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Differential Regulation of Vitamin D Receptor (VDR) By P53, P63 and P73

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DIFFERENTIAL REGULATION OF VITAMIN D RECEPTOR (VDR) BY p53, p63 AND p73

A dissertation submitted in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

By

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SHOOL OF GRADUATE STUDIES

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I HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER MY SUPERVISION BY Ramakrishna Kommagani ENTITLED Differential regulation of Vitamin D receptor (VDR) by p53, p63 and p73 BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTSFOR THE DEGREE OF Doctor of Philosophy.

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ABSTRACT

Kommagani, Ramakrishna. Ph.D., Biomedical Sciences Ph.D. Program, Wright State University, 2009. Differential regulation of vitamin D receptor (VDR) by p53, p63 and p73.

The tumor suppressor *p53* is the single most altered gene in human cancers. p53 homologues, p63 and p73 play a major role in development and in human cancer. Both *p63*and *p73*-null mice exhibit profound developmental abnormalities, suggesting a vital role for p63 and p73 in development. Although the role of p73 in human cancers is well established, the role of p63 still remains to be understood. While p63 plays a major role in development, p73 plays a major role in tumor suppression as well as in development. Although a functional co-operation is evident between each member of p53 family, additional studies are required to understand the functional cross-talk between each member of the p53 family, and their ability to govern multiple biological functions. Identifying common and specific transcriptional networks of p53 family is essential for understanding the existing functional co-operation between each member.

This dissertation focuses on defining the functional relevance of the regulation of vitamin D receptor (VDR) by p53 family members. Secosteroid hormone vitamin D, through its cognate receptor VDR, regulates genes involved in mineral homeostasis, bone formation and in epidermal differentiation. Vitamin D and its analogues also exhibit anti-proliferative activities and are widely used as cancer chemotherapeutic agents. Findings from this study demonstrated VDR as a direct target of p63 and p73; however p53 does not appear to regulate VDR directly or indirectly. One part of this dissertation underpinned the role of VDR in p63 mediated biological functions. Down regulation of endogenous p63 in human epidermoid cancer cells resulted in complete loss of

endogenous VDR expression. In addition, up-regulation of VDR by p63 appears to inhibit the migration and invasion of human epidermoid cancer cells. the second part of this dissertation is involved in understanding the p73 mediated regulation of VDR in vitamin D-mediated differentiation. Findings from this section of study demonstrated that DNA damage-induced expression of VDR is dependent on p73. In addition, p73 was proven to be essential for vitamin D mediated osteoblastic differentiation. Furthermore, we demonstrated that DNA damage sensitized the cells to vitamin D mediated differentiation through p73. Taken together, while understanding the regulation of VDR by p63 will provide new insights on the molecular mechanisms of p63 biology, determining the role of p73 in vitamin D-mediated differentiation may aid in vitamin D based cancer chemotherapeutics.

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I would like to sincerely thank my advisor Dr. Madhavi Kadakia for the opportunity and the enormous scientific freedom I received in her lab. I am especially grateful for the incredible guidance I got through the good as well as hard times of this dissertation work. I am indebt for the support and help I received in and outside the lab from her. I couldn't have wished for a better supervisor; this would not have been completed without her. My sincere thanks to all my committee members: Dr. Steven Berberich, Dr. Michael Leffak, Ph.D., Dr. James McDougal, Ph.D. and Dr. Thomas L Brown, Ph.D. for their valuable time and the suggestions during my committee meetings. I am especially thankful to Drs. Berberich and Leffak for their support and the freedom to utilize their laboratory recourses. I also like to thank all the instructors for the valuable knowledge I got through their courses.

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DEDICATION

To my beloved late brother Srinivas Kommagani whose
memories kept my drive all along

I. INTRODUCTION AND PURPOSE

A. p53 in cancer and chemosensitivity

Transcription factor *p53* is the most altered gene in human cancers and is considered as the guardian of genome (Lane, 1992). Since, mice deficient for p53 are tumor prone and p53 function is lost in a majority of human cancers due to the mutations or inactivation by its negative regulators, p53 is considered as the classical tumor suppressor (Donehower et al., 1992; Hollstein et al., 1991). Primarily, p53 promotes cell cycle arrest, apoptosis, DNA repair, cellular senescence and inhibition of angiogenesis through the transcriptional regulation of target genes (Giaccia and Kastan, 1998); however, the induction of apoptosis and cell cycle arrest by p53 can also occur through non-transcriptional mechanisms (Mihara et al., 2003). Stabilization of p53 protein occurs in response to various stress stimuli including DNA damage, viral infection or oncogenic activation (Schuler and Green, 2001). Once activated and depending on the stimuli, p53 controls the signaling pathways involved in cell cycle arrest (G1 and G2 cell cycle arrest), senesence or apoptosis (Figure 1) (Levine et al., 2004; Vousden, 2000). Strategies involved in activation of the p53 pathway to promote tumor suppression are considered to be effective anticancer therapies and numerous p53 based cancer therapuetics are in preclinical studies. However, alternative p53 independent strategies are required for designing effective cancer therapies for p53 deficient cancers.

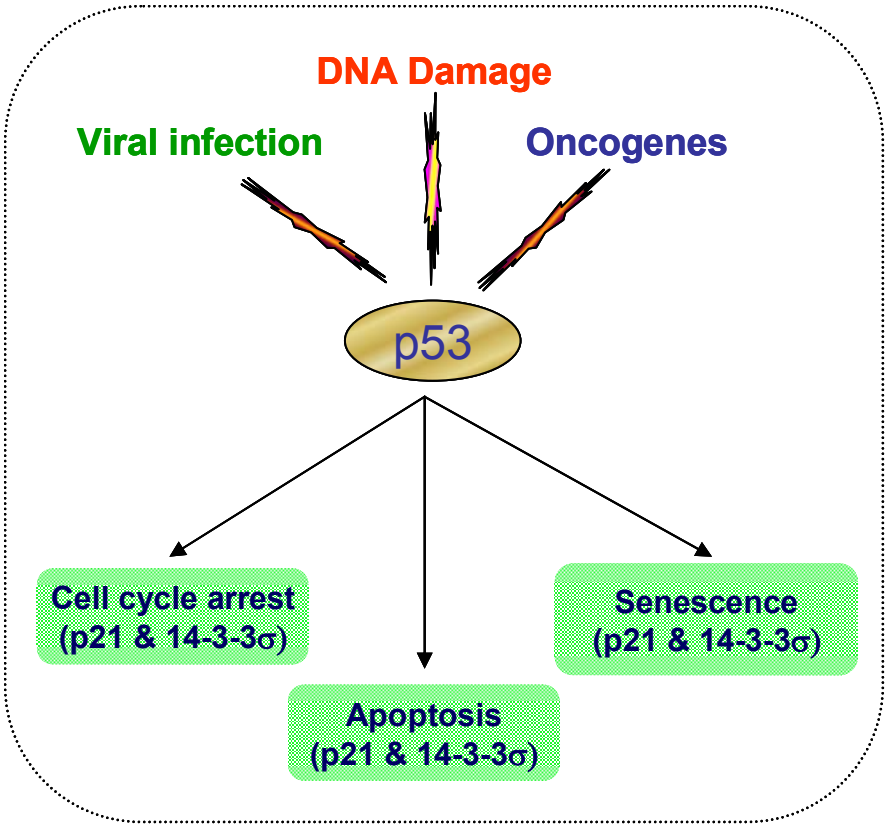
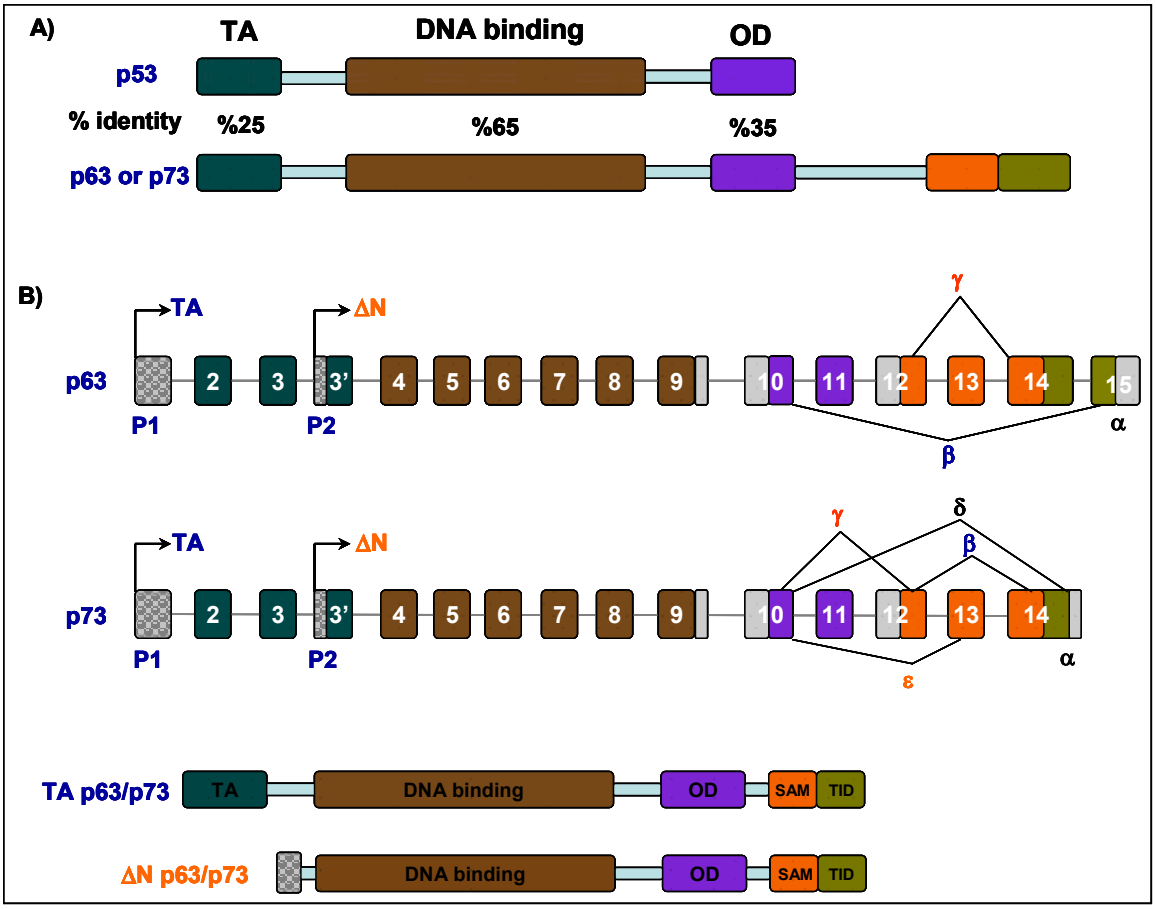


Figure 1: Schematic representation of subset of p53 functions. The activated p53 upon different stimuli promotes the cell cycle arrest, apoptosis and senescence.

B. Structure of p63/p73

Although the functional significance of p53 and its interacting proteins has been studied extensively, it was not until 1997, that the functional homologues of p53, namely p63 and p73 were discovered (Kaghad et al., 1997; Yang et al., 1998). Since their discovery, both these genes gained enormous scientific interest, as proteins encoded by these genes are functionally more similar to each other than to p53 (Deyoung and Ellisen, 2007). All three homologues contain similar functional domains namely N-terminal Transactivation (TA), central DNA binding and C-terminal oligomerization domains (Harms et al., 2004; Levrero et al., 2000). The DNA binding domains of p63 and p73 show the highest homology (65%) with p53 DNA binding domain (De Laurenzi and Melino, 2000). The oligomerization domain of p63 and p73 also show homology (35%) with p53 oligomerization domain and like p53, both p63 and p73 exist as tetramers through the oligomerization domain (Yang and McKeon, 2000). The N-terminal transactivation domain (TA domain) of p63 and p73 show the lowest homology (25%) with p53 transactivation domain. Like p53, the transactivation domain in p63 and p73 is essential for the transcriptional potential, as the TA domain interacts with various co-activators to modulate transcription. Like p53, both p63 and p73 also generate multiple transcripts due to the alternative promoter usage and pre-mRNA splicing (Kaghad et al., 1997; Yang et al., 1998). As a consequence of promoter usage both p63 and p73 generates either transactivation domain containing isoforms (TAp63 and TAp73) or N-terminally truncated isoforms (ΔNp63 and ΔNp73) (Scoumanne et al., 2005). Additionally due to the splicing at the 3'of pre-mRNA both p63 and p73 generate

Figure 2: Schematic representation of p63 and p73 gene structures. A) The degree of homology between the conserved domains of p63/p73 with p53. B) Multiple isoforms of p63/p73 are generated from alternative promoters (P1 and P2) and pre-mRNA splicing. The full length TA isoforms and truncated ΔN isoforms are generated from P1 or P2 promoter respectively. The 3' pre-mRNA splicing results in additional isoforms α,β and γ of p63 and α, β, γ, ε and δ of p73.


A)
TA
DNA binding
OD
p53
% identity
%25
%65
%35
p63 or p73
B)
TA
ΔN
p63
2
3
3'
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6
7
8
9
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11
12
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15
P1
P2
γ
α
β
p73
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ε
TA p63/p73
TA
DNA binding
OD
SAM
TID
ΔN p63/p73

multiple carboxyl-terminal variants, α, β and γ of p63 and α, β, γ, ε and δ of p73 (Kaghad et al., 1997; Yang et al., 1998) (Figure 2). The features that distinguish p63 and p73 from p53 are the presence of two additional domains, a SAM domain (Steric α Motif), and a TID domain (Transactivation Inhibition Domain) in alpha isoforms of both p63 and p73 (Moll, 2003; Moll et al., 2001) (Figure 2B). The precise function of TID domain and SAM domain in p63 or 73 is still unclear; however, the presence of TID domain lowers the transactivation potential of TAp63α and TAp73α isoforms (Serber et al., 2002; Thanos and Bowie, 1999). In general, the SAM domain is observed in proteins involved in development and is believed to be involved in protein-protein or protein – RNA interactions (Kim and Bowie, 2003).

The TA domain containing isoforms of both p63 and p73 are highly homologous to p53, and as a result, TA isoforms act as tumor suppressors like p53 (Harms et al., 2004). Although, ΔNp63 isoforms lack the full length N-terminal transactivation domain, like TAp63 isoforms, ΔNp63 isoforms can also transactivate genes via the presence of a short unique N-terminal TA domain (Helton et al., 2006; Wu et al., 2005). Additionally, both ΔNp63 and ΔNp73 isoforms inhibit TAp63/TAp73 and p53 functions by acting in a dominant negative manner towards TAp73/TAp73 isoforms and p53 (Stiewe et al., 2002b). The inhibitory activity of ΔNp63/ ΔNp73 isoforms involve direct competition of DNA binding through the formation of hetero-oligomeric complexes (Grob et al., 2001; Stiewe et al., 2002a).

C. Roles of p63/p73 in development and differentiation

Gene knock-out studies by multiple groups in mice implicated a broad role for p63 and p73 in development and differentiation. *p63*-null mice are born with severe

developmental defects and die shortly after birth due to dehydration (Mills et al., 1999; Yang et al., 1999). Defects observed in *p63*-null mice include, but are not restricted to, an under-development of the skin, as wells as a distinct lack of distal components of forelimbs, carpals, digits and hind limbs. Although several studies highlighted a critical role for p63 in epithelial stratification program, the precise role of TAp63 and ΔNp63 isoforms in epithelial tissue development and differentiation still remain controversial (Koster et al., 2004; McKeon, 2004). Findings by Senoo et al. (2007) show that ΔNp63α is the only p63 isoform observed during epithelial morphogenesis, and also ΔNp63α is essential for the proliferative potential of already committed epithelial cells, but not for terminal differentiation (Senoo et al., 2007). In contrast, Koster et al. (2004) suggests that TAp63 is essential for the commitment of ectoderm to epithelial tissue and that ΔNp63 is essential for proliferation and terminal differentiation of already committed epithelial cells.

Furthermore evidence from *in vivo* genetic complementation studies in *p63*-null mice suggest the requirement of both TAp63 and ΔNp63 isoforms for commitment and stratification of epithelial tissue (Candi et al., 2006). Consistent with its role in epithelial morphogenesis, mutations in p63 are associated with human developmental defects (Celli et al., 1999; van Bokhoven et al., 2001). Notably, p63 mutations are observed in six different human developmental syndromes; 1) split hand/foot malformation (SHFM), 2) ectrodactyly, ectodermal dysplasia and cleft lip/palate syndrome (EEC), 3) ankyloblepharon-ectodermal defects-cleft lip/palate syndrome (AEC), 4) limb mammary syndrome (LMS), 5) acro-dermato-ungual-lacrimal-tooth syndrome (ADULT) and Rapp-Hodgkin syndrome (RHS) (Figure 3). Primarily, the missense mutations of p63 that cause

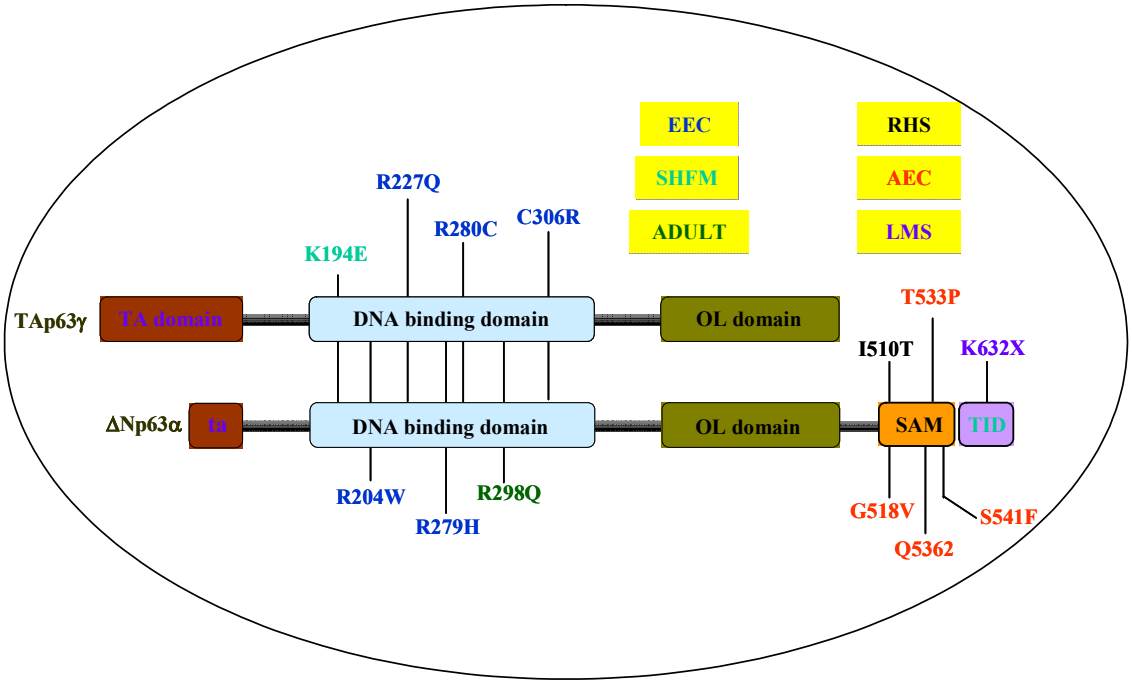


Figure 3: Schematic representation of p63 mutations observed in diseases. Selective germline mutations of p63 are indicated with corresponding human developmental syndromes. Mutations are observed in DNA binding domain or SAM domain or transactivation inhibitory domain.

diseases reside in the DNA binding domain and/or in the SAM/TID domains (Figure 3). Consistent with the role of p63 in epithelial tissue development, the defects observed with p63 mutations are mainly attributed to the aberrations in apical epithelial ridge formation (Rinne et al., 2007; van Bokhoven and McKeon, 2002).

Similar to p63, p73 also plays a distinct role in development, particularly in neurogenesis and sexual behavior (Yang et al., 2000). Unlike p63 null mice, severe neurlogical defects are observed in p73 null mice. p73 null mice have a high mortality rate and show a runting phenotype. The death of *p73* null mice was associated with massive gastrointestinal haemorrhages. In addition, p73 null mice had severe immunological problems distinguished by chronic infections and inflammations. *p73* null mice were born with severe hydrocephalus due to the defects in production or reabsorption of cerebral spinal fluid. Additionally, p73 null mice lacked sexual interest, due to the defects in pheromone detection. Interestingly, TAp73 isoform specific deficient mice showed severe defects in hippocampal and oocyte development but not in neural cell maintenance, indicating an isoform specific function for p73 during embryonic development (Tomasini et al., 2008).

Although, the role of p63 and p73 during embryonic development is well studied, the precise function of p63 and p73 in differentiation has yet to be determined. Several studies indicate the differential effect of TAp63/p73 and ΔNp63/p73 isoforms on differentiation in multiple model systems. TAp73 isoforms promote the differentiation of keratinocytes and neuroblastomas by activating a number of genes including *S100A2* (Lapi et al., 2006), while, suppression of $p57^{kip2}$ expression by ΔNp73 results in a blockade of myogenic differentiation as well as cell cycle exit in murine myoblasts (Li et

al., 2005). TAp63 isoforms promote keratinocyte differentiation by regulating the expression of multiple proteins including loricrin, involucrin, Notch and retinoblastoma (Cam et al., 2006; Carroll et al., 2006; Nguyen et al., 2006). Precise roles of p63 and p73 in differentiation can be understood only by delineating the isoform-specific regulation of multiple signaling pathways and the functional interaction between each isoform.

