p63 and VDR are regulated by Vitamin D (VD3) and UV signaling
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY ANDREW JOHN WHITLATCH ENTITLED **p63 and VDR are regulated by Vitamin D (VD3) and UV signaling** BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF **Master of Science**

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Skin cancers, such as squamous cell carcinoma (SCC), develop from accumulated mutations as a result of excessive exposure to Ultraviolet B (UVB) radiation. Intriguingly, UVB also catalyzes the synthesis of 1α, 25-dihydroxy Vitamin D3 (VD3), the hormonally active form of Vitamin D. Downstream VD3 signaling has been associated with promoting the inhibition of cell cycle progression, regulating calcium homeostasis, and inducing differentiation and apoptosis. VD3 mediates these processes via genomic mechanisms through interaction with its cognate receptor, the Vitamin D Receptor, (VDR). In addition, it was recently discovered that VD3 reduces UVB-mediated phosphorylation of the SAPK/c-Jun N-terminal kinase (JNK), which correlated with a reduction in apoptosis and an increase in cell survival. Furthermore, VD3 treatment of keratinocytes also promoted up-regulation of the pro-survival p63 isoform, ΔNp63α. Expression of ΔNp63α in the basal progenitor layer of the epidermis is required for maintaining epidermal integrity by promoting continual proliferation and early commitment to stratification. VDR has also been shown to be essential for maintaining the integrity of the epidermis as exhibited by VDR null mice, which display skin defects. Although the downstream effects of VDR and ΔNp63α are well documented, their upstream regulation has been underexplored. In this study, we hypothesized that ΔNp63α and VDR are regulated by VD3 and UV signaling. To address this, mouse embryonic
fibroblasts (MEFs) and transformed keratinocyte cell lines were treated with VD3 in the presence or absence of UV radiation. We confirmed that UV treatment resulted in the phosphorylation and activation of the JNK pathway, and that this effect is reduced in keratinocytes pre-treated with VD3. Moreover, we observed that UV exposure resulted in a reduction in VDR protein levels, and led to an electrophoretic mobility shift in ΔNp63α. Although, a mobility shift in ΔNp63α upon UV treatment was previously attributed to phosphorylation by p38, it did not rule out JNK as an alternate kinase and upstream regulator. While VD3 treatment inhibited UV induced p-JNK and VDR degradation, it did not reverse the mobility shift in ΔNp63α. Furthermore, VD3 treatment did not result in a significant elevation of p63 or VDR RNA, but increased the protein levels of VDR and p63. We attributed the increased levels of ΔNp63α and VDR protein to increased stability since VD3 significantly increased the half-life of both proteins. Finally, we observed that siRNA knockdown of VDR did not affect the ability of VD3 to inhibit the formation of p-JNK, but instead resulted in reduced total JNK levels independent of VD3 treatment. Our data shows that UV and VD3 signaling merge to regulate both ΔNp63α and VDR, and the balance between the two signaling pathways could determine whether cells survive or undergo apoptosis following UV-mediated DNA damage.
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I. Introduction

1. Discovery of p63:

The p63 gene was discovered for its significant homology to the tumor suppressor, p53 (Osada, Ohba et al. 1998; Yang, Kaghad et al. 1998). Similarities between p63 and p53 are present in a shared N-terminal transactivation domain (TA), a central DNA binding domain (DBD), and a C-terminal oligomerization domain (OD); however, the p63 gene produces several isoforms with opposing functions due to a cryptic promoter in intron 3 and alternative 3’ splicing (Moll and Slade 2004). Figure 1 shows that p63 isoforms contain (TA) or lack (∆N) the N-terminal transactivation domain, while 3’ splicing generates full length (α) or truncated (β, γ) isoforms (Yang, Kaghad et al. 1998; Courtois, de Fromentel et al. 2004). The full length p63α isoforms contain two additional domains, namely the sterile alpha motif (SAM) and transactivation inhibitory domain (TID), which are responsible for protein-protein interactions and intra-molecular inhibition of the TA domain of p63, respectively (Serber, Lai et al. 2002; Barrera, Poveda et al. 2003). Two recently discovered ∆Np63 isoforms (δ, ε) are also displayed in Figure 1, but their relative physiological contributions are unknown (Mangiulli, Valletti et al. 2009). The most extensive homology between p53 and p63 exists in the DBD, demonstrated by the ability of both TA and ∆N isoforms to interact with p53 response elements in genes involved in proliferation, apoptosis, and differentiation (Westfall, Mays et al. 2003). TAp63 isoforms are most similar to p53 in their ability to transactivate p53 targets and induce apoptosis or senescence, with the TAp63γ and TAp63α isoforms.
displaying the greatest and least potency, respectively (Dietz, Rother et al. 2002). The truncated TA domain reduces the transcriptional activity of ΔNp63 isoforms causing them to act in a dominant negative fashion to antagonize p53 and TAp63-mediated transactivation. However, ΔN isoforms of p63 are capable of inducing expression of select target genes (Senoo, Matsumura et al. 2002; Wu, Osada et al. 2005; Kommagani, Caserta et al. 2006; Ortt, Raveh et al. 2008; Marinari, Ballaro et al. 2009). Despite the extensive homology between p63 proteins and p53, global knockout of the p63 gene does not result in the formation of spontaneous tumors. Instead, p63 knockout mice suffer from impaired limb and epithelial development exemplified by a complete lack of stratified epidermis, prostate, mammary, and sweat glands, suggesting an essential role for p63 in the formation and maintenance of stratified epithelia (Mills, Zheng et al. 1999; Yang, Schweitzer et al. 1999).
The diagram illustrates the domain structure of p63, TAp63α, TAp63β, TAp63γ, ΔNp63α, ΔNp63β, ΔNp63γ, ΔNp63δ, and ΔNp63ε. Each domain includes:

- **TA**: Transactivation domain
- **DNA**: DNA binding domain
- **OD**: Oligomerization domain
- **SAM**: Sterile α motif
- **TID**: Transactivation inhibitory domain

The diagram shows the percentage of DNA binding for each domain:

- p53: 90%
- TAp63α: 25%
- TAp63β: 25%
- TAp63γ: 25%
- ΔNp63α: 25%
- ΔNp63β: 25%
- ΔNp63γ: 25%
- ΔNp63δ: 25%
- ΔNp63ε: 25%

The percentages are indicated on the diagram, with green bars representing DNA binding regions.
Figure 1: *Diagram of p63 gene and protein structure*: Schematic displaying the architecture of the p63 gene and different p63 isoforms. Eight isoforms of p63 are generated from alternative N-terminal promoters and 3’ splicing. The recently discovered ΔNp63 isoforms (δ,ε) are also included in the figure. The homology present between conserved domains of all p63 isoforms and p53 are displayed as percentages (adapted from Mangiulli, Valletti et al. 2009).
2. Contribution of p63 to epidermal integrity:

The ΔNp63α isoform is the most abundant p63 isoform and is predominantly expressed in the basal layer of most epithelial tissues, especially in the epidermis (Pellegrini, Dellambra et al. 2001; Koster, Kim et al. 2004). ΔNp63α expression in the stratum basale of the epidermis maintains the proliferative potential of basal progenitor cells and initiates the early events of epidermal stratification while inhibiting later stages of differentiation (Candi, Rufini et al. 2006; Candi, Dinsdale et al. 2007). In line with its role in proliferation and cell survival, elevated ΔNp63α levels have been observed in a large percentage of SCC cases as a result of amplification of the p63 locus (Hibi, Trink et al. 2000; Sniezek, Matheny et al. 2004; DeYoung, Johannessen et al. 2006).

