of heavily oxidized protein adducts[87]. Aging-related decline in autophagic clearance[88] leads to the accumulation of oxidized phospholipidprotein adducts[89] and oxidized protein aggregates[90]. Adduct accu-

mulation contributes to skin photoaging[89].

Autophagy can be tumor suppressive or protumorigenic, depending on context[91]. Autophagy acts as a tumor suppressor by promoting ROS clearance[92], DNA repair[61, 93, 94] and degrading oncogene p62[95]. Autophagy deficiency causes accumulation of targets bound for degradation, including p62[96], promotes ROS generation[97] and causes genomic instability[60].

Degradation of adaptor protein p62 has been found to be an important tumor-suppressive function of autophagy[95]. In autophagy-deficient conditions or upon transcriptional upregulation by UVA[96], p62 accumulates and acts as a signaling hub by forming interactions with a number of protumorigenic proteins. We have found that p62 binds and stabilizes Twist1, a transcription factor involved in epithelial-mesenchymal transition[98]. In doing so, p62 promotes proliferation and migration of skin cancer cells *in vitro*[98]. In a mouse model of SCC, the p62-Twist1 interaction promotes tumor growth and metastasis[98]. NF-κB signaling is also activated by p62 to promote Ras-mediated tumorigenesis[99].

Evidence implicates both elevated and reduced autophagy in melanoma cells, supporting dual roles for autophagy in melanoma. High levels of autophagy adaptor protein and substrate p62 constitute a prognostic marker of malignant melanoma[100], although other work indicates that p62 expression increases, then decreases late in disease progression[101]. Atg5 expression has also been reported to decrease as melanoma progresses from benign to malignant[102]. Atg5 knockdown promotes proliferation, further suggesting that reduced autophagy at the early stages may contribute to tumorigenesis[102]. Atg5 loss of heterozygosity is found in many advanced melanomas and correlates with poor overall survival[103]. Atg5 LOH increases melanoma metastasis *in vivo* in a $\text{BRAF}^{\text{V600E}}$ and PTEN-deficient mouse model[103].

Expression of the autophagy inducer Beclin1 decreases as melanoma progresses[104, 105]. One study reports that $\text{BRAF}^{\text{V600E}}$ overexpression in melanoma cells decreases basal autophagy levels relative to BRAF wild-type cells through a Beclin1-dependent mechanism[106].

Furthermore, BH3-family proteins Bcl-XL and MCL-1, which disrupt the activation of autophagy by Beclin1, are upregulated in metastatic melanomas[107]. Interactions between BH3-only protein Noxa and MCL-1 have been shown to disrupt inhibition of Beclin1 by MCL-1 and promote autophagy[108]. Recent work has shown that the BH3-only protein Noxa is upregulated in melanoma cells and promotes autophagy to inhibit apoptosis[109].

MITF expression, which correlates with increased lysosome gene expression in melanoma cells [110], decreases with disease progression[107, 111]. LC3 has similarly been reported to decrease during melanoma disease progression[102]. In heavily pigmented melanoma cells, however, LC3 is highly expressed[112]. In these cells, LC3 regulates MITF expression and ultimately, melanin production[112]. As MITF plays a critical role in melanoma growth and metastasis, this link between MITF expression and autophagy may be an important link in melanoma progression.

Autophagy can facilitate tumor development by promoting cell survival in times of genotoxic, oxidative or metabolic stress and by providing the macromolecules necessary to sustain a high rate of proliferation[91]. We have proposed an oncogenic function for autophagy in the development of SCC, in which autophagy promotes survival of SCC cells with extensive DNA damage[57]. Future investigations will help understand the role of autophagy in the regulation of critical signaling pathways in tumorigenesis.

Recent work suggests that malignant melanomas have increased autophagic flux relative to benign nevi[113, 114, 115]. Furthermore, high levels of autophagy in melanoma correlates with metastasis[115], poor response to chemotherapy, and shorter overall survival[113, 115]. Induction of autophagy has been suggested to be a pro-survival mechanism for melanoma cells[114, 116, 117]. Autophagy is also associated with proliferation, invasion, and metastasis [114], as well as promoting ROS accumulation[117].

$BRAF^{V600E}$ mutant melanomas exhibit enhanced autophagy due to chronic ER stress[118] and mTOR inhibition[119]. Increased autophagy has been shown to increase cell survival in $BRAF^{V600E}$ mutant melanomas[120, 121, 122]. In models of $BRAF^{V600E}$ mutant melanoma

with PTEN-deficiency, autophagy is required for tumorigenesis[121]. Knockdown of the essential autophagy gene Atg7 in these mice leads to accumulation of defective mitochondria and ROS, increased senescence, decreased proliferation, and increased apoptosis[121].

Inhibition of $BRAF^{V600E}$ with vemurafenib induces autophagy by inhibition of the mTOR signaling pathway, and autophagy has been shown to promote survival of melanoma cells after vemurafenib[116, 120]. Combined inhibition of autophagy and mTOR signaling enhances cell death[113] and impairs metastasis[123] in $BRAF^{V600E}$ mutant melanomas. Vemurafenib-resistant melanoma cells also have enhanced autophagy, although inhibition or genetic modulation of autophagy was insufficient to regain sensitivity to vemurafenib[124]. Combined inhibition of autophagy and MEK signaling was sufficient to restore vemurafenib sensitivity[124].

UV-Induced DNA Damage

UVB radiation is efficiently absorbed by DNA within the epidermis[17] and damages DNA directly to form photoproducts. The most common UVB-induced photoproducts are cyclopyrimidine dimers (CPDs) and, to a lesser extent, pyrimidine-(6-4)-pyrimidone photoproducts (6-4PPs)[125]. Of these, 6-4PPs are bulkier, but more efficiently repaired than CPDs[126, 127, 128]. As a result of this ineffective repair as well as the abundance of damage, CPDs are responsible for approximately 80% UVB-induced mutations[129, 130].

The ability of UVA to induce photoproduct formation, and the extent to which this contributes to skin damage, remains controversial. UVA causes the formation of far fewer photoproducts than UVB[131, 132] and was previously thought to be harmless. However, multiple studies have reported UVA-induced CPD formation, and in many cases, CPDs were the predominant lesion formed by UVA[131, 133, 134, 135, 136]. UVA does not cause significant formation of 6-4PPs[131, 133, 134, 137]. UVA-induced CPDs are predominantly T-T dimers[138], and these dimers persist longer than UVB-induced CPDs[131]. The abundance of UVA-induced CPDs, and ineffective repair of these lesions, may therefore form a

significant source of UVA-induced skin damage[136].

UV radiation-induced DNA photodamage activates DNA damage repair, cell cycle arrest and apoptotic pathways. Cell cycle arrest allows time for recognition and repair of photoproducts, but if DNA damage is extensive and irreparable, apoptosis is initiated[139]. In surviving cells, proliferation is induced to replace dying cells and maintain tissue homeostasis[134]. Disrupting signaling through these pathways can lead to cancerous expansion of damaged cells.

One of the key signaling mediators is p53, a transcription factor that plays a key role in balancing the pathways activated in response to DNA photodamage (Figure 3). UV-induced photoproduct formation leads to the stabilization of p53[140]. p53 then activates transcription of a complex program of cell cycle inhibitors, DNA damage response genes[141, 142] and apoptotic genes[143, 144]. p53 induces the transcription of cell cycle inhibitor p21 to arrest the cell cycle at the G1 phase following UV exposure[145], and p53-mediated DNA damage response gene induction is required for the repair of UV-induced DNA damage[141]. The decision to induce pro-apoptotic signals by p53 is context-dependent and can be impacted by p53 expression prior to UV exposure[146], skin cell layer[147, 148], length of time since UV exposure[146] and cell type[143].

Mechanisms of UV-Induced DNA Damage Repair

UV-induced photoproducts are repaired by either transcription-coupled (TC-) or global genome (GG-) nucleotide excision repair (NER)[149, 150, 151, 152]. TC-NER allows the rapid recognition and repair of damage in transcriptionally active genes, while GG-NER repairs damage throughout the genome independent of gene transcription. TC-NER begins when RNA polymerase stalls at distorted DNA. Initiation of GG-NER requires recognition of distorted DNA by xeroderma pigmentosum complementation group C (XPC) or damage-specific DNA-binding protein 2 (DDB2, also called XPE)[149]. Transcription of XPC and DDB2/XPE is induced by p53 following UVB irradiation[141].

Upon recognition of DNA damage, both NER subpathways converge on a common repair pathway[149]. Transcription factor II H (TFIIH), a dimer of helicases XPB and XPD, is recruited to the damage site and unwinds DNA around the damage site. XPA and replication protein A (RPA) bind to the damaged strand and undamaged strand, respectively. This binding allows the recruitment of endonucleases XPG and XPF-ERCC1. XPG and XPF-ERCC1 then excise the damaged DNA to create a single strand of DNA complementary to the damage site. Replication factor C (RFC) loads proliferating cell nuclear antigen (PCNA) onto the DNA strand, and a DNA polymerase synthesizes a strand complementary to the damage site. Finally, DNA ligase seals the nicks to complete NER[149].

Cell cycle arrest following UV exposure is critical to provide ample time for DNA damage repair and to prevent proliferation of damaged cells. UV-induced DNA damage activates the sensors ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) to trigger cell cycle arrest via p53 stabilization[144] (Figure 3). ATR recognizes single-strand DNA breaks caused by UV, including RPA-bound ssDNA formed during NER. ATR then phosphorylates checkpoint kinase 1 (Chk1) to activate checkpoints at the G1, S and G2/M phases[153]. ATM, which recognizes double-strand breaks (DSBs), phosphorylates Chk2 to delay the cell cycle[154]. XPC and DDB2 have been shown to facilitate the recruitment of ATM and ATR to sites of DNA damage and promote the activation of cell cycle arrest pathways[155].

Autophagy in UV-Induced DNA Photodamage Response

UVB exposure directly and rapidly activates AMPK[57], UVRAG[156] and p53[157] to activate autophagy (Figure 3). Upon activation by UV, p53 induces transcription of autophagy activators AMPK, TSC2, Sestrin 1 and Sestrin 2[158, 159, 160]. Sestrin 1 and Sestrin 2 have been shown to interact with AMPK, TSC1 and TSC2 to inhibit mTOR signaling in response to genotoxic stress[159].

Genotoxic stress is a trigger for autophagy, and repair of UV-induced DNA damage is

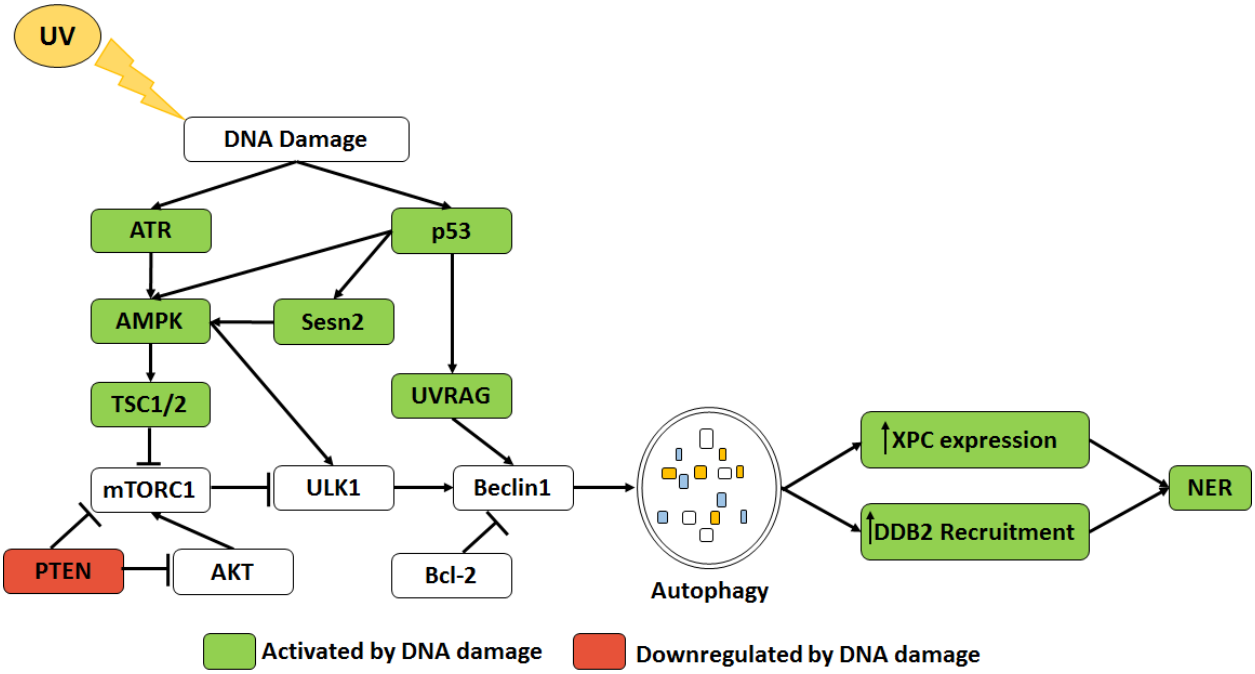


Figure 3: **Autophagy in UVB-Induced DNA Damage Response.** UVB radiation causes direct DNA damage in the form of photoproducts, and autophagy is induced by UVB to promote photoproduct repair. UVB directly induces autophagy activators AMPK, UVRAG and p53. p53, upon stabilization by UVB, activates transcription of AMPK, Sesn2, TSC2 and UVRAG to activate autophagy. ATR is also induced by UVB-induced DNA damage and can activate AMPK signaling. Sesn2 interacts with AMPK, TSC1 and TSC2 to activate autophagy in response to genotoxic stress. Activation of autophagy by these factors leads to the degradation of p62 and thus the decrease in Twist1 stability, which is required for XPC up-regulation following UVB, and recruitment of DDB2 to sites of UVB-induced DNA damage. Conversely, autophagy activator PTEN is inhibited in response to UVB, and this impairs NER by down-regulating XPC.

regulated by autophagy. Knockdown of key autophagy genes AMPK, Atg5, Atg7, Atg12 and Atg14 impairs the repair of UVB-induced DNA damage[161, 94]. Recent work has identified multiple pathways through which autophagy regulates UV-induced DNA damage repair.

First, we have shown that autophagy positively regulates the recognition of DNA damage by XPC and DDB2. Knockdown of essential autophagy gene Atg5 decreases XPC transcription following UVB radiation by a Twist1-dependent mechanism[94]. Autophagy deficiency leads to the accumulation of Twist1, which in turn activates transcriptional repressor complex E2F4-RBL2 through AKT signaling. The E2F4-RBL2 complex represses XPC transcription in autophagy-defective cells, leading to an accumulation of DNA damage[94].

Recognition of UV-induced DNA damage by DDB2 is also dependent on autophagy. Autophagy deficiency impairs DDB2 recruitment to CPD sites following UVB exposure[94] (Figure 3). This defect was found to be caused by Twist1 binding to and inhibiting p300, a key factor in DDB2 recruitment[94]. We have previously shown that Twist1 is stabilized by p62 in autophagy-deficient conditions[162], and autophagy-mediated degradation of Twist1 can facilitate DDB2 recruitment and CPD repair[94]. Collectively, this work suggests a tumor-suppressive role for autophagy in the promotion of DNA damage repair.

UVB rapidly induces autophagy through activation of AMPK to regulate DNA damage repair[57, 161] (Figure 3). AMPK activation by UVB increases XPC protein levels and increases CPD repair. Knockdown of AMPK reduces CPD repair following UVB, but has no effect on 6-4PP repair[161]. This work further links autophagy to the positive regulation of UVB-induced DNA damage repair.

Autophagy activator UVRAG was initially identified in a genetic screen as a protein able to partially complement the UV sensitivity of xeroderma pigmentosum (XP) cells defective in GG-NER[163]. Until recently, however the function of UVRAG in DNA damage response was unknown. UVRAG has recently been shown to be essential for both autophagy and GG-NER in response to UV[164]. UVRAG was found to facilitate the recruitment of DDB1 and DDB2 to sites of UV-induced DNA damage by binding DDB1. Knockdown of UVRAG

inhibits transfer of damaged DNA from DDB1 to XPC during NER initiation. However, UVRAG activates autophagy and GG-NER independently[164], suggesting UVRAG may act as a signaling hub for concurrent activation of DNA damage repair and autophagy (Figure 3).

Conversely, autophagy activator PTEN is inhibited by Sestrin 2[165] and in response to UVB[166] (Figure 3). PTEN downregulation impairs GG-NER by downregulating XPC transcription[167]. It is unclear, however, whether downregulation of autophagy has a significant role in PTEN-regulated DNA damage repair in response to UVB.

UV-Induced DNA Damage in Disease

UVA was initially believed to be nontumorigenic due to its poor ability to cause direct DNA damage. This indirectly promoted the development of UVB-specific sunscreens[168] and the use of high-powered UVA lamps in indoor tanning beds[169]. It has since been shown that UVA induces skin carcinogenesis *in vivo*[170, 171, 172]. In mouse models of childhood UV exposure, UVA is capable of inducing melanoma in pigmented C57BL/6 mice, but not albino mice. Furthermore, an increased risk of melanoma has also been linked to psoralen and UVA (PUVA) therapy. Indoor tanning, even intermittently, significantly increases skin cancer risk[173, 174].

UVB has been shown to induce melanomagenesis in childhood models of UV exposure and UVB induces carcinogenesis of NMSCs. UVB radiation has been thought to contribute mostly to NMSC formation by causing direct DNA photodamage. UVB signature TC-TT and CC-TT mutations are commonly found in the p53 gene of skin cancer patients[175], suggesting the importance of p53-mediated DNA damage response in preventing skin carcinogenesis. Defects in UV-induced DNA damage repair greatly accelerate skin cancer development, as is seen in XP patients, who have a 10,000-fold increased susceptibility to UV-induced NMSC and 2,000-fold increased risk of melanoma[20].

There is conflicting evidence regarding the ability of melanoma cells to respond to

DNA damage compared to normal melanocytes. Some research has shown that melanoma cells exhibit reduced DNA damage repair[176] and that UVB exposure further lowered their XPC, DDB1, and DDB2 expression[176]. UVA similarly lowered XPC expression in melanoma cells, and they also show impaired repair of UVA-induced CPDs relative to normal melanocytes[177]. We have found that Sestrin2, a stress-inducible protein, is induced by UVB in melanoma cells and negatively regulates DNA damage repair[160]. Knockdown of Sestrin2 increased UVB-induced apoptosis and decreased tumor formation *in vivo*[165].