D. p63/p73 roles in tumorigenesis

Both p63 and p73 are not considered to be classical tumor suppressors, however, both p63 and p73 might contribute to p53-mediated tumor suppression (Flores, 2007). Mouse embryonic fibroblasts (MEF's) from either p73 or p63 ablated mice show reduced cell death upon genotoxic stress, suggesting the requirement of p63 and/or p73 for p53 mediated apoptosis (Flores et al., 2002). Given that DNA damage induces the expression of TAp63/p73 isoforms in these MEFs, the reduced apoptosis observed in p63-and/or p73-null MEF's can be attributed to the lack of TAp63/p73 isoforms. An independent study using reconstituted thymocytes from different genetically defined mice suggested that p63 and p73 are not required for p53 dependant apoptosis, at least in T- cells (Senoo et al., 2004). Further monitoring the lymphoma development in same model system showed that loss of p63 or p73 do not underpin any growth advantage to p53 deficient mice, suggesting that p53 mediated tumor suppression is independent of p63 and/or p73 (Perez-Losada et al., 2005).

Additionally, studies by Keyes et.al. (2005) demonstrated that p63 heterozygous mice are not prone to the development of spontaneous tumors or to chemically induced tumors. Moreover, fewer tumors were observed in p53+/- and p63+/- compound mice than p53+/- mice (Keyes et al., 2006). These observations suggest that p63 might in fact

promote tumor progression. Contrastingly, an affirmative role for p63 and p73 in tumor suppression came from mice with single allele deletions of p63 and/or p73. Both p63 and p73 heterozygous mice have spontaneous tumors in multiple organs, including the colon and prostate (Flores et al., 2005). Therefore, the higher frequency of tumor occurrence in p63 and p73 heterozygous mice was attributed to the impairment in p53 mediated apoptosis. The obvious differences in findings by these two groups might be due to the genetic background variation in creating p63 heterozygous mice. Although the role of p63 in tumorigenesis still needs to addressed, recent findings have confirmed the role of TAp73 in tumorigenesis. Notably, findings by Tomasini et. al. (2008) indicated that the TAp73 isoform specific knock out mice were prone to spontaneous as well as chemically induced tumors (Tomasini et al., 2008). However, complete lack of p63 and/or p73 mutations in human cancers refutes the tumor suppressive functions for p63 and p73 (Sunahara et al., 1999; Yoshikawa et al., 1999). Thus, additional isoform specific knock out mice studies are required for both p63 and p73 to further define their roles in tumorigenesis.

E. p63/p73 and human cancers

The expression of both p63 and p73 in normal tissue and in human cancers is well studied. The expression of p63 is mainly limited to basal layers of normal epithelial tissues including prostate, skin, urothelia and cervix (Yang et al., 1998). The majority of the normal human tissues studied express p73 at low levels (Kovalev et al., 1998). In the majority of human cancers studied, the expression of p63 and/or p73 is either amplified or lost. The amplified levels of p63, particularly ΔNp63 isoforms are observed in various human cancers including head and neck squamous cell carcinoma (HNSCC), lung

carcinoma, esophageal adenocarcinoma, bladder cancers and cervical carcinomas (Glickman et al., 2001; Hara et al., 2004; Massion et al., 2003; Park et al., 2000; Quade et al., 2001; Sniezek et al., 2004; Wang et al., 2001; Yamaguchi et al., 2000). Although yet to be resolved, the overexpression of p63 in these cancers appears to be both dependant and independent of genomic amplification (Redon et al., 2001; Yamaguchi et al., 2000). In fact, the *p63* gene resides in 3q27–28 locus, which is frequently amplified in squamous cells carcinomas (Bjorkqvist et al., 1998). While, ΔNp63 isoform expression is amplified in squamous cells carcinomas, the expression of TAp63 isoforms is fairly undetectable (DeYoung et al., 2006). Moreover, loss of p63 expression was correlated with poor prognosis in multiple carcinomas including bladder cancers (Koga et al., 2003).

In contrast to p63, the expression of p73 was observed in a majority of the human cancers studied (Melino et al., 2002; Moll and Slade, 2004). Particularly, the up regulation of the oncogenic ΔNp73 isoform was observed in a large variety of human cancers including, neuroblastoma, glioma, breast, colon, ovarian, liver, squamous cell carcinomas, chronic lymphoid leukemia (CLL) and acute myelogenous leukemia (AML) (Concin et al., 2004; Cui et al., 2005; Dominguez et al., 2006; Dominguez et al., 2001; Douc-Rasy et al., 2002; Leupin et al., 2004; Muller et al., 2005; Rizzo et al., 2004; Wager et al., 2006). Although the pro-apoptotic TAp73 isoform expression is observed in a subset of human tumors, the oncogenic ΔNp73 inhibits TAp73 function to promote tumor progression (Muller et al., 2005). Consistent with its oncogenic function, the preferential up regulation of ΔNp73 in a subset of breast tumors was correlated with poor prognosis (Dominguez et al., 2006). Moreover, inactivation of the TAp73 promoter by methylation was reported in lymphoma (Corn et al., 1999; Kawano et al., 1999).Taken together, these

findings implicate oncogenic function for ΔNp73 isoforms and a potential tumor suppressive function for TAp73 isoforms.

F. Role of p63 and p73 in apoptosis and chemosensitivity

In general, while TAp63 and TAp73 function similar to p53 in promoting cell cycle arrest and apoptosis, ΔNp63 and ΔNp73 isoforms functions opposite of p53 to promote cell growth. Ectopically overexpressed TAp63 isoforms induce apoptosis and cell cycle arrest in cell culture systems (Shimada et al., 1999). TAp63 isoforms can promote the inhibition of cell growth in multiple cancer cell lines by inducing pro-apoptotic genes and at the same time inhibiting pro-survival genes (Kommagani et al., 2006; Senoo et al., 2002; Shimada et al., 1999; Spiesbach et al., 2005; Wu et al., 2005). TAp63 isoform levels are undetected in most human cancers, however, the induction of TAp63 levels upon genotoxic stress are shown to determine the chemosensitivity of hepatoma cell lines (Gressner et al., 2005; Petitjean et al., 2005).

Similar to p63, TAp73 isoforms are also capable of inducing cell cycle arrest and apoptosis in multiple cancer cell lines. Both ectopic TAp73β and TAp73α isoforms promote apoptosis, although TAp73β is shown to be slightly more potent than the TAp73α isoform (Melino et al., 2002). Unlike p63, endogenous expression of p73 is induced upon treatment with a wide variety of chemotherapeutic agents or cytotoxic drugs including, adriamycin, cisplatin, etoposide, doxorubicin and bleomycin (Agami et al., 1999; Costanzo et al., 2002; Gong et al., 1999; Urist et al., 2004; Vayssade et al., 2005). Notably, up-regulation of both TAp73β and TAp73α isoforms was observed upon DNA damage, however, the predominant p73 isoform induced upon DNA damage is TAp73α (Urist et al., 2004). Given the robust response to a wide variety of DNA

damaging agents, inactivation of p73 confers chemoresistance to multiple cancer cell lines (Irwin et al., 2003). Moreover, TAp73 isoforms play a pivotal role in p53-independent apoptosis particularly TAp73α, which is vital for E2F1-mediated p53-independent apoptosis of T-cells (Lissy et al., 2000). Since chemotherapeutic agent mediated induction of TAp73α and its expression determines the chemoresistance and chemosensitivity, TAp73α is considered a potential target for cancer therapeutics (Irwin et al., 2003).

In contrast to TA isoforms, amplified levels of ΔNp63α and/or ΔNp73α promote chemo-resistance in multiple carcinomas by interfering with pro-apoptotic pathways (Leong et al., 2007; Rocco et al., 2006). In support of this view, direct physical association of ΔNp63α with TAp73 led to a dramatic down regulation of TAp73 mediated apoptosis in squamous cell carcinomas (Rocco et al., 2006). Taken together, these findings underline complex molecular interactions associated with p63 and p73 isoforms in governing the chemo resistance and chemosensitivity of multiple cancers. Nevertheless, additional functional studies are essential to delineate the contribution of individual isoforms of p63 and p73 and their interaction in human cancers.

G. Vitamin D and VDR

The secosteroid hormone 1α, 25 Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) (vitamin D) plays a vital role in maintaining calcium and phosphate homeostasis, in order to protect skeletal integrity. Apart from its classical functions, vitamin D also plays a major role in differentiation, immunosuppression, and growth inhibition (Bikle, 2004) (Figure 4). The human body obtains vitamin D either by nutritional intake or through UV

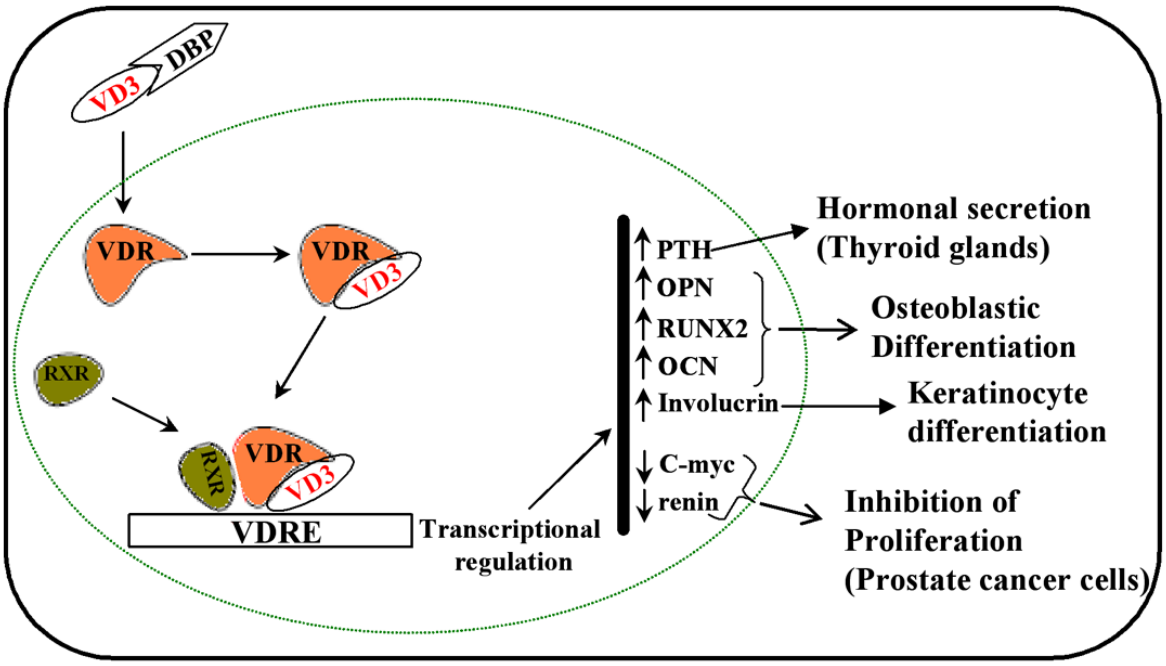


Figure 4: Schematic representation of vitamin D signaling pathway. The binding of active form of vitamin D to VDR results in activation of VDR. Once activated VDR hetrodimerizes with RXR and binds to VDRE within target gene promoters and modulates the transcription. The subset of VDR target genes and their tissue specific functions are listed.

mediated production in skin. The active form of vitamin D, 1α, 25 Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) is synthesized through multiple steps in skin (Holick et al., 1980a; Holick et al., 1980b). First, the exposure of skin to UVB irradiation results in production of pro-vitamin D from 7-dehydrocholesterol, subsequently 25-hydoxyvitamin D3 ($25(OH)D_3$) is synthesized by the hydroxylation of vitamin D_3 using vitamin D 25-hydroxylase in liver; finally, hydroxylation of 25(OH) D_3 by CYP27b1 in kidney results in production of active form $1,25(OH)_2D_3$ (Lawson et al., 1971; Masumoto et al., 1988). The vitamin D active form $1,25(OH)_2D_3$ will be refereed to as vitamin D (VD or VD3) in rest of the document. Circulating vitamin D in serum is carried by the vitamin D binding protein (DBP) to the nucleus of target tissues (Cooke and David, 1985; Cooke and Haddad, 1989). Biological functions of vitamin D are mediated by its cognate receptor Vitamin D receptor (VDR), a member of the nuclear receptor family. Principally, the *vdr* gene is composed of 14 exons of which two exons cover a 5'-non coding region and eight exons (2-9) produce the classical VDR protein (Baker et al., 1988) (Figure 5). Primarily, binding of vitamin D to VDR results in hetero-dimerization of VDR with RXR (Retinoid X Receptor), subsequently activating VDR. Activated VDR regulates the target genes by directly binding to the vitamin D responsive elements (VDRE) in their promoters (Figure 4) (Nezbedova and Brtko, 2004).

H. Vitamin D and VDR functions

The anti-proliferative actions of vitamin D and its analogues are demonstrated in many different human cancer cell lines (Gombart et al., 2006), notably, cancer cell lines from colon, prostate and breast (Guzey et al., 2002; Kane et al., 1996; Koike et al., 1997). Generally, VDR exert anti-proliferative effects by up regulating the genes involved in

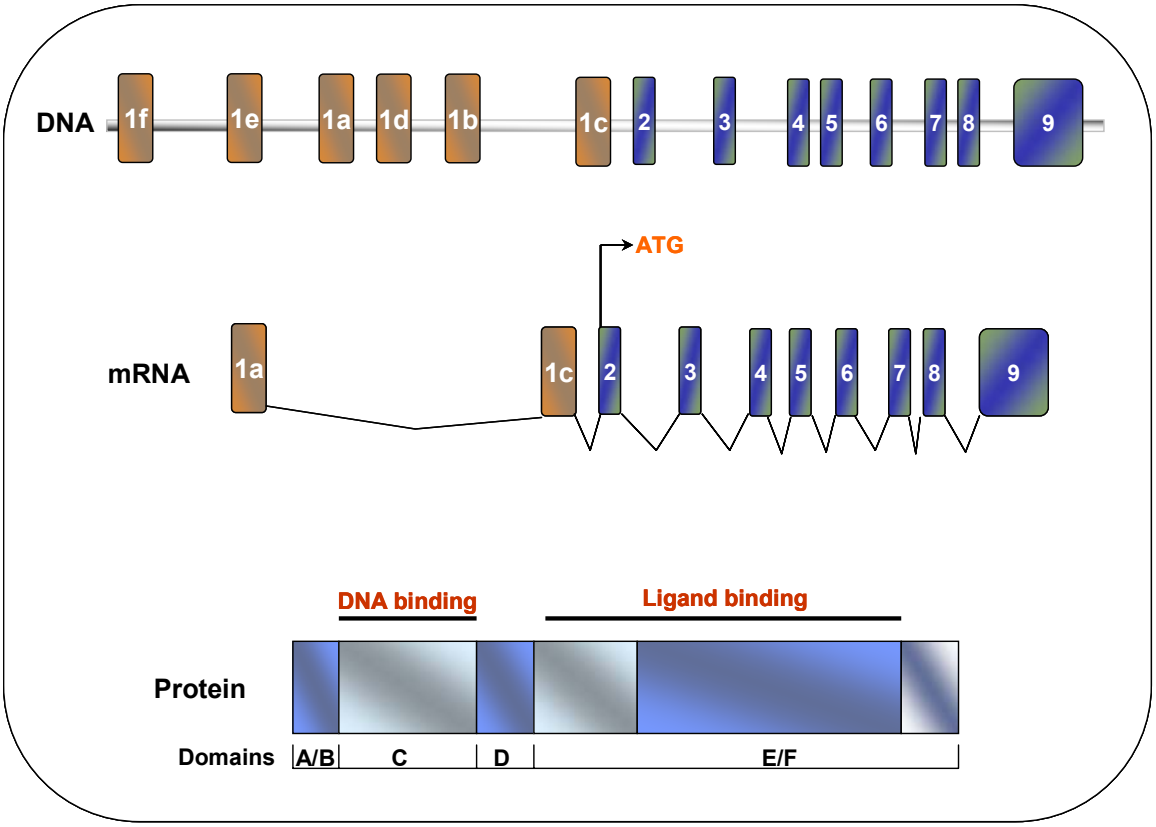


Figure 5: Schematic representation of vitamin D receptor gene, mRNA and protein. The structural organization of the human VDR gene (DNA) and the exons of corresponding full length transcript (mRNA). Exons 1f, 1e, 1d and 1d are untranscribed and exons 1a and 1c are part of 5' untranslated region. Modular structure of VDR protein (protein) with functional domains and the location of DNA and ligand binding domains are illustrated.

growth inhibition, while down-regulating pro-proliferative genes (Banerjee and Chatterjee, 2003); Vitamin D and VDR regulate genes involved in cycle arrest including *p21, p27, PTEN* and *Rb* (Bouillon et al., 2006; Hisatake et al., 2001; Huang et al., 2004; Liu et al., 1996; Moffatt et al., 2001). Several other genes involved in growth inhibition are also regulated by VDR including, *BRAC1, GADD45* and *IGFBP-3* (Boyle et al., 2001; Campbell et al., 2000; Campbell et al., 1997; Jiang et al., 2003; Stewart and Weigel, 2005). Since VDR expression is observed in virtually all tissues, vitamin D exhibits anti-proliferative actions in most human cancer cell lines. Thus, vitamin D and its analogous are extensively used for cancer chemotherapy, specifically for prostate, breast and colon cancers. Combinatorial use of vitamin D and its analogues with chemotherapeutic agents have shown to be more potent in promoting growth inhibition than using either of them alone (Hershberger et al., 2002; Moffatt et al., 1999). In fact, clinical trials are underway testing the combinatorial use of vitamin D analogues and chemotherapeutic agents for cancer chemotherapy (Fakih et al., 2007; Trump et al., 2006).

Vitamin D and VDR promote differentiation of several human cancer cell lines by regulating multiple signaling pathways, including the β-catenin pathway (Palmer et al., 2001; Palmer et al., 2003). Vitamin D and its analogues also promote osteoblastic differentiation, particularly, the differentiation of osteosarcoma cell lines including MG-63 (Finch et al., 2001). The molecular mechanism of vitamin D-mediated osteoblastic differentiation involves the activation of cell cycle inhibitors (p21) and differentiation markers (Zenmyo et al., 2001), specifically, the direct transcriptional regulation of osteopontin and osteocalcin (Paredes et al., 2004; Shen and Christakos, 2005).

Extensive *in vitro* studies implicate a novel role for VDR in promoting calcium mediated keratinocyte differentiation (Bikle, 2004; Bikle et al., 2004). An increase in intracellular calcium levels is a critical molecular event during keratinocyte differentiation and is modulated by elevated levels of calcium receptors and phopholipase C. Since functional VDRE elements are found in the calcium receptor and in phopholipase C promoters, VDR-mediated induction of calcium receptor and phopholipase C is believed to be essential for keratinocyte differentiation (Pillai et al., 1995; Xie and Bikle, 1997; Xie and Bikle, 2001). Additionally, depletion of VDR expression levels in primary keratinocytes results in concomitant decrease in the expression of keratinocyte differentiation markers including involucrin, profilaggrin, and loricrin (Bikle et al., 2002; Hawker et al., 2007). *In vivo* studies from VDR-deficient mice further strengthen the role of VDR in epidermal differentiation; the ablation of VDR leads to development of alopecia due to the defects in hair follicle regeneration (Xie et al., 2002), and reduced expression of epidermal differentiation markers including involucrin, proflaggrin, and loricrin (Xie et al., 2002).

I. Regulation of VDR

Although numerous studies characterized the functional significance of VDR in multiple cellular processes, the molecular regulation or the transcriptional regulation of VDR is poorly studied. Transcript levels of VDR are modulated by several growth factors and cytokines including parathyroid hormone (PTH), thyroid hormone, glucocorticoids and retinoic acid (Chen et al., 1983; Chen and Feldman, 1985; Krishnan et al., 1995; Krishnan and Feldman, 1991b; Mahonen et al., 1991; Petkovich et al., 1984). Interestingly, while activated protein kinase A induces the VDR expression, activated

protein kinase C down regulates VDR expression, indicating a homeostatic regulation of VDR mediated responses (Krishnan and Feldman, 1991a; Krishnan and Feldman, 1992). Additionally, recent studies showed the transcriptional regulation of VDR by several transcription factors including Wilms' tumor suppressor protein (WT1), Caudal-type homeobox protein 2 (Cdx-2), SNAIL, and ZEB, SP1 and ß-catenin (Lazarova et al., 2001; Maurer et al., 2001; Palmer et al., 2004; Shah et al., 2006; Wietzke et al., 2005; Yamamoto et al., 1999). Interestingly, transcriptional regulation of VDR through an auto feedback mechanism by 1,25-dihydroxyvitamin D3 was reported in a tissue specific manner (Zella et al., 2006). Although all these studies gave a preliminary insight on the regulation of VDR, additional studies are essential in understanding the cell and tissue specific regulation of VDR by key signaling components.

J. Rationale and Significance

Identifying new transcriptional networks of p53 family members will provide significant insight into the functional diversities of each member. Furthermore, understanding the functional cross-talk between each member of p53 family, based on the regulation of common and unique target genes, will be crucial to deciphering their role in tumorigenesis and development. Understanding the diverse biological affects exerted by p63 and p73 through common target genes will shed light on the functional co-operation between p63 and p73. The secosteroid hormone vitamin D exerts its biological actions through its cognate receptor VDR. Although extensive work has been carried out on the biological function of vitamin D and its analogs, little is known about the transcriptional regulation of VDR. This dissertation work focuses on the transcriptional regulation of VDR by members of p53 family. The initial studies from this dissertation demonstrate that VDR is induced by p63 and p73, but not by p53. My hypothesis is that, 1) regulation

of VDR by p63 is coupled with the inhibition of invasiveness of squamous cell carcinoma cells 2) p73 mediated regulation of VDR is essential for vitamin D-mediated osteoblastic differentiation. Thus, understanding the transcriptional regulation of VDR by p63 and p73 and their implication on vitamin D-mediated biological activities, will aid in vitamin D based cancer chemotherapeutic strategies. Moreover, understanding the role of VDR in p63 and/or p73 mediated biological activities will shed light on the molecular mechanism behind p63 and/or p73-specific biological functions.