The epidermis is composed of several layers, from the highly proliferative stratum basale to the terminally differentiated, enucleated stratum corneum (Figure 2). The stratum basale contains the progenitor cells responsible for maintaining epidermal stratification. Cells in this layer are capable of undergoing mitosis and express the ΔNp63α isoform, although minimal expression of TAp63α has been detected (Ichikawa, Suenaga et al. 2008; Su, Paris et al. 2009). In the stratum spinosum, early differentiation markers, such as the keratins K1 and K10, are expressed as well as the miRNA, mir-203. Keratinocytes are closer to terminal differentiation in the stratum granulosum and produce lipid-filled lamellar bodies and keratohyalin granules containing pro-fillaggrin and loricrin, both essential for the cornification process. Terminally differentiated, enucleated keratinocytes
comprise the stratum corneum, which provides a barrier to water loss and microorganism invasion. As cells proceed through the stratification process, they lose their proliferative capacity as a result of the loss of ΔNp63α expression through several mechanisms, including the suprabasal presence of the miRNA, mir-203 (Lena, Shalom-Feuerstein et al. 2008; Yi, Poy et al. 2008). mir-203 post-transcriptionally inhibits the stability of p63 RNA, relegating ΔNp63α to the stratum basale. Intriguingly, our laboratory discovered that the Vitamin D Receptor (VDR) is a transcriptional target of all p63 isoforms, including ΔNp63α (Kommagani, Caserta et al. 2006). The expression of ΔNp63α strongly correlates with expression of VDR in the stratum basale and in the hair follicle, both of which display the highest levels of VDR expression in the epidermis (Kommagani, Leonard et al. 2009).
stratum basale
K5/K14, p63, VDR

stratum spinosum
K1/K10, TG1, VDR, mir-203

stratum granulosum
Loricrin, Profilaggrin

stratum corneum
Filaggrin

stratum corneum
Filaggrin

Proliferation

Differentiation

VD3 production
Figure 2: *Diagram of epidermal stratification:* The epidermis is composed of four layers which become progressively more differentiated upwards from the highly proliferative basal layer (stratum basale) to the terminally differentiated cells of the stratum corneum. Included in the diagram are differentiation markers characteristically expressed in each stratum. In addition, the epidermis is capable of metabolizing 7-dehydrocholesterol to VD3. VD3 production is greatest in the proliferating layer of the stratum basale, where VDR is also highly expressed (adapted from Bikle and Xie et al 2004).
3. VD3/VDR pathway:

1α, 25-dihydroxyvitamin D3 (VD3), the active metabolite of Vitamin D, is produced by the conversion of 7-dehydrocholesterol to pre-Vitamin D3 by UVB radiation and finally to VD3 by two hydroxylation reactions that can be catalyzed in the epidermis, as depicted in Figure 3. The final hydroxylation reactions can also be carried out in the liver and kidneys through the passage of Vitamin D3 in the blood (Masumoto, Ohyama et al. 1988; Lehmann, Tiebel et al. 1999). The final hydroxylation is tightly regulated by two enzymes; CYP27B1 which catalyzes the formation of VD3 by hydroxylating the C1 position, and CYP24 which antagonizes this reaction by hydroxylating C24 (Bikle, Nemanic et al. 1986). Elevated VD3 production initiates a negative feedback pathway consisting of VD3-mediated induction of the CYP24 gene and transcriptional inhibition of the CYP27B1 gene, effectively inhibiting further VD3 synthesis (Issa, Leong et al. 1998). Genomic actions of VD3 are mediated by its interaction with its receptor, VDR. More precisely, VD3 interaction with VDR induces its heterodimerization with the retinoid X Receptor and interaction with Vitamin D response elements (VDRE). VD3 also influences cellular processes non-genomically by activating downstream signaling pathways (Losel and Wehling 2003). Interestingly, VDR levels can be increased by VD3 through transcriptional activation or VDR stabilization in a cell-line specific manner (Solvsten, Svendsen et al. 1997; Healy, Frahm et al. 2005).

In addition, VD3 has been shown to promote apoptosis in several cancer cell lines. Due to its ability to induce differentiation as well as apoptosis, VD3 and VD3 analogues have
been explored as potential chemotherapeutic adjuvants (Wigington, Urben et al. 2004). VD3 also plays an essential role in epidermal homeostasis by promoting differentiation through the induction of several proteins involved in the differentiation process (Bikle 2010). Moreover, studies from mice lacking the CYP27B1 enzyme display reduced epidermal differentiation and altered permeability, suggesting that VD3 production is essential for maintaining epidermal integrity (Bikle, Chang et al. 2004).

4. VDR background:

The Vitamin D receptor (VDR) is a member of the nuclear receptor family, possessing the greatest homology to the thyroid receptor (T\(_3\)R) and retinoic acid receptors (RAR). The VDR protein consists of 3 major domains, an N-terminal DNA-binding domain, a ligand binding domain, and a C-terminal transaction domain which can mediate co-factor interaction (Issa, Leong et al. 1998). VD3 predominantly interacts with VDR in the nucleus, although this interaction has been shown to occur in the cytosol, which promotes nuclear accumulation of the VD3/VDR complex (Stambolsky, Tabach et al.). VDR interaction with VD3 causes VDR to heterodimerize with the retinoid x receptor (RXR) and induce or inhibit transcription of the gene targets shown in Figure 4. Although VDR is capable of dimerizing with other nuclear receptors, it has been observed to prefer interaction with RXR (Mangelsdorf and Evans 1995). Intriguingly, XPE, an essential component of the nucleotide excision repair pathway involved in removing UV-induced DNA lesions, was identified in a microarray experiment as a potential VDR target, but
7-dehydrocholesterol $\xrightarrow{UV}$ Pre-Vitamin D3 $\xrightarrow{Heat}$ Vitamin D3 $\xrightarrow{CYP27}$ 25-hydroxyvitamin D3 (VD3) $\xrightarrow{CYP27B1}$ 1α, 25-dihydroxyvitamin D3 (VD3) (active) $\xrightarrow{P450}$ 24,25- dihydroxy Vitamin D3 (inactive)
Figure 3: *Diagram of VD3 metabolism:* Diagram depicting the synthesis of 1α, 25-dihydroxy Vitamin D3 (VD3) from 7-dehydrocholesterol. UV light and heat induces a rearrangement of the double bonds in 7-dehydrocholesterol, to form Vitamin D3.

Conversion of Vitamin D3 to VD3 occurs through hydroxylation reactions via the CYP27 family. The CYP24 enzyme hydroxylates and functionally inactivates 25-hydroxy Vitamin D3 at the C24 position, preventing further VD3 synthesis (adapted from (Dusso, Brown et al. 2005)).
Figure 4: *VD3/VDR pathway*: Diagram depicting the signaling effects of VD3 treatment.

VD3 interacts with its cognate receptor, the Vitamin D Receptor (VDR) in both the nucleus and cytoplasm. Interaction of VDR with VD3 induces its heterodimerization with the retinoid X receptor (RXR), another member of the nuclear receptor family. The VDR/RXR heterodimer interacts with VDR/RXR response elements in target gene promoters involved in calcium homeostasis, osteoblast and epidermal differentiation and cell cycle regulation (adapted from Nezbedova and Brtko 2004)
this has not been investigated further (Moll, Sander et al. 2007). Data obtained from VDR knockout mice has been instrumental in elucidating the physiological actions of VDR. The phenotypes of these mice suggest an essential role for VDR in bone and epidermal development as the mice display stunted bone growth, reduced epidermal differentiation, and altered hair follicle cycling leading to alopecia (Li, Pirro et al. 1997; Yoshizawa, Handa et al. 1997; Xie, Komuves et al. 2002). Interestingly, mice unable to produce VD3 do not develop alopecia, demonstrating a potential ligand-independent role for VDR in hair follicle maintenance (Bikle, Chang et al. 2004). Additional VD3-independent actions of VDR are seen in the development of UV-induced tumors in VDR -/- mice versus the absence of UV-induced tumors in CYP27B1 -/- mice. The tumor and alopecia phenotypes suggest that the relationship between VDR and VD3 is more complex than previously considered (Ellison, Smith et al. 2008).

5. Effects of UV damage on p63 and VDR:

One of the primary functions of the epidermis is to protect the progenitor cells residing in the basal layer from UV exposure. Maintaining epidermal stratification is essential for this function, and has been found to be mediated by both ∆Np63α and VDR. UV exposure causes the formation of DNA lesions, such as cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts. In normal keratinocytes, accumulation of these UV-induced lesions results in apoptosis mediated by the c-Jun N-terminal kinase (JNK) and p38 pathways (Hibi, Lin et al. 1993; Derijard, Hibi et al. 1994; Raingeaud, Gupta et al. 1995). In addition, UV-mediated down-regulation of the ∆Np63α isoform is required for
apoptosis to occur in keratinocytes (Liefer, Koster et al. 2000). Furthermore, phosphorylation of p63 was found to occur following UV treatment (Katoh, Aisaki et al. 2000; Okada, Osada et al. 2002; Papoutsaki, Moretti et al. 2005; Westfall, Joyner et al. 2005). UV-mediated phosphorylation has differential effects on the p63 family as TAp63 proteins accumulate; whereas ΔNp63α is degraded and loses its ability to bind DNA (Katoh, Aisaki et al. 2000; Okada, Osada et al. 2002; Papoutsaki, Moretti et al. 2005; Westfall, Joyner et al. 2005). Phosphorylation of ΔNp63α was suggested to be mediated by the p38 kinase; however, JNK has not been eliminated as a candidate kinase (Papoutsaki, Moretti et al. 2005). UV-induced DNA damage activates both the p38 and JNK pathways, which are essential mediators of UV-induced apoptosis; however, only the p38 kinase was investigated as an upstream regulator of ΔNp63α (Papoutsaki, Moretti et al. 2005). UVB radiation has the additional affect of initiating VD3 synthesis in the epidermis. VD3 pre-treatment was found to protect keratinocytes from apoptosis following UV damage by inhibiting the activation of JNK (De Haes, Garmyn et al. 2003). In addition, VD3 has also been shown to up-regulate ΔNp63α (Langberg, Rotem et al. 2009). The pro-apoptotic tendencies of VD3 are thus, in direct conflict with these results and suggest that the keratinocyte may be a unique system in which to model the effects of VD3 signaling.
6. Rationale:

UVB radiation has the effect of inducing VD3 production while simultaneously generating DNA lesions, the accumulation of which can result in cancer-causing mutations. Cells are normally protected from these mutations by undergoing apoptosis as a result of JNK and p38 activation, but amplification of the p63 locus can allow tumorigenic cells to escape apoptosis through elevated pro-survival signals mediated by ΔNp63α, as seen in SCC tumors. VD3 treatment of keratinocytes also leads to up-regulated ΔNp63α and a reduction in JNK activation, both of which could contribute to the observed reduction in apoptosis generated by VD3 pre-treatment. In addition, VDR null mice are susceptible to UV-induced tumorigenesis, suggesting that VDR is a potential tumor suppressor in the epidermis. This has been attributed to a VD3-independent function of VDR in the skin, as mice lacking the CYP27B1 gene do not develop skin tumors following UV treatment. The mechanism of maintaining epidermal integrity upon UV insult has yet to be discovered, and may lie in the regulation of VDR and p63 by UV and VD3 signaling. As both pathways can be activated simultaneously, they are intimately tied to epidermal homeostasis. We examined the effect of VD3 and UV signaling on p63 and VDR by first investigating whether UV damage regulates p63 and VDR. We hypothesized that UVC treatment would result in a mobility shift in ΔNp63α, as previously observed, and that VDR would also be reduced, as UVC radiation does not promote VD3 synthesis in keratinocytes. Secondly, we investigated whether VD3 signaling directly modulates expression of VDR and ΔNp63α. We hypothesized that VD3 treatment would result in an increase in both VDR and ΔNp63α through
transcriptional activation or increasing protein stability based on previously reported data (Solvsten, Svendsen et al. 1997; Healy, Frahm et al. 2005; Langberg, Rotem et al. 2009) Exploring both of these aims would provide critical information concerning both UV and VD3 signaling and how these pathways affect epidermal integrity by regulating ∆Np63α and VDR.
II. Materials and Methods:

1. Cell lines and chemicals: The H1299 non-small cell lung carcinoma and A431 squamous cell carcinoma (SCC) derived cell lines were purchased from ATCC. The immortalized keratinocyte cell line, HaCaT, was generously provided by Dr. Dori Germolec from the National Institute of Environmental Health Sciences (NIEHS). Mouse embryonic fibroblasts (MEF) cell lines expressing wild type JNK (WT), lacking JNK (JNK -/-), or JNK activators (MKK 4/7 DKO), were obtained from Dr. Anja Jaeschke from the University of Cincinnati. All cell lines were maintained in Dulbecco Modified Eagle’s Media (DMEM) supplemented with 10% FBS and 1% Penicillin/Streptomycin. 1α, 25-dihydroxyvitamin D3 (VD3) was purchased from Sigma (St. Louis, MO, USA) and diluted to a stock concentration of 10 µM in 100% ethanol. Emetine dihydrochloride was obtained from Calbiochem (San Diego, CA, USA) and diluted to a stock concentration of 50 mM in DMSO.

2. VD3 pre-treatment and UV irradiation: HaCaT and A431 cells were transferred from normal growth media to media supplemented with charcoal treated serum (CTS) in which all lipids are removed. At the time of media change, cells were treated with either vehicle (100% ethanol), or the indicated concentration of VD3. Following incubation, cells were washed twice and covered with a thin layer of PBS prior to UVC treatment. For UVC treatment, dishes were placed in a UV Stratalinker 1800 with lids removed and subjected to 50 or 150 J/m2 UVC radiation. PBS was removed and replaced with normal growth media for the indicated times following UVC irradiation. To determine the stability of
ΔNp63α and VDR in the presence of VD3, HaCaT cells were pre-treated for 24 hrs with 100 nM VD3 or vehicle. Cells were then placed in fresh CTS containing 2 µM emetine plus vehicle or VD3.

3. siRNA and plasmid transfections: Non-silencing control and VDR-specific siRNA containing the sense sequence r(GCGUCAGUGACGUGACCAA)dTdT and antisense r(UUGGUCACGUCACUGACGC)dGdG were purchased from Qiagen (Valencia, CA, USA). siRNA treatments consisted of 1 round of reverse transfection followed by a second round of forward transfection. For reverse transfections, cells were counted and plated to a density of 3x10^5 per 6 cm plate in antibiotic-free media. For each condition, the transfection mixture consisted of 112 pmol siRNA (5.6 µl) in 400 µl Opti-MEM with an equal volume (5.6 µl) of Oligofectamine transfection reagent obtained from Invitrogen (Foster City, CA, USA). Transfection mixes were vortexed thoroughly and incubated for twenty minutes prior to adding to the cells. Whole cell extracts were harvested 24-36 hours following the second round of transfection. Plasmid transfections were performed in the same manner as siRNA treatments, but with the use of the Lipofectamine 2000 reagent (Invitrogen Foster City, CA, USA). The JNK1 plasmid was kindly provided by Dr. Anja Jaeschke from the University of Cincinnati.

4. Western blot analysis: Cells were collected by trypsinization and underwent centrifugation for 2 min at 4⁰ at max rpm (14,000 x G). Media was removed and the cells were re-suspended in PBS to wash away residual media. Cells were re-pelleted for 2 min at max speed at 4⁰. PBS was then removed and the cell pellet was frozen in -80⁰ or in
liquid nitrogen. Cells were thawed and re-suspended in phosphatase inhibitor buffer (PhIB) (50 mM Tris pH 8, 120 mM NaCl, 5mM EGTA, 1mM EDTA, 5mM sodium pyrophosphate decahydrate, 10mM NaF, 30mM para-nitro phenyl phosphate, 1mM benzamidine, 0.1% NP-40, and 1x sodium vanadate ) supplemented with protease inhibitor cocktail (PIC) at a 1:100 dilution and lysed with rotation at 4°C for ~30 min. Protein quantitation was performed using a BCA kit obtained from Thermo Scientific (Rockford, IL, USA) using a Safire 2 plate reader. Following quantitation, equivalent amounts of protein was run on an SDS-denaturing gel and transferred to a PVDF membrane. Membrane blocking and primary antibody incubation was performed in 1% BSA in TTBS w/ 0.1% Tween for phospho-JNK and phospho-c-Jun detection (Cell Signaling Boston, MA, USA) and 5% Non-fat Milk buffer in TTBS w/ 0.05% Tween for all non phospho-proteins. Monoclonal anti-VDR (D-6), p63 (4A4), c-Jun (sc-44) (Santa Cruz Technology) and anti-β-actin (Sigma, St. Louis, MO, USA) were used for immunodetection of VDR, p63, c-Jun, and β-actin, respectively. Basal total JNK detection was performed using a polyclonal anti-JNK antibody purchased from Cell Signaling. Quantitation of band intensity was performed using MultiGauge Image Software.

5. RNA extraction: RNA was extracted from cells using the ezRNA kit according to the manufacturer’s protocol (Omega Bio-Tek, Norcross, GA, USA). Cells were harvested in TRK Lysis Buffer supplemented with 1:50 β-mercaptoethanol and collected in 1.5 ml Eppendorf tubes containing an equal volume of 70% ethanol. The suspension was vortexed and transferred to Hi-bind RNA spin columns. Columns underwent
centrifugation at 10,500 rcf from 1 min., followed by washing in 500 µl RNA Wash buffer I before centrifugation at 10,000 rcf. Hi-Bind columns were then placed into new 2 ml collection tubes and washed twice with 500 µl RNA Wash Buffer II diluted with absolute ethanol. Following the second wash, columns were spin-dried for 2 minutes at maximum speed. RNA was eluted from columns with pre-heated DEPC-treated water and spun for 1.5 min at 10,500 rcf. Collected RNA was immediately placed on ice and diluted 1:50 in TE buffer. Quantitation and purity of RNA was determined by a Genesys 6 Spectrophotometer.