DNA repair is critical for suppressing melanomagenesis. In melanoma patients, low levels of XPC have been shown to correlate with poor survival[176]. In a genetically engineered mouse model of melanoma featuring deletion of Arf and expression of $BRAF^{V600E}$, UVB exposure accelerated melanomagenesis by inhibiting NER[33]. Further analysis concluded that Arf deletion induces XPC promoter hypermethylation and repression, as well as E2F4/DP1 inhibition in this model[33]. $BRAF^{V600E}$ mutation alone was also sufficient to repress UVB-induced XPC[33]. Melanocyte-specific deletion of Arf alone *in vivo* reduced repair of UVB-induced DNA damage[178]. Deletion of XPC alongside Arf knockout accelerated UVB-induced melanomagenesis *in vivo*[51].

However, other studies suggest that there are no differences in repair of UV-induced DNA damage between melanocytes and melanoma cell lines[179]. While several studies found an association between DNA damage response gene upregulation and melanoma progression, the upregulated genes did not include NER genes[180, 181]. Arf-deficient mice with XPA deletion were sensitive to UVB-induced nevus formation, but developed fewer melanomas than mice with Arf deletion alone[182]. This work suggests that UVB-driven progression from nevus to melanoma may depend on specific NER pathways in some genetic backgrounds. Similarly, loss of cell cycle regulator RhoA led to defective repair of UV-induced DNA damage, which decreased proliferation and reduced survival of melanoma cells[183].

αMSH treatment is sufficient to induce nucleotide excision repair (NER) in melanocytes with wild-type MC1R[184](Figure 1). αMSH signaling activates NER by upregulating XPC

and inducing the phosphorylation of DNA damage sensors ATR and ATM[184]. Furthermore, αMSH signaling through MC1R increases the recruitment of XPA to UV-induced DNA damage sites by phosphorylating ATR, thus improving DNA repair[185, 186]. Activation of NER by αMSH requires functional MC1R[39, 187, 184, 188].

UV-Induced Oxidative Stress

The most established effect of UVA is causing oxidative damage to DNA, proteins and lipids (Figure 5). UVA is absorbed by cellular photosensitizers, which transfer energy in either type I or type II photosensitization reactions[189]. In type I reactions, energy is transferred directly to DNA through free radical formation, resulting in oxidative modifications. In type II reactions, photosensitizers transfer energy to molecular oxygen, creating singlet oxygen[190]. Singlet oxygen reacts preferentially with guanine and can cause a number of oxidative DNA modifications. Of these, 8-oxo-2'-deoxyguanosine (8-oxo-dG) is the most common oxidative lesion caused by UVA[135]. 8-oxo-dG can be repaired through base excision repair by 8-oxo-guanine glycosylase (OGG1)-mediated excision[191]. However, defective repair of 8-oxo-dG lesions following UVA exposure can cause GC-TA transversion. UVB can also cause ROS production and oxidative damage[192], although this effect is likely secondary to the direct DNA damage caused by UVB.

ROS and reactive nitrogen species (RNS) generated in melanocytes in response to UVA radiation lead to the production of dark CPDs hours after UVA exposure[136]. The accumulation of oxidatively modified DNA only in pigmented, and not albino, mice with UVA-induced melanoma[46] suggests that melanin could play a role in UVA-induced oxidative stress. Loss of MC1R reduced repair of UV-induced CPDs in melanocytes, leading to increased UV-induced apoptosis[39]. Accordingly, melanin content was inversely correlated with UV-induced apoptosis and CPD formation[39]. Melanomas featuring disruptive mutations in MC1R are associated with a 42% increase in UV-signature mutations over those in MC1R wild-type melanomas[193]. αMSH treatment is sufficient to reduce

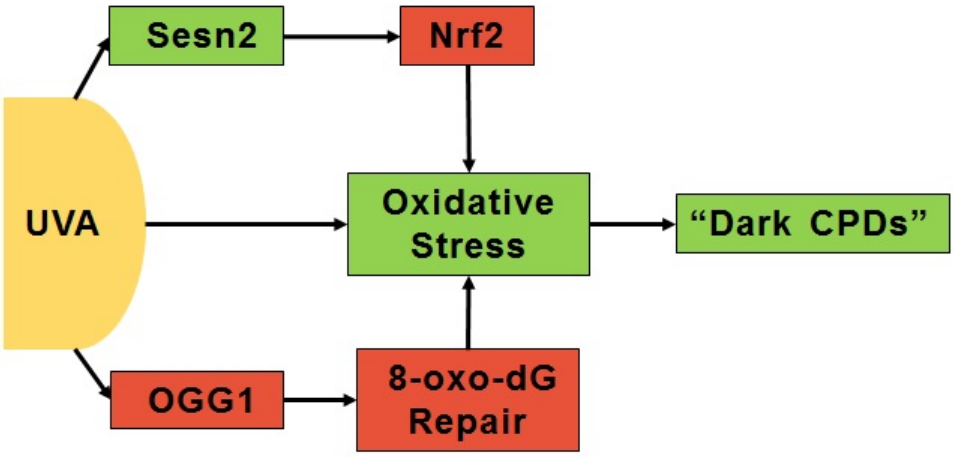


Figure 4: **Mechanisms of UVA Response in Melanoma.**
UVA is known to directly induce oxidative stress in melanocytes, but recent work suggests that UVA perpetuates the accumulation of oxidative stress through several mechanisms. UVA induces Sestrin2, a negative regulator of Nrf2 to promote oxidative stress accumulation in melanocytes. Furthermore, UVA suppresses OGG1, impairing repair of oxidative lesions and furthering oxidative stress. Oxidative stress can ultimately lead to the accumulation of dark CPDs hours after UVA exposure.

UV-induced oxidative stress[187]. Mutant MC1R increases levels of UV-induced oxidative stress[39, 187, 184, 188].

Autophagy in UV-Induced Oxidative Stress Response

UVA-induced oxidative stress damages lipids and proteins in addition to DNA. UVA induces the oxidation of phospholipids in keratinocytes[58, 194], and these oxidized phospholipids form adducts with proteins[195, 196]. Accumulation of oxidized phospholipidprotein adducts is found in a number of degenerative diseases and also in photoaged skin[192].

Accumulation of proteins with oxidative modifications is also seen in the dermis after UVA exposure[192], likely due to a reduced expression of antioxidant enzymes[192, 197]. Recently, UVA has been shown to target OGG1 for oxidation[198] and, in doing so, compromises BER of oxidized DNA[198]. Furthermore, UVA has been shown to cause oxidative damage to single-strand DNA-binding factor RPA, impairing NER[199, 200]. This work suggests that there are combinatorial effects of UVA and UVB in inducing and perpetuating DNA damage.

ROS generation in response to UVA triggers the antioxidant response pathway, beginning with the stabilization of antioxidant response factor Nrf2[201]. ROS formation triggers the dissociation of Nrf2 from KEAP1, stabilizing Nrf2 and allowing its nuclear translocation[202]. Nuclear Nrf2 activates an antioxidant response program through binding to antioxidant response elements in gene promoters[202]. Nrf2 also activates the transcription of genes involved in DNA damage response, including 8-oxo-dG-excising enzyme OGG1[203, 204].

The function of autophagy is critical for oxidative stress response[205]. However, little work has been done to establish the role of autophagy in the response to UVA-induced oxidative stress. UVA-induced oxidative stress triggers autophagy to clear oxidized lipids and proteins[58] (Figure 5). Autophagy deficiency leads to the accumulation of oxidized phospholipids and protein aggregates following UVA exposure[58]. Furthermore, autophagy deficiency increases the Nrf2-dependent antioxidant response in keratinocytes, even prior to UVA exposure[58]. We have found that in melanocytes, the autophagy activator Sestrin 2

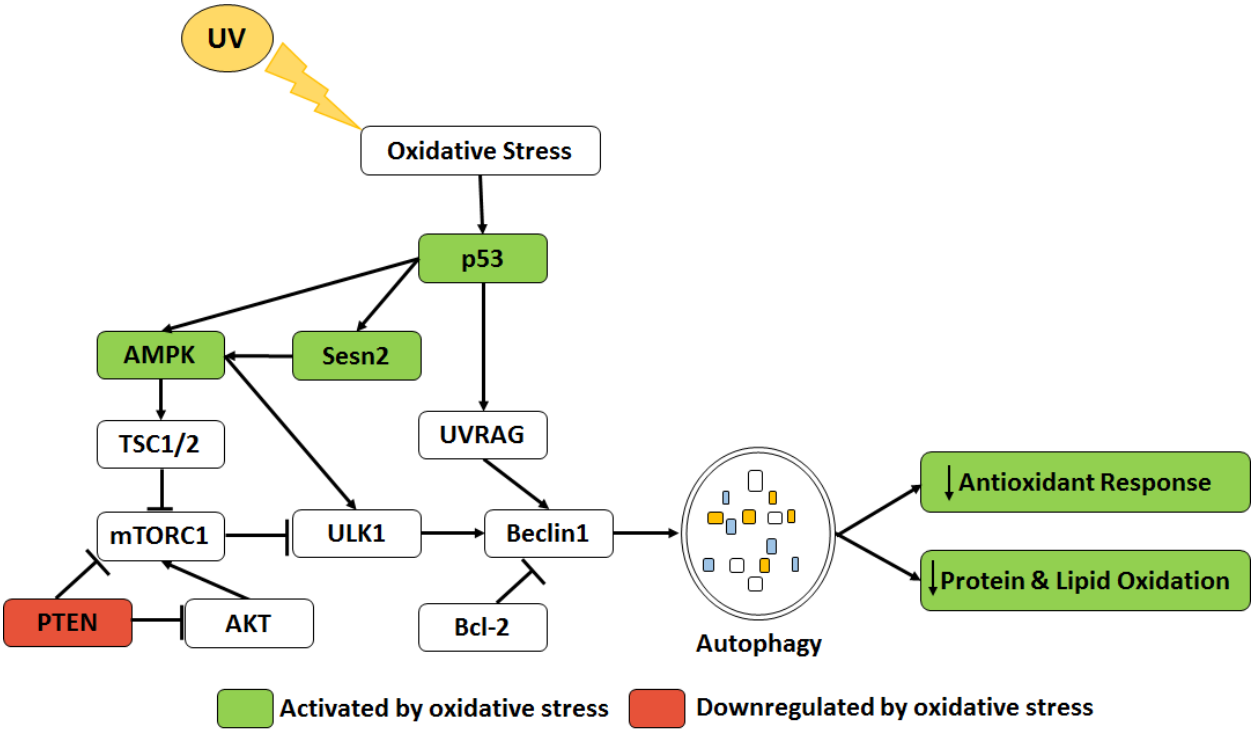


Figure 5: **Autophagy in UV-Induced Oxidative Stress Response.** Autophagy in UVA response. UVA-induced ROS production stimulates autophagy, and autophagy regulates the oxidative stress response following UVA. UVA stabilizes p53, which induces transcription of autophagy activators AMPK and Sesn2. Conversely, UVA suppresses PTEN expression, which may negatively impact autophagy induction following UVA. Autophagy clears oxidized proteins and lipids following UVA exposure, but suppresses Nrf2-mediated antioxidant response following UVA.

reduces Nrf2 levels upon induction by UVA and increases UVA-induced ROS production[160]. Autophagy induction also serves to inhibit Nrf2 stabilization by degrading p62[58]. This work indicates that autophagy plays a complex role in UVA-induced oxidative stress response, by clearing oxidized proteins and lipids, while minimizing antioxidant response in different cell types.

UV-Induced Oxidative Stress in Photoaging

Photoaging (also called extrinsic aging) is premature aging of the skin caused by environmental effects, primarily UV exposure. Photoaging differs from intrinsic (chronological) aging, which affects the skin in ways similar to other organs and can be superimposed on photoaging[206]. While intrinsically aged skin is thin and smooth, photoaging can cause a leathery thickening, sagging and wrinkling of skin[207, 208]. Histologically, photoaged skin is characterized by a loss of dermal collagen[209], induction of matrix metalloproteinases (MMPs)[210] and accumulation of elastin[211].

Both UVA and UVB have been shown to cause photoaging by inducing ROS production and subsequent oxidative damage to DNA, lipids and proteins[211]. UVA-induced alterations to skin lipid composition[212] and phospholipid oxidation[58] cause skin sagging characteristic of photoaging. Furthermore, UV exposure leads to the accumulation of oxidatively modified proteins and depletes antioxidant enzymes in photoaged skin[192, 213].

UV-Induced Oxidative Stress in Skin Cancer

Oxidative modification of DNA is an important mechanism of UVA-induced skin damage and carcinogenesis (Figure 4). Melanocytes have diminished repair of UVA-induced oxidative damage, as melanin acts as a photosensitizer to UVA[214]. Dysplastic nevi have increased ROS levels relative to normal melanocytes, supporting a role for ROS accumulation in melanomagenesis[215]. Several potential mechanisms of ROS accumulation in melanomas have been suggested. LOH mutations in hOGG1, an enzyme that repairs oxidative DNA

damage, have been demonstrated in a small number of melanomas[216, 217]. Additionally, we have found that UVA induces Sestrin2 in melanocytes and melanoma cells, which in turn suppresses antioxidant response factor Nrf2 and increases ROS production[160]. Deletion of p16 could also contribute to UV-induced ROS accumulation and oxidative DNA damage in melanocytes[218]. However, one study has found that OGG1 is overexpressed in some metastatic melanomas[219].

The effect of antioxidants on melanomagenesis has been explored in mouse models. The antioxidant N-Acetylcysteine (NAC) has been found to delay the onset of UV-induced melanoma *in vivo*[220]. In a mouse model with $BRAF^{V600E}$ mutation and PTEN deletion, NAC increased metastasis, but had no effect on primary tumors[221]. NAC treatment also increased migration and invasion of melanoma cell lines *in vitro* by activating RhoA[221].

UV-Induced Cell Proliferation and Apoptosis

UV radiation exposure can induce apoptosis and proliferation[134], often within the same sun-damaged tissue[222, 223]. In normal skin, compensatory hyperproliferation is induced to replace the cells cleared by apoptosis and maintain homeostasis. The mechanisms governing UV-induced proliferation and apoptosis are tightly regulated to prevent cancerous expansion of cells damaged by UV.

Autophagy in UV-Induced Cell Proliferation and Apoptosis

UVA and UVB induce apoptosis of epidermal cells by increasing p53 and Bax expression, while decreasing expression of Bcl-2[223, 224]. Furthermore, UVA and UVB can induce apoptosis through p38 activation[225, 226, 57]. UVA has been reported to downregulate PTEN and upregulate AKT signaling to protect against apoptosis[227]. Conversely, UVB-induced UV radiation resistanceassociated gene (UVRAG) expression suppresses apoptosis by sequestering Bax away from the mitochondria, where it induces apoptosis[228]. As UVRAG is

a p53 target gene[62], this would suggest that UVRAG serves as a possible negative feedback loop in the regulation of UV-induced apoptosis.

Another essential mediator of both UV-induced proliferation and apoptosis is cyclooxygenase 2 (COX-2). Both UVA and UVB upregulate COX-2[229, 230, 231], an inducible prostaglandin synthase which catalyzes the rate-limiting step in prostaglandin E2 (PGE_2) synthesis. PGE_2 signals through autocrine and paracrine mechanisms to promote cell proliferation[232] and suppress apoptosis[233]. COX-2 is induced in the skin of hairless mice following UV radiation to increase proliferation[234], and induction of COX-2 protects against UVB-induced apoptosis[234, 235, 230].

In addition, the mTOR signaling pathway regulates cell growth and proliferation in response to stress. Sestrin 2, a stress-inducible protein that is activated following UVB exposure[165], inhibits cell proliferation through the negative regulation of mTOR signaling [159]. Similarly, AMPK, an inhibitor of mTOR signaling, suppresses cell proliferation in response to UVB[161]. UVB activates the AMPK signaling pathway to induce autophagy[57, 161] and suppress apoptosis[57, 236]. Autophagy induction by UVB limits p62 levels to prevent p62-mediated p38 activation and subsequent apoptosis[57]. UVB similarly induces autophagy activator Sestrin 2 to promote cell survival[165]. This work suggests that autophagy is activated by UVB to promote cell survival and likely contributes to UVB-induced tumorigenesis. Furthermore, we have previously shown that autophagy activator AMPK regulates expression of XPC and consequently UVB-induced DNA damage repair[161]. AMPK-mediated DNA damage repair suppressed cell proliferation in response to UVB[161], suggesting a key role for AMPK in the regulation of autophagy, proliferation and apoptosis.

UV exposure induces apoptosis and cell proliferation to maintain tissue homeostasis. Research suggests that autophagy plays key roles in the regulation of apoptosis and proliferation, but the contributions of autophagy are likely context-dependent. Knockdown of the autophagy activators AMPK[57, 160], Sestrin 2[165], Beclin1[237], Atg5[57, 237] and

UVRAG[238] sensitizes skin and skin cancer cells to UVB-induced apoptosis, suggesting a protective role of autophagy. However, other research suggests that autophagy or AMPK signaling promotes UVB-induced apoptosis[239, 240].

The anti-apoptotic Beclin1-binding protein Bcl-2 is downregulated following UVB exposure [223], which may free Beclin1 to bind UVRAG and induce autophagy[80]. UVB-induced UVRAG expression suppresses apoptosis by sequestering Bax[238]. independent of its role in autophagy induction[238]. Furthermore, UVRAG is key to suppressing proliferation after UVB, independent of its function in autophagy activation[80]. As the domains of UVRAG required for activating autophagy, promoting proliferation and suppressing apoptosis differ, it is possible that UVRAG can activate these processes simultaneously, therefore forming a critical hub in the response to UV radiation.

PTEN, which has been shown to negatively regulate autophagy, is involved in the regulation of cell proliferation and survival following UV exposure. Both UVA and UVB suppress PTEN expression to promote survival of keratinocytes[241, 227, 242]. Sestrin 2 promotes the suppression of PTEN and subsequent AKT activation to promote survival in response to UVB[165]. PTEN suppression by UVA was accompanied by upregulation of AKT signaling and an increase in proliferation[243]. PTEN is therefore central to the regulation of proliferation and apoptosis after UV, and has previously been linked to autophagy. It is unclear whether PTEN regulates UV-induced autophagy, however.