II. MATERIALS AND METHODS

A. Cell Lines, Reagents and plasmids

Human non-small cell lung carcinoma cell line (H1299), human cervical carcinoma cell line (HeLa), human osteosarcoma cell line (SaOS2) and human epidermoid carcinoma cell line (A431) were obtained from ATCC. All the cells were maintained in Dulbecco's modified eagle medium (DMEM) with 8% fetal bovine calf serum (FBS) and 250 U of penicillin and 250 μg of streptomycin (PS) at 37°C in 5% CO_2. Primary mouse embryonic fibroblasts (MEF) obtained from wild-type or p63–/– mice were kindly provided by Dr. Elsa Flores (University of Texas M.D. Anderson Cancer Center, Houston, TX). The active form of vitamin D, 1α, 25-dihydroxyvitamin D_3 (VD3) (Sigma, Inc.) was prepared as 10 mM solution in 100 % ethanol. Doxorubicin hydrochloride (Sigma, Inc.) was prepared in water as 100 mM solution and etoposide (Sigma, Inc.) was prepared as 50 mM solution in dimethyl sulfoxide (DMSO) (Sigma, Inc.). Expression plasmids encoding TAp63γ, TAp63γ (R279H) mutant and TAp63α were constructed in pcDNA3.1A vector as described earlier (Caserta et al., 2006). Expression plasmids encoding ΔNp63γ and ΔNp63α were kindly provided by Dr. Frank McKeon (Harvard Medical School, Boston, MA) and TAp73β, ΔNp73β and TAp73α, TAp63β and ΔNp63β were kindly provided by Dr. Xinbin Chen (University of Alabama,

Birmingham, AL). p53 and $p14^{ARF}$ expression plasmids were a generous gift from Dr. Steven Berberich (Wright State University). VDRE-Luc reporter was a generous gift from Dr. Alberto Munoz (University of Madrid).

VDR-Luc reporter construct was constructed by amplifying the -1500 to -1 region (Accession no # AB002168) upstream of the VDR open reading frame using BAC clone RP11-254E3 (BACPAC Resources) and subsequently the -1500 to -1 region was cloned into promoter-less pGL3 Basic vector (Promega, Inc.) into the Kpn I and Hind III sites. The primers used for amplifying -1500 to -1 region, are as follows, sense 5'- CGG GGT ACC CGA TGC TTT GGG CAA GG –'3 and anti sense 5'- CCC AAG CTT AGA CAG CCC AGC ACC TGG CC –'3. The three different VDR promoter deletion constructs were generated by amplifying the desired region within VDR promoter region from VDR-Luc construct and subsequently cloned the region into promoter-less pGL3 Basic vector (Promega, Inc.) at Kpn I and Hind III sites. The primers used for amplifying -1267 to -1 region of VDR promoter were as follows, sense 5'- CGG GGT ACC TGT TGG AAG GTT CTT CCT TC –'3 and anti sense 5'- CCC AAG CTT AGA CAG CCC AGC ACC TGG CC –'3. The primers used for amplifying -1060 to -1 region of VDR promoter were as follows, sense 5'- CGG GGT ACC TGT TGG AAG GTT CTT CCT TC –'3 and anti sense 5'- CCC AAG CTT AGA CAG CCC AGC ACC TGG CC –'3. The primers used for amplifying -765 to -1 region of VDR promoter were as follows, sense 5'- CGG GGT ACC TGT TGG AAG GTT CTT CCT TC –'3 and anti sense 5'- CCC AAG CTT AGA CAG CCC AGC ACC TGG CC –'3. The -631/-585 region of VDR promoter containing luciferase construct, VDR shRNA and NSC (non silencing control) shRNA

constructs were generated by Dr. Madhavi Kadakia (Kommagani et al., 2006; Kommagani et al., 2007).

B. Transient transfections

Cells were seeded onto 6-well plates prior to day of transfections unless otherwise indicated. At around 80% confluency, transient transfections were performed using Lipofectamine 2000 reagent (Invitrogen, Inc.) with desired plasmids in serum free DMEM. After 5 hr incubation the medium was replaced with DMEM supplemented with FBS and PS. For all the studies a total of 3 μg of desired expression plasmids were used and in a given experiment total amount of plasmids was adjusted to 3 μg using empty vector.

C. Real time quantitative PCR

Total RNA from cells was extracted by using either RNAeasy kit (Qiagen, Inc.) or eZNA RNA isolation kit (Omega Bio-tek**,** Inc.) as per manufacture's protocol. To determine the concentration of RNA, the RNA was diluted in TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) and quantified on a spectrophotometer by measuring the absorbance at 260 nm. A total 1μg of RNA was used to synthesize cDNA by using TaqMan reverse transcription kit (Applied Biosystems, Inc.). The conditions used for cDNA synthesis are as follows; 10 minute at 25^{o}C, 30 minute at 48^{o}C and 5 minute at 95^{o}C. Quantitative Real-Time PCR analysis was performed in a 96 well micro titer plate format on a ABI Prism7900HT sequence detection system using TaqMan Universal master mix or TaqMan 2X master mix and gene specific Assays on Demand. Assays on Demand (AOD) used are VDR (Hs_0017213_m1), p21 (Hs_00355782_m1), p63 (Hs_00978340_m1), IGFBP-3 (Hs_00426287_m1), CYP27B1 (Hs_ 00168017_m1), p73

(Hs_ 00232088_m1), Osteopontin (OPN) (Hs_ 00167093_m1), Osteocalcin (OCN) (Hs_01587813_m1), Runx2 (Hs_00231692), murine p63 (mm_00495788_m1), murine vdr (mm_00437297_m1) and murine id2 (mm_00711781_m1) (PE Applied Biosystems, Inc.). A total 40 ng of cDNA was used for a single PCR reaction and each cDNA sample was run in triplicates and GAPDH was used as endogenous control for normalizing the Ct values. Relative mRNA quantitation was performed by using the comparative ΔΔCt method on SDS 2.0 software (Pfaffl, 2001) and error bars represent the standard deviation of RQ (relative quantification) values from triplicates.

D. Western blot analysis

At appropriate times, cells were first washed in PBS and whole cell extracts were made using RIPA buffer (0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS, phosphate buffered saline, pH 7.4). Total protein concentrations were measured by using BCA protein detection method and bovine serum albumin (BSA) was used as a standard. Equal amounts of protein were mixed with appropriate amount of 5X SDS loading dye (0.5 M DTT, 0.3 M Tris (pH 6.8), 10% SDS, 50% glycerol and 0.05% bromophenol blue) to obtain 1X SDS loading dye containing protein samples. Subsequently, protein were run on a 10% SDS-PAGE gel and transferred onto PVDF membrane and blocked with blocking solution (5% non dry fat milk powder in TTBS (1X TBS, 0.1% Tween-20)). Membranes were subsequently probed with antibodies to detect specific proteins. Monoclonal anti-VDR, rabbit polyclonal anti-p21, monoclonal anti-p63 4A4 (Santa Cruz Biotechnology, Inc.) and monoclonal anti-β-actin (Sigma) antibodies were used to detect VDR, p21, p63 and β-actin expression respectively. Rabbit polyclonal anti-p73 antibody (Bethyl laboratories Inc., Montgomery, TX) was used to detect p73 expression. Mouse

monoclonal anti-p63 C-12 antibody was used for detecting exogenous ΔNp63α protien. Monoclonal anti-p53 Ab-6 (Calbiochem, San Diego, CA) was used to detect p53. Appropriate horseradish peroxidase-conjugated antibodies (Promega, Madison, WI) were used as secondary antibodies and Supersignal Westpico Chemiluminescent Substrate kit (Pierce, Inc.) was used to detect chemiluminescence signal on a FUJI FILM LAS3000 image reader.

E. Immunoprecipitation for endogenous p73

Cells were harvested for total protein using RIPA buffer and 1 mg of total protein was used for immunoprecipitation. Total protein was pre cleared with 20 μl of recombinant-protein G-Sepharose beads (Invitrogen, Inc.) for 1 hr at 4^{o}C. After pre clearing beads were removed and the protein was incubated with rotation for O/N at 4^{o}C with 1 μg of monoclonal anti-p73 antibody (Ab-4 Lab Vision Corporation). The next day pre-cleared protein samples were incubated with 25 μl of rec-protein G-Sepharose beads for an hour followed by 4X washes with RIPA buffer to remove the unbound proteins. Immunoprecipitated samples with beads were resuspended in appropriate 5X SDS loading dye (0.5 M DTT, 0.3 M Tris (pH 6.8), 10% SDS, 50% glycerol and 0.05% bromophenol blue) to obtain 1X SDS loading dye containing protein samples and were run on a 10% SDS PAGE gel. Subsequently, immunoblot analysis was performed with rabbit polyclonal anti-p73 antibody (Bethyl laboratories Inc.) and images were developed as explained above.

F. Transactivation assays

Transactivation assays were performed by two different methods, single luciferase reporter assay method and dual luciferase assay method. For single luciferase reporter

based transactivation assay, cells were washed once with PBS and harvested in Single Lysis buffer (1XPBS, 0.15mM EDTA and 0.1% Triton-X 100). Luciferase reporter activity was measured by using Luciferase activity assay (Promega, Inc.) and Bradford assay (Bio Rad, Inc.) was performed to detect total protein concentration. Relative luciferase units were calculated by normalizing luciferase activity to total protein concentrations. For Dual luciferase assays, cells were transfected with desired firefly reporter construct and 5 ng of Renilla luciferase reporter construct (Promega, Inc.) along with desired plasmids. At 24 hr post transfection cells were washed once with PBS and harvested in passive lysis buffer (Promega, Inc.). Dual luciferase assay were performed to detect both firefly and Renilla luciferase activity using Dual-Luciferase Reporter 1000 Assay System as per manufacturer's protocol (Promega, Inc.). The relative luciferase activity (RLU) was measured by calculating ratio of Renilla luciferase activity to Firefly luciferase activity. The relative fold change in RLU activity was calculated by comparing to control vector transfected cells. The error bars represent the standard deviation from duplicate samples from same experiment.

G. Chromatin Immunoprecipitation assays

Chromatin Immunoprecipitation assays (ChIP) were carried out using a ChIP kit (Active motif, Upstate Cell Signaling Solutions, Inc.). ChIP assay was performed either on H1299 cells transiently transfected with p63 or p53 and on normal H1299 or A431 cells as required. For all purposes, three 15 cm dishes with cells at around 70-80% confluency were processed for ChIP assay. First cells were fixed on plate by using 10 % formaldehyde for 10 min. After fixation cells were scraped and cell pellet were resuspended in 1X lysis buffer. Subsequently cells were homogenized for 10 strokes

using a homogenizer and tight pestle. The homogenized cells were centrifuged to obtain nuclei and nuclei were resuspended in chromatin shearing buffer and subjected to sonication to shear the DNA. For the chromatin from H1299 cells overexpressed with p63 or p53, sonication was performed on Fisher scientific model 550 sonic dismembrator for total 8 cycles with 20-25 sec pulses for each cycle with 3 min resting time. For the chromatin from normal H1299 and normal A431 cells, sonication was performed on Fisher scientific model 500 sonic dismembrator for total 4 cycles each with 15 times of 5 sec pulses with 15 sec off resting between each pulse. Sonicated chromatin was centrifuged at 14000 RPM for 12 min at 4 ^{o}C to obtain the fragmented chromatin. The fragmented chromatin in all the ChIP assays was in the size range of 400 bp to 600 bp. The fragmented chromatin was pre-cleared using protein G beads for 1.5 hrs and the pre-cleared chromatin was subjected to overnight immunoprecipitation with appropriate primary antibodies. Part of pre-cleared chromatin (10%) which was not subjected to immunoprecipitation was used as input DNA. After immunoprecipitation both input DNA and immunoprecipitated chromatin were reverse cross linked at 65 ^{o}C for 16 hrs using 5M NaCl and DNA was eluted. The fragmented chromatin from H1299 cells overexpressed with p53 or p63 was immunoprecipitated with anti-p63 rabbit polyclonal (H-137), ant-p53 rabbit polyclonal (FL393) antibody and normal rabbit IgG antibodies (Santa Cruz Biotechnology Inc.). PCR amplification was performed by using Pfx polymerase (Invitrogen Inc.) with the following PCR conditions. For VDR, 38 cycles were performed each consisting of 30 sec at 94^{0}, 30 sec at 58^{0} and 45 sec at 68^{0} ; for 14-3-3, 38 cycles were performed each consisting 30 sec at 94^{0}, 30 sec at 58^{0} and 45 sec at 68^{0}. Primers used for amplification are as follows: VDR, forward 5'-CGGGGTACCCAG

TAACAGGTTGCGACGGAG-3' and reverse 5'-CCCAAGCTTGATGATTATAGGTGCG GATACCCG-3` For 14-3-3σ, forward 5'-CTCACTACCTCAAGATACCC-3' and reverse 5'-CACAGGCCTGTGTCTCCC-3'. To determine the occupancy of endogenous p73 protein on target genes fragmented chromatin from intact H1299 cells was immunoprecipitated with mouse mono clonal anti-p73 Ab-4 (Lab Vision Corporation) or normal rabbit IgG antibodies (Santa Cruz Biotechnology Inc. The eluted DNA was subjected to PCR amplification using GoTaq Green PCR master mix as per manufacture's protocol (Promega, Madison, WI) using primers specific for VDR (forward 5'- CGGGGTACCCAG TAACAGGTTGCGACGGAG -3' and reverse 5'-CCCAAGCTTGATGATTATAGGTGCGGATACCCG -3') and p21 (forward 5'-GGTACCGGCACTCTTGTTCCC CC AGGC TG-3' and reverse 5'-CTCGAGACCATC CCCTTCCTCACCTGAAAA-3'). PCR conditions used for both VDR and p21 are as follows, a total of 40 cycles were performed each consisting 30 sec at 94^0 C, 30 sec at 60^0C and 45 sec at 68^0 C. To determine the binding of endogenous p63 protein on target genes, fragmented chromatin from intact A431 cells was immunoprecipitated with monoclonal anti-p63 4A4, rabbit polyclonal H-129 and normal mouse monoclonal IgG antibodies (Santa Cruz Biotechnology Inc.). Eluted DNA was subjected to PCR amplification for two regions (RE1 and RE2) within VDR promoter, p21 promoter and β-actin gene using GoTaq Green PCR master mix (Promega, Madison, WI). Primers used for PCR amplification as follows VDR RE1 (forward 5'-CGGGGTACCTTTTCCGCAGCCATCCACA -3' and reverse 5'-CCCAAGCTTCTGCAGAACGCCAGGAAGCTC-3'), VDR RE2 (forward 5'-

CGGGGTACCCAG TAACAGGTTGCGACGGAG -3' and reverse 5'-CCCAAGCTTGATGATTATAGGTGCGGATACCCG -3'), and p21 (forward 5'-GGTACCGGCACTCTTGTTCCC CC AGGC TG-3' and reverse 5'-CTCGAGACCATC CCCTTCCTCACCTGAAAA-3'). The conditions used are as follows, a total of 38 cycles were performed each consisting 30 sec at 94^0 C, 30 sec at 60^0C and 45 sec at 68^0 C.

H. siRNA transfections

Calcium phosphate based transfections were performed for transfecting siRNA oligo into A431, H1299 and SaOS2 cells. Cells were plated in 6 well plates prior to day of transfections and at around 30- 40 % confluency, cells were transfected with total 110 pico moles of siRNA oligo. Total two rounds of siRNA transfections were carried out at 24 and 48 hr post seeding. One hour before onset of siRNA transfections the medium on the cells was replaced with fresh medium and incubated in an incubator with 3% CO_2. Following that transfection reaction mix was prepared by mixing siRNA with $CaCl_2$ (125mM final concentration) and 2X BBS (50mM BES (pH 7), 280mM NaCl, 1.5mM Na_2HPO_4). The transfection mixture was incubated for 3 minutes at room temperature and then added onto respective wells. After transfections, cells were kept back in incubator with 3% CO_2. At 6 hr post transfections medium was replaced with fresh medium and cells were kept back in incubator with 5% CO_2. All the siRNA used for studies were purchased from Qiagen (Qiagen, Valencia, CA) and the target Sequences used for siRNA are as follows, p63 siRNA 1 (CACCCTTATAGTCTAAGACTA), p63 siRNA 2 (CCAGATGACATCCATCAAGAA), p73 siRNA1 (CCCGGGATGCTCAACAACCAT), p73 siRNA2

(CTCGGGAGGGACTTCAACGAA) and VDR siRNA (CCGCGTCAGTGACGTGACCAA).

I. Wound healing assays

For the wound healing assay A431 cells were transfected twice with desired siRNA as described earlier. At 24 hr post transfection a 200 µl pipette tip was used for creating an artificial wound or a cleared area on monolayer cells. Scratched cells were washed with 1X PBS and were re fed with fresh medium and allowed to grow for 24 hr. Immediately after the scratching (0 hr) and after 24 hrs, cell images were taken to visualize migrated cells and wound healing.

J. Matrigel based invasion assay

The matrigel membrane based invasion assays were performed using 24 well BD BioCoat Matrigel Invasion chambers (BD Biosciences). The matrigel invasion chambers contain 8 micron pore size PET membrane with a layer of basement membrane made of matrigel. While the basement membrane blocks the migration of non-invading cells through the PET membrane, the invading cells can invade through the basement membrane and PET membrane, thus distinguishing the invading cells from non-invading cells. The control insert chambers lack the basement membrane and are used for ruling out any inconsistencies in cell seeding into the chambers. First, both invasion chambers and control inserts were placed in 24 well plates and were re-hydrated by adding 500 µl of serum free DMEM inside and outside of chambers. After medium was added onto chambers plates were incubated for 2 hrs at 37 ^{o}C in an incubator with 5% CO_2. After chambers were re-hydrated, a total of 1×10^5 cells from each condition were resuspended in 500 µl of DMEM medium and seeded onto both invasion chambers and control inserts.

Fetal bovine serum was used as chemo-attractant and total 750 µl of medium containing FBS was added onto the lower part of invasion chambers. After cells were seeded onto chambers, plates were kept in an incubator with 37 ^{o}C and 5% CO_2. After 24 hrs chambers were retrieved from the incubator and subjected to the staining process. First, the cells present inside the invasion chambers were removed using cotton swabs and the invaded cells present on the bottom of the lower membrane were stained with Diff-Quik stain kit. The Diff-Quik staining method involves staining the cells with three different staining solutions, solution I for fixing, solution II for cytoplasmic staining and solution III for nuclear staining. A total 500 µl of each solution was added in 24 well plates and cells were stained by sequentially incubating the chambers in each staining solution for 2 mins. After cells were stained, chambers were air dried overnight. The next day, membranes were removed gently from chambers and were mounted on glass slides and dried for a day. Stained cell images were captured at 20X magnification using phase contrast microscopy and at least 9 images were captured from multiple fields. The number of cells from each field was counted to calculate the number of invading cells.

K. Generation of A431 stable cells using lentiviral expression system.

The ΔNp63α cDNA containing lentiviral expression plasmid was constructed by amplifying the ΔNp63α cDNA sequence from ΔNp63α plasmid and then cloning it into modified pLentiV6 vector at BamHI and XhoI sites. The primers used for amplifying the ΔNp63α cDNA as follows, sense 5'-GGAGGATCCATGTTGTACCTGGAAAACA-'3 and anti-sense 5'-AAACTCGAGTCACTCCCCCTCCCTTT-'3. The modified pLentiV6 vector contains additional restriction sites and was a generous gift from Dr. Thomas Brown. The pLentiV6-eGFP construct was a generous gift from Dr. Steven Berberich..

To prepare the lentivirus expressing eGFP or ΔNp63α, 293FT cells were transfected with 9 μg of lentiviral expression plasmids with 7 μg of lentiviral packaging mix using Lipofectamine 2000 (Invitrogen, Inc.). At 24 hr post transfections medium was replaced with fresh medium. After 48 hrs medium was collected and centrifuged to remove debris, then the lentiviral supernatant was collected and stored at -80 ^{0}C as 1 mL stocks. The A431-eGFP and A431-p63 stable cells were generated by infecting the parental A431 cells with lentivirus expressing eGFP or ΔNp63α respectively. Briefly, prior to the day of infections A431 cells were seeded onto 6 well dishes. The next day lentiviral supernatant (1 mL) was thawed and was diluted in 500 μl medium containing polybrene (1.4 μg/mL). The medium on wells was removed and 1.5 ml of diluted lentiviral supernatant was added to appropriate wells. At 24 hr post infections lentivirus containing medium was replaced with fresh medium. After 24 hrs medium was replaced with fresh medium containing blasticidin (10 μg/mL) antibiotic to select the transduced cells. After 48 hrs cells were released from selection and as explained above another round of selections were carried out to obtain the final stable pool of A431 cells expressing ΔNp63α or eGFP. The stable expression of ΔNp63α was confirmed by performing immunoblot analysis with p63 specific antibody.