6. cDNA synthesis and RT-PCR: Synthesis of cDNA from 1 µg of total RNA was performed using a TaqMan-based reverse-transcription kit from ABI (Foster City, CA, USA) according to the manufacturer’s protocol. Each reaction contained the following: (2.5 µl 10x Buffer, 5.5 µl 25 mM MgCl2, 5 µl dNTP’s, 1.25 µl random hexamers, 0.5 µl RNAse inhibitors, and 0.625 µl Taq reverse transcriptase). Quantitative real-time-PCR was performed using TaqMan 2x assay buffer and ABI assays on demand (AOD). Detection of murine and human transcripts was performed using the following AOD’s: murine VDR (Mm_00437297_m1) murine p63(Mm_00495788_s1) human VDR (Hs_00172117_m1), human p63 (Hs_00978340_m1). AOD’s for and murine β-actin (Mm-00607939_s1) and human GAPDH were used as endogenous controls. An ABI 7900 HT RT-PCR machine was used for RT-PCR analysis.
III. Results:

*JNK is phosphorylated in response to UV damage*

Both ΔNp63α and VDR are required to maintain the integrity of the stratified epidermis, yet little is known regarding their upstream regulation in response to stimuli, such as exposure to UV radiation. We began to investigate the effect of UV exposure on VDR levels in WT MEFs, as this cell line has been used in UV damage studies (Fang, Liu et al. 2006; Mohanty, Town et al. 2008) As shown in Figure 5, UV treatment of wild type MEFs results in a dose and time-dependent increase in the phosphorylation of both isoforms of the c-Jun N-terminal kinase (JNK), referred to as JNK1 and JNK2. Both the JNK isoforms were detected and specified according to the information provided by the antibody manufacturer. The JNK1 and JNK2 isoforms are ubiquitously expressed in human tissue and are essential for epidermal homeostasis (Weston, Wong et al. 2004). UV-induction of p-JNK also resulted in phosphorylation of the canonical downstream JNK substrate, c-Jun. Although the β-actin is noticeably uneven, we noticed that UV treatment resulted in a modest decrease in VDR protein. Normalization of the VDR band to β-actin (Fig 5B) demonstrated that exposure to 50 J/m2 UV lead to a modest reduction in VDR, yet this was not consistently observed at the higher UV treatment. This discrepancy suggests the effect of UV treatment on VDR may have additional complexities that need to be investigated. However, the reduction in VDR appeared to correlate with activation of the JNK pathway, implying that JNK may negatively regulate VDR levels. While utilizing UVC radiation may not have direct physiological relevance, exposure to UVC wavelengths produces similar DNA lesions to UVB exposure, and
activates the p38 and JNK pathways, two essential mediators of UV-damage signaling. In addition, UVC does not activate VD3 synthesis in keratinocytes, allowing us to investigate the effects of UV damage on VDR independent of VD3 production and thus, activation of the VD3 pathway. Therefore, we confined all of our future treatments to UVC wavelengths.
Figure 5: *JNK is phosphorylated by UV in NIH 3T3 MEFs.* A) WT NIH 3T3 MEFs were plated at a density of $4.4 \times 10^5$ cells in 6 cm dishes. Cells were then washed and kept in a thin layer of PBS and irradiated with increasing doses of UV. Whole cell extracts were harvested at the indicated intervals following UV treatment and were subjected to Western blot analysis with antibodies specific for p-JNK, JNK, p-c-Jun, c-Jun, VDR, and β-actin. B) Fold change in relative intensity of VDR normalized to endogenous β-actin in A). Fold change was determined relative to the untreated control.
Figure 6: *VDR is down-regulated by UV damage in keratinocytes.* A) The SCC-derived A431 and B) immortalized HaCaT cell lines were seeded at densities of 7.5x10^5 and 7x10^5, respectively, in 6 cm dishes 24 hours prior to UV-irradiation. Cells were washed and covered in a thin layer of PBS prior to UV exposure. Whole cell extracts were harvested at the indicated intervals following irradiation and subjected to immunoblot analysis for p-JNK, JNK, p-c-Jun, c-Jun, VDR, and β-actin. C) Fold change in the relative intensity of VDR to endogenous β-actin in A) and B). Fold change was calculated relative to the untreated control for each cell line.
Next, we examined the effects of UV exposure on more physiologically relevant cell lines. We treated the squamous cell carcinoma-derived A431 and spontaneously immortalized HaCaT cell lines with 50 or 150 J/m², and analyzed its effect on VDR (Figure 6). The results confirm that UV induces the JNK pathway and higher levels of UV radiation lead to a sustained increase in p-JNK and p-c-Jun levels. Quantitation of VDR relative to β-actin in Figure 6C demonstrated reduction in VDR levels upon UV exposure in HaCaT cells; however, this was not evident in the A431 line. It is likely that this demonstrates a difference between the two cell lines, especially since HaCaT cells more closely resemble primary keratinocytes than A431 (Boukamp, Petrussevska et al. 1988).

*JNK affects VDR expression, but not UV-mediated down-regulation*

JNK is an essential downstream mediator of UV-mediated DNA damage signaling (Chen, Wang et al. 1996). Our initial observation that UV-mediated activation of JNK correlated with reduced VDR levels led us to speculate that JNK may down-regulate VDR. To further examine this hypothesis, we utilized MEF cell lines obtained from our collaborator, Dr. Anja Jaeschke. These cells express WT JNK (WT), lack both JNK isoforms (JNK -/-), or lack upstream MAPKKs responsible for phosphorylating JNK (MKK 4/7 DKO). We first examined VDR and p63 expression at the transcript level in all cell lines. As illustrated in Figure 7A, VDR transcript increased ~ 3 fold in JNK -/- and 4 fold in MKK4/7 DKO MEFs, indicating that lack of JNK signaling increases VDR
Figure 7: *Inactive JNK signaling increases VDR expression, but does not inhibit UV-mediated degradation.*

A) RNA harvested from MEF cell lines expressing WT JNK (WT), lacking JNK (JNK -/-), or JNK upstream kinases (MKK 4/7 DKO) was subjected to Q-RT-PCR analysis with Taqman-based assays on demand (AOD) specific for murine p63, and VDR. The Y-axis represents the fold change in p63 and VDR relative to β-actin.

B) MEF cell lines as indicated were treated with 150 J/m2 UV and harvested for whole cell extracts 4 hrs following treatment. Protein was subjected to Western blot analysis using antibodies specific for VDR and β-actin.

C) Fold change in the relative intensity of VDR normalized to endogenous β-actin. Fold change was determined relative to the untreated controls for each cell line.
expression. The JNK -/- line also exhibited a 6 fold elevation in p63 transcript; however, we have not been able to detect p63 protein in these cell lines (data not shown).

To monitor differences in UV-mediated VDR down-regulation among the MEF lines, we treated them with 150 J/m2 UV. The results in Figure 7B confirmed the transcript results that increased VDR protein levels were observed in JNK-/- and MKK4/7 DKO MEFs. However, VDR down-regulation following UV treatment was still observed in all cell lines. Quantitation of the VDR bands in Figure 7C demonstrates that VDR is down-regulated in cell lines with inactive JNK pathways, whereas no down-regulation was observed in WT MEFs. This down-regulation could be due to the compensatory activation of additional signaling pathways. Additional experiments involving inhibition UV-induced signaling pathways, such as JNK, will provide further insight into the mechanism behind UV-mediated VDR down-regulation.

**UV-mediated JNK activation correlates with a mobility shift in $\Delta Np63\alpha$**

Phosphorylation of $\Delta Np63\alpha$ has been shown to occur following UV treatment (Papoutsaki, Moretti et al. 2005; Westfall, Joyner et al. 2005). This phosphorylation was attributed to activation of the p38 pathway; however, UV is also a potent activator of JNK signaling and JNK was not definitively eliminated as a candidate kinase. In addition, both p53 and TAp73$\alpha$ have been identified as JNK as substrates. Due to the high
Figure 8: ΔNp63α exhibits a mobility shift upon UV treatment. A) A431 cells were plated at a density of $6.5 \times 10^5$ in 6 cm dishes and treated with increasing concentrations of UV. Whole cell extracts were harvested at the indicated time points following UV treatment. Protein was analyzed via Western blotting with antibodies specific for p-JNK, JNK, p63, and β-actin. B) HaCaT cells were plated at a density of $6 \times 10^5$ in 6 cm dishes and were treated the same as in A).
ΔNp63α H. sapien

ΔNp63α M. musculus

TAp73α H. sapien

\[ \begin{array}{ccccccccc}
53 & 66 & 319 & 332 & 369 & 397 & 444 \\
\end{array} \]

...APSP...DALSP...MLL KIKESLELMQY...GNSSPP...NALTP...HCTPPP...