UV-Induced Inflammation

UV radiation is known to induce a "sunburn" response, an inflammatory response characterized by increased vascular dilation and permeability in the upper dermis. Sensitivity to UV-induced sunburn correlates with skin cancer risk. Furthermore, UVB induces infiltration of inflammatory cells, including macrophages, CD4+ T cells, neutrophils, and mast cells, into the skin[244].

We found that hairless mice with epidermal deletion of Atg7 (Atg7 KO) were resistant

to UVB-induced sunburn relative to wild-type (WT) mice. Atg7 KO mice had reduced epidermal vasodilation, angiogenesis, and lymphangiogenesis following UVB exposure relative to WT mice. Atg7 deletion in the epidermis impairs UVB-induced inflammatory cell infiltration and cytokine secretion. Furthermore, Atg7 KO mice were resistant to UVB-induced hyperplasia and tumorigenesis. Atg5 deletion also regulates UVB-induced inflammatory gene expression, suggesting autophagy, rather than Atg7 expression is critical for UVB response[244].

Both UVA and UVB have been shown to induce expression of COX-2. By catalyzing the production of PGE_2, which can be secreted and signal through paracrine mechanisms to promote inflammation and recruit immune cells. We have shown that COX-2 induction by UVB is autophagy-dependent. By regulating COX-2, Atg7 controls PGE_2 production in response to UVB. This regulation of PGE_2 allows Atg7 to control vascular dilation, permeability, epidermal hyperplasia[244].

Atg7 regulates COX-2 through two mechanisms. First, Atg7 regulates COX-2 transcription through a cell-intrinsic CREB1/CRE-dependent mechanism. CREB1 transcriptional co-activators EP300 and CRTC1 were explored as possible regulators of UVB-induced CREB. EP300 regulates COX-2 expression in an Atg7-dependent matter, and this is enhanced by UVB treatment. However, EP300 and CREB1 are primarily localized in the nucleus, while Atg7 is cytosolic, and this localization was unchanged in Atg7-deficient cells. CRTC1, on the other hand, was differentially localized in Atg7-deficient cells. CRTC1 localization and activation is regulated by phosphorylation. Therefore, we explored changes in CRTC1 phosphorylation by known regulator AMPK in Atg7-deficient cells. Atg7 KO also activates AMPK signaling, increasing CRTC1 phosphorylation and inactivation, reducing COX-2 induction by CREB. CRTC1, and consequently COX-2 expression, was also regulated by cytosolic calcium and ER stress. Inducing ER stress and calcium mobilization with thapsigargin was sufficient to rescue UVB-induced inflammation and skin tumorigenesis in Atg7 KO mice.In addition, Atg7 regulates COX-2 by a non-cell autonomous mechanism through regulation

of IL-1β secretion. Atg7 regulates both basal and UVB-induced COX-2 through promoting IL-1β secretion[244].

Overexpression of COX-2 occurs in many cancers, including skin cancer, and correlates with poor prognosis[245]. COX-2 overexpression is sufficient to induce spontaneous skin carcinogenesis *in vivo*[246]. We and others have shown that COX-2 promotes epidermal hyperplasia, invasion, and metastasis in response to UV *in vivo*[246, 234, 247, 244]. Knockdown of COX-2 reduces susceptibility to experimentally-induced tumorigenesis[246, 248]. Furthermore, inhibition of COX-2 prevents UV-induced skin tumorigenesis in humans, even in patients at high risk of NMSCs[249].

UV-Induced Inflammation in Melanoma

Inflammation and immune response to melanomagenic conditions are critical for melanoma development and therapeutic response. UVB regulates the recruitment of inflammatory cells into the skin, including macrophages[250, 251] and neutrophils[252, 253]. UVB induces macrophage infiltration of melanomas by upregulating Ccr2[250] and ATF2[251]. Upon recruitment, IFN-γ signaling from macrophages triggers further upregulation of Ccl8, a Ccr2 ligand, in melanocytes[250]. This positive feedback loop increases melanoma growth *in vivo* by reducing melanoma cell death[250]. Depletion of macrophages inhibits UV-induced melanocyte proliferation in mouse skin[254]. Melanoma-derived factors also trigger the upregulation of CCL2 and MMP-9 in macrophages, which in turn promote invasion of melanoma cells[255]. UVB-mediated neutrophil recruitment further promotes melanoma metastasis by stimulating angiogenesis and increasing migration of melanoma cells toward blood vessels[252].

Melanoma-associated inflammation involves multiple regulatory pathways. For example, interleukin 23 (IL-23) induces DNA damage repair in melanocytes, including XPC and XPA expression and γ-H2AX foci formation[256]. DNA damage repair induced by IL-23 inhibits melanomagenesis[256]. IL-23 also inhibited regulatory T cell expansion and limited IFN-γ

production[256]. The IFN-γ receptor on melanocytes inhibits UV-induced apoptosis[257], suggesting that suppression of IFN-γ by IL-23 suppresses melanomagenesis by clearing damaged cells.

Another critical inflammation and immunological regulatory pathway in melanoma is Programmed cell death 1 (PD1) and its ligand PD-L1. Anti-PD-1 immunotherapy has shown efficacy in melanoma, but fewer than half of all melanoma patients treated with anti-PD-1 immunotherapy have a prolonged response[9]. Current efforts are aimed at understanding the differences between immunotherapy responders and non-responders. PD-L1 expression in melanoma cells is not associated with $BRAF^{V600E}$ mutation, and cells expressing PD-L1 recruit tumor-infiltrating lymphocytes (TILs) independent of BRAF status[258]. BRAF inhibitor-resistant melanoma cells increase PD-L1 expression in a MEK-dependent manner, and inhibition of MEK and BRAF increases apoptosis and decreases PD-L1 expression[259]. COX-2 expression is also correlates with PD-L1 expression in primary melanomas, and inhibition of COX-2 by celecoxib downregulates PD-L1 in melanoma cells[260].

Inflammation and response to immune therapy are also regulated by PTEN status. Loss of PTEN in melanoma cells allows PI3K-mediated activation of immunosuppressive cytokines[261, 262]. PTEN expression represses PD-L1 expression, promoting an immune response against tumor cells. PTEN loss was associated with non-brisk (localized) immune response in tumors[261, 262]. Other work shows that PTEN loss in melanoma cells inhibits both T cell recruitment into tumors and targeted killing of tumor cells by T cells[262]. PTEN loss is also associated with poor response to anti-PD-1 therapy[262]. Treatment with a PI3K inhibitor improved response to immune therapies *in vivo*, further supporting a role for PTEN loss in immunosuppression in melanoma[262].

Oncogenic Autophagy Adaptor p62

p62 is a multidomain adaptor protein which acts as a signaling hub, activating a number of key oncogenic signaling pathways(Figure6). p62 is overexpressed in a number of cancers[263,

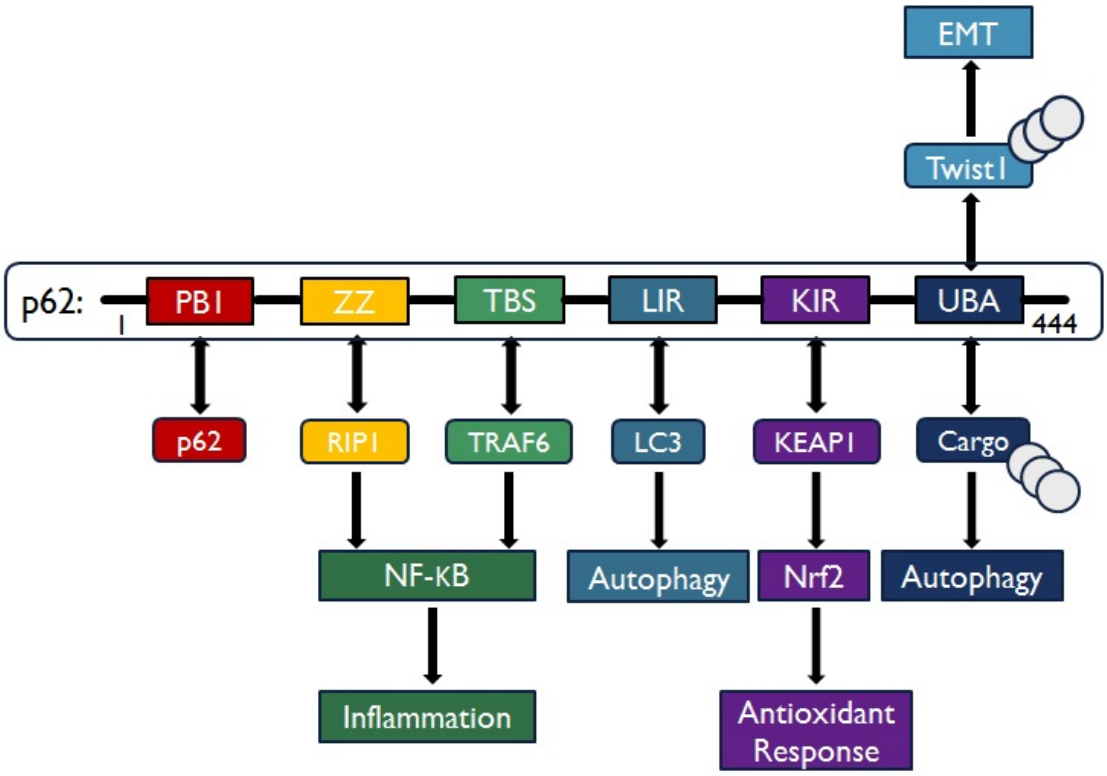


Figure 6: **p62 is a multidomain adaptor protein.**
p62 acts as an adaptor for a number of signaling pathways. p62 can form self-oligomers through the N-terminal phox and Bhem1 (PB1) domain. p62 can also bind to receptor-interacting protein 1 (RIP1) through the zinc finger (ZZ) domain to activate NF-KB signaling. Interactions between tumor necrosis factor receptor-associated factor 6 (TRAF6) and the TRAF6-binding domain (TBS) of p62 can additionally activate NF-KB signaling to promote inflammation and cancer progression. The LC3-interacting region (LIR) of p62 facilitates inclusion of p62 into the autophagosome by binding to LC3. The Keap1-interacting region (KIR) allows p62 to bind Keap1 and displace it from Nrf2, leading to Nrf2 stabilization and antioxidant response. Finally, the ubiquitin-associated (UBA) domain of p62 interacts with polyubiquitinated proteins to be degraded in autophagy, but as we have shown, can also stabilize polyubiquitinated Twist1 to promote epithelial-mesenchymal transition (EMT).

264, 265]. This includes NMSCs[162] and melanoma[100], where its expression correlates with poor prognosis[100]. In kidney cancer, the p62 gene is amplified and drives kidney cancer growth[266]. Degradation of p62 is one mechanism by which autophagy suppresses tumorigenesis[95].

Most famously, p62 is an adaptor and substrate for selective autophagy. By binding to LC3-II in the autophagosomal membrane and polyubiquitinated cargo in the cytosol, p62 facilitates cargo degradation and is degraded in the process (Figure 2). p62 interacts with LC3 via the LC3-interacting region (LIR) and binds to polyubiquitinated cargo via the ubiquitin-associated (UBA) domain. We were the first to report that p62 binding to polyubiquitinated proteins through the UBA domain can stabilize, rather than degrade proteins[162]. Interaction between p62 and polyubiquitinated Twist1 leads to Twist1 stabilization and subsequent activation of epithelial-mesenchymal transition (EMT)[162]. Twist1 stabilization by p62 leads to increased proliferation and migration of SCC cells *in vitro* and increases tumor growth and metastasis *in vivo*[162].

p62 also activates NF-κB signaling in a feed-forward loop. p62 interacts with TRAF6 through the TRAF6-binding domain (TBS)[267, 99, 268] to activate NF-κB. Furthermore, p62 binds to death domain serine/threonine kinase RIP[269] via the zinc-finger (ZZ) domain and with atypical PKC (aPKC) via the phox and Bem1 (PB1) domain to activate NF-κB signaling[270]. NF-κB signaling in turn upregulates p62 transcription[271] and induces inflammation. p62 also activates the antioxidant response by regulating the stability of Nrf2. p62 sequesters KEAP1 to prevent degradation of Nrf2[272, 273, 274, 275].

p62 interacts with Raptor, a part of the mTOR complex, adjacent to the LIR[276]. As mTOR activation suppresses autophagy, and this interaction blocks p62 binding to LC3 and subsequent degradation, p62 binding to Raptor has been suggested to positively regulate p62 levels. Finally, p62 binding to ERK via the PB1 domain inhibits ERK activity[267].

UV-Induced Disease Prevention and Treatment

Childhood exposure to UV radiation is a major risk factor for skin cancer development, particularly at doses high enough to achieve sunburn[277]. Some studies suggest that childhood sunburns could as much as double the risk of melanoma[278]. This effect is highly dependent on skin tone, however. In red haired and freckled individuals, childhood UV exposure is a particularly potent risk factor for melanoma development[279]. Conversely, in light-skinned individuals prone to tanning, childhood UV exposure can be protective against melanoma[280]. Exposure to UV early in life is associated with the development of BRAF mutant melanomas, while NRAS mutation is more commonly associated with high UV exposure later in life[281].

In addition to childhood UV exposure, ease of access to indoor tanning has provided teenagers and young adults with further opportunities to increase UV radiation exposure. Indoor tanning at a young age increases melanoma risk[282]. Use of indoor tanning beds increases as children enter adolescence, and this shift is accompanied by a 50% drop in sunscreen use[283]. In the US, it is estimated that 40-50% of teenagers have utilized tanning beds[284]. Furthermore, approximately 70% of tanning salon customers are females under 30[285], and indoor tanning before age 30 leads to a 75% increase in melanoma risk[286]. Data suggest that melanoma rates in women ages 15-39 are nearly double that of men of the same age group[287].

Despite links between early age sunburn and melanoma, one study has found that melanoma risk was associated with the number of sunburns throughout life[288]. Some studies have found a similar dose-dependent effect of indoor tanning independent of age[173], although another found that tanning increased risk for young women[289]. Indoor tanning also likely contributed to an epidemic of melanomas on the trunk in young Icelandic women in the early 2000s[290]. Furthermore, misunderstandings persist about the ability of an all-year tan to protect against melanoma[278].

Sunscreen has been linked to a paradoxical increase in sun exposure and sunburns.

The sun protection factor (SPF) of sunscreen correlates with increased intentional sun exposure[291]. A similar paradoxical increase in risk is seen in indoor workers, who have a higher risk of melanoma than outdoor workers[292]. UVB exposure has also been linked to decreased rates of melanoma mortality[293], and in mouse models, sunscreen is ineffective at preventing melanomagenesis[294]. Recent meta-analyses of published epidemiological data have found no link between sunscreen use and melanoma risk, however[295, 296]. In the United States, use of sunscreen and physical barriers, such as clothing and sunglasses, is increasing[282], but many Americans still report receiving at least one sunburn in the last year[282].

Skin Cancer Prevention

Given the extremely high incidence of skin cancers, making significant strides in prevention will require large-scale public health campaigns. Australia implemented one such campaign in the 1980s, which has led to a shift in the behavior and attitude toward UV exposure[297, 298], particularly in young adults[298, 299]. Incidence of melanoma on the trunk and shoulders, sites subject to intermittent UV exposure if left unprotected, was significantly decreased in Australian young adults[299].

Recent efforts have aimed to reach teenagers and young adults via social media[300, 301] and texting[302], in addition to determining the efficacy of positive vs negative/fear-based messaging[303]. Targeted messaging to parents of adolescents was effective at starting conversations between mothers and daughters about the concerns of indoor tanning[304]. In households receiving these messages, fewer daughters reported a desire to go indoor tanning than non-intervention households[304]. Fathers and sons were largely unaware of the messaging, however, suggesting that additional avenues are needed to engage men in awareness of the dangers of UV radiation exposure. A survey of young women who indoor tan indicates that, while they are overwhelmingly supportive of policies limiting indoor tanning for minors and placing stronger warnings of indoor tanning risks on tanning beds, they do not support

a total ban[305].

Tanning-related regulations vary across the US. Many states have age-based tanning bans, some requiring parental consent, and others simply require warning signs to be placed in tanning salons (186). As of 2014, FDA regulations require displays on indoor tanning devices warning of skin cancer risk[306]. Similar restrictions exist worldwide, with many countries banning indoor tanning under the age of 18[306]. Enforcement of these laws is lax, however, and there are likely high rates of non-compliance by users and owners of tanning salons[306].

A nationwide 10% tax on indoor tanning was implemented in the US in 2010 with the passing of the Affordable Care Act in an attempt to curb tanning bed use. A drop of approximately 25% in tanning salon patronage accompanied the tanning bed tax, although other salons reported customers were indifferent to the tax[307].

Even more recently there has been a push to name tanning bed use an addiction, as frequent users can exhibit many of the classic signs of addiction[308, 309]. A study of indoor tanning users found that those who met standards for addiction to indoor tanning exhibited higher anxiety-related symptoms and substance abuse than those who did not[309]. This suggests that for some indoor tanning salon users, taxes and regulations will be insufficient to prevent tanning.

Autophagy in UV-Induced Disease Prevention and Treatment

Autophagy modulators are of clinical interest for the treatment and prevention of skin cancer and many other diseases. However, the context-dependent and often opposing functions of autophagy make it difficult to predict response to autophagy modulators in the clinic. Here, we examine the current understanding of autophagy modulation in UV-induced skin cancer.

We have reported that AMPK is activated by UVB to induce autophagy[57], promote DNA repair[161], impair cell proliferation[161] and inhibit apoptosis[57, 236] under stress

conditions. AMPK activation is reduced in human skin cancer samples[161], and therefore, targeting AMPK for activation in skin cancers could provide an opportunity to block tumor growth. Our data support the use of AMPK activators metformin and AICAR to block the growth of UVB-induced skin tumors *in vivo*[161].

Autophagy activator rapamycin reduces UV-induced skin tumor formation and progression [94, 310]. Rapamycin treatment increases XPC levels through Twist1 downregulation[94] and consequently reduces the number of UV-induced mutations in p53 in skin tumors[310]. Inhibition of autophagy by Spautin-1 increases Twist1, decreases XPC and increases tumor growth induced by UVB[94]. Collectively, this work suggests that autophagy plays a tumor-suppressive function in UVB response. Understanding the mechanisms underlying UV-induced disease will provide new opportunities for the development of novel targeted therapies.