III. RESULTS

A. TAp63γ up-regulates VDR expression in human cancer cell lines.

The role of p63 in epithelial morphogenesis and differentiation is well documented; however, the underlying p63 downstream signaling pathways that are responsible for p63 biology are still understudied. In an effort to identify such p63-specific gene targets, our lab performed a microarray-based gene expression analysis using H1299 cells transiently transfected with TAp63γ. Extensive data mining analysis showed up-regulation of a significant number of genes by p63 and interestingly the vitamin D receptor (VDR) gene was observed in the list of genes upregulated by p63 (data not shown). To determine whether VDR is specifically activated by TAp63γ, the gene chip results were validated by performing the transient transfection experiments. H1299 and HeLa cells were transfected with control vector, TAp63γ and p53 expression plasmids and at 24 hr post transfection cells were harvested for either total RNA or whole cell extracts. TaqMan based real time PCR was performed to detect the transcript levels of VDR and p21 and western blot analysis was performed to detect the endogenous VDR and p21 protein expression levels. Both p63 and p53 have been shown to activate p21 expression and the detection of p21 expression was used as a positive control for both p53 and p63. As expected, exogenous p63 and p53 significantly up-regulated the p21

Figure 6: Induction of VDR expression by TAp63γ. H1299 and HeLa cells were transfected with control vector, p63γ or p53 as indicated. At 24 hr post transfection, total RNA (A) or whole cell lysates (B) were extracted. A) TaqMan based Reverse transcriptase PCR was performed to quantitate the transcript levels of Vitamin D receptor (VDR) (upper panel) and p21 (lower panel) in H1299 (left) and HeLa (right) cell lines as indicated. Y-axis represents fold change in expression levels in p63γ and p53 transfected cells compared to empty vector transfected cells. B) Immunoblot analysis was performed to detect the endogenous VDR and p21 protein levels and also over expressed p63 and p53. Immunoblotting for β-actin was performed to confirm equal protein loading.

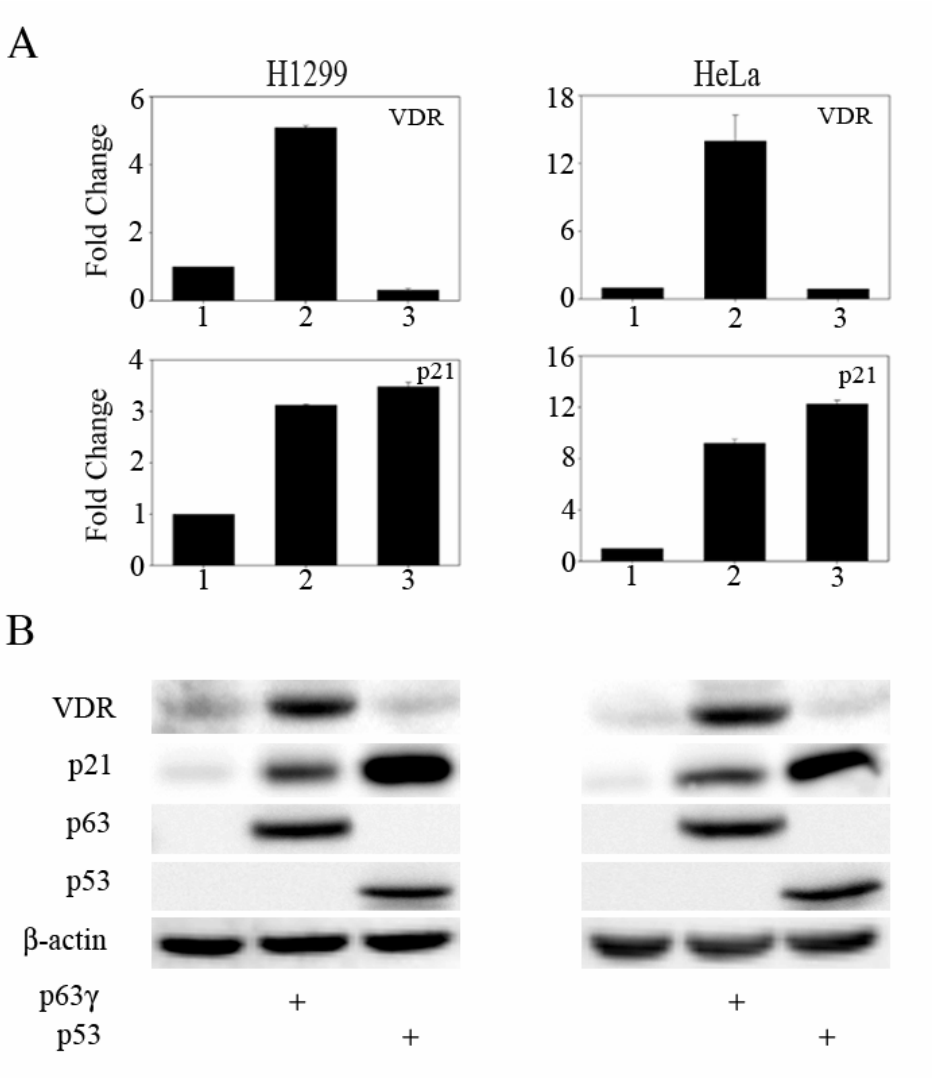
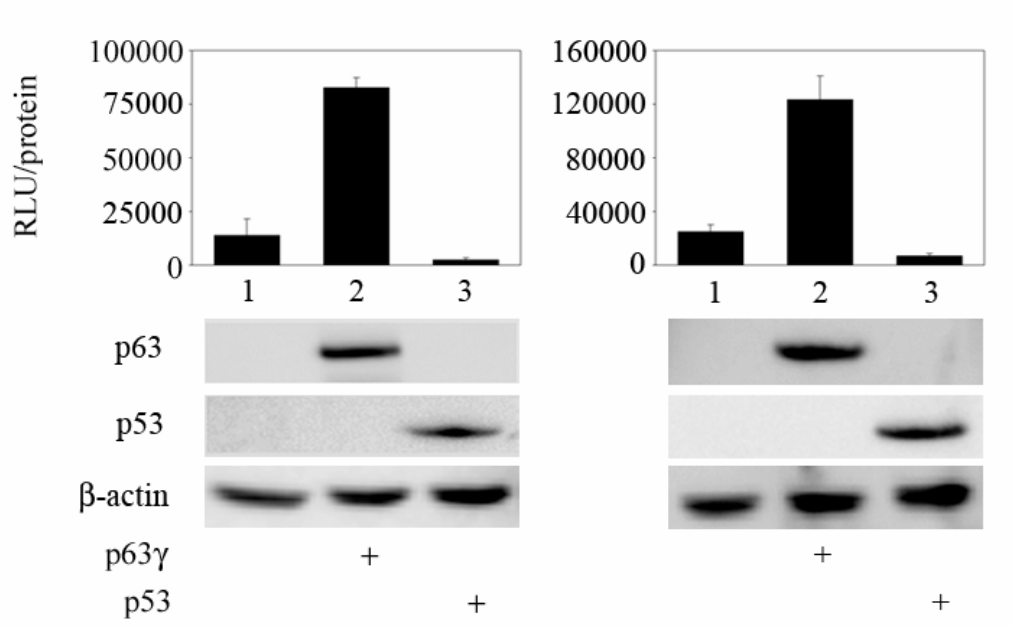

A
H1299
HeLa
VDR
p21
Fold Change
B
VDR
p21
p63
p53
β-actin
p63γ
p53
+

Figure 7: Transactivation of VDR by TAp63γ. H1299 (left) HeLa (right) cells were transfected with 250 ng of VDR-Luc reporter alone or with p63γ and p53 expression plasmids as indicated. At 24 hr post transfection cells were harvested and luciferase assays were performed on the whole cell extracts. Y axis represents relative luciferase units normalized to protein concentration. Over expression of p63 and p53 proteins was confirmed by immunoblotting using anti-p63 and anti-p53 antibodies


RLU/protein
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160000
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1
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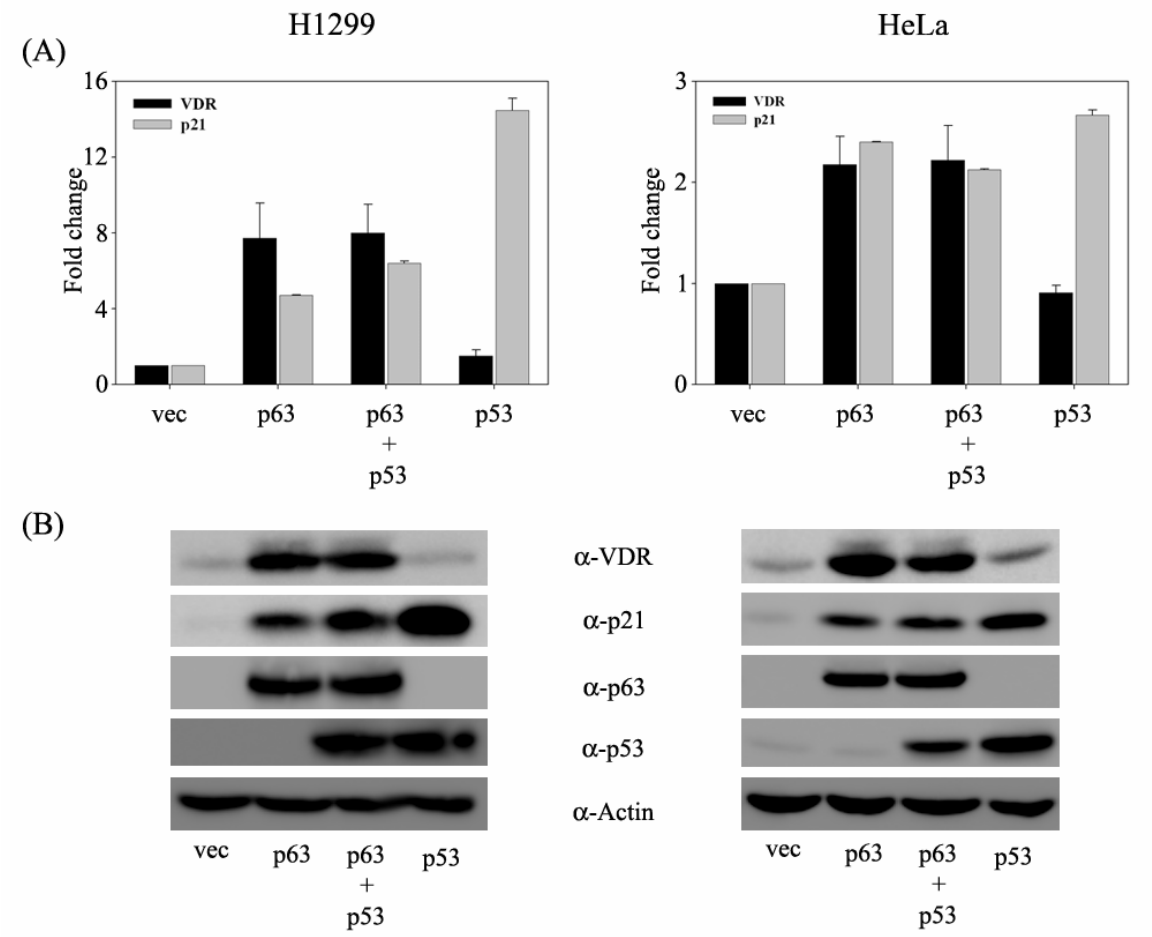
3
p63
p53
β-actin
p63γ
+
+
p53
+
+

transcript and protein levels in both cell lines (Figure 6). While a significant increase in VDR transcript and protein levels was observed with exogenous TAp63γ, over expression of p53 did not result in up-regulation of VDR expression at transcript or protein level (Figure 6). To determine whether TAp63γ activates VDR transcriptionally, a VDR-Luc reporter was constructed by cloning reported 1500 bp VDR promoter region in pGL3-basic luciferase reporter vector. H1299 cells were transiently transfected with VDR-Luc reporter alone or with TAp63γ or p53 expression plasmids and the extracts from same samples were run on SDS-PAGE gel to confirm the overexpression of TAp63γ and p53 proteins (Figure 7). As shown in Figure 7, TAp63γ transfected cells showed a significant increase in VDR-Luc reporter activity when compared to the control vector transfected cells. Consistent with transcript and protein levels results, p53 was unable to induce VDR-Luc activity, suggesting VDR as a specific target of TAp63γ (Figure 7).

Given p53 was unable to activate VDR expression and that both p63 and p53 bind to the similar responsive elements, next we determined whether p53 affects the TAp63γ mediated induction of VDR. H1299 and HeLa cells were over expressed with p63 alone, p53 alone or together and expression of VDR and p21 were determined at the transcript and protein levels. Although p53 had no effect on the TAp63γ mediated activation of VDR, p53 by itself induced the expression levels of p21 at transcript and protein levels, suggesting that p53 does not affect VDR expression directly or indirectly (Figure 8). To further confirm the specificity of TAp63 mediated activation of VDR, studies were performed by inhibiting the TAp63 using the negative regulators of p63. Since, the p63γ (R279H) point mutant observed in EEC syndrome acts in dominant negative manner

Figure 8: p53 does not affect p63 mediated induction of VDR. H1299 and HeLa cells were transfected with empty vector, p63 γ (1.5 μg) or along with p53 (1.5μg) and p53 alone (1.5μg) as indicated. (A) At 24 hr post-transfection, total RNA was harvested and subjected to TaqMan Reverse transcriptase PCR. Y-axis represents fold change in VDR and p21 transcript levels relative to empty vector transfected cells. (B) Immunoblot analysis was performed to detect the endogenous VDR and p21 protein levels and also over expressed p63 and p53 proteins. Immunoblotting for β-actin was performed to confirm equal protein loading.


(A)
H1299
HeLa
VDR
p21
Fold change
16
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8
4
0
3
2
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0
vec
p63
p63 + p53
p53
(B)
α-VDR
α-p21
α-p63
α-p53
α-Actin

Figure 9: Down regulation of WT p63 mediated induction of VDR by mutant p63. H1299 and HeLa cells were co-transfected WT p63γ (1.5 μg (lane 2)) or along with p63γ (R279H) mutant (1.5μg (lane 3)) and mutant alone (1.5μg (lane 4)) as indicated. (A) TaqMan Reverse transcriptase PCR analysis was performed to detect expression levels of VDR (upper panel) and p21 (lower panel) as indicated. Y-axis represents fold change in VDR and p21 transcript levels relative to empty vector transfected cells (lane 1). (B) Immunoblot analysis performed to detect the endogenous VDR and p21 protein levels and to confirm the over expression of wild type p63γ and mutant p63γ (R279H).

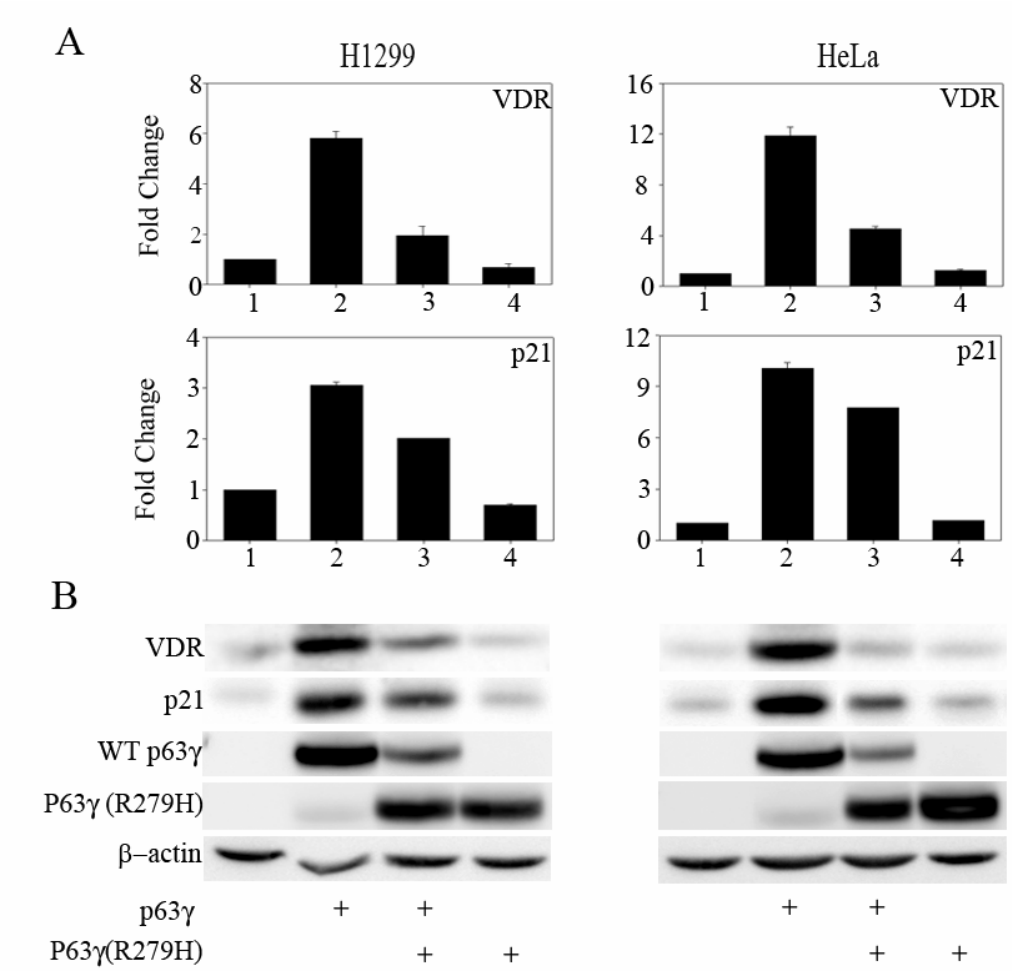
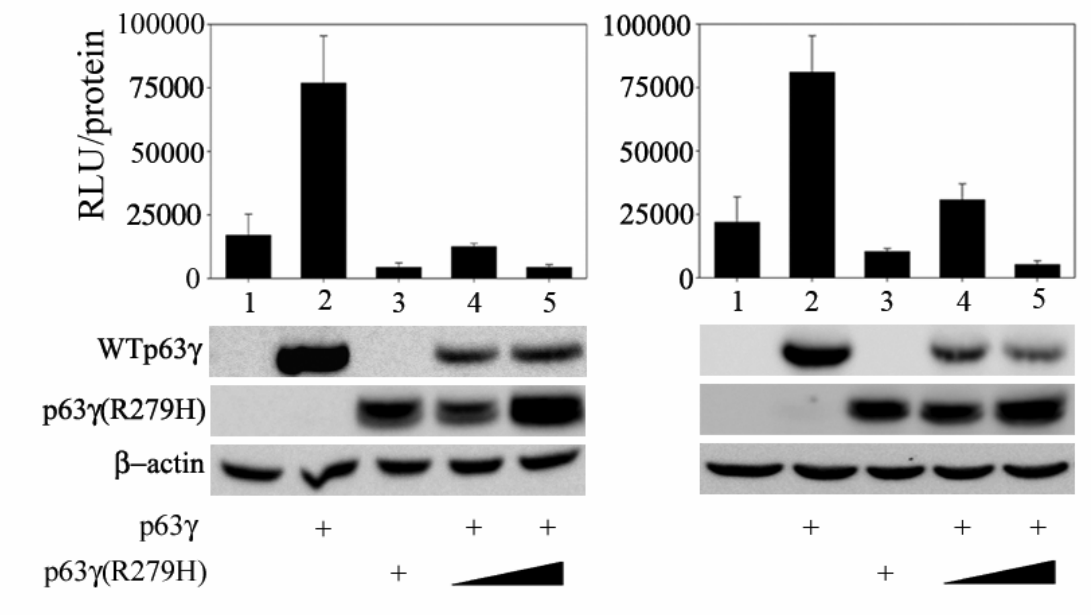

A
H1299
HeLa
VDR
p21
Fold Change
B
VDR
p21
WT p63γ
P63γ (R279H)
β–actin
p63γ
P63γ(R279H)

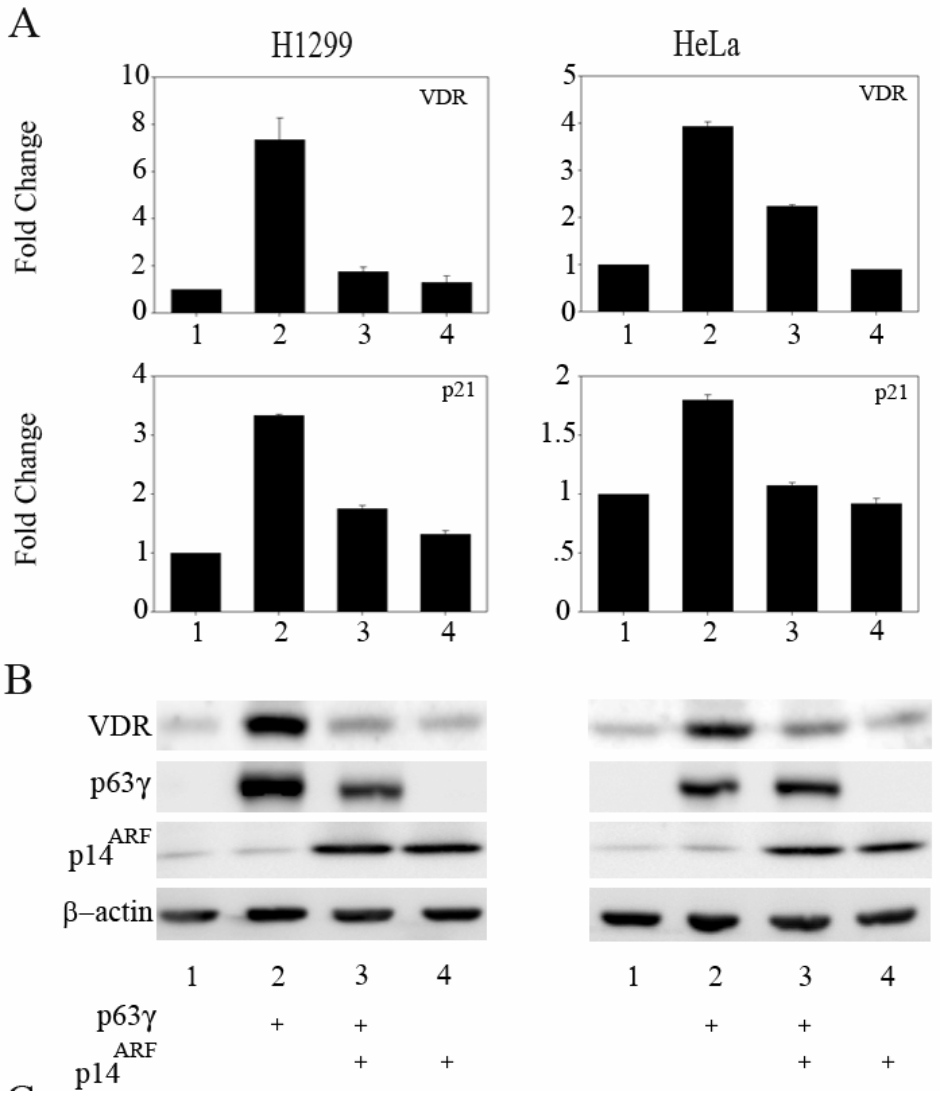
Figure 10: Mutant p63 down regulates WT p63 mediated transactivation of VDR. H1299 (left) and HeLa (right) cells were transfected with 250 ng of VDR-Luc reporter alone (lane 1) or co-transfected with expression plasmids encoding WT p63γ (0.5 μg) (lane 2) or along with p63γ (R279H) mutant (0.5μg (lane 4) and 1.0 μg (lane 5) or mutant alone (lane 3) as indicated. Cells were harvested at 24 hr post-transfection and luciferase assays were performed. Y axis represents relative luciferase units normalized to protein concentration. Immunoblot analysis was performed to confirm the over expression of WT p63γ and mutant p63γ (R279H) proteins using anti-p63 antibody.