...APSP...DALSP...MLL KIKESLELMQY...GNSSPP...NALTP...HCTPPP...

...THSPY...DTMSPAP...ILM KIKESLELMEL...PVLSPM...SAATP...HCTPPP...
Figure 9: JNK phosphorylation sites are conserved between ΔNp63α and TAp73α.

The amino acid sequences for murine and human ΔNp63α and human TAp73α were obtained from the Uniprot website and uploaded to ClustalW. Putative Ser/Thr sites with the neighboring proline are shown in bold. A putative JNK interaction site is shown underlined and in bold.
homology among p53 family members, it is possible that p63 is also phosphorylated by JNK. To determine if JNK phosphorylates endogenous ΔNp63α following UV exposure, we treated both A431 and HaCaT cells with increasing concentrations of UV and harvested for whole cell extracts at different intervals (Figure 8). We observed a significant mobility shift in ΔNp63α in extracts harvested as early as 30 minutes following UV treatment with both 50 and 150 J/m² UV. Moreover, the shift appeared to be slightly enhanced in both cell lines with the higher UV treatment, indicating that ΔNp63α may have undergone phosphorylation (Lanes 2-4 vs. Lanes 5-7). Moreover, phosphorylation of JNK correlated with the mobility shift in ΔNp63α, suggesting that it might be a potential JNK substrate. Several putative JNK phosphorylation sites were identified on TAp73α. The homology between p73 and p63 caused us to investigate if they are conserved on ΔNp63α. We compared the amino acid sequence of murine and human ΔNp63α with human TAp73α, as displayed in Figure 9. We determined that several of the putative Ser/Thr phosphorylation sites identified on TAp73α are conserved on both human and murine ΔNp63α. In addition, we identified a putative JNK interaction site present in all three proteins, as illustrated by the underlined sequence in Figure 9.

*Ectopic ΔNp63α exhibits a mobility shift when co-overexpressed with the JNK1 isoform*

Both the JNK1 and JNK2 isoforms are phosphorylated following UV damage; however, the JNK1 isoform has been described as the primary mediator of UV-induced JNK signaling (Chen, Wang et al. 1996). To monitor the effect of the JNK1 isoform on the mobility observed in ΔNp63α following UV treatment, we co-expressed both proteins in H1299 lung cancer cells, and treated with 150 J/m² UV. As shown in Figure 10, ectopic
ΔNp63α does show a slight mobility shift upon treatment with UV; however, UV-mediated phosphorylation of endogenous JNK was relatively small compared to the robust p-JNK levels seen in A431 and HaCaT (Figure 6). Co-transfection of both ΔNp63α and JNK1; however, resulted in a robust increase in JNK1 phosphorylation which resulted in a more noticeable mobility shift in ΔNp63α, indicating it is potentially phosphorylated by JNK1.

**VD3 inhibits UV-induced JNK activation**

VD3 treatment of HaCaT cells was found to inhibit induction of p-JNK and apoptosis; however, the dose of VD3 required for this effect was not physiologically relevant (De Haes, Garmyn et al. 2003). In addition, we observed that UV exposure lead to VDR degradation (Figures 5 and 6). To monitor the effect of VD3 on UV signaling, we first examined the effect of VD3 on UV-mediated JNK phosphorylation (Figure 11). Pretreatment of A431 and HaCaT cell lines with increasing concentrations of VD3 resulted in a dose-dependent reduction in p-JNK following UV treatment. In addition, a more robust inhibition of p-JNK occurred in HaCaT cells, suggesting that this cell line may be more sensitive than A431 to VD3 treatment. This effect was also not the result of a decrease in the overall levels of either the JNK1 or the JNK2 isoform, as measured by total JNK levels.
Figure 10: *Ectopic ΔNp63α displays a mobility shift upon UV treatment when coexpressed with JNK1.*

H1299 cells were plated at a density of 3x10^5 in 6 well dishes and reverse transfected with 2 µg of the indicated plasmids. The following day, the UV treated conditions were washed twice and kept in PBS and treated with 150 J/m2 UV. Cells were then put back in normal growth media and harvested for whole cell extracts 2 hours following UV exposure. Protein was examined using Western blot analysis with antibodies specific for p-JNK, JNK, p63, and β-actin.
Figure 11: *VD3 pre-treatment results in a reduction in UV-mediated p-JNK in a dose-dependent manner.*

A) A431 and B) HaCaT cells were plated at a density of $5 \times 10^5$ and $4 \times 10^5$, respectively, in 6 cm culture dishes. 24 hours after seeding, cells were incubated in media containing charcoal treated serum containing either vehicle or increasing concentrations of VD3. The following day, cells were washed and kept in a thin layer of PBS and exposed to 150 J/m² UV radiation. Immediately following radiation, cells were put back in normal growth media and harvested 2 hours later. Whole cell extracts were processed and subjected to immunoblot analysis for p-JNK, JNK, and $\beta$-actin.
In addition to studying the effect of increasing concentrations of VD3 on p-JNK inhibition, we wanted to see if inhibition of p-JNK upon VD3 exposure is dependent on the duration for which the cells are exposed to VD3. The results in Figure 12 demonstrate that pre-treatment of both A431 and HaCaT cell lines resulted in a significant reduction in UV-mediated p-JNK as early as 2 hours following VD3 treatment. Moreover, JNK phosphorylation was not reduced any further with longer intervals of VD3 exposure compared to shorter intervals (Lane 4 vs. Lane 12).
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- JNK2
- JNK1
- β-actin

B) HaCaT

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- p-JNK2
- p-JNK1
- JNK2
- JNK1
- β-actin
Figure 12: *VD3-mediated inhibition of UV-induced p-JNK is not time-dependent.* A) A431 and B) HaCaT cells were pre-treated with Vehicle or 50 nM VD3 for the indicated time points prior to UV treatment. Cells were washed and kept in PBS and irradiated with 150 J/m² UV. Following UV treatment, cells were put back in normal growth media and harvested for whole cell extracts 2 hours after irradiation. Protein was subjected to Western blot analysis for p-JNK, JNK, and β-actin.
**VD3 inhibits basal p-JNK**

We confirmed previous reports that VD3 pre-treatment inhibits UV-induced JNK activation and have also observed that this effect is reproducible with more physiological concentrations of VD3 (Figure 11). Furthermore, this inhibition is observed within 2 hours of VD3 exposure (Figure 12). Next, we decided to extend our investigation to see if VD3 is also capable of inhibiting endogenous JNK phosphorylation. To monitor the ability of VD3 to inhibit basal p-JNK, we treated both A431 and HaCaT cells with 10 and 100 nM VD3 for 24 hours (Figure 13). We observed, in both cell lines, that VD3 reduces basal levels of p-JNK. Notably, phosphorylation of the JNK2 isoform was reduced in both cell lines, but inhibition of JNK1 phosphorylation was observed at both VD3 concentrations in only the A431 cell line. The higher concentration of VD3 was able to reduce p-JNK1 in HaCaT cells; however. This suggests that VD3 is capable of inhibiting JNK phosphorylation in both A431 and HaCaT cells, but further investigation into the downstream effects of this inhibition will need to be performed.