MATERIALS AND METHODS

Cell Lines and Treatments

Normal human epidermal keratinocytes (NHEKs) were grown in KGM-Gold BulletKit medium (Lonza) according to manufacturers protocol. NHEKs were cultured for less than 4 passages. Normal human epidermal melanocytes (NHEM) were obtained from Invitrogen and cultured according to the manufacturers instructions for less than 4 passages. iMC23 and iMC65 melanocytes were generated and cultured as described previously[311].

A375 human amelanotic melanoma cells, shNC mouse embryonic fibroblasts (MEFs), and shp62 MEFs were kindly provided by Dr. Seungmin Hwang at the University of Chicago. MEFs, A375 cells, PAM212 (squamous cell carcinoma) cells and Hela cells were maintained in Dulbeccos modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone), 1% non-essential amino acids (Invitrogen), 100 U/mL penicillin and 100 g/ml streptomycin (Invitrogen). Cells were maintained in monolayer culture at 37^{o}C and in 95% air/5% CO (vol/vol). WT, shNC, and shAtg5 iBMK (immortalized mouse baby kidney epithelium) were kindly provided by Dr. Eileen White at The Cancer Institute of New Jersey, NJ, USA. iBMK cells were maintained at 38.5^{o}C in 8.5% CO_2.

Inducible expression of PTEN in WM793TRPTEN cells was obtained by treatment of cultures with doxycycline (Sigma) at a final concentration of 100 ng/ml. Cells were maintained in DMEM with GlutaMAX (Invitrogen) supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 g/ml streptomycin, and 4 g/ml insulin (Sigma). Other melanoma cells were generously provided by Dr. Meenhard Herlyn (Wistar Institute, Philadelphia) and cultured as described previously[312].

To inhibit transcription, NHEKs were treated with 1 μg/ml actinomycin D (Fisher) for 1 hour prior to sham or UVA radiation. To inhibit autophagic flux, cells were treated with 25 nM Bafilomycin A1 (Sigma) for 1 hour prior to UVA radiation. Protein stability was assessed by treating cells with 100 ng/μl cyclohexamide (CHX) for the indicated times.

Cells were treated with 10 μM MG132 (Sigma) for 6 hours before lysing cells for co-IP. To study oxidative stress, cells were pretreated with 10 mM NaN3 for 1 h, 2.5 μM mitoCP for 1.5h, 1 mM NAC overnight, or 1 mM GSH-ester overnight prior to UV exposure.

Plasmid Transfection and siRNA

NHEKs were transiently transfected with siRNA targeting TFEB (Santa Cruz) or p62 (Dharmacon) using Amaxa Nucleofector kit according to manufacturers protocol. NHEM were transfected with siNC and siNrf2 siRNA (Dharmacon) using Amaxa nucleofector according to the manufacturers protocol. Mouse shCon, shp62, shAtg5, and shAtg7 constructs in pLKO.1 vector were purchased from Sigma. Lentivirus was produced by co-transfecting HEK-293T cells with the lentiviral construct, pCMV8.2 packaging plasmid, and pVSV-G envelope plasmid. Supernatant was collected 24-48 hours after transfection and used to infect cells in the presence of 8 μg/ml Polybrene (Sigma). Stable clones were selected using 2 μg/ml puromycin (Santa Cruz) treatment for 2 weeks as described previously[57].

HA-p62 in pcDNA4 was obtained from Qing Zhong (University of California, Berkeley) (Addgene plasmid 28027). Mutant HA-p62-dUBA was generated from HA-p62-pCDNA4 as described previously[57]. HeLa cells were transfected with HA-p62 and HA-p62-dUBA constructs using X-tremeGENE 9 (Roche) as described previously[94]. COX-2 (human) in pCDNA5 vector was generously provided by William Smith (University of Michigan)[313]. pCMV6-AC-GFP COX-2 (mouse) was purchased from Origene. COX-2 constructs were transiently transfected into HeLa cells (human COX-2) and PAM212 cells (mouse COX-2) using X-tremegene 9 HP transfection reagent (Roche) as described previously[94].

UVA Treatments

For UVA irradiation, four parallel PUVA lamps were used and doses were measured using a Goldilux UV meter equipped with UVA and UVB detector (Oriel). Contamination from

UVB irradiation was eliminated using a 0.13 mm Mylar filter material from Cope Plastics. This filter limits UVB exposure to 0.003% of total emitted UV radiation[314, 315]. We have found these lamps emit no UVC radiation (100-280 nm). The UVA dosages used here are relevant to human exposure. The dose of UVA which will cause erythema is 30 J/cm^2 for human skin and the UVA dose (20 J/cm^2) used in the *in vitro* studies equates to about 1 hour in the midday sun during the summer at latitude 48^{o}N in Paris, France[316]. In our laboratory, obtaining 20 J/cm^2 of UVA irradiation requires approximately 1 hour. Therefore, the UVA dose used in our application is relevant to human exposure.

RNA-Seq Analysis

Normal human epidermal keratinocytes (NHEKs) were exposed to sham or UVA irradiation (20 J/cm^2). Six hours after irradiation, RNA was prepared from these cells using an RNeasy Plus Kit (Qiagen), according to manufacturer's protocol. Two biological repeats were included for each treatment group. RNA quality assessment, library preparation, and sequencing were performed by the University of Chicago Functional Genomics Facility. RNA quality assessment was performed using an Agilent Bioanalyzer. An oligo dT-selected library was prepared and sequencing was performed on an Illumina HiSeq2000 platform with 50 bp single-end reads.

The established Tuxedo protocol was used to analyze the RNA-Seq data[317]. In this pipeline, quality control analysis of raw RNA-Seq data was performed in FastQ Groomer. Reads were then aligned to the human reference genome (hg38) using Tophat2. Transcript assembly was performed using Cufflinks. Cufflinks assemblies were combined using CuffMerge for all treatments. CuffDiff was used to calculate the difference in gene expression between sham- and UVA-irradiated samples, with a false-discovery rate of 10% and $p<0.05$ as a standard of significance. CummeRbund was used to perform analyses of differentially expressed genes. Gene ontology and KEGG pathway analysis were performed using DAVID.

Real-Time PCR

Quantitative real-time PCR assays were performed using a CFX Connect real-time system (Bio-Rad) using Bio-Rad iQ SYBR Green Supermix (Bio-Rad, 1708880). The threshold cycle number (CT) for each sample was determined in triplicate. The CT values were normalized against Gapdh as described previously[162, 94]. Amplification primers are as follows: GAPDH, 5-ACC ACA GTC CAT GCC ATC AC-3 (Forward, F) and 5-TCC ACC ACC CTG TTG CTG TA-3 (Reverse, R). TFEB, 5-GCT GAT CCC CAA GGC CAA T-3 (F) and 5-TCT CCA GCT CCC TGG ACT TT-3 (R). p62, 5-CAG AGA AGC CCA TGG ACA G-3 (F) and 5-AGC TGC CTT GTA CCC ACA TC-3 (R). LC3, 5-AGA CCT TCA AGC AGC GCC G-3 (F) and 5-ACA CTG ACA ATT TCA TCC CG-3 (R). ULK1, 5-TCG AGT TCT CCC GCA AGG-3 (F) and 5-CGT CTG AGA CTT GGC GAG GT-3 (R). ULK2, 5-TGG GTC CTC CCA ACT ATC TAC AAG T-3 (F) and 5-CGA GAT GTT GTG TGG CAC CAA-3 (R). LAMP1, 5-TCT CAG TGA ACT ACG ACA CCA-3 (F) and 5-AGT GTA TGT CCT CTT CCA AAA GC-3 (R). LAMP2, 5-GAA AAT GCC ACT TGC CTT TAT GC-3 (F) and 5-AGG AAA AGC CAG GTC CGA AC-3 (R). COX-2, 5'-CCC TTG GGT GTC AAA GGT AA-3' (F) and 5'-GCC CTG GCT TAT GAT CTG TC(R).

Western Blot

Prior to Western blot analysis, cells were treated as indicated, then lysed using RIPA lysis buffer containing protease and phosphatase inhibitors (Thermo). Supernatant was removed for analysis or frozen for later use. Normalization of total protein levels was performed using the BCA assay. Lysate was separated on 4-12% gradient SDS-PAGE gels (Novex) and blotted onto nitrocellulose membranes (Novex). For expression analysis, the following antibodies were used: p62 (GP62-C, Progen), GAPDH (FL-335, Santa Cruz), COX-2 (ab15191, Abcam), LC3A/B (4108, Cell Signaling), Nrf2 (C-20, Santa Cruz), PTEN (Cell Signaling) and TFEB (A303-673A-M). Anti-horseradish peroxidase (HRP) secondary antibodies (Cell

Signaling) were used for visualization of proteins. Film was used for visualization.

ChIP

IP was performed on 5x10^6 cells following treatment with sham or UVA (20 J/cm^2) irradiation. ChIP was performed using an EZ-Magna ChIP A Kit (Millipore), according to manufacturer's protocol. Lysate was sonicated 12 times for 10s each, with a 30s rest between each sonication. IP was performed with TFEB (ab2636, Abcam) antibody, as well as with positive and negative control antibodies included in the EZ-Magna ChIP Kit. qPCR analysis of IP samples was performed using primers described in Supplementary Table 1.

Immunofluorescence Microscopy

For immunofluorescence analysis, cells were seeded on glass coverslips overnight prior to treatment. Following treatment, cells were fixed using 10% neutral buffered formalin permeabilized using Triton X-100, and incubated with primary antibody overnight. AlexaFluor fluorescence-conjugated secondary antibodies were added for 2h before imaging samples. Samples were examined using an inverted microscope with fluorescence function and dedicated analysis software (Olympus, Model IX71).

PGE_2 Production Assay

PGE_2 production was measured using a PGE_2 Parameter Assay Kit (KGE004B, RD). HeLa cells were transiently transfected with empty vector (EV), EV and HA-tagged p62, EV and COX-2, HA-p62 and COX-2 or HA-p62-dUBA and COX-2 using X-tremegene 9 transfection reagent. 48 hours after transfection, cells were serum starved for 24 hours before taking samples for this assay. PAM212 cells with stable knockdown of p62 (shp62) were transiently transfected with mouse COX-2 (shp62-COX-2). 48h after transfection, cells were serum starved for 24h prior to taking medium for this assay.

Migration and Proliferation Assays

Cell proliferation was measured using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (MTS) (Promega) according to manufacturer's protocol. Cell viability was measured using the Cell Counting Kit-8 (CCK-8) assay (Sigma) according to manufacturers protocol. Migration was measured using Transwell inserts (Corning) with serum-free medium above and below insert, with medium supplemented with 10% FBS below insert, and with serum-free medium containing PGE_2 below insert. Migrated cells were measured 16 hours after seeding.

ROS Assays

To measure glutathione oxidation following UVA exposure, cells were first transfected and treated as indicated. Total glutathione (GSH) and oxidized glutathione (GSSG) levels were assessed using the GSH/GSSG-Glo assay (Promega) according to manufacturers protocol. Briefly, cells were lysed using total glutathione or oxidized glutathione lysis reagent, then treated with luciferin generation reagent followed by luciferin detection reagent. Luciferase activity was measured using a luminometer and GSH/GSSG calculated from a glutathione standard curve.

Cells were treated with or without UVA radiation for 24h and then incubated with CM-DCFH-DA (2 M, Invitrogen) for 30 min at 37C. Cells were washed with PBS three times, and oxidative stress was analyzed by flow cytometry, as described previously[318].

Mouse Studies

All animal procedures were approved by the University of Chicago Institutional Animal Care and Use Committee. BALB/c mice were purchased from Charles River Laboratories. 5 million PAM212 cells, which are syngeneic with BALB/c mice, were injected subcutaneously into the flank of BALB/c mice. Tumor growth was measured over 10-12 weeks using a

caliper and at $1cm^3$ volume, tumors were harvested along with lungs for histological analysis. Tumors and lungs were fixed in 10% formalin for IHC analysis. For UVA treatment studies in mice, SKH1 hairless mice received sham or 15 J/cm^2 UVA irradiation 3 times, every other day, then skin samples were harvested 72 h after the final treatment for IHC.

Immunohistochemistry

For HE, myeloperoxidase (MPO), and keratin 10 (K10) staining, tumors and lungs (or skin in UVA experiments) were harvested and fixed in 10% formalin. Paraffin-embedding, sectioning, and HE staining were performed by the Human Tissue Resource Center at the University of Chicago. MPO (Abcam ab45977, 1:500) and K10 (Covance MMS-159S, 1:1000) staining was performed as described previously46. Stained samples were scanned by the Integrated Light Microscopy Core Facility at the University of Chicago.

Human Skin Samples

All human specimens were studied after approval by the University of Chicago Institutional Review Board. Formalin-fixed, paraffin-embedded tissue blocks were obtained under consent (Dept. of Medicine, University of Chicago). Non-sun-exposed normal skin, nevus, and malignant and metastatic melanoma tissues were used for immunohistochemical analysis of p62 protein levels.

Statistical Analysis

Statistical analyses for qPCR, and migration, proliferation, viability and PGE_2 production assays were performed using GraphPad Prism 5. Data were expressed as mean of at least three independent experiments and analyzed by Student's t test. Error bars indicate standard deviation of the mean. $P<0.05$ is considered statistically significant.

UVA REGULATES P62 IN MELANOCYTES AND MELANOMA CELLS

Summary

To understand the effects of UVA radiation on melanocytes and melanoma, we examined levels of adaptor protein p62 in pigmented and amelanotic pigmented cells. We also identified a feedback loop between p62 and PTEN which could impact melanomagenesis by clearing ROS.

Introduction

Melanomas account for only 4% of skin cancer diagnoses, but cause nearly half of all skin cancer deaths[7, 3]. Recent advances in melanoma therapy, such as targeted therapy and immunotherapy, have benefitted a growing number of melanoma patients[319, 320, 321, 322]. However, many patients fail to have a sustained response, and better therapeutic advancements for melanoma are urgently needed. A better understanding of melanoma development and progression is required. [2]

60-70% of all melanoma cases are attributed to UV exposure[11]. UVA is the most abundant form of UV in sunlight and indoor tanning beds[13]. UVA penetrates deep into the dermis and has been linked to oxidative stress-induced skin damage[16]. UVA is absorbed by cellular photosensitizers, such as melanin in melanocytes, leading to the production of reactive oxygen species (ROS)[18, 323]. Evidence suggests that the absorption of UVA by melanin is photoprotective, as both UVA exposure and reduced levels of melanin are associated with melanoma risk[323, 324, 325].

UVA-induced ROS production triggers an antioxidant response, led by Nrf2[204, 201]. KEAP1, which facilitates the degradation of Nrf2 at the proteasome, is sequestered away

[2]This chapter and the associated methods in the previous chapter are reproduced with little modification from an article in press at *Photochemistry and Photobiology*. Modification is limited to minimal reorganization of figures to improve image quality.

from Nrf2 by p62 to allow Nrf2 stabilization[326, 273]. Nrf2 in turn controls p62 transcription in a positive feedback loop that perpetuates antioxidant response[327].

p62 functions as an adaptor and substrate for selective autophagy, and as an oncogenic signaling adaptor[328]. We and others have found that overexpression of p62 induces proliferation, invasion, and ultimately, tumor growth and metastasis[98, 329, 266, 99]. Overexpression of p62 is seen in a number of cancers, including melanoma, and is a marker of poor prognosis[98, 100]. Transcription of p62 is induced by UVA in skin fibroblasts[96], further implicating p62 in the UVA response.

PTEN is a crucial tumor suppressor and undergoes loss-of-heterozygosity (LOH) in a proportion of melanomas[330]. Loss of PTEN promotes tumorigenesis by increasing cell survival[330]. High levels of p62 have been found to be associated with PTEN deletion in prostate cancer, and loss of PTEN coupled with high p62 levels were associated with poor prognosis[264]. In breast cancer, p62 knockdown leads to PTEN accumulation concurrent with reduced AKT activation[331]. However, little is currently known regarding the function and regulation of p62 in UVA response and melanoma. Here, we examine the regulation of p62 by UVA and the functional implications for melanoma.

Results

UVA induces p62 up-regulation in melanocytes and melanoma cells

To determine the effects of UV radiation on p62 expression, we treated lightly pigmented adult human epidermal melanocytes with UVA (20 J/cm^2) or UVB (20 mJ/cm^2) for 6 and 24 hours. In these cells, p62 was induced by UVA, but not UVB irradiation (Figure 7A). Similarly, in lightly pigmented melanocyte progenitor iMC23 cells, p62 was induced by UVA in a dose-dependent manner 24h post-irradiation (Figure 7B). In darkly pigmented and differentiated iMC65 melanocytes, however, p62 induction by UVA was reduced at

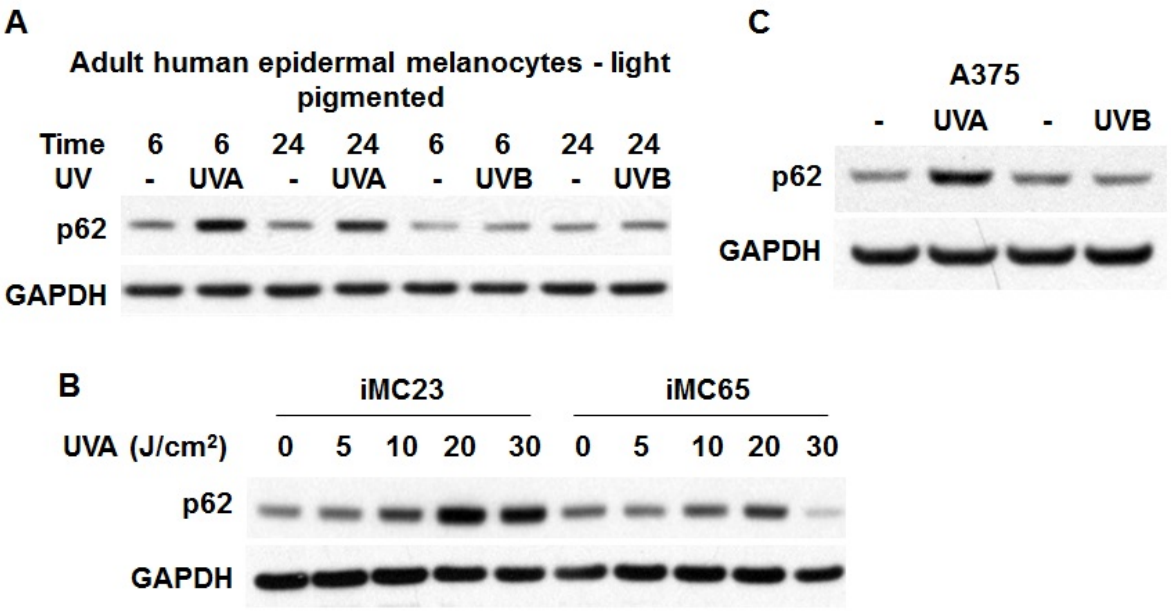


Figure 7: **UVA, but not UVB induces p62 in melanocytes.**
(A) Immunoblot analysis of p62 and GAPDH in normal human epidermal melanocytes(NHEM) with light pigmentation treated with sham, UVA (20 J/cm^2) or UVB (20 mJ/cm^2) irradiation and then harvested at 6h or 24h post-irradiation. (B) Immunoblot analysis of p62 and GAPDH in iMC23 and iMC65 melanocytes treated with UVA radiation and collected at 6h post-irradiation. (C) Immunoblot analysis of p62 and GAPDH in A375 melanoma cells treated with sham, UVA, or UVB irradiation and collected at 6h post-irradiation.

higher doses as compared with iMC23 cells (Figure 7B). This suggests that pigmentation is protective against UVA-induced p62.