RLU/protein
100000
75000
50000
25000
0
1
2
3
4
5
WTp63γ
p63γ(R279H)
β–actin
p63γ
+
+
+
p63γ(R279H)
+
+

towards wildtype (Celli et al., 1999; South et al., 2002;), the effect of p63γ (R279H) mutant on wildtype (WT) p63 mediated activation of VDR was studied. Co-transfection of p63γ (R279H) along with WT TAp63γ led to a significant reduction in WT TAp63γ mediated induction of VDR and p21 at both transcript and protein levels (Figure 9). Co-transfection of TAp63γ with increasing concentrations of p63γ (R279H) mutant resulted in a dose dependent down regulation of WT TAp63γ mediated transactivation of VDR-Luc reporter activity (Figure 10). However, over expression of p63γ (R279H) mutant by itself had no effect on VDR expression at protein or transcript levels or on VDR-Luc activity (Figure 9 & 10).

Since the discovery of p63, numerous studies were centered on identifying the functional similarities and dissimilarities between p53 and p63. Particularly, studies were carried out to understand the effect of proteins that regulate p53 on p63 function. Recently, p63 mediated transactivation of its responsive genes was shown to be inhibited by $p14^{ARF}$ (Calabro et al., 2004). Tumor suppressor $p14^{ARF}$ promote G_1 cell cycle arrest by activating p53 signaling pathway. Co-transfection of $p14^{ARF}$ along with TAp63γ in both H1299 and HeLa cells resulted in significant reduction in p63 mediated induction of VDR expression at both transcript and protein levels (Figure 11). Additionally, $p14^{ARF}$ inhibited TAp63γ mediated transactivation of VDR-Luc reporter activity in both H1299 and HeLa cell lines (Figure 12). Co-transfection of $p14^{ARF}$ with TAp63γ also led to a significant inhibition in TAp63γ mediated activation of p21. As expected, exogenous $p14^{ARF}$ by itself had no effect on VDR expression at transcript or protein levels (Figure 11). Thus inhibition of p63 mediated induction of VDR by both $p14^{ARF}$ and p63γ (R279H) mutant validates VDR as a potential target of p63.

Figure 11. $p14^{ARF}$ inhibits TAp63γ mediated up-regulation of VDR. H1299 and HeLa cells were transfected with either empty vector, p63γ alone or along with $p14^{ARF}$ or $p14^{ARF}$ alone as indicated. (A) At 24 hr post-transfection, total RNA was extracted and subjected to TaqMan Reverse transcriptase PCR to quantitate VDR (upper panel) and p21(lower panel) transcript levels. Y-axis represents fold change in VDR and p21 transcript levels relative to empty vector transfected cells (lane 1). (B) At 24 hr post-transfection, total protein was extracted and immunoblot analysis was performed to detect the endogenous VDR expression and to confirm the over expression of p63 and $p14^{ARF}$.


A
H1299
HeLa
Fold Change
VDR
p21
B
VDR
p63γ
p14ARF
β–actin
1
2
3
4
p63γ
p14ARF

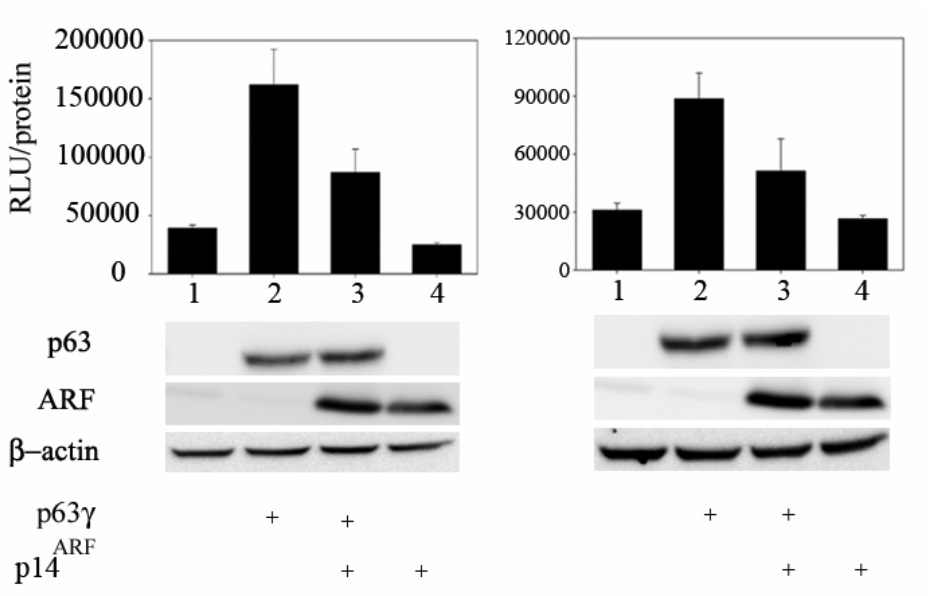


Figure 12. p14^{ARF} down regulates TAp63γ mediated transactivation of VDR. H1299 (left) HeLa (right) cells were transfected with 250 ng of VDR-Luc reporter alone or with p63γ alone or along with p14^{ARF} or p14^{ARF} alone as indicated. At 24 hr post-transfection cells were harvested and luciferase assays were performed. Y axis indicates the VDR promoter activity and represents relative luciferase units normalized to protein concentration. Immunoblot analysis was performed to confirm the over-expression of p63 and p14^{ARF} proteins using p63 and p14^{ARF} specific antibodies.

B. VDR is direct target of p63 and identification of p63 responsive element within VDR promoter.

Chromatin immunoprecipitation assay (ChIP) was performed to demonstrate that VDR is a direct target of p63. H1299 cells were transfected with either p53 or TAp63γ plasmids and at 24 hr post transfection cells were subjected to ChIP assay. The sonicated chromatin was immunoprecipitated with control IgG and p63 or IgG and p53 specific antibodies for cells over expressed with p63 or p53 respectively (Figure 13). DNA from the immunoprecipitated chromatin was then PCR amplified with primers specific for VDR and 14-3-3σ promoters. 14-3-3σ promoter amplification was used as a positive control for both p63 and p53 as both p63 and p53 are known to bind the 14-3-3σ promoter (Westfall et al., 2003). A substantial abundance of TAp63γ protein on a specific region within the VDR promoter was observed (Figure 13), but the occupancy of p53 protein on the VDR promoter region was not observed. These results clearly demonstrate that VDR is direct transcriptional target of TAp63γ.

A recent report indicated that TAp63γ can induce genes by binding to both p63-specific and p63/p53-specific consensus binding sites (Osada et al., 2005). Since VDR is up regulated only by TAp63γ but not by p53, studies were conducted to identify the p63 specific responsive element within the VDR promoter. To delineate the p63 responsive element required for the induction of VDR by p63, a series of promoter deletion reporters were constructed. VDR promoter regions (-1267 to 1), (-1020 to 1), (-765 to 1) and (-631 to -585) were cloned upstream of the pGL3 basic vector. The -631 to -585 region of the VDR promoter was specifically cloned as our *in-silico* based analysis indicated the possibility of a potential p63 responsive element within that region (Figure 14). H1299

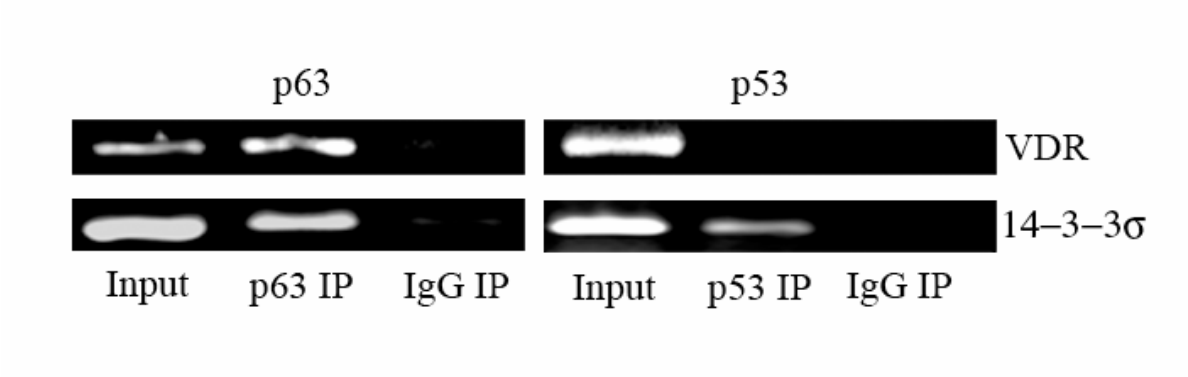


Figure 13. TAp63γ binds to VDR promoter *in vivo.* H1299 cells were transfected with p63γ or p53 expression plasmids and at 24 hr post-transfection cells were subjected to Chromatin Immunoprecipitation analysis. Formaldehyde cross-linked chromatin was immunoprecipitated with anti-p63, anti-p53 or normal IgG antibodies as indicated. Eluted DNA was PCR amplified with primers specific for VDR (RE2) and 14-3-3σ promoter.

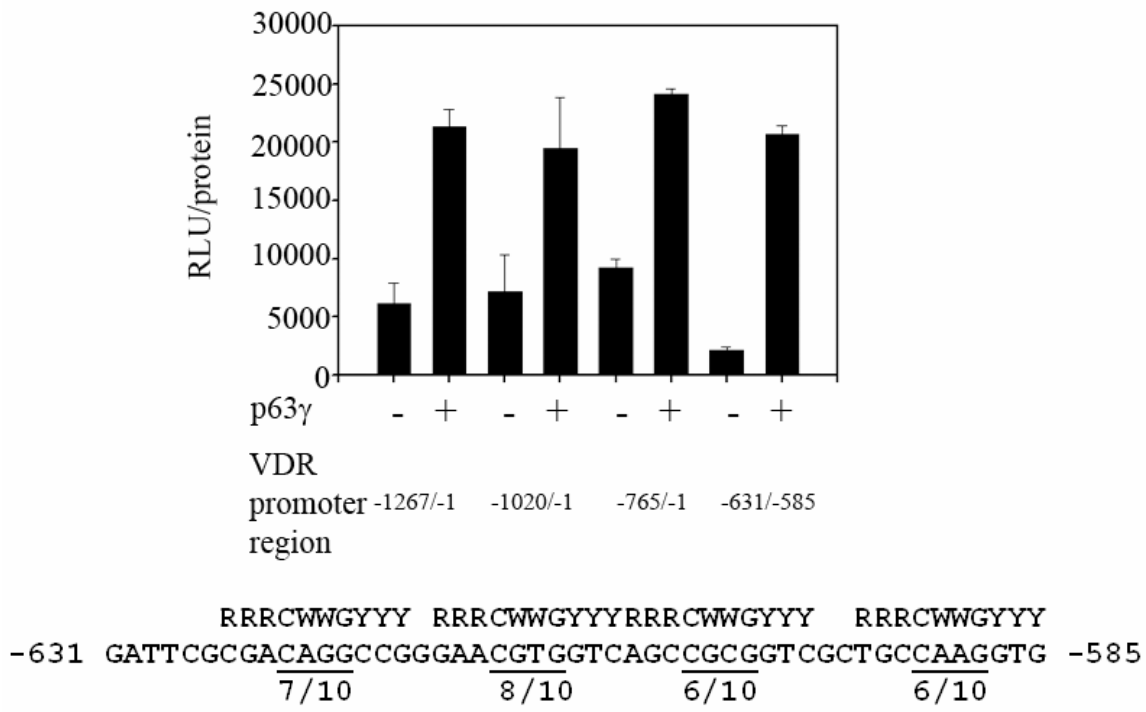


Figure 14. Identification of p63 responsive element within the VDR promoter. The indicated regions within VDR promoter were constructed and transfected into H1299 cells along with control vector or p63. At 24 hr post-transfection cells were harvested to subject for luciferase assays. Y axis represents relative luciferase units normalized to protein concentration. Lower Panel represents a schematic representation of the VDR promoter region containing the putative p63 binding elements. The nucleotide sequences that match the reported p63 consensus binding sequences are indicated.

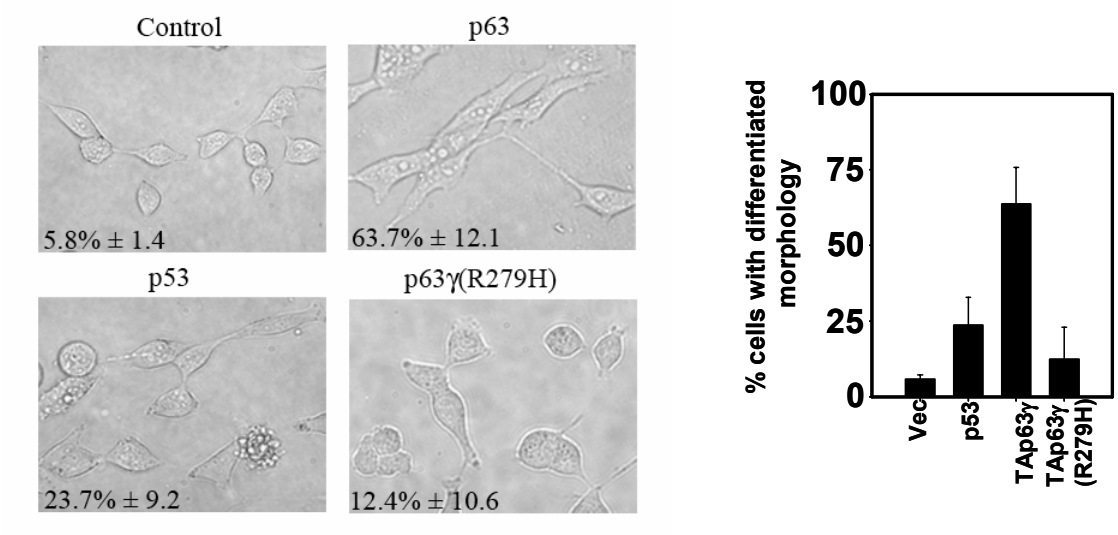


Figure 15. TAp63γ promotes the differentiation of SaOS2 osteosarcoma cells. SaOS2 cells were transfected with control vector, p63γ, p53 or p63γ(R279H) expression plasmids as indicated. At 48 hr post transfection morphological changes of cells were analyzed by phase contrast microscopy. For each condition images of cells were taken from six different fields randomly in unbiased manner. Percentage of differentiated cells (differentiated cells/total number of counted cells from several fields), as determined by morphological changes are indicated. The left panel graph represents the percent of differentiated cells measured from several fields.

cells were co-transfected with different VDR promoter deletion constructs along with either control vector or TAp63γ expression plasmids. The deletion of first 736 nucleotides (-1500 to -765) from the transcription start site did not abolish the p63 mediated increase in VDR-Luc activity (Figure 14). TAp63γ also significantly increased the VDR (-631 to -585) reporter activity which contains a potential p63 responsive element. These results suggest that VDR is direct target of p63 and that p63 stimulates the expression of VDR from a p63 responsive element within the VDR promoter.

C. Exogenous TAp63γ promotes differentiation by inducing VDR.

Vitamin D receptor has been shown to play major role in bone formation and osteoblastic differentiation. Therefore, studies were carried out to determine whether VDR induction by p63 promotes osteoblastic differentiation and whether p63 potentiates vitamin D-mediated osteoblastic differentiation. Osteosarcoma cell line SaOS2, a well studied model system for osteoblastic differentiation was employed for these studies. SaOS2 cells were transfected with a control vector, TAp63γ, p53 or TAp63γ (R279H) expression plasmids and morphological changes observed upon differentiation were monitored. The morphological features observed upon SaOS2 cells differentiation include elongated cytoplasmic processes and flattened fibroblastic appearances. A total of 63% of the cells transfected with TAp63γ showed the differentiation morphology, whereas cells transfected with p53 or TAp63γ (R279H) mutant showed only 23% and 12% of differentiation morphology respectively (Figure 15). p63 mediated induction of osteoblastic differentiation was further validated by monitoring the well studied differentiation markers osteopontin (OPN), RUNX2 and osteocalcin (OCN). In correlation with the morphology results, over expression of TAp63γ in SaOS2 cells led to

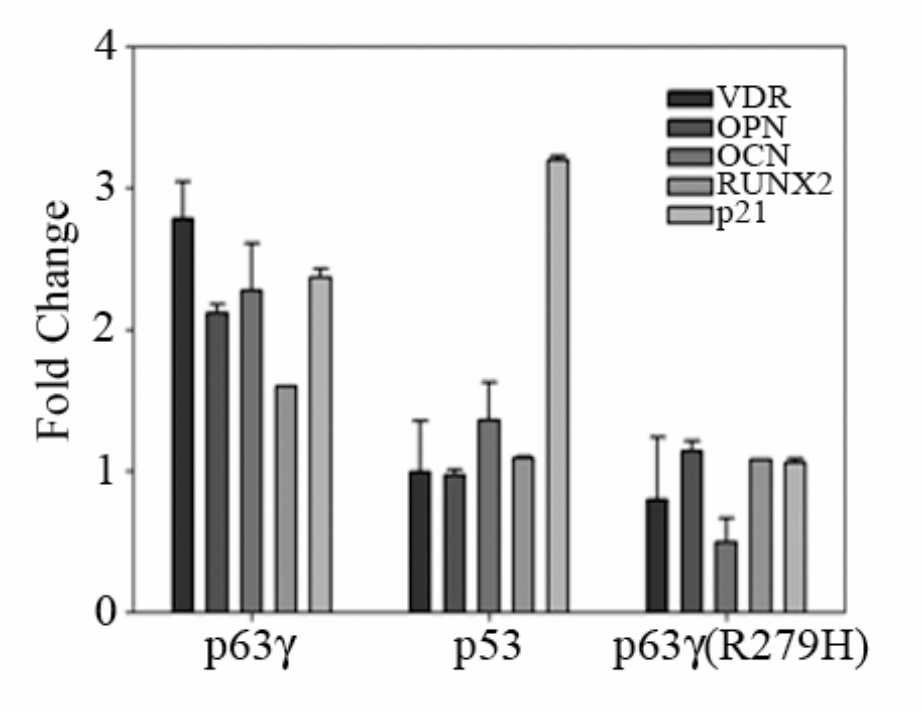


Figure 16. Induction of VDR and osteoblastic differentiation markers by p63 in SaOS2 cells. SaOS2 cells were transfected with control vector or TAp63γ or p53 or p63γ (R279H) mutant expression plasmids as indicated. After 48 hrs, transcript levels of VDR, OPN, OCN, RUNX2 and p21 were detected by performing TaqMan Reverse transcriptase PCR. Y-axis represents the relative fold change in expression levels in p63γ, p53 and p63γ (R279H) mutant transfected cells compared with cells transfected with control vector.

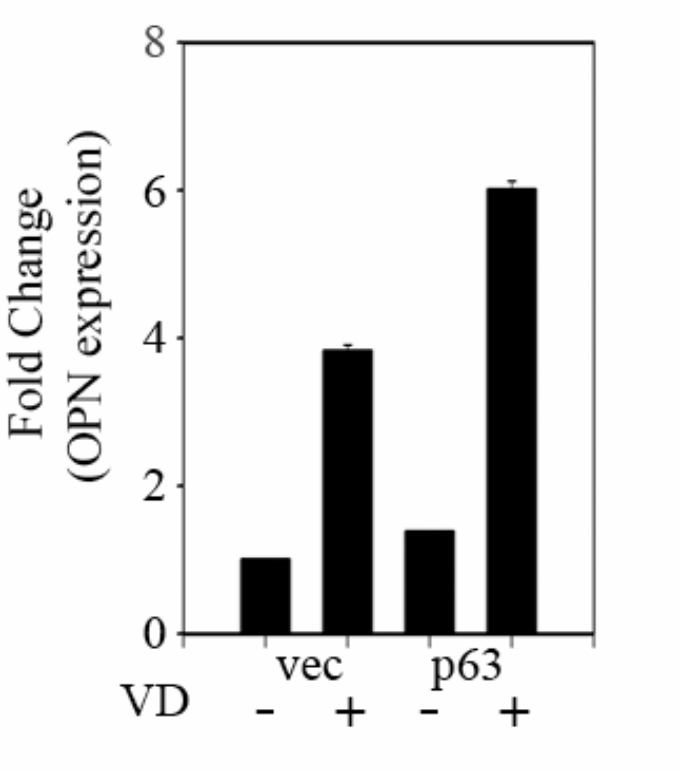


Figure 17. TAp63γ enhances the vitamin D mediated differentiation of SaOS2 cells.