**VD3 treatment reduces UV-mediated VDR degradation**

VD3 has been shown to increase the levels of VDR through either transcriptional activation or receptor stabilization (Solvsten, Svendsen et al. 1997; Healy, Frahm et al. 2005). Since UV does not activate VD3 synthesis, the reduction in VDR we observed could be the result of the inability of keratinocytes to produce VD3. To monitor the effect of VD3 on VDR levels following UV exposure, we pre-treated HaCaT cells with 100 nM
Figure 13: VD3 inhibits basal p-JNK levels. A) A431 cells were plated at a density of 3x10^5 in 6 cm dishes and subjected to treatment with vehicle, 10, or 100 nM VD3 for 24 hours. Following treatment, conditions were harvested for whole cell extracts and subjected to Western blot analysis using antibodies specific for p-JNK, JNK, and β-actin. B) Fold change in relative intensity of p-JNK normalized to total JNK levels. Total JNK was first normalized to endogenous β-actin followed by normalization of p-JNK to the corrected JNK level. Fold change was determined relative to the untreated control. C) HaCaT cells were plated as and processed as in A). D) Quantitation of p-JNK in HaCaT cells was performed as in B).
Figure 14: VD3 pre-treatment reduces UV-mediated VDR degradation. A) HaCaT cells were treated with vehicle or VD3 for 24 hours prior to UV irradiation. Immediately before irradiation, cells were washed and kept in PBS and treated with 150 J/m² UV. Whole cell extracts were harvested 2 hours following UV exposure and Western blot analysis was performed using antibodies specific for p-JNK, JNK, VDR, and β-actin. B) Fold change in relative VDR intensity normalized to endogenous β-actin. Fold change was determined relative to the untreated control.
VD3 for 24 hours prior to UV exposure. As the results show in Figure 14, UV treatment resulted in VDR down-regulation, which was further confirmed by quantitation of the VDR band (Figure 14B). Interestingly, VDR reduction following UV exposure was inhibited when pre-treated with VD3. Additionally, the VD3-mediated inhibition of p-JNK was not as significant as we previously observed (Figures 11-13), yet VDR was still increased. This suggests that VD3 may directly stabilize VDR levels, rather than indirectly through p-JNK inhibition.

**VD3 does not inhibit the gel shift in ΔNp63α**

We determined that UV-mediated increases in p-JNK correlate with mobility shifts seen in ΔNp63α and identified conserved Ser/Thr residues and a JNK interaction site shared between TAp73α and ΔNp63α (Figures 8, 9, respectively). Since we also determined that VD3 inhibits UV-induced p-JNK, we wanted to see if this inhibition removed the mobility shift in ΔNp63α (Figure 15). We pre-treated HaCaT cells with 100 nM VD3 24 hours prior to treatment with 150 J/m2 UVC. As we previously observed, UV treatment resulted in increased p-JNK, which correlated with a mobility shift in ΔNp63α. VD3 pre-treatment inhibited p-JNK, yet this did not affect the retarded mobility of ΔNp63α (Lane 4 vs. Lane 2). The p38 pathway is also activated in response to UV damage and has been suggested to be the kinase responsible for the mobility shift in ΔNp63α following UV exposure (Papoutsaki, Moretti et al. 2005). Since VD3 pre-treatment does not inhibit the p38 pathway following UV exposure, this explains the inability of VD3 to affect the shift in ΔNp63α (De Haes, Garmyn et al. 2003; Langberg, Rotem et al. 2009).
Figure 15: *VD3 does not abolish the mobility shift in ΔNp63α.*

HaCaT cells were pre-treated with Vehicle or 100 nM VD3 for 24 hours prior to UV exposure. Cells were washed twice and kept in a thin layer of PBS and exposed to 150 J/m² UV. Whole cell extracts were harvested 2 hours after irradiation and analyzed via Western blotting using antibodies specific for p-JNK, JNK, p63, and β-actin.
While this does not definitively eliminate ΔNp63α as a JNK substrate, it indicates that additional pathways can compensate to phosphorylate ΔNp63α following UV exposure.

**VD3 treatment stabilizes p63 and VDR**

VD3 treatment of HaCaT cells has been shown to increase ΔNp63α protein (Langberg, Rotem et al. 2009). In addition, we demonstrated that VD3 treatment inhibited UV-mediated down-regulation of VDR, which could be the result of transcriptional or stabilization mechanisms (Solvsten, Svendsen et al. 1997; Healy, Frahm et al. 2005). Based on these observations, we wanted to assess whether VD3 directly affects VDR or ΔNp63α levels. To examine this, we treated HaCaT cells with increasing concentrations of VD3 for 24 hours. As shown in Figure 16A and B, increasing concentrations of VD3 resulted in increased ΔNp63α and VDR protein levels, which was slightly increased with increasing concentrations of VD3. VD3 treatment can result in effects attributed to genomic or non-genomic actions. Effects occurring briefly after VD3 exposure are classified as non-genomic actions and occur as the result of activation of signaling pathways (Losel and Wehling 2003). Genomic VD3 actions typically occur after longer intervals of treatment and are the result of transcriptional regulation (Dusso, Brown et al. 2005). Most of our studies utilized a 24-hour VD3 incubation period indicating that the results could be due to genomic VD3 activities. To test whether genomic or non-genomic mechanisms are responsible for the increases in VDR and ΔNp63α, we performed a VD3 time-point study (Figure 16B). We treated HaCaT cells with 100 nM VD3 for the
Figure 16: *VDR and ΔNp63α are increased by VD3.*

A) HaCaT cells were plated at a density of $4 \times 10^5$ cells in 6 cm dishes and treated with Vehicle or increasing concentrations of VD3 for 24 hours. Following treatment, conditions were harvested for whole cell extracts. Protein was analyzed via Western blotting using antibodies specific for p63, VDR, and β-actin. B) Fold change in relative p63 and VDR band intensity normalized to endogenous β-actin. Fold change was determined relative to the untreated control. C) HaCaT cells were treated with either vehicle or 100 nM VD3 for the indicated time periods. Following VD3 treatment, whole cell extracts were harvested and processed as in A). D) Normalization of p63 and VDR was performed as in B).
indicated time-points and examined the protein levels of VDR and ∆Np63α. We discovered that both VDR and ∆Np63α are modestly increased as early as 30 minutes following VD3 treatment and continued up to 4 hours of VD3 exposure. Interestingly, the quantitation of the p63 and VDR bands demonstrated that there is a slight increase in both proteins as the duration of VD3 incubation is increased. The rapid elevation of both proteins suggests that VD3 induces both proteins through non-genomic mechanisms.

**VD3 treatment does not increase p63 or VDR transcripts**

We observed that VD3 increases both VDR and ∆Np63α at the protein level and does so, briefly after VD3 exposure (Figure 16). We wanted to test if, indeed, VD3 treatment does not mediate this increase via transcriptional activation. We examined this by incubating HaCaT cells with either vehicle or increasing concentrations of VD3 for 24 hours and measuring p63 and VDR RNA levels through quantitative real-time PCR (Q-RT-PCR). The results shown in Figure 17 illustrate that treatment with either VD3 concentration does not significantly increase the levels of p63 at the transcript level, yet VDR levels were modestly elevated in a dose-dependent manner. However, the increase in VDR RNA does not reflect the changes observed in Figure 16A or 16B, but may slightly contribute to VDR accumulation following VD3 treatment.
Figure 17: *VD3 treatment does not significantly inhibit p63 or VDR transcript.*

HaCaT cells were treated with vehicle or the indicated dose of VD3 for 24 hours.

Following the incubation period, RNA was harvested and converted to cDNA. 2 µg of cDNA was used for Q-RT-PCR analysis with Taqman-based AODs specific for p63 and VDR. The Y-axis shows the fold change in transcript relative to GAPDH.
VD3 increases the stability of both ∆Np63α and VDR.

Due to our initial observations represented in Figures 16 and 17, we believe the mechanism for the VD3-mediated increase in both ∆Np63α and VDR is the result of increased protein stabilization. We tested this hypothesis by treating the HaCaT cell line with the protein synthesis inhibitor, emetine dihydrochloride, in the presence and absence of 100 nM VD3. We utilized emetine to eliminate off-target activation of the JNK pathway that has been observed with the other protein synthesis inhibitors, cycloheximide and anisomycin (Shaulian and Karin 1999; Topisirovic, Gutierrez et al. 2009). As the results in Figure 18 demonstrate, the levels of both ∆Np63α and VDR decrease with emetine treatment in the absence of VD3. However, emetine treatment in the presence of VD3 maintained ∆Np63α and VDR levels indicating that, in absence of de novo protein synthesis, VD3 increases the stability of both proteins, likely contributing to the up-regulation seen in Figure 16A and B.
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Figure 18: *VD3 increases the stability of both VDR and ΔNp63α.*

HaCaT cells were plated at a density of $4 \times 10^5$ in 6 cm plates prior to VD3 treatment. Following seeding, cells were treated with either vehicle or 100 nM VD3 for 24 hours. Cells were then treated with 2 µM emetine in the presence of fresh vehicle or 100 nM VD3. Whole cell extracts were harvested at the indicated intervals following emetine treatment and subjected to Western blot analysis with p63, VDR, and β-actin-specific antibodies.
**VDR reduction decreases JNK levels independent of VD3**