In amelanotic A375 melanoma cells, treatment with 20 J/cm^2 UVA was found to induce p62 expression 6h post-irradiation, while UVB exposure (20 mJ/cm^2) did not (Figure 7C).

ROS and Nrf2 are required for UVA-induced p62 up-regulation

One known effect of UVA radiation is the generation of reactive oxygen species (ROS). To determine whether oxidative stress has a role in UVA-induced p62 up-regulation, we treated cells with sodium azide (NaN3), a known scavenger of the ROS species singlet oxygen. Treatment with NaN3 prior to UVA exposure inhibited UVA-induced p62 up-regulation as well as Nrf2 accumulation in iMC23 cells (Figure 8A). Pretreatment with the antioxidant mitochondria-targeted carboxy-proxyl (mito-CP) similarly inhibited UVA-induced p62 up-regulation as well as Nrf2 accumulation (Figure 8B). Knockdown of the antioxidant response factor Nrf2 in normal melanocytes impaired the up-regulation of p62 by UVA, suggesting that p62 is up-regulated in part by Nrf2 in response to UVA (Figure 8C).

However, pretreatment with the antioxidant N-acetyl cysteine (NAC) overnight enhanced UVA-induced expression of p62 and Nrf2 (Figure 8D). The inability of antioxidants to suppress Nrf2 and p62 induction by UVA in these cells suggests that pigmentation may play an oxidative stress-independent role in the regulation of UVA response. Glutathione ester (GSH-ester) also caused a slight increase in UVA-induced p62 expression in iMC23 melanocytes (Figure 8E). These data indicate that NAC and glutathione fail to protect the cells from UVA-induced p62 up-regulation and Nrf2 accumulation.

p62 promotes UVA-induced ROS production and Nrf2 up-regulation

Given the regulation of Nrf2 by p62, we next investigated whether p62 regulates UVA-induced oxidative stress. p62 deletion did not affect basal levels of ROS (Figure 9A). In WT cells, UVA radiation increased the ROS level (Figure 9A). However, ROS were not signifi-

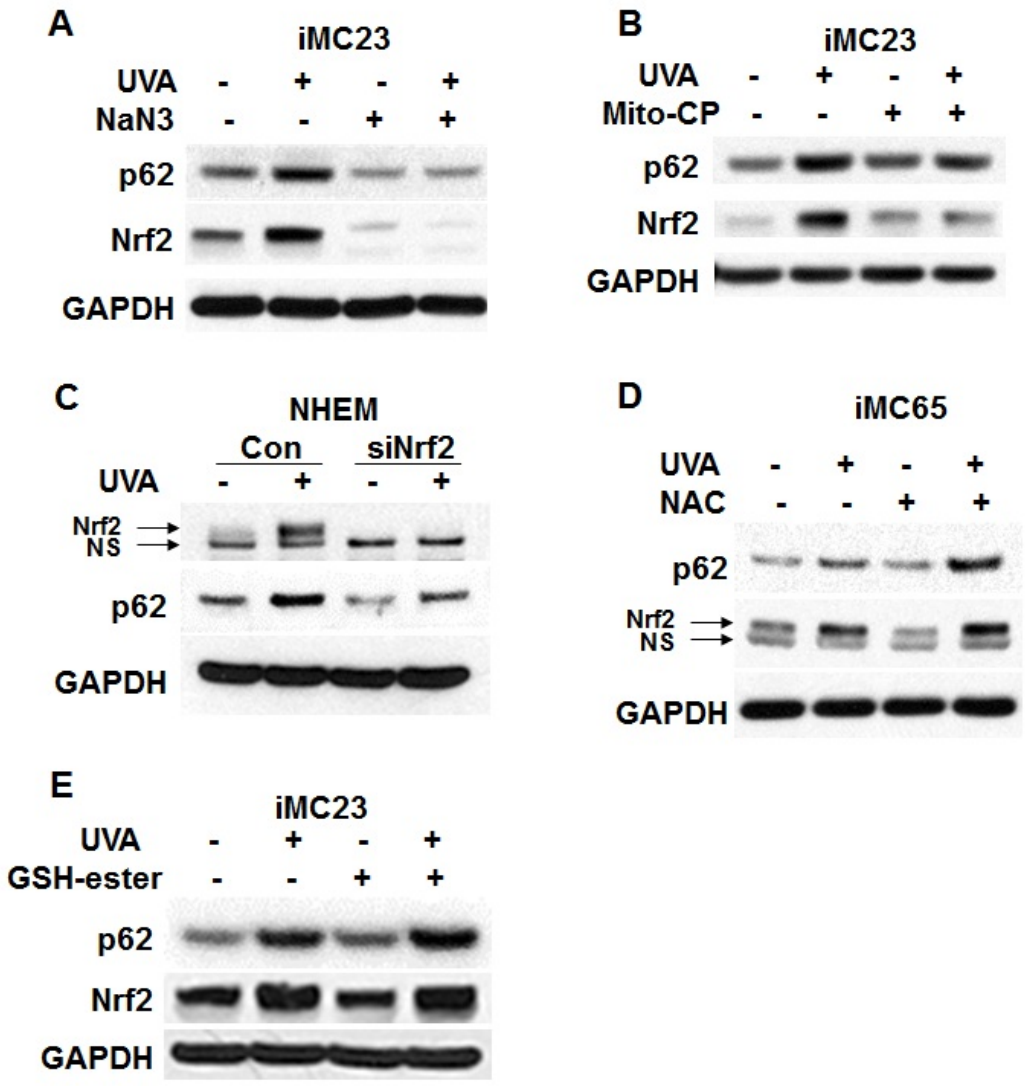


Figure 8: **UVA-induced p62 upregulation requires ROS and Nrf2.** (A) Immunoblot analysis of p62, Nrf2, and GAPDH in iMC23 melanocytes treated with 10 mM sodium azide (NaN3) overnight prior to UVA irradiation (20 J/cm^2). (B) Immunoblot analysis of p62, Nrf2, and GAPDH in iMC23 melanocytes treated with 2.5M mito-CP for 1.5h prior to UVA exposure. (C) Immunoblot analysis of p62, Nrf2, and GAPDH in NHEM cells transfected with siRNA targeting negative control (siCon) or Nrf2 (siNrf2) for 48h and then irradiated with UVA. (D) Immunoblot analysis of p62, Nrf2, and GAPDH in iMC65 melanocytes treated with 1 mM NAC overnight and then irradiated with UVA. (E) Immunoblot analysis of p62, Nrf2, and GAPDH in iMC23 cells treated with 1 mM GSH-ester overnight and then irradiated with UVA exposure. NS, non-specific band.

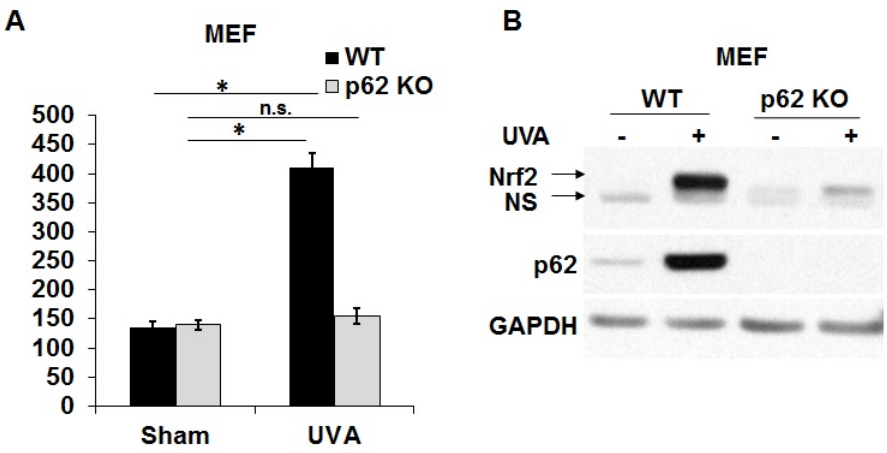


Figure 9: **p62 regulates UVA-induced ROS and antioxidant response.** (A) Flow cytometric analysis of reactive oxygen species (ROS) levels in WT and p62 KO MEFs at 0h post-sham or -UVA (20 J/cm^2) using DCFH-DA assay. (B) Immunoblot analysis of p62, Nrf2, and GAPDH in WT and p62 KO MEFs at 6 h post-sham or -UVA (20 J/cm^2). NS, non-specific band.

cantly induced in p62-deficient cells following UVA radiation (Figure 9A). p62 knockdown in mouse embryonic fibroblasts (MEFs) inhibited the induction of Nrf2 in response to UVA (Figure 9B), indicating that UVA-induced p62 up-regulation is required for ROS generation and Nrf2 accumulation.

p62 and PTEN form a feedback loop in melanoma cells

Given that previous work had suggested a negative association between p62 and PTEN expression in the context of prostate cancer[264], we next examined whether a similar feedback loop between p62 and PTEN exists in melanoma cells. In the malignant melanoma cell line WM793, doxycycline-induced PTEN expression decreased p62 expression (Figure 10A). Knockdown of p62 in A375 melanoma cells increased PTEN expression (Figure 10B), indicating that p62 and PTEN form a feedback loop in melanoma cells.

p62 is up-regulated in human melanomas and melanoma cells

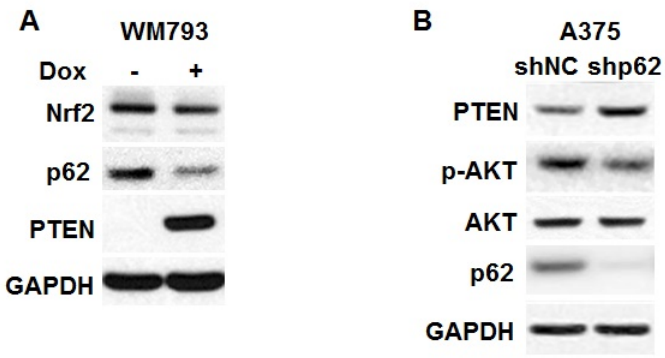


Figure 10: **p62 and PTEN form a feedback loop in melanocytes.** (A) Immunoblot analysis of Nrf2, p62, PTEN, and GAPDH in WM793 melanoma cells treated with doxycycline for 48h to induce PTEN overexpression. (B) Immunoblot analysis of PTEN, p-AKT, AKT, p62, and GAPDH in A375 melanoma cells stably infected with a lentiviral vector expressing shRNA targeting negative control (shNC) or p62 (shp62).

Given the importance of p62 for UVA response in melanocytes and melanoma cells *in vitro*, we next examined the presence of p62 in melanoma patient samples. As compared with normal skin, p62 protein levels increased in nevus, malignant melanoma, and metastatic melanoma (Figure 11A, 11B). 60% of melanomas express a mutant form of $BRAF^{V600E}$ and 20% of melanomas express mutant NRAS. In melanomas with varying BRAF and NRAS mutation states, p62 was up-regulated compared to normal melanocytes (Figure 11C), independent of BRAF or NRAS mutation status (Figure 11C), suggesting that p62 acts as an oncogene in melanoma development.

Conclusions

Here we report that UVA induces p62 in melanocytes and melanoma cells via ROS- and Nrf2-dependent mechanisms. UVA is thought to act primarily through ROS generation to promote tumorigenesis. Here we show that UVA induces p62 expression through ROS production, as antioxidants and ROS scavengers inhibit p62 induction by UVA. Interestingly, the antioxidant response factor Nrf2, which is known to positively regulate p62 transcription, is also required for p62 induction by UVA. This work suggests that UVA could induce p62

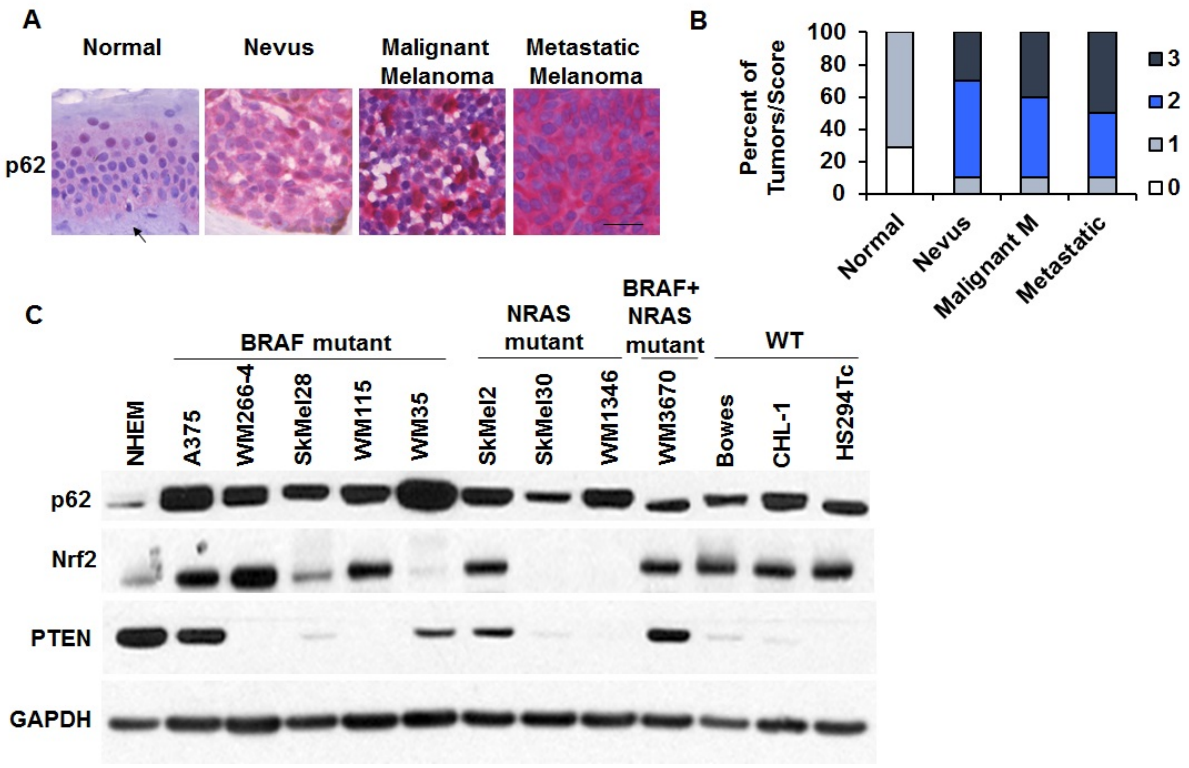


Figure 11: **p62 is up-regulated in human melanomas.**
(A) Immunohistochemical analysis of p62 protein levels in normal human skin, nevus, malignant melanoma, and metastatic melanoma. Scale bar: 25 μM. Arrow indicates a melanocyte. (B) Percentage of tumors (in stacked column format) for each score of p62 expression. (C) Immunoblot analysis of p62, Nrf2, PTEN and GAPDH in NHEM and human melanoma cells lines with $BRAF^{V600E}$ mutation, NRAS mutation, BRAF+NRAS mutations, or wild-type (WT)BRAF/NRAS.

through Nrf2 as a protective mechanism against ROS. p62 knockdown reduced UVA-induced ROS production and Nrf2 accumulation, suggesting that p62 is crucial for UVA-induced oxidative stress. p62 knockdown also positively regulated PTEN abundance, and PTEN overexpression decreased p62 levels, indicating a feedback loop between p62 and PTEN. Our findings demonstrate that UVA induces p62 accumulation as part of the oxidative stress response and suggest that p62 functions as an oncogene in melanoma pathogenesis.

We show here that Nrf2 is required for UVA-induced p62 up-regulation. Nrf2 is a transcription factor that can induce p62 transcription[327]. In turn, p62 can bind to the Nrf2 negative regulator KEAP1 to stabilize Nrf2[326, 273]. In response to UVA irradiation, p62 and Nrf2 form a positive feedback loop and are likely to enhance the Nrf2-dependent antioxidant transcription program in the face of oxidative stress. Nrf2 is known to mediate gene expression of many antioxidant genes. Perhaps the p62-Nrf2 axis serves as an oxidative stress response mechanism for the melanocytes or melanoma cells to adopt and survive. However, we cannot exclude the role of autophagy and autophagic flux in UVA-induced p62 up-regulation. p62 is known to be a selective autophagy receptor and substrate, and it can be degraded via the autophagy pathway[332, 86]. Future studies will elucidate the role of autophagy in UVA-induced p62 up-regulation.

It is unclear why different antioxidants had distinct effects on UVA-induced p62 accumulation. The singlet oxygen quencher sodium azide and the mitochondria-targeted antioxidant Mito-CP abolished UVA-induced p62 up-regulation, whereas NAC and GSH had no effect. Indeed, recently, using a genetically modified melanoma mouse model, Gal and colleagues have shown that NAC increases lymph node metastases of melanoma[221]. *In vitro*, NAC and Trolox, the soluble vitamin E analog, increased cell migration and invasive properties[221]. These findings suggest that antioxidants such as NAC at the dose tested may not be protective against melanoma progression. Future studies are essential to define the role of different types of antioxidants on melanoma initiation and progression.