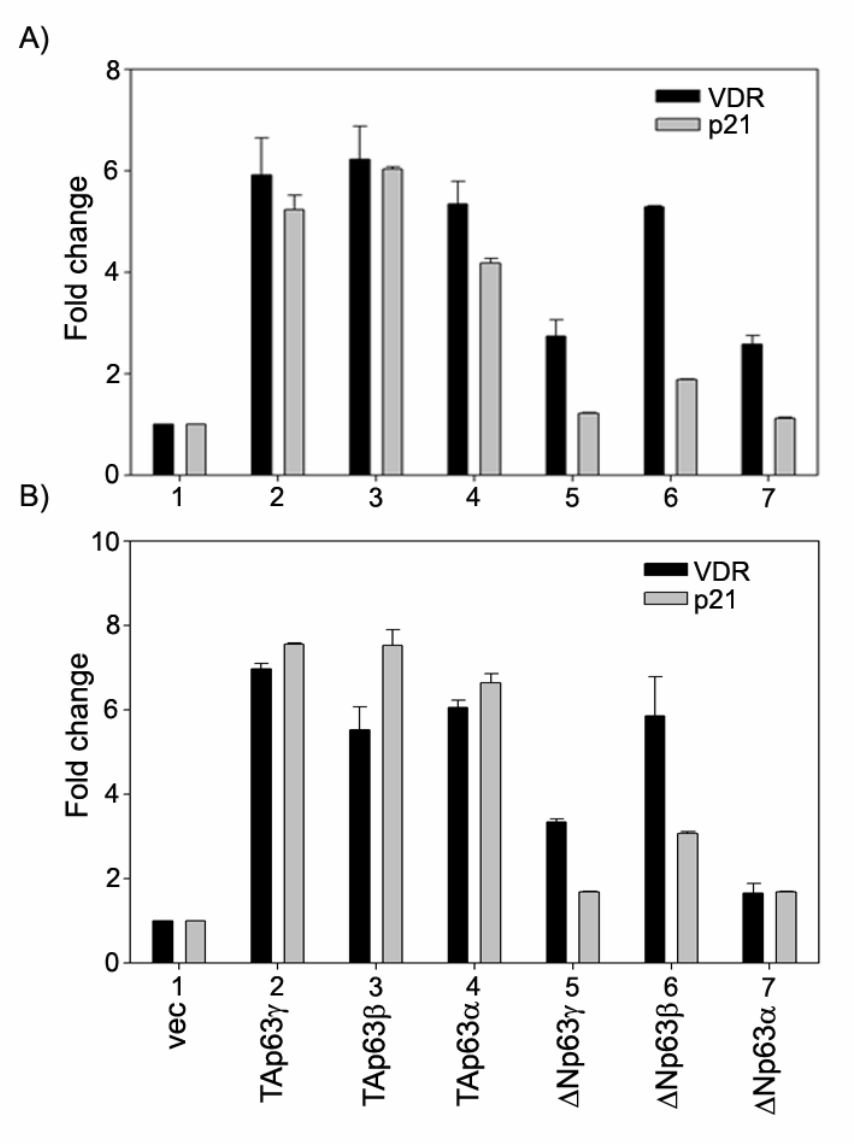
SaOS2 cells were transfected with p63γ or vector as indicated and at 24 hr post-transfection, cells were either treated with control vehicle or with VD (100 nM). After 48 hrs, TaqMan Reverse transcriptase PCR was performed to detect the transcript levels of OPN. Y-axis represents the relative fold change in OPN expression levels relative to vector transfected cells with no VD treatment.

a significant induction in OPN, OCN, RUNX2 and VDR transcript levels (Figure 16). Both p53 and p63γ (R279H) mutant were unable to induce the expression of OPN, OCN, RUNX2 and VDR (Figure 16). These results suggest that activation of VDR by p63 is involved in differentiation of SaOS2 osteosarcoma cells. To test whether exogenous p63 potentiates vitamin D mediated differentiation, SaOS2 cells were transfected with either control vector or TAp63γ and transcript levels osteopontin were monitored in presence of vitamin D. Compared to vector transfected cells, cells transfected with TAp63γ showed an increase in vitamin D mediated increase in OPN transcript levels. These results suggest that p63 enhances the vitamin D mediated function by inducing the VDR expression levels (Figure 17).

D. Transcriptional activation of VDR by all the isoforms of p63.

Having shown the transcriptional activation of VDR by TAp63γ, the regulation of VDR by all the isoforms of p63 was studied. This is important since the most abundant isoform of p63 expressed in basal epithelial cells of the skin is ΔNp63α and VDR is also shown to be expressed in basal and upper layers of differentiating skin (Cianferotti et al., 2007). To determine the effect of different p63 isoforms on endogenous VDR expression, six different isoforms of p63 (TAp63γ, TAp63β, TAp63α, ΔNp63γ, ΔNp63β and ΔNp63α,) were overexpressed in H1299 and HeLa cell lines and the expression of VDR at transcript and protein levels was monitored. As a positive control the transcript and protein levels of p21 were monitored as p21 was shown to be positively regulated by all the isoforms of p63 except ΔNp63α (Barbieri et al., 2005; Petitjean et al., 2008). A significant increase in VDR transcript levels were observed in cells transfected with all the isoforms of p63 when compared to control vector transfected cells (Figure 18). As

Figure 18. Up-regulation of VDR transcript levels by p63 isoforms. H1299 (A) and HeLa (B) cells were transfected with either empty vector, or expression plasmids encoding all the isoforms of p63 as indicated. TaqMan based Reverse transcriptase PCR was performed to detect the transcript levels of VDR and p21. Y-axis represents the relative fold change in VDR and p21 transcript levels relative to vector transfected cells.


A)
Fold change
VDR
p21
0
2
4
6
8
1
2
3
4
5
6
7
B)
Fold change
VDR
p21
0
2
4
6
8
10
vec 1
TAp63γ 2
TAp63β 3
TAp63α 4
ΔNp63γ 5
ΔNp63β 6
ΔNp63α 7

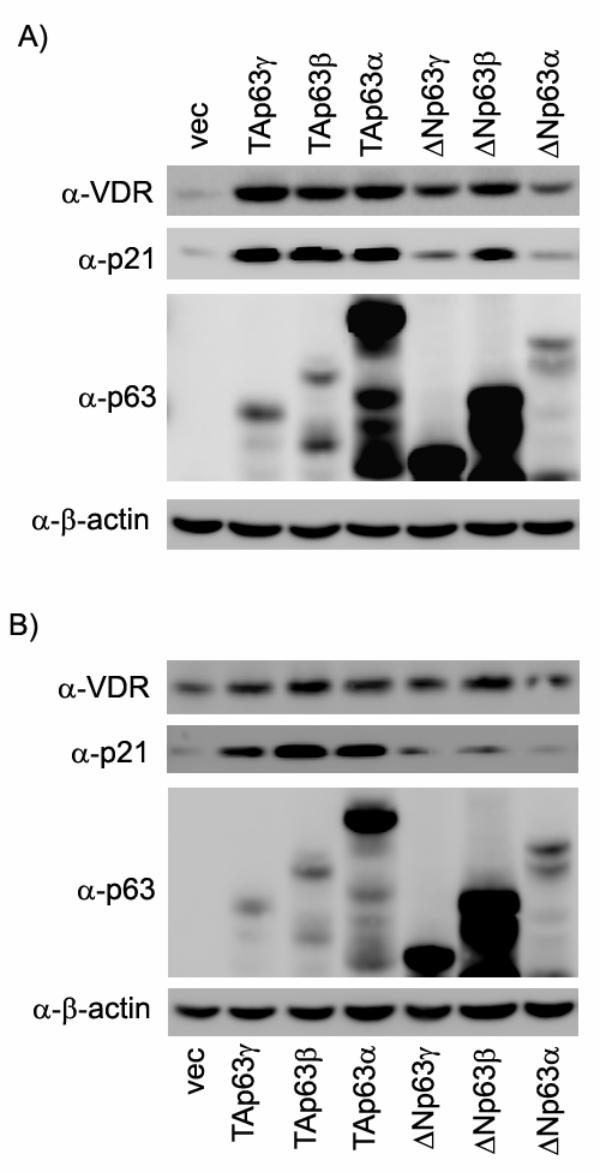
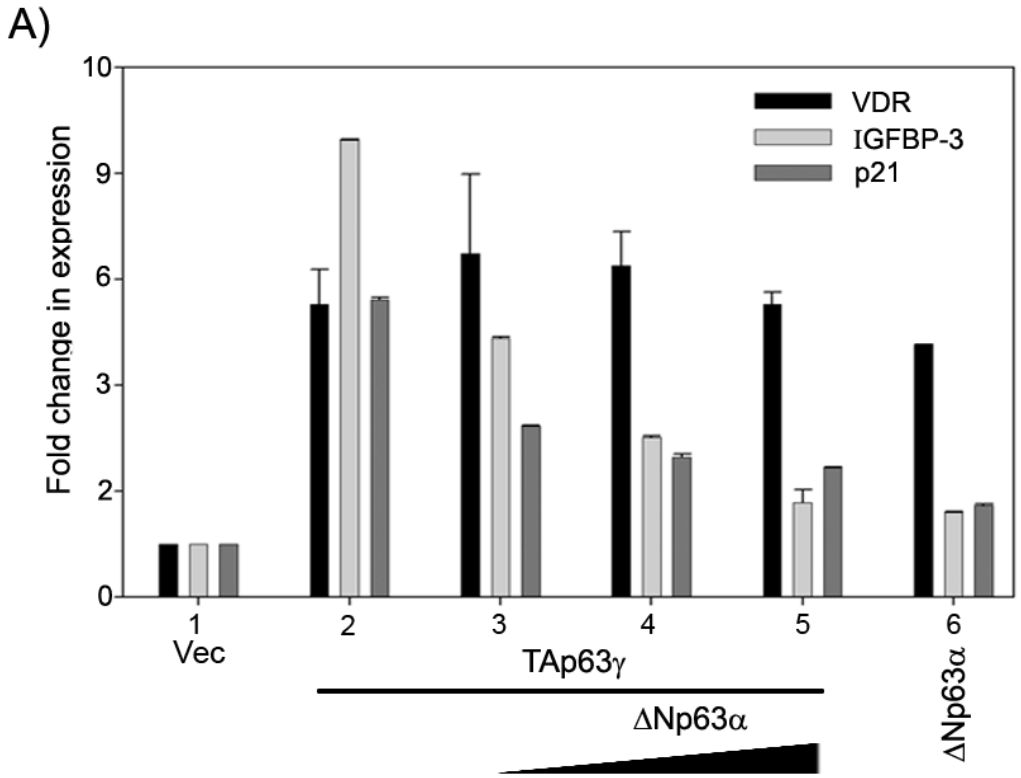


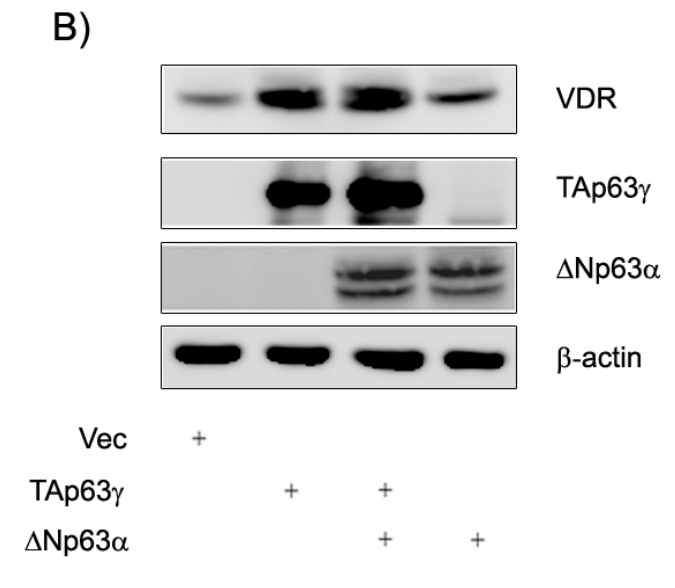
Figure 19. Induction of VDR protein levels by p63 isoforms. H1299 (A) and HeLa (B) cells were transfected with either empty vector, or expression plasmids encoding all the isoforms of p63 as indicated. Western blot analysis was performed to determine the protein levels of VDR and p21.

expected a significant increase in p21 transcript levels by TAp63γ, TAp63β and TAp63α, and ΔNp63β isoforms but not ΔNp63γ and ΔNp63α was observed. Consistent with transcript levels results, all the isoforms of p63 elevated the VDR protein levels. However, TAp63γ, TAp63β, TAp63α and ΔNp63β isoforms induced the VDR protein levels much higher than that of ΔNp63γ and ΔNp63α isoforms (Figure 19). Except the ΔNp63α isoform, all the isoforms of p63 induced the p21 protein levels, albeit a modest increase by ΔNp63γ (Figure 19). These results clearly show that, all p63 isoforms including ΔNp63α can transcriptionally activate VDR.

A report indicated that ΔNp63α isoform exerts a dominant negative effect towards p53 and TAp63 isoforms (Senoo et al., 2001). Since the induction of VDR by both ΔNp63α and TAp63 isoforms was observed, the effect of ΔNp63α isoform on TAp63 mediated activation of VDR was examined. H1229 cells were transfected with TAp63γ alone or along with increasing concentrations of ΔNp63α and transcript levels of VDR, p21 and IGFBP-3 (Insulin growth factor binding protein-3) were monitored. Earlier reports have shown that, ΔNp63α represses the expression of both p21 and IGFBP-3 (Barbieri et al., 2005; Westfall et al., 2003), therefore the expression of p21 and IGFBP-3 were used as controls for ΔNp63α mediated dominant negative effect on TAp63. As expected TAp63γ but not ΔNp63α upregulated the transcript levels of both p21 and IGFBP-3. While ΔNp63α clearly downregulated TAp63 mediated induction of p21 and IGFBP-3, it had no affect TAp63γ mediated induction of VDR (Figure 20). Consistent with the transcript levels results, co-transfection of ΔNp63α did not lead to a significant down regulation of TAp63γ mediated induction of VDR protein levels (Figure

Figure 20. ΔNp63α does not inhibit TAp63 mediated induction of VDR. A) H1299 cells were transfected with either empty vector or TAp63γ alone or along with increasing concentrations of ΔNp63α as indicated. After 24 hrs TaqMan based real time PCR was performed to detect the transcript levels of VDR, p21 and IGFBP-3. Y-axis represents fold change in VDR, IGFBP-3 and p21 transcript levels relative to empty vector transfected cells. B) H1299 cells were transfected with either empty vector or TAp63γ alone or along with ΔNp63α or ΔNp63α alone as indicated. Immunoblot analysis was performed using VDR and p63 isoform specific antibodies.


A)
Fold change in expression
10
9
6
3
2
0
VDR
IGFBP-3
p21
1
Vec
2
3
4
5
6
TAp63γ
ΔNp63α
ΔNp63α


B)
VDR
TAp63γ
ΔNp63α
β-actin
Vec
TAp63γ
ΔNp63α

20). These results clearly demonstrate that ΔNp63α does not act in dominant negative manner towards TAp63 mediated induction of VDR in H1299 cells.

E. VDR is a direct target of ΔNp63α.

Since VDR expression was induced by ΔNp63α, next we determined whether VDR is a direct target of ΔNp63α. First, the ability of ΔNp63α to transactivate full length VDR promoter was studied. Co-transfection of full length VDR promoter construct (VDR-Luc) along with increasing concentrations of ΔNp63α led to a dose dependant increase in VDR-Luc activity (Figure 21A). To unequivocally confirm that VDR is a direct target of ΔNp63α, ChIP assay was performed on intact A431 cells. Human epidermoid cancer cell line A431 was employed for ChIP assay, as detectable endogenous expression levels of both ΔNp63α and VDR are observed in these cells. ChIP assay was performed using two different antibodies against p63 (4A4 and H-127) to determine the occupancy of endogenous ΔNp63α protein on the VDR promoter. Since ΔNp63α isoform is the only isoform observed in A431 cells, anti-p63 monoclonal 4A4 antibody which detects all the isoforms of p63 and anti-p63 polyclonal H-129 antibody which detects only alpha isoforms of p63 were used. As shown in figure 21B, endogenous ΔNp63α protein bound to two different regions (RE1 and RE2) within VDR promoter region. Since the size of fragmented chromatin was around 500 bp and the 250 bp RE1 and RE2 regions are right next each other, it is possible that ΔNp63α binds to the VDR promoter on a single region. ΔNp63α is known to transrepress p21 (Westfall et al., 2003) and as expected the binding of ΔNp63α on the p21 promoter was observed.

Given, the occupancy of endogenous ΔNp63α on the VDR promoter, we determined whether endogenous VDR expression levels are under control of ΔNp63α.

Figure 21. ΔNp63α transactivates VDR and binds to VDR promoter in vivo. A) H1299 cells were co-transfected with full length VDR promoter construct along with either control vector or increasing concentrations of ΔNp63α as indicated. At 24 hr post transfection, cells were subjected to dual luciferase assay. Y-axis represents the fold change in relative luciferase units compared to empty vector transfected cells. B) A431 cells were subjected to chromatin immunoprecipitation assay and crosslinked chromatin was immunoprecipitated using two different antibodies for p63 and control IgG as indicated. Eluted DNA was used for PCR amplification of two different regions within VDR promoter (RE1 and RE2) and amplification of p21 promoter was used as positive control for p63.

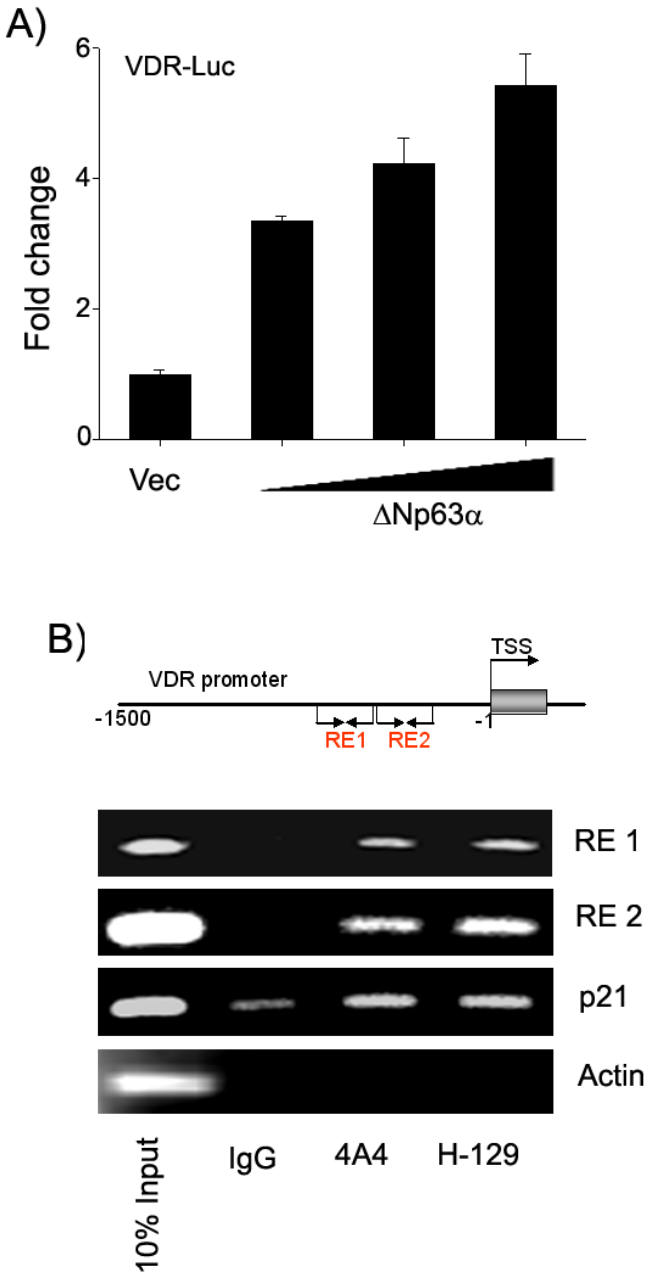
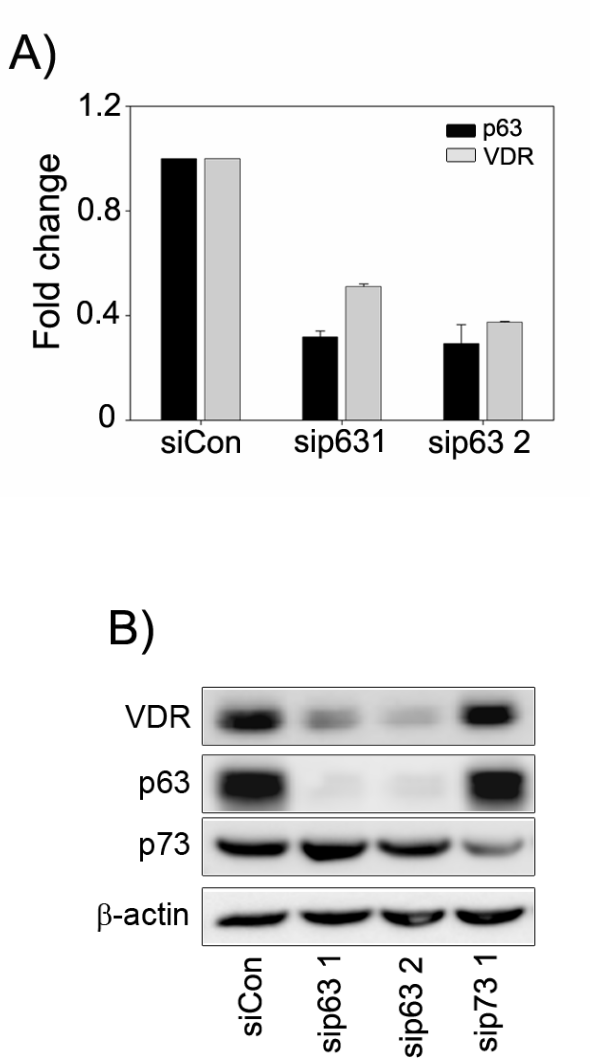

A)
VDR-Luc
Fold change
6
4
2
0
Vec
ΔNp63α
B)
VDR promoter
TSS
-1500
RE1
RE2
-1
RE 1
RE 2
p21
Actin
10% Input
IgG
4A4
H-129

Figure 22. Silencing ΔNp63α results in reduced expression of endogenous VDR in A431 cell. A) A431 cells were transfected with either control siRNA (siCon) or two different siRNA against p63 as indicated. At 48 hr post transfection, TaqMan based Reverse transcriptase PCR was performed to detect the transcript levels of VDR and p63. Y-axis represents the fold change in VDR and p63 transcript levels relative to control siRNA transfected cells. B) A431 cells were transfected with either control siRNA or two different siRNA against p63 or p73 siRNA as indicated. At 48 hr post transfections, whole cell lysates were made and were subjected to immunoblot analysis. The endogenous protein expression levels of VDR, p63 and p73 were detected using anti-VDR, anti-p63 and anti-p73 antibodies respectively.