There has been increased investigation of VD3-independent effects displayed by VDR as a result of the differing phenotypes of VDR -/- mice versus CYP27B1 -/- mice (Bikle 2004). This information leads us to believe that VDR may have a VD3-independent effect on the JNK pathway. To investigate this possibility, we utilized control or VDR-specific siRNA transfections and observed the effect of reducing VDR on JNK (Figure 19A). We confirmed a significant reduction in VDR and found that this resulted in a reduction in the total levels of JNK, most noticeably the JNK2 isoform. As basal levels of JNK1 are minimal in keratinocytes, it is difficult to definitively conclude that this isoform was reduced following VDR silencing. This observation suggests that in the absence of VD3, VDR may maintain expression of JNK in keratinocytes. To further ascertain the role of VD3 in JNK expression, we performed the same siRNA transfection with the addition of 100 nM VD3 for 24 hours. The data presented in Figure 19B confirms that compared to treatment with control siRNA, VDR knockdown leads to a reduction in JNK levels. Curiously, VD3 treatment following transfection with control siRNA modestly reduced JNK levels. However, VD3 treatment appeared to slightly restore JNK2 levels following VDR silencing. Taken together, the data in Figure 19 indicates that VDR may have a VD3-independent role in maintaining JNK2 expression.
Figure 19: *VDR loss reduces JNK levels independent of VD3.*

A) HaCaT cells were plated at a density of 3x10^5 in 6 cm dishes and transfected with 2 rounds of control or VDR-specific siRNAs. 36 hours following the second round of transfection, protein was harvested and subjected to immunoblot analysis with antibodies specific for JNK, VDR, and β-actin. B) HaCaT cells were subjected to siRNA transfections as in A). 24 hours following the second round of transfection, cells were treated with either vehicle or 100 nM VD3 for an additional 24 hours. Following the incubation period, cells were harvested for whole cell extracts and analyzed via immunoblotting using JNK, VDR, and β-actin-specific antibodies. C) Fold change in relative JNK2 intensity normalized to endogenous β-actin. Fold change was determined relative to the control siRNA-transfected condition.
**JNK down-regulation following VDR reduction inhibits UV-mediated p-JNK**

Our observations in Figure 19 suggested that VDR maintains JNK expression independent of VD3. JNK reduction as a result of VDR knockdown should, therefore, affect the amount of JNK phosphorylated following UV exposure. We examined this by transfecting HaCaT cells with control or VDR-specific siRNA followed by incubation with vehicle or VD3 24 hours prior to UV exposure. The results in Figure 20 confirm our previous results that VDR knockdown resulted in a significant reduction the total levels of JNK. This down-regulation, in turn, correlated with decreased p-JNK levels following UV exposure (Lanes 2 & 6). Additionally, VD3 treatment did not significantly enhance the inhibition of JNK phosphorylation, but did slightly increase the levels of total JNK in cells transfected with VDR siRNA; however, this could be due to unequal protein loading as evidenced by the β-actin levels.
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![Western Blot Image](image_url)

- **p-JNK2**
- **p-JNK1**
- **JNK2**
- **JNK1**
- **β-actin**

67
Figure 20: JNK down-regulation via VDR loss inhibits UV-mediated p-JNK levels.

HaCaT cells were plated at a density of 3x10^5 in 6 cm plates and subjected to 2 rounds of siRNA transfections. 24 hours following the second round of transfection, cells were treated with either vehicle or 100 nM VD3 for an additional 24 hours prior to UV treatment. Cells were exposed to 150 J/m2 and harvested for whole cell extracts 2 hours after treatment. Protein was analyzed via Western blotting using antibodies specific for p-JNK, JNK, VDR, and β-actin.
IV. Discussion:

Excessive exposure to UVB radiation causes the formation of DNA lesions which can develop into cancer-causing mutations. Contrasting the harmful behavior of UVB exposure is its beneficial ability to promote VD3 synthesis in the epidermis, a process that is essential for epidermal stratification and maintenance (Bikle, Chang et al. 2004). The harmful affects of UV signaling are, therefore, coupled to activation of the VD3 pathway, suggesting that dual exploration of both pathways is critical to understanding the mechanism of skin cancer formation. Additionally, both VDR and the ΔNp63α isoform play crucial roles in maintaining epidermal integrity, and have been implicated to participate in either epidermal tumor suppression or oncogenesis, respectively. The upstream regulation of both proteins has been unexplored and could result in greater understanding of the balance between apoptosis and survival in the epidermis.

UVB promotes VD3 synthesis through the initial conversion of 7-dehydrocholesterol to Vitamin D3 and finally by a series of hydroxylation reactions to the hormonally active 1α, 25-dihydroxy Vitamin D3 (VD3). The epidermis has been demonstrated to possess the enzymes responsible for VD3 production, suggesting that local VD3 production is necessary for normal epidermal processes (Bikle 2008). In addition, VD3 has been found to increase the levels of its receptor, VDR, through either transcriptional activation or protein stabilization mechanisms in a cell-line specific manner (Solvsten, Svendsen et al. 1997; Healy, Frahm et al. 2005). Downstream modulation of VDR by UV signaling, however, has not been examined due to the ability of UVB to synthesize VD3.
Lack of VD3 production via UVC irradiation enables us to distinguish the effects of UV mediated modulation of VDR independent from VD3 synthesis. In addition, utilization of UVC radiation activates the p38 and JNK pathways, two of the main signaling mediators of UV-induced DNA damage. Our initial investigation demonstrated that exposure of WT MEFs and HaCaT cells to increasing levels of UV down-regulated VDR, suggesting that VDR is reduced upon UV-induced DNA damage (Figures 5 and 6). In HaCaT cells, VDR down-regulation appeared to correlate with activation of JNK signaling, a pathway potently activated by UV damage.

Sustained JNK activation following UV treatment results in the activation of apoptotic programs (Chen, Wang et al. 1996). VDR down-regulation could, thus, result from the initiation of apoptosis due to activation of JNK signaling. However, utilization of MEF cell lines possessing inactive JNK pathways resulted in VDR reduction following UV irradiation (Figure 7B). Interestingly, VDR was down-regulated to a more significant extent in JNK-inactive MEFs than was observed in WT MEFs, implying that JNK signaling may promote VDR expression rather than contribute to UV-induced down-regulation. As UV-induced DNA damage activates multiple signaling pathways, including the p38 pathway, it is also possible that JNK may be dispensable for VDR down-regulation due to the activation of additional pathways. Instead, basal JNK signaling may inhibit VDR expression as MEF cell lines with inactive JNK pathways expressed elevated basal VDR transcripts (Figure 7A). Additional experiments involving inhibition of the JNK and p38 pathways in HaCaT cells or primary keratinocytes in the
presence of UV damage will resolve the role JNK plays in UV-induced VDR down-regulation.

The presence of the ΔNp63α isoform in the stratum basale of the epidermis maintains the proliferative potential of the basal progenitor cells (Senoo, Pinto et al. 2007). UVB-mediated DNA damage results in apoptosis of progenitor cells through down-regulation of ΔNp63α (Liefer, Koster et al. 2000). This down-regulation was attributed to phosphorylation of ΔNp63α by the p38 MAPK, leading to the reduced stability and ability of ΔNp63α to interact with DNA (Papoutsaki, Moretti et al. 2005; Westfall, Joyner et al. 2005). Intriguingly, activation of both the p38 and JNK pathways occurs following UV exposure, yet JNK was not definitively eliminated as a potential ΔNp63α candidate kinase. Moreover, the identification of both TAp73α and p53 as JNK substrates, further implied that ΔNp63α may be phosphorylated by JNK due to the considerable homology between p53 family members (Buschmann, Potapova et al. 2001; Jones, Dickman et al. 2007). Treatment of keratinocytes with increasing concentrations of UV confirmed the phosphorylation of ΔNp63α by the presence of a mobility shift (Figure 8). Moreover, higher levels of UV radiation resulted in a more robust shift in ΔNp63α, suggesting that the increased accumulation of DNA damage could lead to an increase in the phosphorylation status of ΔNp63α (Figure 8). A comparison of the amino acid sequences of human TAp73α with human and murine ΔNp63α demonstrated that the phosphorylation sites identified on TAp73α were conserved on ΔNp63α (Figure 9). In addition, we discovered a putative JNK interaction site is conserved between all the proteins displayed in Figure 9.
The activation of downstream JNK signaling by UV exposure is mediated primarily by the JNK1 isoform (Chen, Wang et al. 1996; Katagiri, Negishi et al. 2006). Phosphorylation of JNK1 should, therefore, reproduce the mobility shift in ΔNp63α. Co-expression of ΔNp63α and JNK1 in p63-null H1299 cells in the presence of UV damage resulted in the appearance of a mobility shift in ΔNp63α (Figure 10). In summary, correlating the mobility shift in ΔNp63α with activation of the JNK pathway and the identification of conserved phosphorylation sites and a putative JNK interaction motif between ΔNp63α and TAp73α suggest ΔNp63α is a potential JNK substrate. Further confirmation could be determined via an in vitro kinase assay, or through chemical or JNK-specific siRNA-mediated inhibition of the JNK pathway in the presence of UV damage. Inhibition of p38 signaling could additionally be used to confirm that JNK contributes to the UV-induced shift in ΔNp63α.