In addition, we found that p62 knockdown prevented UVA-induced ROS production and

oxidative stress. The reduced ROS production in p62 knockdown cells can also contribute to the reduction in UVA-induced Nrf2 accumulation. However, the underlying mechanism is unclear. It is possible that the accumulation of p62 led to p62-mediated protein aggregation in the cells in response to UVA damage. It is also possible that p62 induction disrupted mitochondrial function, which can lead to increased ROS formation. Further investigation is required to elucidate the underlying mechanism by which p62 induction regulates oxidative stress.

Consistent with previous studies[100], we found that p62 is up-regulated in human nevus, malignant melanoma, and metastatic melanoma, as compared with normal human skin melanocytes. In addition, as compared with normal human epidermal melanocytes, p62 is up-regulated in a panel of human melanoma cell lines. These findings suggest that p62 acts as an oncogene. p62 may regulate melanoma development through multiple pathways, such as Twist1, NF-κB, or Nrf2, or a combination of these pathways[326, 273, 328, 99, 333, 61, 334]. Our recent work showed that p62 stabilizes Twist1 to promote cell proliferation and migration *in vitro* and increases melanoma tumor growth *in vivo*[98]. Future studies are needed to delineate the role of other p62-controlled pathways in melanoma development.

Our work also establishes a feedback loop between p62 and PTEN in melanoma, in which forced expression of PTEN suppresses p62 expression and knockdown of p62 increases PTEN. We and others have shown that p62 is upregulated in melanoma and correlates with poor prognosis[98, 100]. The existence of a p62-PTEN regulatory loop suggests an important role for p62 in melanoma progression. Loss of PTEN in melanoma patients, therefore, may compound the up-regulation of p62 throughout disease progression. Collectively, this work implicates p62 up-regulation as an important factor in UVA response in melanocytes and in melanoma pathogenesis.

UVA REGULATES P62 IN KERATINOCYTES AND SCC

Summary

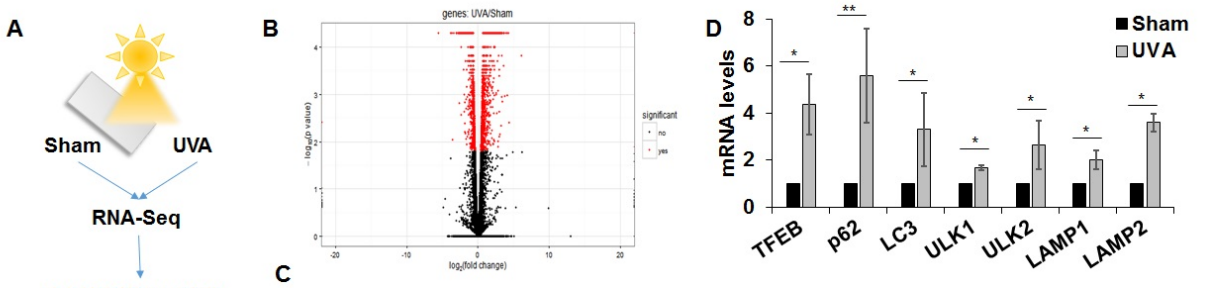
To examine the effects of UVA radiation, we studied the affects of UVA radiation exposure on keratinocytes using RNA-Seq. Upon UVA exposure, we found that keratinocytes upregulated a number of autophagy and lysosomal genes through master autophagy regulator TFEB. Autophagy adaptor protein p62 transcription was up-regulated by TFEB in response to UVA.

Introduction

Squamous cell carcinoma (SCC) affects more than half a million Americans each year[5], and causes an estimated 9,000 deaths[6]. 80-90% of SCCs are attributed to UV radiation exposure[11, 12]. UVA induces skin carcinogenesis *in vivo*[170, 171, 172] and indoor tanning, even intermittently, significantly increases skin cancer risk[173, 174]. However, the mechanism of UVAs contribution to skin cancer remains unclear.

Transcription factor EB (TFEB) is a master regulator of autophagy and lysosomal gene expression. One TFEB target gene, oncogenic autophagy adaptor p62, plays a number of crucial roles in tumorigenesis and tumor progression. TFEB activates transcription of autophagy genes such as p62 through a phosphorylation-dependent mechanism. Under nutrient-rich conditions, TFEB is primarily cytosolic, phosphorylated, and inactive. Upon nutrient deprivation, TFEB rapidly translocates to the nucleus to induce transcription of autophagy and lysosomal genes. However, the role of TFEB in UVA response is unknown. Identifying the underlying molecular and cellular mechanisms of p62 regulation and function may elucidate mechanisms key to skin tumorigenesis and tumor progression.

Results



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Pathway:	Genes Upregulated:
Pathways in Cancer	COX-2, c-myc, EGF, MDM2, Bcl-XL, p21, JNK, HIF1α, CEBPα
MAPK Signaling Pathway	EGFR, FGFR, PDGF, Ras, PKA, CREB, RSK2, TNFR, TGFBR, Fas, IL-1
Cell Adhesion Molecules	CLDN, OCLN, JAM1, IGSF4, NRCAM, CDH2
Lysosome	LAMP1, LAMP2, LIMP, cathepsins, MANB, HYAL1
Additional Autophagy Genes	TFEB, p62, ULK1, ULK2, LC3, NBR1, Atg14, Atg16L2

Figure 12: **UVA induces expression of autophagy and lysosomal genes.** (A) Schematic of RNA-Seq analysis of normal human epidermal keratinocytes (NHEK) at 6 h post -sham or -UVA (20 J/cm^2) radiation. Gene expression and pathway analysis were performed using the Tuxedo pipeline and DAVID. (B) Volcano plot representing genes significantly differentially expressed between sham and UVA-irradiated NHEKs as determined by RNA-Seq. $p<0.05$. (C) Pathway analysis of the significantly upregulated genes by RNA-Seq identified the highly enriched pathways following UVA irradiation in NHEKs. (D) Real-time PCR validation of RNA-Seq results in NHEKs treated with UVA. *$p<0.05$, **$p<0.01$ (Student's *t*-test).

UVA radiation activates the autophagy-lysosome pathway

To identify genes regulated by UVA radiation, RNA-Seq was performed on sham or UVA-irradiated normal human epidermal keratinocytes (NHEK). A comparison of sham or UVA-irradiated NHEKs identified more than 4,000 differentially expressed genes (Figure 12A, 12B). Pathway analysis shows that one of the upregulated pathways following UVA exposure is the autophagy-lysosome pathway (Figure 12C). UVA induced expression of a number of autophagy-related genes, including TFEB, p62, LC3, ULK1, ULK2, LAMP1 and LAMP2 (Figure 12D). These data indicate that UVA activates the transcription program of the autophagy-lysosomal pathway, including the expression of p62.

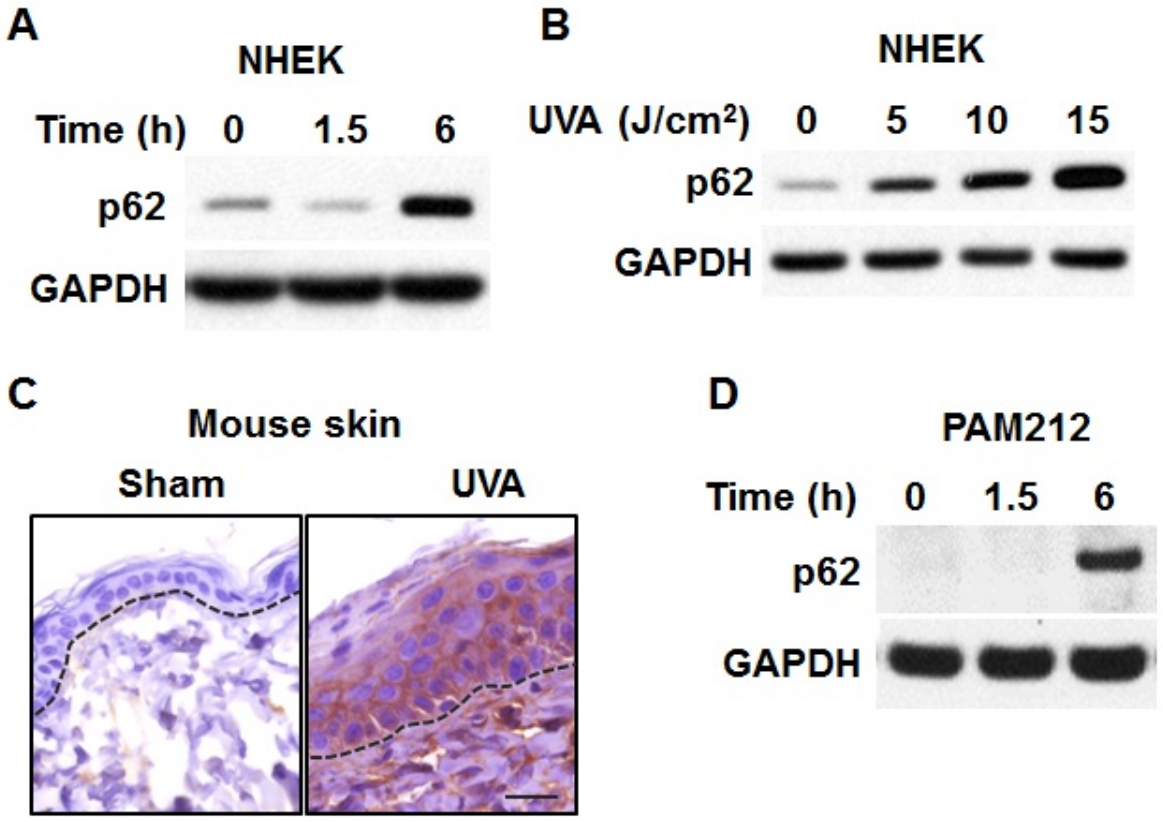

Figure 13: **UVA induces p62.**
(A) p62 protein levels in NHEKs 0, 1.5, and 6h after UVA radiation (10 J/cm^2). (B) p62 protein levels 6h after irradiation with 0, 5, 10 or 15 J/cm^2 UVA. (C) Immunohistochemical staining of p62 in mouse skin after UVA irradiation (30 J/cm^2) three times every other day and collected at 72 h post-the final UVA irradiation. (D) p62 protein levels in SCC cell line PAM212 0, 1.5, and 6h after UVA irradiation.

p62 regulation is independent of autophagy in UVA response

Since p62 is up-regulated in skin cancer and regulates skin tumor growth and metastasis [98], we elected to focus on the p62 induction by UVA. UVA irradiation increased the protein levels of p62 in a time- (Figure 13A) and dose-dependent (Figure 13B) manner. In addition, UVA also increased p62 protein levels in mouse epidermis *in vivo* (Figure 13C) and skin cancer cells (Figure 13D). To determine the mechanism by which UVA regulates p62 expression, we assessed the role of autophagy, as p62 is a selective autophagy substrate and thus inhibition of autophagy leads to an increase in p62 protein levels[332, 86]. We next

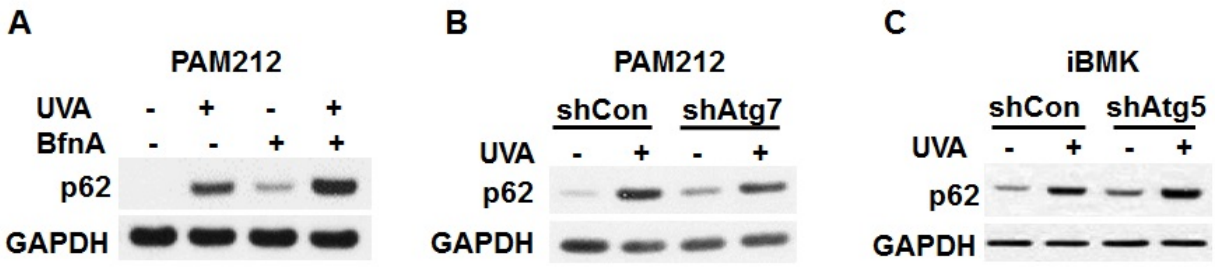

Figure 14: **UVA induces p62 independent of autophagy.**
(A) p62 protein levels in irradiated PAM212 cells treated with vehicle or autophagic flux inhibitor bafilomycin A1. (B) p62 protein levels in PAM212 cells transfected with shCon or shAtg7 after sham or UVA irradiation. (C) p62 levels in iBMK cells transfected with shCon or shAtg5 after sham or UVA irradiation.

assessed whether p62 upregulation was dependent on an inhibition of autophagic flux. Treatment with the lysosome bafilomycin A1, which inhibits autophagic flux, increased basal and UVA-induced p62 protein levels (Figure 14A). In addition, knockdown of essential autophagy genes Atg7 (Figure 14B) or Atg5 (Figure 14C) increased both basal and UVA-induce p62 up-regulation. These data indicate that UVA-induced p62 up-regulation is independent of autophagy.

TFEB is a UVA-responsive transcription factor regulating p62 expression

To determine the mechanism by which UVA regulates p62 expression, we assessed the role of the transcription factor TFEB (Figure 12C), as UVA induced TFEB expression in our RNA-Seq analysis (Figure 12C). TFEB activation and activation is negatively regulated by phosphorylation, with dephosphorylation triggering the nuclear translocation of TFEB and activation of CLEAR network/TFEB target genes. Western blot of TFEB protein levels following UVA exposure showed a shift in the molecular weight suggestive of TFEB dephosphorylation (Figure 15A). Furthermore, there was also an increase in TFEB nuclear localization after UVA (Figure 15B), supporting an increase in TFEB activation after UVA. Treatment with phosphatase inhibitor (PI) calyculin A inhibited the TFEB band shift induced by UVA

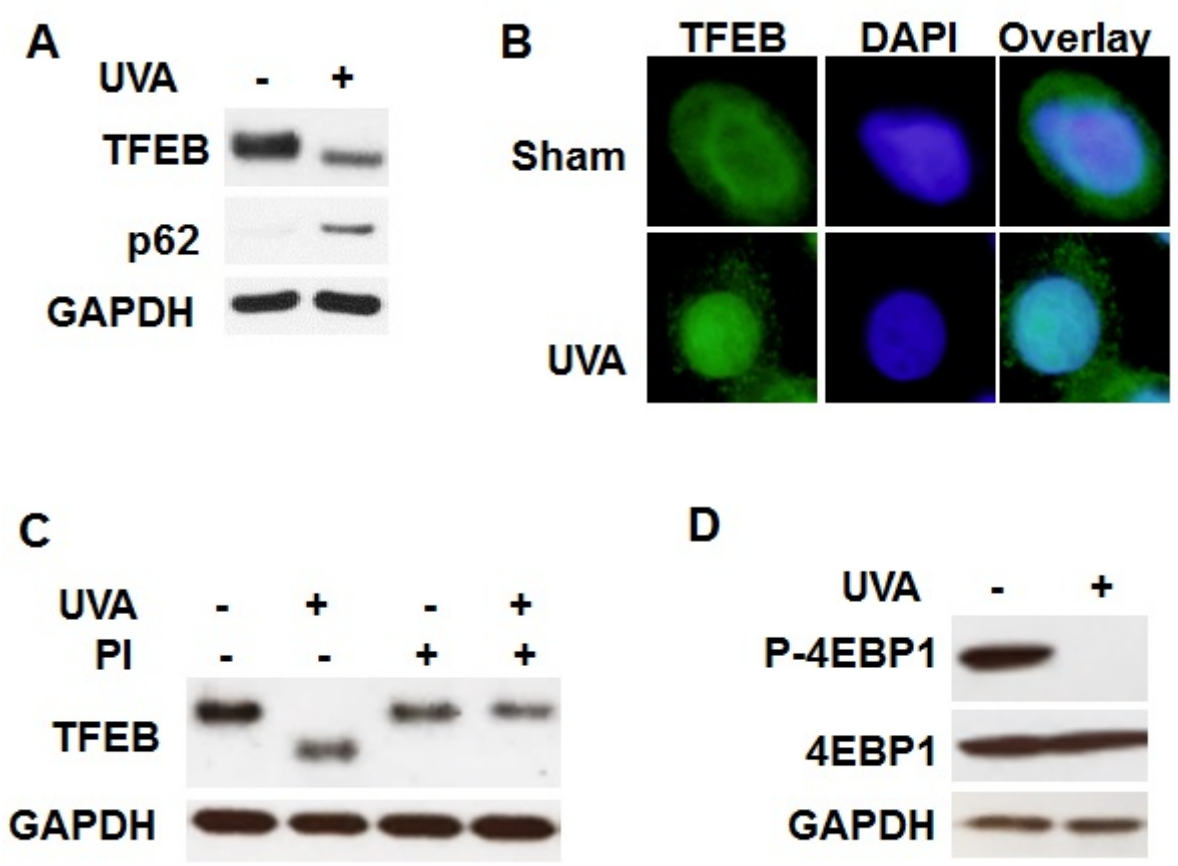

Figure 15: **TFEB is activated by UVA.**
(A) TFEB and p62 protein expression in NHEKs 6h after treatment with sham or UVA (20 J/cm^2) irradiation. (B) Immunofluorescence analysis of TFEB localization in NHEKs 6h after sham or UVA irradiation. (C) TFEB protein expression in NHEKs treated with phosphatase inhibitor (PI) calyculin A (50 nM) after sham or UVA irradiation. (D) Phosphorylation of mTOR target gene, 4EBP1, in response to UVA (20 J/cm^2) in NHEKs.

(Figure 15C), further supporting the hypothesis that TFEB is dephosphorylated and activated in response to UVA. Decreases in phosphorylation of 4EBP1, a mTOR target gene, are seen after UVA (Figure 15D), suggesting mTOR is suppressed by UVA. Suppression of mTOR signaling could be one mechanism of TFEB activation by UVA, as mTOR-mediated phosphorylation of TFEB inhibits TFEB activation.