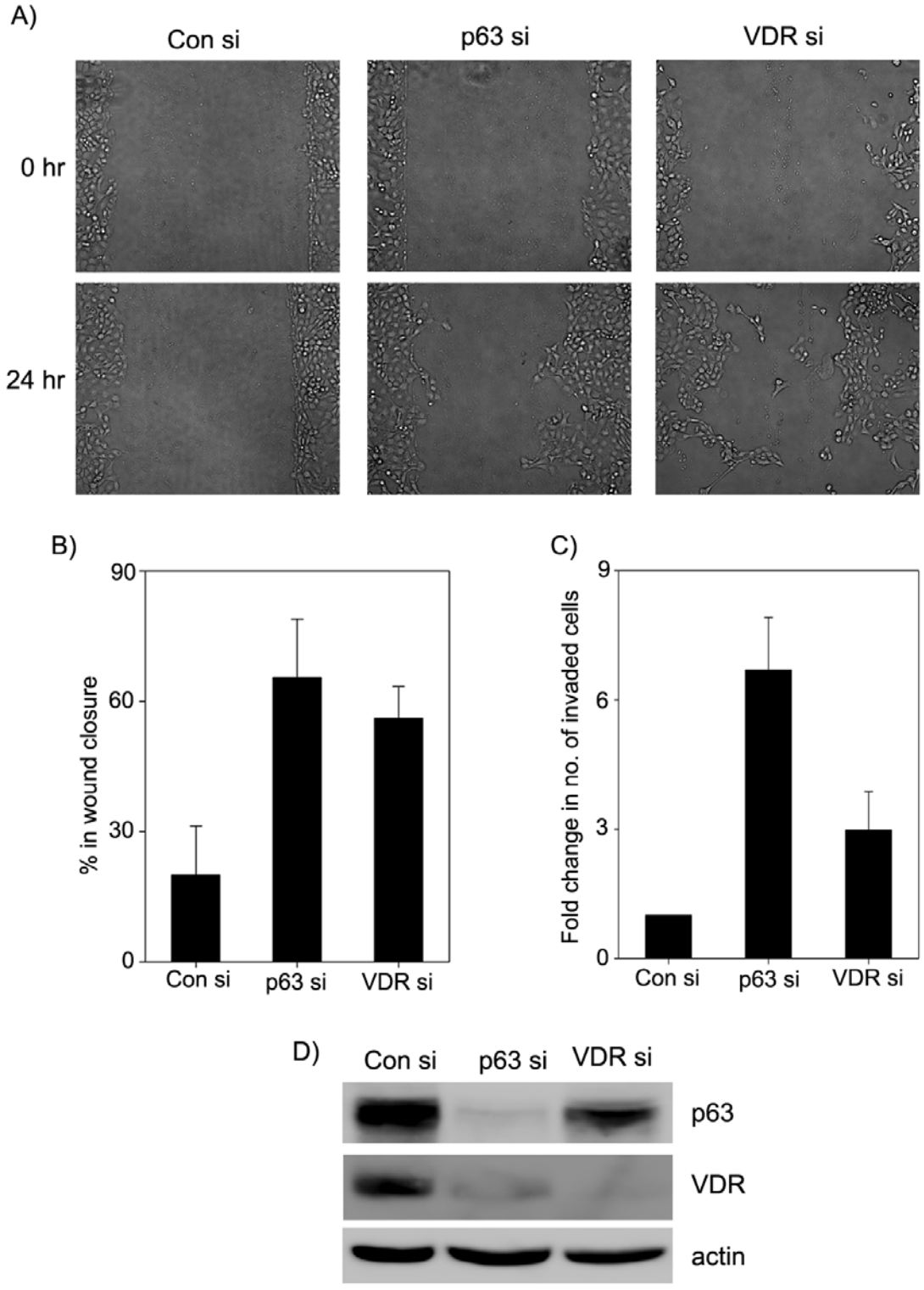

A)
1.2
0.8
0.4
0
Fold change
p63
VDR
siCon
sip631
sip63 2
B)
VDR
p63
p73
β-actin
siCon
sip63 1
sip63 2
sip73 1

A431 cells were transfected with two different siRNA's against p63 to silence the endogenous expression of ΔNp63α. Compared to control siRNA transfected cells, cells transfected with p63 siRNA_1 or p63 siRNA_2 showed a complete reduction in p63 transcript and protein levels (Figure 22). Silencing endogenous ΔNp63α led to a complete abolishment in endogenous VDR expression at transcript and protein levels, indicating the requirement of endogenous ΔNp63α for the basal expression of VDR (Figure 22). Eventhough VDR can be activated by multiple isoforms of p73 (Kommagani et al., 2007), down regulation of endogenous ΔNp73α in A431 cells did not result in reduction in endogenous VDR expression. These results indicate that VDR is a direct target of ΔNp63α and that ΔNp63α is essential for basal VDR expression.

F. The role of VDR regulation by ΔNp63α in cell migration and invasiveness.

A recent report demonstrated that loss of p63 expression lead to an increase in invasiveness of squamous cell carcinoma cells including A431 cells (Higashikawa et al., 2007). Since silencing p63 led to a down regulation in VDR expression and vitamin D is shown to regulate genes involved in metastasis (Tokar and Webber, 2005), the role of ΔNp63α mediated regulation of VDR in inhibition of invasiveness was determined. A431 cells were silenced for either p63 or VDR and cell migration and cell invasion were assessed by wound healing and matrigel based assays, respectively. As shown in Figure 23A & B, silencing either p63 or VDR in A431 cells led to an increase in wound closure, compared to control siRNA transfected cells, indicating enhanced cell migration. The matrigel membrane based invasion assays accurately measures the invasiveness and migration of cancer cell lines. Interestingly, knockdown of either p63 or VDR resulted in significant increase in number of invading cells (Figure 23C).

Figure 23. Down regulation of p63 or VDR results in increased cell migration and cell invasion in A431 cells. A431 cells were transfected with either control siRNA or p63 siRNA or VDR siRNA as indicated. A) After 24 hrs cells were initiated for wound healing assay and microscopic pictures were taken at 0 hr and 24 hr post initiation of wound healing assay. B) The extent of wound closure after 24 hr was measured and normalized to 100% at 0 hr. Y-axis represents the percent in wound closure compared to 0 hr and error bars represents the standard deviation from two independent experiments. C) Cells were subjected to matrigel based invasion assay as explained in methods section. After 24 hrs micrographic pictures of cells migrated through transwell membranes were taken and total number cells migrated through membranes were counted. Y-axis represents the number of cells invaded through membrane in p63 or VDR siRNA transfected cells compared to control siRNA transfected cells and error bars represent the standard deviation from two independent experiments. D) Immunoblot analysis was performed to confirm the effective silencing of p63 and VDR.


A)
Con si
p63 si
VDR si
0 hr
24 hr
B)
% in wound closure
90
60
30
0
Con si
p63 si
VDR si
C)
Fold change in no. of invaded cells
9
6
3
0
Con si
p63 si
VDR si
D)
Con si
p63 si
VDR si
p63
VDR
actin

Since the loss of either VDR or p63 results in enhanced invasiveness, we next tested whether induction of VDR by ectopic p63 results in diminished invasiveness. A431 cells stably expressing p63 or control eGFP were established and as expected, compared to A431 eGFP stable pool cells, a significant increase in both p63 and VDR expression levels were observed in A431-p63 stable pool cells (Figure 24A). Compared to A431-eGFP stable pool cells, a significant reduction in number of invading cells was observed in A431-p63 stable pool cells, indicating that overexpression of p63 and VDR abrogates the normal invasion of A431 cells (Figure 24A). Additionally, to demonstrate that p63 mediated inhibition of cell invasiveness occurs via VDR, the control A431-eGFP and A431-p63 stable pool cells were transfected with control siRNA or p63 siRNA and matrigel based invasion assays were performed. Since the p63 siRNA used for these studies targets the 3'UTR of p63 mRNA, the A431-p63 stable cells expressing only the coding region of p63 will not be silenced, therefore detectable expression of VDR can be observed even in absence of p63 (Figure 24B, right panel compare lane 2 and 4). Compared to control siRNA transfected cells, silencing p63 led to an increase in number of invading cells only in A431-eGFP cells but not in A431-p63 stable cells (Figure 24B). These results provide evidence that p63 mediated regulation of VDR play a role in inhibiting the migration and invasiveness of A431 cells.

G. Significance of VDR regulation by p63 during embryonic development.

The functional roles of p63 and VDR in epidermal development are well established, VDR has been shown to induce terminal epidermal differentiation markers and VDR null mice had severe alopecia conditions (Xie et al., 2002). To determine the role of p63 in regulating VDR expression during embryonic development, transcript

Figure 24. Re-expression of p63 and VDR results in reduced cell migration and cell invasion of A431 cells. A) A431-eGFP and A431-ΔNp63α stable pool cells were subjected to matrigel based invasion assay (right panel). Y-axis represent the fold change in number of A431-ΔNp63α stable pool cells invaded, compared to A431-eGFP stable pool cells. Immunoblot analysis was performed to detect the expression of ΔNp63α and VDR in both A431-eGFP and A431-ΔNp63α stable pool cells (left panel). B & C) A431-eGFP and A431-ΔNp63α stable pool cells were transfected with either control siRNA or p63 siRNA as indicated. 24 hr post siRNA transfections cells were subjected to Matrigel based invasion assay (right panel). After 24 hrs cell images were taken from multiple fields and the invading cells were counted. B) Y-axis represent the fold change in number of cells invaded compared to control siRNA transfected A431-eGFP cells and error bars represent the standard deviation from two independent experiments. Immunoblot analysis was performed to detect the expression of ΔNp63α and VDR in both A431-eGFP and A431-ΔNp63α stable pool cells transfected with control siRNA or p63 siRNA as indicated (left panel). C) Representative cell images from A431-eGFP and A431-ΔNp63α stable pool cells that were transfected with either control siRNA or p63 siRNA as indicated.

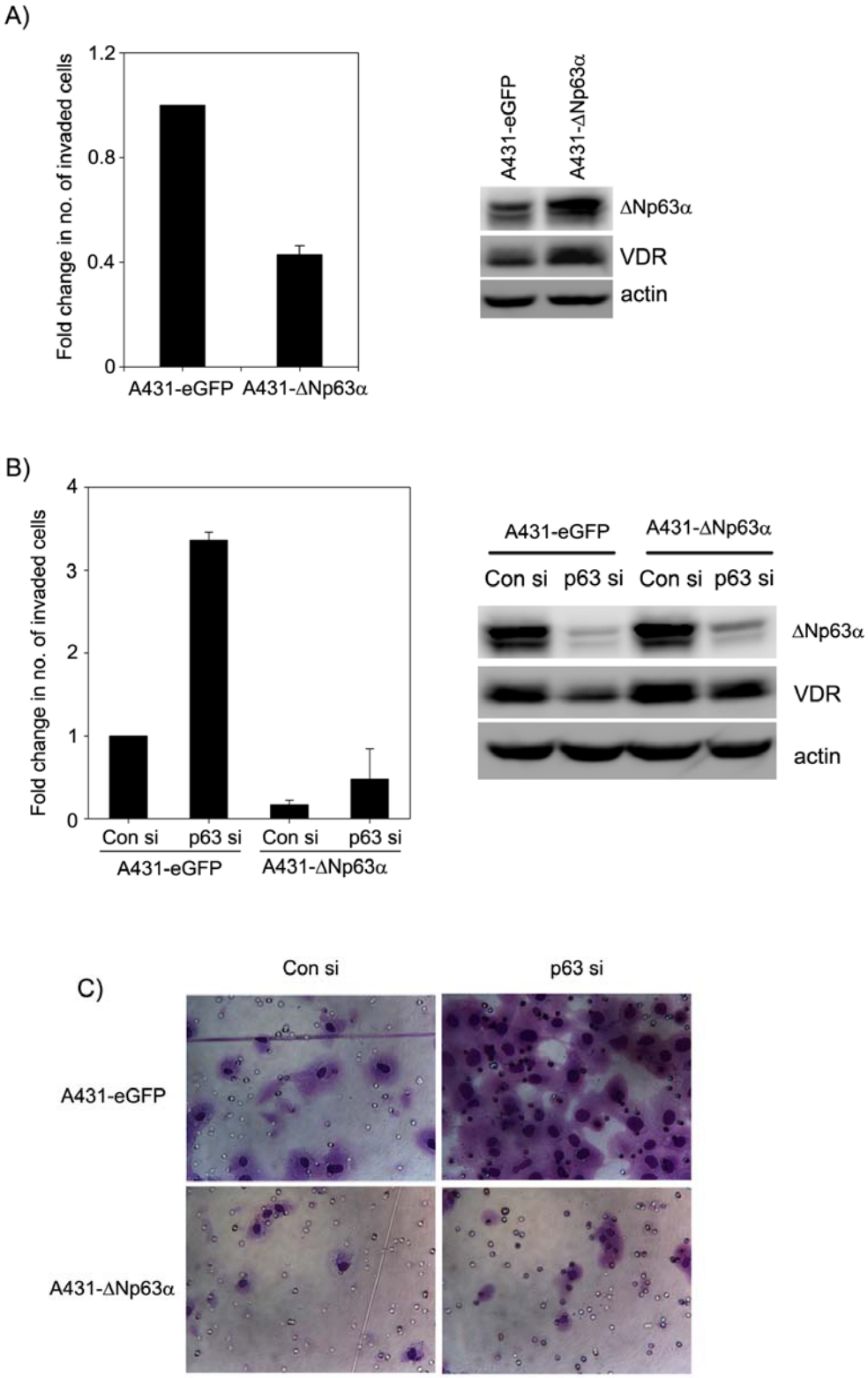
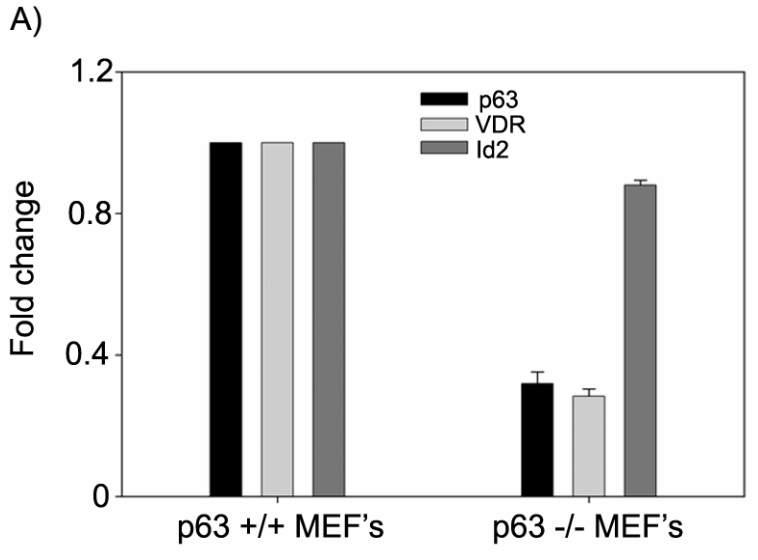

A)
Fold change in no. of invaded cells
1.2
0.8
0.4
0
A431-eGFP
A431-ΔNp63α
A431-eGFP
A431-ΔNp63α
ΔNp63α
VDR
actin
B)
Fold change in no. of invaded cells
4
3
2
1
0
Con si
p63 si
Con si
p63 si
A431-eGFP
A431-ΔNp63α
A431-eGFP
A431-ΔNp63α
Con si
p63 si
Con si
p63 si
ΔNp63α
VDR
actin
C)
Con si
p63 si
A431-eGFP
A431-ΔNp63α

Figure 25- p63 is essential for VDR expression during embryonic development.

Total RNA from 18.5 embryonic day wild type MEFs and p63-/- MEFs were extracted and transcript levels of p63, VDR and Id2 were determined by using TaqMan based real time PCR analysis. Y-axis represents fold change in VDR, Id2 and p63 transcript levels relative to wild type MEFs. Detection of Id2 expression was used as negative control for p63.


A)
1.2
0.8
0.4
0
Fold change
p63
VDR
Id2
p63 +/+ MEF's
p63 -/- MEF's

levels of both p63 and VDR were assessed in mouse embryonic fibroblast (MEF) from both wildtype and p63 null mice. Compared with wildtype MEF's a significant reduction in VDR transcript levels were observed in p63 null MEF's (Figure 25). Histochemical analysis of new born (NB), postnatal day 4 (P4) and day 16 (P16) wildtype mice skin showed the similar VDR and p63 expression pattern and the single layered skin of p63 null mice showed a complete lack in VDR expression (data not shown (obtained by Dr. Satrajit Sinha)). Since the p63 mutations are observed in developmental diseases, the effect of several naturally occurring p63 mutants on VDR expression was determined. As shown in figure 26, a differential effect of p63 mutants on VDR expression was observed; while TAp63γ (R227C) and TAp63γ (R298Q) mutants were able to induce the VDR expression, all other tested mutants showed no effect on VDR expression.

H. DNA damage induced expression of VDR is independent of p53 and correlates with p73 expression.

The results from our earlier studies clearly demonstrated that p63, but not p53, induces VDR expression (Kommagani et al., 2006). Recently, Maruyama et. al. (2006) demonstrated that p53 induces VDR expression and that VDR expression was induced upon DNA damage in p53 positive cell lines (Maruyama et al., 2006). One possible explanation for this discrepancy could be the differences in the amount of p53 employed for the over expression studies. Towards understanding the possible dose dependent effect of p53 on VDR expression, studies were carried out to test the effect of increasing concentrations of p53 on endogenous VDR expression. H1299 and HeLa cells were over expressed with increasing concentrations of p53 alone or p63 alone and transcript and protein levels of VDR and p21 were monitored. Regulation of p21 was used as a

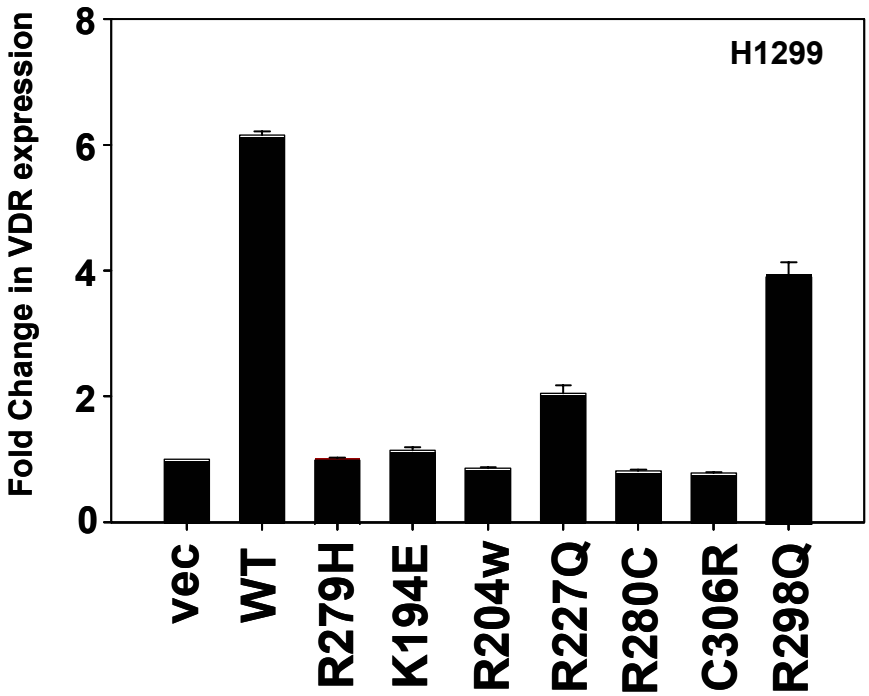
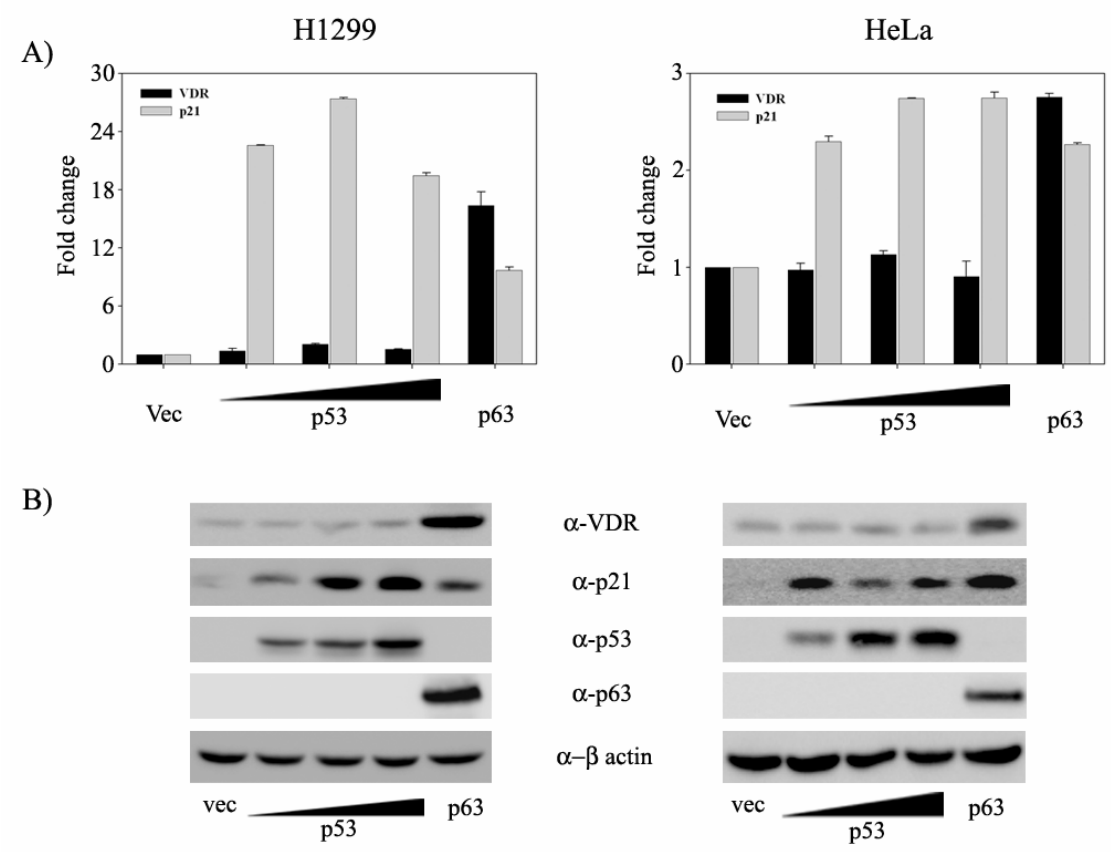


Figure 26. Differential effect of naturally occurring p63 mutants on VDR. H1299 cells were transfected with either empty vector or expression plasmids encoding WT p63 or indicated p63 mutants alone. At 24 hr post-transfection, total RNA was extracted and subjected to TaqMan Reverse transcriptase to detect the VDR transcript levels. Y-axis represents fold change in VDR transcript levels relative to empty vector transfected cells.