The role of VD3 in promoting differentiation and apoptosis has been extensively characterized (Blutt, McDonnell et al. 2000; Wang, Lee et al. 2001; Bikle, Chang et al. 2004). Recent reports suggesting that VD3 promotes cell survival following UV and IR-induced stress are in direct conflict with previously identified VD3 activities. Specifically, VD3-mediated inhibition of the JNK, but not p38, pathway resulted in a reduction in apoptosis (De Haes, Garmyn et al. 2003; Langberg, Rotem et al. 2009). However, the pro-survival effects of VD3 following UV treatment were observed with elevated VD3 concentrations (1 µM) and for extended durations of VD3 exposure (24 hours) (De Haes, Garmyn et al. 2003). Confirming these observations with lower concentrations of VD3 and determining the rate of response to VD3 exposure are...
important for ascertaining the in vivo relevance of past studies. We confirmed that VD3-inhibited UV-induced JNK phosphorylation at reduced concentrations of VD3 and that this was not due to a diminished pool of total JNK (Figure 11). In addition, the VD3-mediated reduction in UV-induced p-JNK occurred following brief intervals of VD3 incubation, with significant effects observed after only 2 hours of treatment (Figure 12). Further examination revealed that VD3 also inhibited basal levels of JNK phosphorylation at concentrations as low as 10 nM (Figure 13). These results strengthen the previous VD3 studies and imply in vivo significance to the reduction in p-JNK following VD3 treatment.

VD3 treatment up-regulates VDR through multiple mechanisms in addition to inhibiting p-JNK (Solvsten, Svendsen et al. 1997; Healy, Frahm et al. 2005). Pre-treating keratinocytes with VD3 could compensate for the VDR down-regulation and erase the mobility shift in ΔNp63α observed after UVC irradiation. VD3 pre-treatment successfully inhibited VDR reduction subsequent to UV treatment (Figure 14A and B). In this instance, VD3-mediated p-JNK inhibition was less robust than was previously observed suggesting that VD3 increases VDR directly rather than indirectly via JNK inhibition. Additionally, inhibition of p-JNK did not ablate the retarded mobility of ΔNp63α after exposure to UV radiation (Figure 15). Since the p38 pathway has already been linked to ΔNp63α phosphorylation and VD3 does not inhibit p38 signaling, this clarifies the inability of VD3 to abolish the shift in ΔNp63α (Papoutsaki, Moretti et al. 2005; Langberg, Rotem et al. 2009). Future studies separating the JNK and p38 pathways will determine whether ΔNp63α is a downstream substrate of JNK signaling.
VD3 signaling has been observed to increase both VDR and ∆Np63α levels (Solvsten, Svendsen et al. 1997; Healy, Frahm et al. 2005; Langberg, Rotem et al. 2009). Understanding the mechanism of VD3-mediated elevation of ∆Np63α would complement the current information concerning the pro-survival behavior perpetuated by VD3 in UV-treated keratinocytes. The underlying cause of this effect has not been examined, but could be the result of either transcriptional activation of protein stabilization. Specifically, genomic mechanisms propagated by VD3 exposure are attributed to transcriptional activation and are observed at prolonged intervals subsequent to VD3 incubation, whereas non-genomic mechanisms of VD3 actions occur rapidly following treatment. Treatment of keratinocytes with increasing concentrations of VD3 resulted in an increase in both ∆Np63α and VDR (Figure 16A and B). We initially attributed this to non-genomic mechanisms of VD3 signaling as both proteins were modestly induced following 30 minutes of exposure to VD3 (Figure 16C and D). By investigating the transcript levels of both p63 and VDR, we concluded that VD3 treatment does not significantly induce transcription of either protein (Figure 17). In contrast, we observed that VD3 treatment increased the stability of both ∆Np63α and VDR when coupled to treatment with the protein synthesis inhibitor, emetine dihydrochloride (Figure 18). By preventing ∆Np63α degradation, VD3 may inhibit UV-mediated apoptosis by perpetuating cell survival signals. VD3-induced stabilization of ∆Np63α, thus provides an additional mechanism by which VD3 initiates cell survival.

The generation of both VDR -/- and CYP27B1 -/- mice lead to the discovery of VD3-independent functions for VDR in the epidermis. In particular, mice lacking VDR have a
propensity to develop both UV and chemically-induced skin tumors, a phenotype that is absent in CYP27B1 -/- mice (Zinser, Sundberg et al. 2002; Ellison, Smith et al. 2008). The skin cancer-prone phenotype of VDR -/- mice compared to the absence of tumors in VD3-deficient mice implies that VDR plays an important VD3-independent role in the epidermis. These VD3-independent activities of VDR are only beginning to be understood. To determine a VD3-independent contribution of VDR in regulating the JNK pathway, we observed JNK expression upon VDR knockdown (19A). JNK expression, specifically the JNK2 isoform, was reduced following VDR loss. A reduction in JNK1 was not determinable due to the low basal JNK1 levels in keratinocytes. The addition of VD3 following VDR silencing slightly recovered the decrease in JNK expression, suggesting that VDR may maintain JNK2 levels independent of VD3 (19B).

VDR maintenance of JNK could additionally dictate the ability of keratinocytes to respond to the effects of UV treatment. Specifically, decreased JNK levels would diminish the amount of JNK phosphorylated after UV exposure. VDR loss coupled with UV treatment did result in a reduction in p-JNK levels. VD3-pre-treatment significantly inhibited JNK phosphorylation, but there was no further reduction in p-JNK with VDR silencing together with VD3 treatment (Lane 6 vs. 8). Taken together, these results demonstrate that in the absence of either UV or VD3 signaling, VDR maintains normal JNK expression. The high occurrence of UV-induced tumors in VDR -/- could, in part, be explained by a suppressed p-JNK response resulting in reduced apoptosis following the accumulation of UV-induced DNA lesions, eventually leading to mutations and the development of skin cancer.
Our data provides novel insight into the upstream regulation of VDR and ΔNp63α, an area that has presently been underexplored. We identified ΔNp63α as a potential substrate for JNK, although additional investigation is required to definitively demonstrate this effect. Furthermore, the promotion of cell survival with VD3 pre-treatment could be the result of VD3-mediated inhibition of p-JNK and its ability to increase the stabilization of ΔNp63α. Lastly, we discovered a VD3-independent role for VDR in maintaining JNK expression in unstressed keratinocytes, an effect that could determine the response of keratinocytes to UV irradiation.

The combined study of both UV and VD3 signaling are directly relevant to mechanisms contributing to cancer development and the maintenance of epidermal homeostasis. In addition, the recent VD3-independent roles discovered for VDR should be further determined. The initial indication is that VD3 and UV signaling activate the response of keratinocytes to UV damage. The joint regulation of ΔNp63α and VDR by both pathways, therefore, implicates them as essential contributors in the response to UV damage. The balance between survival and apoptosis, and even the formation of cancer, may reside in the ability of cells to either repair UV-induced DNA lesions or to undergo apoptosis with the regulation of VDR and ΔNp63α determining the final response.

In conclusion, in unstressed HaCaT cells, we demonstrated that VDR maintains JNK expression, and thereby, may regulate the JNK pathway response to UV exposure. UV-mediated DNA damage reduced the levels of VDR and activated the JNK pathway, which corresponded to a mobility shift in ΔNp63α, suggesting that ΔNp63α may be a
JNK substrate. UV exposure also induces VD3 production, which can decrease activation of the JNK pathway. We confirmed this occurs with lower concentrations of VD3 and that VD3 acts directly on VDR and ΔNp63α to increase their stability which could stabilize their levels following UV-mediated DNA damage. VD3-mediated stabilization of VDR and ΔNp63α, coupled with its ability to inhibit the formation of p-JNK could promote keratinocyte survival in keratinocytes following UV-induced DNA damage. However, accumulated levels of UV-mediated damage could overwhelm the effects of VD3 and induce apoptosis. Future studies should more intimately define the response of both the UV and VD3 pathways in regards to their downstream effects on ΔNp63α and VDR and cell survival.
V. References:


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