TFEB knockdown prevented UVA-induced p62 up-regulation at the protein level (Figure 16A). Knockdown of TFEB prevented UVA-induced expression of p62 as well as LC3 (Figure 16B-16D). Inhibition of RNA synthesis by actinomycin D (Figure 16E) block UVA-induced

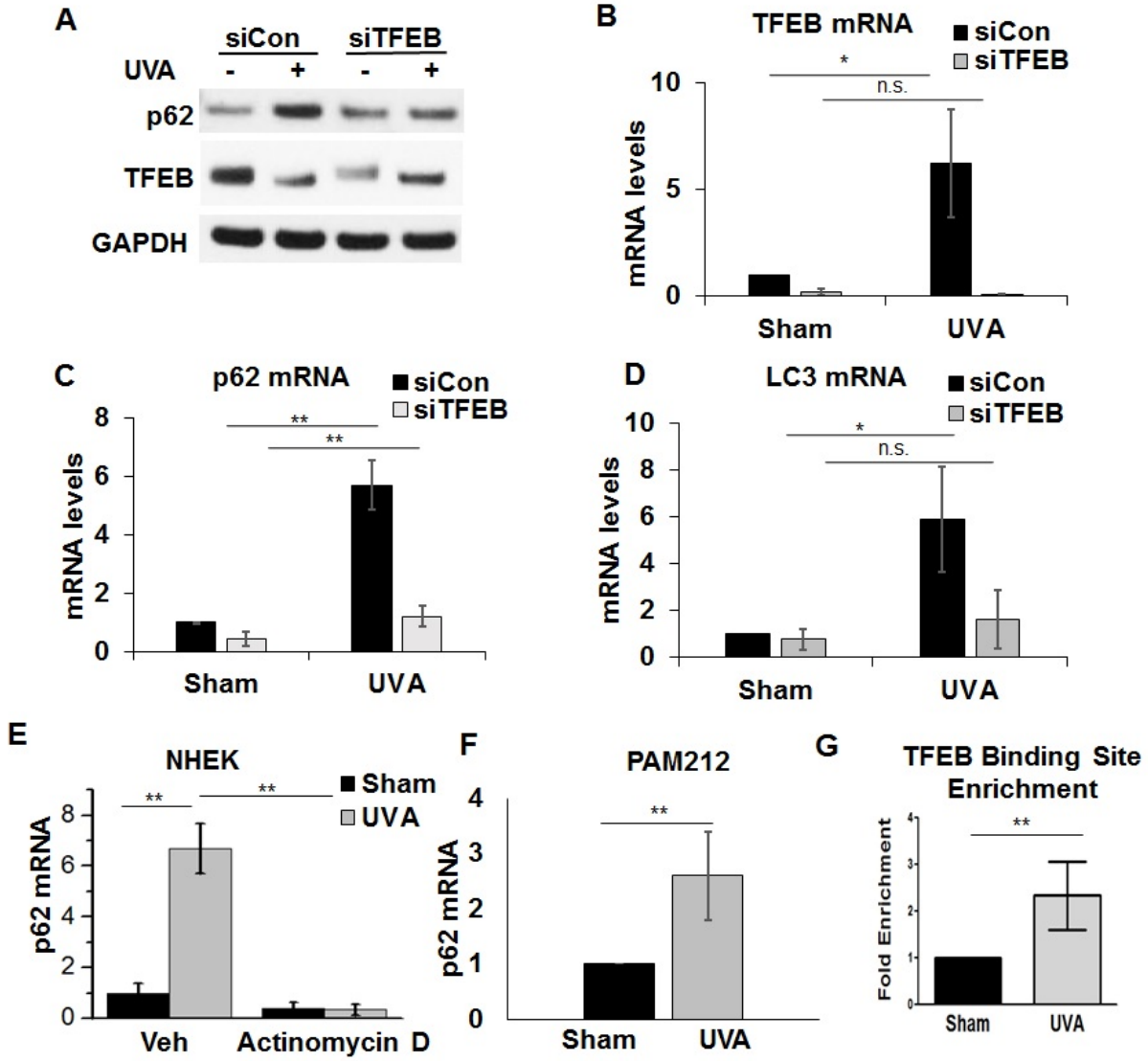

Figure 16: **TFEB is activated by UVA to induce p62 transcription.** (A) TFEB and p62 protein levels in NHEKs transfected with siCon or siTFEB treated with sham or UVA irradiation. (B) TFEB, (C) p62, and (D) LC3 mRNA in NHEKs transfected with siCon or siTFEB and treated with sham or UVA irradiation. n.s.=not significant; *p<0.05, **p<0.01. (E) qPCR analysis of p62 mRNA in NHEKs treated with sham or UVA radiation with or without actinomycin D (1 μg/ml) for 6h. (F) p62 mRNA levels in PAM212 squamous cell carcinoma cells 6h after sham or UVA irradiation. (G) Chromatin immunoprecipitation (ChIP) analysis of TFEB binding to the p62 promoter in NHEKs treated with sham or UVA irradiation. **p<0.01.

up-regulation of p62 mRNA, indicating that p62 is transcriptionally upregulated by UVA. Furthermore, UVA induced p62 up-regulation in PAM212 skin cancer cells (Figure 16F). Chromatin immunoprecipitation (ChIP) analysis of the p62 promoter indicated that UVA increased the binding of TFEB to the p62 promoter (Figure 16G). These findings have demonstrated that TFEB activation is required for UVA-induced p62 expression.

Conclusions

UVA is a complete carcinogen that is known to cause skin cancer. However, the signaling pathways underlying UVA response are unknown. Here we show that UVA radiation activates the transcription of an autophagy and lysosomal gene program including p62 through transcription factor TFEB. We found that UVA irradiation up-regulates p62 at the transcription levels, independent of autophagy, since (1) UVA increases p62 mRNA, (2) inhibiting RNA synthesis abolished UVA-induced p62 mRNA increase, and (3) UVA induced p62 up-regulation in lysosome-inhibited cells and cells with genetic autophagy inhibition. Previous studies have show that in keratinocytes UVA induces autophagy[58]. In contrary, in fibroblasts UVA has been shown to block autophagic flux[96]. Thus it is possible that the effect of UVA on autophagy is cell-type dependent. We anticipate the activation of autophagy by UVA will degrade p62 and reduces the induction of p62 at the protein levels. The role of autophagy-p62 interaction in UVA response requires further investigation.

In addition to p62, our RNA-Seq analysis in primary human keratinocytes identified multiple genes in the autophagy and lysosomal pathways. While we have shown that p62 is required for the transcription and protein stability of COX-2, and for skin tumor growth and metastasis *in vivo*, other UVA-regulated genes may have important roles in UVA-associated skin cancers. In particular, the function of the autophagy-lysosomal genes is unknown and needs to be investigated *in vitro* and *in vivo*. In addition to p62, UVA-induced TFEB nuclear translocation also mediates LC3 up-regulation at the mRNA levels.

P62 REGULATES COX-2

Summary

Having established the importance of p62 in UVA radiation response, we next evaluated its contribution to skin cancer progression. We found that p62 regulates COX-2 transcription and protein stability, and promotes skin tumor growth and metastasis.

Introduction

COX-2 is a critical oncogene in skin cancer. COX-2 expression is induced by a number of stimuli, including UVA[231], and is negatively regulated by the ubiquitin-proteasome system[335]. COX-2 acts through PGE_2 signaling to promote proliferation[232], invasion[247], and inflammation[336, 337]. Overexpression of COX-2 occurs in many cancers, including skin cancer[245, 229], and is correlated with poor prognosis[245]. Transgenic mice with overexpression of COX-2 are highly susceptible to spontaneous skin tumor formation[246], and knockdown of COX-2 reduces susceptibility to experimentally-induced tumorigenesis. Furthermore, inhibition of COX-2 prevents UV-induced skin tumorigenesis in humans[248, 249], even in patients at high risk of NMSCs[249].

Here, we show that p62 bound to and stabilized COX-2 through p62's UBA domain. p62-mediated COX-2 stabilization promotes increased PGE_2 production, and may be responsible for increased tumor growth and metastasis *in vivo*. Elucidating this link between p62 and COX-2 has therefore uncovered a novel oncogenic pathway key to the development and metastasis of skin cancer.

Results

p62 regulates COX-2 expression

To determine the function of p62 induction in UVA response, we explored its relationship

to COX-2. We found that cyclooxygenase-2 (COX-2) was upregulated in response to UVA, in parallel with p62 induction in NHEK cells (Figure 17A) and PAM212 skin cancer cells (Figure 17B). This was accompanied by a moderate increase in COX-2 transcription following UVA (Figure 17C and 17D). As previous reports have shown that COX-2 induction is required for the development of skin cancer[246], we next asked whether p62 regulates COX-2 in skin cancer cells. Knockdown of p62 led to a decrease in COX-2 protein levels in PAM212 cells (Figure 17E). COX-2 basal transcript levels were also reduced in p62-knockdown cells (Figure 17F). These data indicate that p62 regulates COX-2 protein and transcription.

p62 binds to COX-2 and regulates COX-2 stability

As increased COX-2 protein levels and activity are required for skin cancer development [246, 248, 249], we further examined the regulation of COX-2 protein levels by p62. We have shown that p62 stabilizes Twist1 via the direct interaction of p62s ubiquitin-associated (UBA) domain with polyubiquitinated Twist1[98]. Therefore, we asked whether p62 similarly regulated COX-2 protein stability through p62s UBA domain. Expression of p62 in HeLa cells, which lack endogenous p62, COX-2, and Twist1 expression, was sufficient to increase the protein stability of exogenously expressed COX-2 (Figure 18A, 18B). This increase in COX-2 protein stability was lost in cells transfected with a mutant p62 construct that lacks the UBA domain (Figure 18C), suggesting the regulation of COX-2 by p62 was dependent on p62's UBA domain. Co-immunoprecipitation (CoIP) of endogenous p62 and COX-2 in skin cancer cells shows that p62 interacts with COX-2 (Figure 18D). p62 also regulates COX-2 transcription through the UBA domain (Figure 18E). These results suggest that p62 regulates COX-2 transcription and protein stability.

p62 regulates PGE_2 production

COX-2 activity leads to the production of PGE_2, which signals through autocrine and paracrine signaling to promote tumorigenesis. To determine whether p62 regulates PGE_2

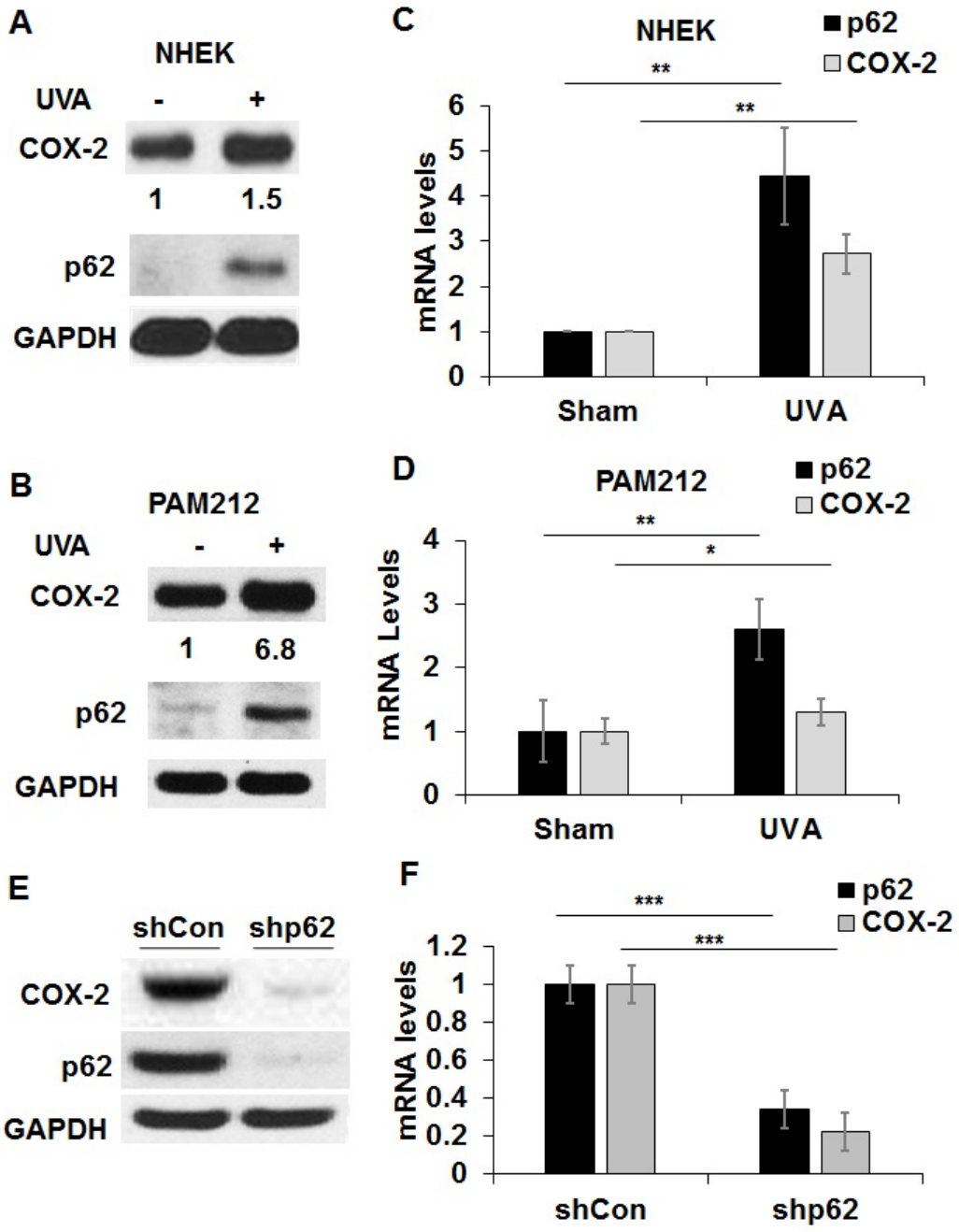

Figure 17: **p62 regulates COX-2 expression.**
(A) COX-2 and p62 protein levels in NHEKs treated with sham or UVA irradiation (20 J/cm^2). (B) p62 and COX-2 protein in PAM212 cells after UVA irradiation (10 J/cm^2). qPCR analysis of p62 and COX-2 protein in NHEKs (C) and PAM212 cells (D). *$p<0.05$, **$p<0.01$.(E) COX-2 protein levels in PAM212 cells transfected with shCon and shp62. (F) qPCR analysis of COX-2 RNA levels in PAM212 cells transfected with shCon and shp62. ***$p<0.001$.

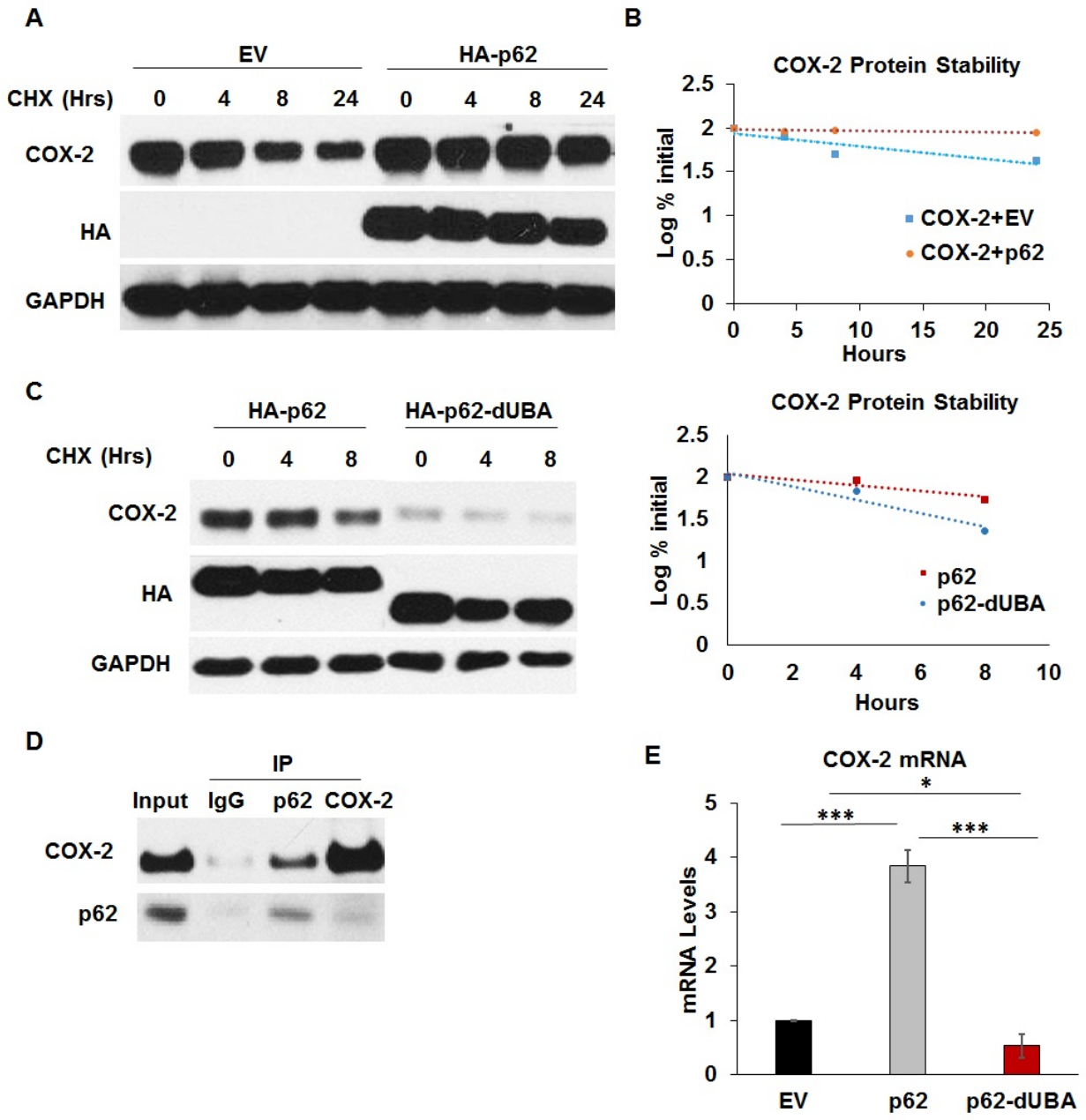

Figure 18: **p62 binds to COX-2 and regulates COX-2 stability.**
(A) HeLa cells were transiently transfected with COX-2 and either empty vector or HA-p62. COX-2 stability was measured over time by treating with cyclohexamide (CHX) for the time indicated. (B) Quantification of the stability of COX-2 protein in (A). (C) COX-2 stability was measured over time after treating with CHX for 0, 4, or 8h and quantified (right). (D) Co-immunoprecipitation of p62 and COX-2 in PAM212 cells treated with the proteasome inhibitor MG132 (10μM) for 6h. (E) COX-2 mRNA levels were measured in Hela cells transfected with the indicated HA-p62 constructs 48h after transfection. n=3, *p<0.05, ***p<0.001.

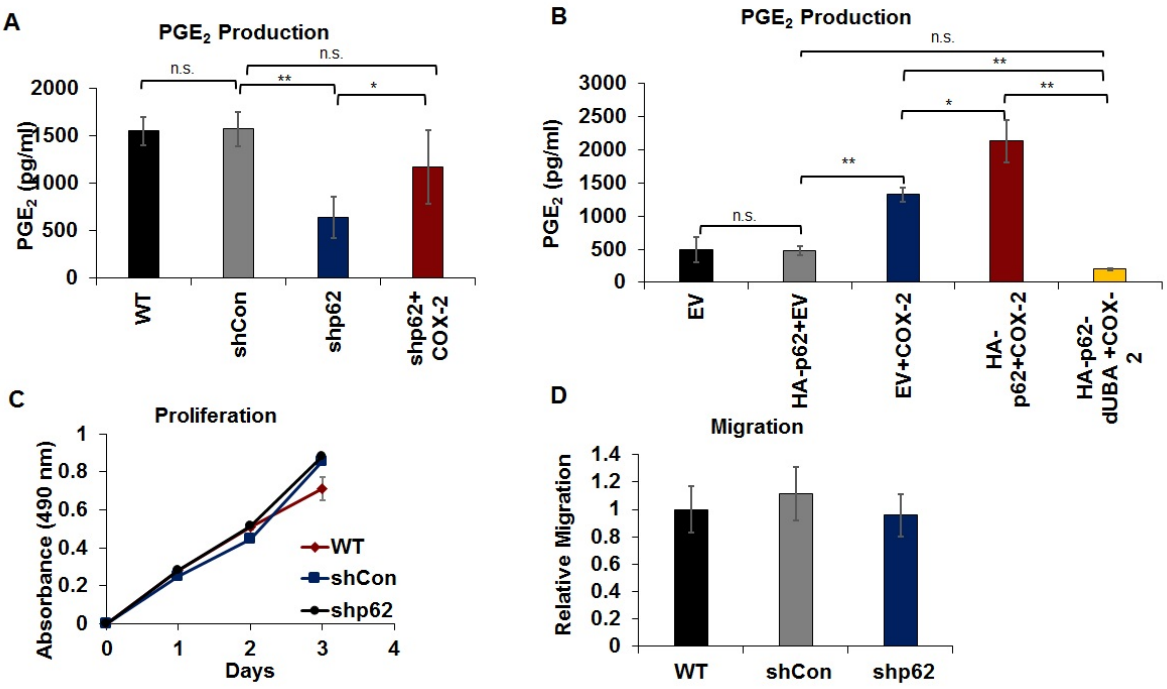

Figure 19: **p62 regulates PGE_2 production, but not proliferation or migration *in vitro*.**
(A) PGE_2 production in PAM212 cells stably transfected with shCon or shp62, and shp62 cells were transiently transfected with COX-2. n.s.=not significant; *p<0.05; **p<0.01. (B) PGE_2 production in HeLa cells transiently transfected with empty vector (EV), HA-p62, COX-2, and HA-p62-dUBA as indicated. n.s.=not significant; *p<0.05; **p<0.01. (C) Proliferation of PAM212 cells stably transfected with shCon or shp62 measured over 3 days using MTS proliferation assay. (D) Migration of WT, shCon,and shp62 PAM212 cells measured using Transwell migration assay in serum-free medium.

production as a result of COX-2 regulation, we examined PGE_2 levels in skin cancer cells following p62 knockdown. We found that p62 knockdown decreased PGE_2 production (Figure 19A), and this deficit was rescued by re-expression of COX-2 (Figure 19A). Similarly, expression of both HA-p62 and COX-2 in HeLa cells increased PGE_2 levels beyond PGE_2 levels in cells expressing COX-2 alone (Figure 19B). Co-expression of COX-2 with mutant p62 lacking the UBA domain reduced PGE_2 levels to that of cells lacking COX-2 (Figure 19B). Therefore, p62 regulates PGE_2 production by stabilizing COX-2 through p62s UBA domain.

p62 does not regulate proliferation or migration in vitro

Considering the known role of PGE_2 production in proliferation and migration, we assessed whether p62 knockdown impacted these functions in skin cancer cells. In both CCK8 (Figure 19C) and MTS (data not shown) assays, p62 knockdown in skin cancer cells had no effect on proliferation. Similarly, p62 knockdown had no effect on migration in Transwell migration assays (Figure 19D), even when PGE_2 was used as a chemoattractant (data not shown). While this data suggests that p62-mediated effects on PGE_2 production in skin cancer cells do not affect the cancer cells themselves, PGE_2 is a known paracrine signaling mediator and may affect neighboring cells in the tumor microenvironment.

p62 is required for tumor growth and metastasis in vivo

To test the requirement for p62 in skin tumor growth and progression, we utilized a syngeneic mouse model of skin cancer. In this model, we injected control and p62-knockdown PAM212 skin cancer cells into BALB/c mice. Tumor growth measurement over 10 weeks showed that p62 knockdown inhibited tumor growth (Figure 20A) and metastasis to the lung (Figure 20B). These data indicate that p62 is required for tumor growth and metastasis, Histological analysis of tumor samples indicated that p62-knockdown tumors exhibit decreased immune cell infiltration and increased cell differentiation (Figure 20C, arrows).

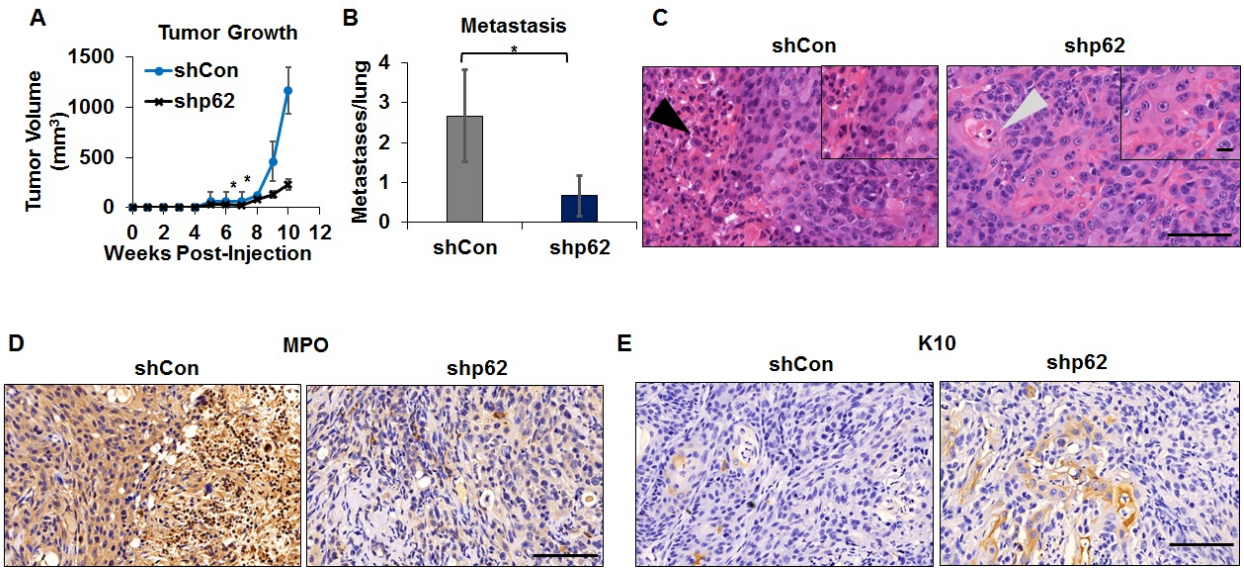

Figure 20: **p62 is required for tumor growth and metastasis *in vivo*.** (A) shCon and shp62 PAM212 cells were injected into syngeneic BALB/c mice and tumor growth measured over 10 weeks. N=6 per group. (B) Metastasis to the lungs of mice injected with shCon and shp62 PAM212 cells was measured using H&E staining. N=6 mice per group. (C) H&E staining of tumors from mice injected with shCon and shp62 PAM212 cells. Black arrow: immune cell infiltration. Gray arrow: differentiated keratinocytes. 20X magnification, scale bar=100 μm. Inset: 40X magnification, scale bar=50 μm. IHC staining for (D) MPO and K10 (E) in tumors from shCon and shp62 PAM212-injected mice. 20X magnification, scale bar=100 μm.

Therefore, we next performed IHC using immune and differentiation markers. Staining for myeloperoxidase (MPO), a marker of myeloid cells, showed that p62-deficient tumors exhibited lower levels of MPO-positive cells (Figure 20D). Staining for differentiation marker K10 also showed that p62-deficient tumors had an increase in K10-positive differentiated cells relative to controls (Figure 20E). These findings support an oncogenic role of p62 in tumor growth and progression.

Conclusions

We report here that p62 regulates COX-2 by two mechanisms: (1) promoting COX-2 expression, and (3) stabilizing COX-2 protein by binding to COX-2. Knockdown of p62 inhibits skin tumor growth and metastasis, in association with a decrease in immune cell

infiltration in the tumors. Our findings suggest that targeting p62 may be an effective method to prevent tumor growth and metastasis after UVA.

In examining potential functions of p62 in UVA response, we identified a novel relationship between p62 and COX-2. As we previously reported with Twist1[162], p62 binds and stabilizes polyubiquitinated COX-2. This stabilization was mediated by the UBA domain of p62, which is more commonly linked to the degradation of polyubiquitinated proteins at the autophagosome. COX-2 is primarily degraded at the proteasome via ER-associated degradation (ERAD)[338, 313]. The interaction between COX-2 and the UBA domain of p62 may prevent this degradation of COX-2 at the proteasome.

COX-2 stabilization by p62 also increases the production of PGE_2. PGE_2 is the key effector of COX-2 activity and acts to promote proliferation[232], invasion[247], and protumorigenic immune infiltration[336, 337], while inhibiting cancer cell differentiation. Knockdown of p62 phenocopies COX-2 knockdown[246], with decreased tumor growth, metastasis, and immune infiltration, as well as increased cancer cell differentiation. As these cells lack Twist1 expression, it is possible that p62 is acting through COX-2-mediated PGE_2 production to promote skin cancer growth and metastasis. Further study of the p62-COX-2 signaling axis could provide a novel target for the prevention and treatment of skin cancers.

DISCUSSION AND FUTURE DIRECTIONS

p62 up-regulation in melanoma

We report here that UVA induces p62 in melanocytes and melanoma, independent of BRAF or NRAS mutation status. This work supports previous research which has established that p62 is up-regulated and a prognostic marker in melanoma[100]. We showed that p62 is up-regulated through a ROS- and Nrf2-dependent mechanism. ROS are known to activate Nrf2 to induce the antioxidant response[201], and Nrf2 can activate p62 transcription [327]. As p62 forms a positive feedback loop with Nrf2 by interacting with KEAP1[272], p62 up-regulation could serve to perpetuate oxidative stress response. The UVA-induced oxidative stress response mediated by p62 is complex, however, as it induces both ROS and the Nrf2-mediated antioxidant response. This induction of oxidative stress in combination with an antioxidant response may serve to promote melanomagenesis by allowing cells to survive cancer-causing oxidative damage to proteins, lipids, and DNA. Further research could determine the whether p62 up-regulation serves to balance the oxidative stress and antioxidant response in melanoma.

Both p62 and autophagy are induced in response to UVA, despite autophagy being a negative regulator of p62. The sustained up-regulation of p62 by UVA alongside autophagy, suggests that the two have divergent, but complementary functions. It is possible that autophagy functions to clear proteins and protein aggregates damaged by oxidative stress, while p62 functions to suppress PTEN and modulate the oxidative stress response through Nrf2. Additional experiments could be performed to compare levels of oxidatively modified proteins, Nrf2 expression, and PTEN expression in autophagy-deficient melanoma cells and p62 knockdown cells. Understanding these complementary functions of autophagy and p62 could identify mechanisms key to melanomagenesis.

Clarifying the p62-PTEN feedback loop

We report here a novel feedback loop between p62 and PTEN. However, the mechanisms underlying this loop remain unclear. p62 may negatively regulate PTEN expression by facilitating its degradation via autophagy. Furthermore, negative regulation of p62 expression by PTEN occurs through an unknown mechanism.

Further studies should determine whether the p62-PTEN regulation occurs at the transcript level or protein level. If regulation is transcriptional, the signaling pathways responsible, such as the PI3K/Akt pathway, could be identified. If p62 regulates PTEN at the protein-level, possible interactions should be interrogated, the domain through which p62 binds to PTEN identified, and degradation through autophagy examined as a potential mechanism. Finally, we could assess whether this negative feedback loop between p62 and PTEN is seen in other contexts, including other types of skin cancer and cancer of other organs.

Identifying the mechanism by which UVA regulates TFEB

We show here that TFEB is induced at the transcriptional level and activated at the post-translational level by UVA. Little work has been done to establish the mechanisms controlling TFEB transcription, and the significance of this regulation for regulating TFEB activity. TFEB is regulated by a number of transcription factors, including CREB, CEBPA, Jun, and myc. We have identified these transcription factors as being activated by UVA in keratinocytes. Further work could elucidate whether any of these transcription factors are required for TFEB induction by UVA, and the functional impact of this regulation in UVA response.

Furthermore, TFEB activation is induced by UVA through dephosphorylation. Inhibition of phosphatase activity, thus inhibiting the dephosphorylation of TFEB, precludes its activation by UVA. Two key mechanisms have been found to govern TFEB activation and

phosphorylation. Phosphorylation of TFEB by mTOR or ERK suppresses TFEB activation. We show here that mTOR is suppressed following UVA, suggesting UVA may relieve mTOR-mediated TFEB suppression. Further work could clarify the phosphosite regulated in response to UVA, the kinase and phosphatase pair responsible for regulating TFEB activation in response to UVA, and whether these sites are responsible for autophagy and lysosomal gene expression after UVA. TFEB mutant constructs, featuring serine to alanine substitutions of known phosphosites in TFEB, could be used to study this response. These mutants could also be used to establish the function of TFEB in skin cancer.

The roles of TFEB and LC3 in skin cancer are unknown and deserve to be investigated in the future. Recent studies have shown that TFEB is up-regulated in pancreatic cancers and the MiT/TFE family of transcription factors including TFEB mediates cancer cell metabolic reprogramming to maintain amino acid pool and is required for pancreatic cancer growth[339, 340]. LC3 has been shown to be up-regulated in several cancers including esophageal, 38 gastric and 19 colorectal cancers[341]. However, future studies are required to investigate the mechanism and function of LC3 up-regulation in these cancers and skin cancers.

Finally, we could also evaluate whether TFEB is activated by UVA in melanocytes and melanoma cells. If so, the mechanisms of activation could be evaluated as in keratinocytes and the functional implications of TFEB activation in these cells. Given the impact of pigmentation on UVA response, we could then assess whether the regulation of TFEB varies by pigmentation state in response to UVA.

p62-COX-2 mechanism

We report here that p62 positively regulates COX-2 stability through the UBA domain. p62 similarly regulates Twist1 stability, suggesting a pattern of p62 function in positively regulating polyubiquitinated proteins. The mechanism by which p62 differentiates between polyubiquitinated proteins bound for degradation in autophagy and those to be stabilized is unclear. Further work will determine how p62 differentiates between these two sets of

proteins and whether other oncogenic proteins are similarly stabilized by p62.

p62 also regulates COX-2 transcript levels in a UBA domain-dependent manner. COX-2 mRNA stability is regulated by p38 in response to UVA. COX-2 transcription is also regulated by UVA. Further work will determine whether p62 regulates COX-2 mRNA stability or transcription, and identify the other factors critical for regulating COX-2.

COX-2 is critical for skin cancer development and by identifying a novel mechanism of COX-2 regulation by p62 in skin cancer cells, we have identified a targetable interaction for therapeutic intervention. Further work is needed to clarify the regulatory mechanism underlying this phenomenon. Immunoprecipitation of p62 and COX-2 in cells expressing mutant p62 lacking the UBA domain was unsuccessful, likely due to the dramatic reduction in COX-2 mRNA and protein in the absence of wild-type p62. IP of both COX-2 mutants lacking the domain required for degradation via ERAD, and p62 mutants with a point mutation rendering them defective in binding ubiquitin moieties were unsuccessful due to these technical limitations.

p62-COX-2 in skin cancer

Our work shows that p62 is critical for skin cancer growth and metastasis in a syngeneic model of SCC. The reduction in growth and metastasis is accompanied by a reduction in myeloid immune cell infiltration and an increase in cell differentiation. We hypothesize that this effect is due to reduced levels of COX-2 and further work in our lab will test this hypothesis. We will first re-express COX-2 in shp62 PAM212 cells, test this expression to ensure stable expression, and use the syngeneic mouse model to investigate the role of p62-dependent COX-2 regulation in skin cancer development. Mutant constructs, including the p62-dUBA mutant, could be expressed to further link the effects on tumorigenesis to p62-dependent COX-2 regulation. We have studied the impact of PGE_2 treatment on the development of skin cancer in this model, but experiments were discontinued before tumor development due to an inflammatory response in mice where treated.

We found that p62 knockdown tumors showed reduced immune cell infiltration relative to control tumors. These cells were myeloid-derived, MPO-positive cells, which are known to be recruited in response to PGE_2. Further work could identify the cell type(s) recruited and whether this recruitment is p62- and PGE_2-dependent. It is possible that this phenomenon is dependent on p62, but not PGE_2, as p62 also regulates NF-κB signaling through a number of mechanisms to promote inflammation. Furthermore, p62-deficient tumors had an increase in cancer cell differentiation, as shown by K10 staining. This effect may be due to a reduction in PGE_2 production and further work will establish this link. Further study of the p62-COX-2 signaling axis is essential to evaluate its potential as a target for the prevention and treatment of skin cancers.

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