Figure 27. p53 does not induce VDR expression. H1299 and HeLa cells were transfected with either empty vector, increasing doses of expression plasmids encoding p53 (1 μg, 2 μg and 3 μg) and p63 alone (3 μg) as indicated. A) At 24 hr post-transfection, total RNA was harvested and subjected to TaqMan reverse transcriptase PCR. Y-axis represents fold change in VDR and p21 mRNA levels relative to empty vector transfected cells. B) Whole cell lysates were subjected to immunoblot analysis to determine the protein expression levels of VDR, p21, p53, β-actin and p63.


A)
H1299
HeLa
VDR
p21
Fold change
30
24
18
12
6
0
3
2
1
0
Vec
p53
p63
B)
α-VDR
α-p21
α-p53
α-p63
α–β actin
vec
p53
p63

positive control for the transactivation of both p53 and p63. As seen earlier, exogenous p63 up-regulated the expression of both VDR and p21 at transcript and protein levels (Figure 27). In contrast, over expression of p53 at any tested dose did not result in induction of VDR at transcript or protein levels in both the cell lines (Figure 27). As expected, p21 was significantly induced by p53 at all the tested doses, thus confirming the transactivation potential of p53. Further, similar studies were performed to test the ability of p53 to transactivate VDR promoter using VDR-Luc reporter. Consistent with protein and transcript results, ectopic p53 had no effect on VDR-Luc reporter activity (data not shown).

Since ectopic p53 was unable to induce VDR expression in p53-/- cell lines (H1299 and HeLa), studies were carried out to determine whether DNA damage induced expression of VDR is dependent or independent of p53. H1299 and SaOS2 cells were examined for the effects of two DNA damaging agents, doxorubicin and etoposide, on VDR expression. An in depth multiple dose analysis was conducted prior to choosing appropriate doses for doxorubicin (0.2 μM and 0.4 μM) and etoposide (6 μM, 8 μM for H1299 and 2 μM, 4 μM for SaOS2). At 24 hr post etoposide and doxorubicin treatments transcript levels of VDR and CYP27B1 were measured by performing TaqMan based real time PCR. Measuring the transcript levels of CYP27B1, a known down stream target of VDR, was used as a positive control for VDR mediated induction of its target genes. In both cell lines, compared with untreated cells, a significant increase in VDR and CYP27B1 transcript levels were observed with etoposide and doxorubicin treatments (Figure 28), which suggests that transcriptional activation of VDR upon DNA damage occurs independent of p53.

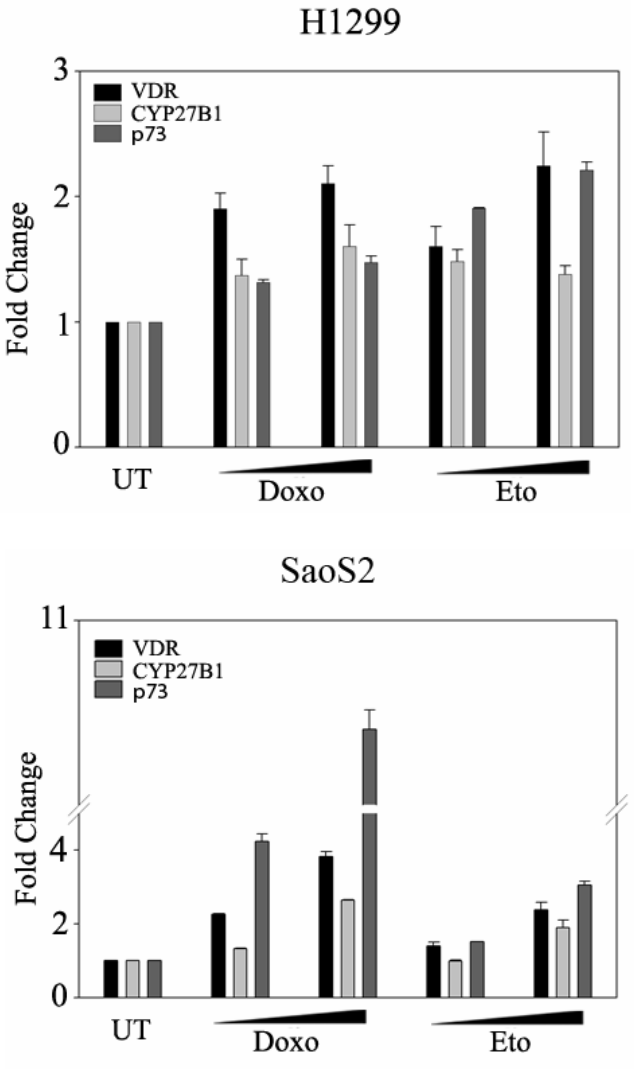
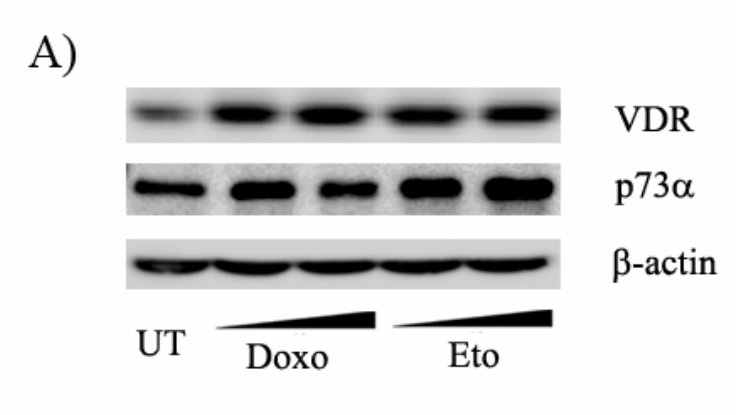
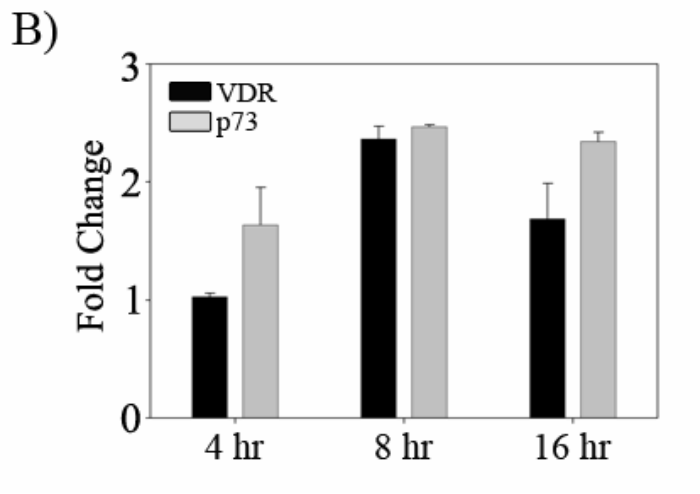


Figure 28. DNA damage induced expression of VDR is independent of p53. H1299 and SaOS2 cells were either untreated or treated with Doxorubicin (0.2 μM and 0.4 μM) or Etoposide (6 μM, 8 μM for H1299 and 2 μM, 4 μM for SaOS2). After 24 hr TaqMan real time PCR was performed to determine the transcript levels of VDR, CYP27B1 and p73. Y axis indicates change in transcript levels compared with untreated cells.

Figure 29. Induction of VDR expression upon DNA damage correlates with p73 expression and p73 up-regulation precedes VDR expression upon DNA damage. A) H1299 cells were either untreated or treated with doxorubicin or etoposide for 24 hr following which immunoblot analysis was performed to detect the protein levels of VDR and p73. For the detection of p73 protein levels whole cell lysates were first immunoprecipitated with monoclonal anti-p73 antibody and immunoblotted with polyclonal anti-p73 antibody. B) H1299 cells were treated with 8 μM etoposide and total RNA was extracted at indicated post-treatment time points. TaqMan based reverse transcriptase PCR was performed to detect the transcript levels of p73 and VDR. Y-axis represents the fold change in VDR and p73 transcript levels relative to untreated cells at respective time points.


A)
VDR
p73α
β-actin
UT
Doxo
Eto

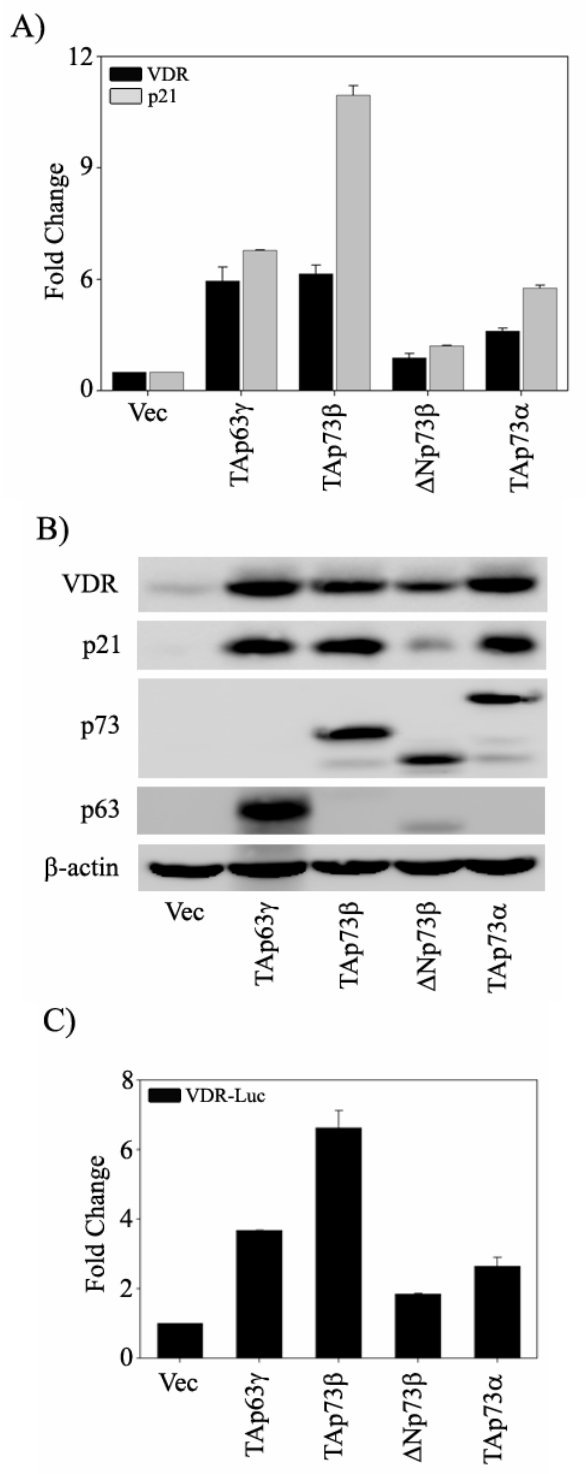

B)
VDR
p73
Fold Change
0
1
2
3
4 hr
8 hr
16 hr

Previous reports demonstrated that both these cell lines have endogenous p73, and p73 is induced upon DNA damage in various cancer cell lines (Chen et al., 2001; Urist et al., 2004), therefore, the correlation between p73 and VDR expression levels upon DNA damage was studied. A considerable correlation between p73 and VDR transcript levels were observed with etoposide and doxorubicin treatment in both cell lines (Figure 28). Consistent with the transcript levels, endogenous protein levels of both VDR and p73 were also significantly up-regulated upon etoposide and doxorubicin treatments in H1299 cells (Figure 29A). Given the correlation between p73 and VDR expression levels upon DNA damage, we tested whether p73 induction precedes the VDR induction upon DNA damage. H1299 cells were treated with 8 μM etoposide then at 4, 6 and 16 hr post treatment transcript levels of VDR and p73 were measured. A significant increase in p73 transcript levels were observed at 4 hr post etoposide treatment and VDR induction was observed only at 8 hr post etoposide treatment (Figure 29B), suggesting that p73 induction precedes VDR induction upon DNA damage.

I. Direct transcriptional regulation of VDR by p73.

To determine the possible direct transcriptional activation of VDR by p73, studies were carried out to determine whether p73 activates VDR and whether basal VDR expression levels are regulated by p73. To address this, the transcriptional activation of VDR by multiple p73 isoforms was determined. H1299 cells were transfected with different p73 isoforms or TAp63γ alone and the expression of VDR and p21 at transcript and protein levels were determined. TAp63γ was used as a positive control for both VDR and p21 induction. Over expression of TAp73β and TAp73α in H1299 cells led to a significant increase in VDR and p21 expression at both transcript and protein levels

Figure 30. Multiple isoforms of p73 up regulates VDR expression. H1299 cells were transfected with control vector, TAp63γ, TAp73β, ΔNp73β, or TAp73α as indicated. A) At 24 hr post transfection, transcript levels of VDR and p21 were determined by Real time PCR and Y-axis represents the fold change in transcript levels of VDR and p21 compared to empty vector transfected cells. B) At 24 hr post-transfection, immunoblot analysis was performed to detect the endogenous VDR and p21 protein levels and to detect the ectopic expression of p63 and p73 isoforms. C) H1299 cells were co-transfected with full length VDR promoter reporter along with indicated plasmids. At 24 hr post transfection, cells were subjected to dual luciferase assays. RLU/R-Luc ratios were calculated to normalize for transfection efficiency. Y-axis represents fold change relative to empty vector control.


A)
12
9
6
0
Fold Change
VDR
p21
Vec
TAp63γ
TAp73β
ΔNp73β
TAp73α
B)
VDR
p21
p73
p63
β-actin
Vec
TAp63γ
TAp73β
ΔNp73β
TAp73α
C)
8
6
4
2
0
Fold Change
VDR-Luc
Vec
TAp63γ
TAp73β
ΔNp73β
TAp73α

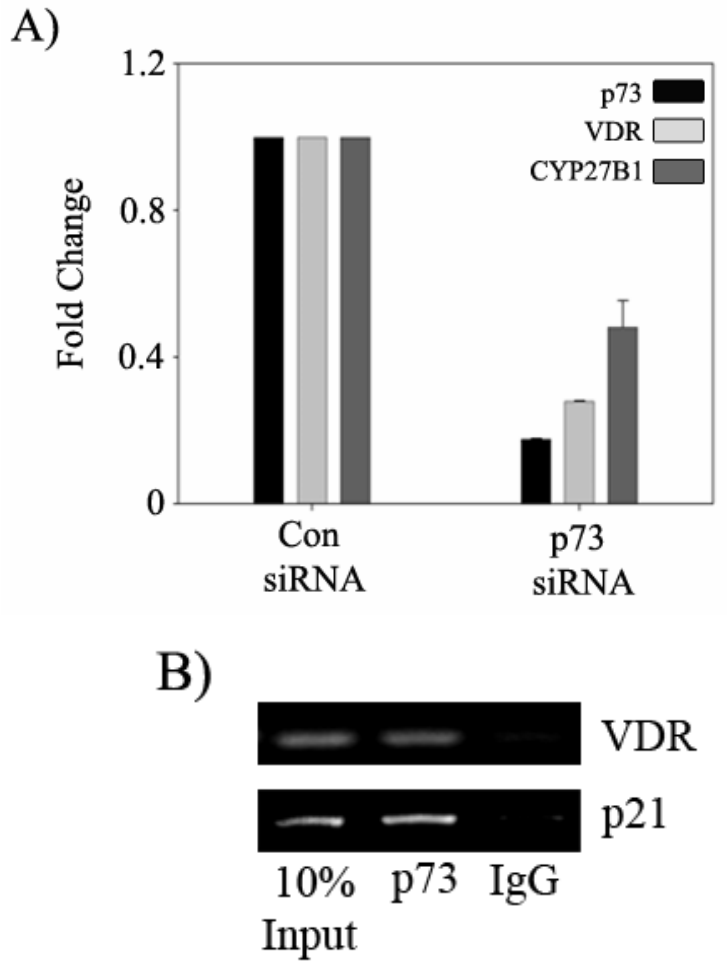


Figure 31. Endogenous p73 is essential for basal VDR expression and binds to VDR promoter *in vivo*. A) H1299 cells were transfected with either control siRNA or p73 siRNA and at 48 hr post transfection, Real time PCR was performed to detect the transcript levels of p73, VDR and CYP27B1. Y-axis represents fold change in transcript levels of VDR, CYP27B1 and p73 compared to control siRNA transfected cells. B) ChIP analysis on H1299 cells. Formaldehyde cross-linked chromatin was immunoprecipitated with anti-p73 or normal IgG antibodies as indicated. Eluted DNA was PCR amplified with primers specific for VDR and p21.

(Figure 30 A & B). In contrast, ΔNp73β led to a modest increase in both VDR and p21 expression levels when compared to the other TAp73 isoforms (Figure 30A & B). Next the ability of different p73 isoforms in transactivating full length VDR promoter was tested. As expected, both TAp73β and TAp73α isoforms significantly increased the VDR-Luc reporter activity (Figure 30C).

Additionally, we determined whether endogenous p73 binds to the VDR promoter and controls the basal VDR expression levels. To test this, endogenous p73 was silenced in H1299 cells and VDR and CYP27B1 transcript levels were monitored. As shown in Figure 31A, compared with control siRNA, 80% reduction in p73 transcript levels were observed with p73 siRNA. Silencing p73 led to a concomitant decrease in the basal transcript levels of VDR as well as CYP27B1 (Figure 31A). Finally, ChIP analysis on intact H1299 cells demonstrated that p73 binds onto the VDR promoter *in vivo*. A significant abundance of endogenous p73 protein was observed on the VDR and p21 promoters (Figure 31B). These results clearly suggest that VDR is transcriptionally activated by p73 and that endogenous p73 is essential for basal VDR expression in H1299 cells.

J. p73 is required for DNA damage induced expression of VDR.

The requirement of p73 for the induction of VDR upon DNA damage was determined by either inhibiting the transcriptional activity of p73 or by silencing p73 in presence of DNA damage. To demonstrate that the transcriptional activity of p73 is required for VDR induction upon DNA damage, a dominant negative p53 (R248W) mutant shown to inhibit the transcriptional activity of p73 was employed. Since TAp73α is the predominant p73 isoform observed upon DNA damage, the inhibition of

Figure 32. Mutant p53 but not WT p53 inhibits p73 mediated induction of VDR upon DNA damage. A) H1299 cells were transfected with control vector or expression plasmids encoding TAp73α, WT p53 and p53 (R248W) mutant alone or in combination as indicated. Immunoblot analysis was performed to detect the endogenous VDR and p21 protein levels and over expressed p73 and p53 proteins. B) H1299 cells were transfected with empty vector or p53 (R248W) or WT p53 as indicated. Next day cells were either untreated or treated with 8 μM etoposide as indicated. Immunoblot analysis was performed to detect the endogenous VDR protein levels and to confirm the over expression of WT and mutant p53. p73 protein levels were detected by performing immunoprecipitation with monoclonal anti-p73 antibody and immunoblotted with polyclonal anti-p73 antibody.

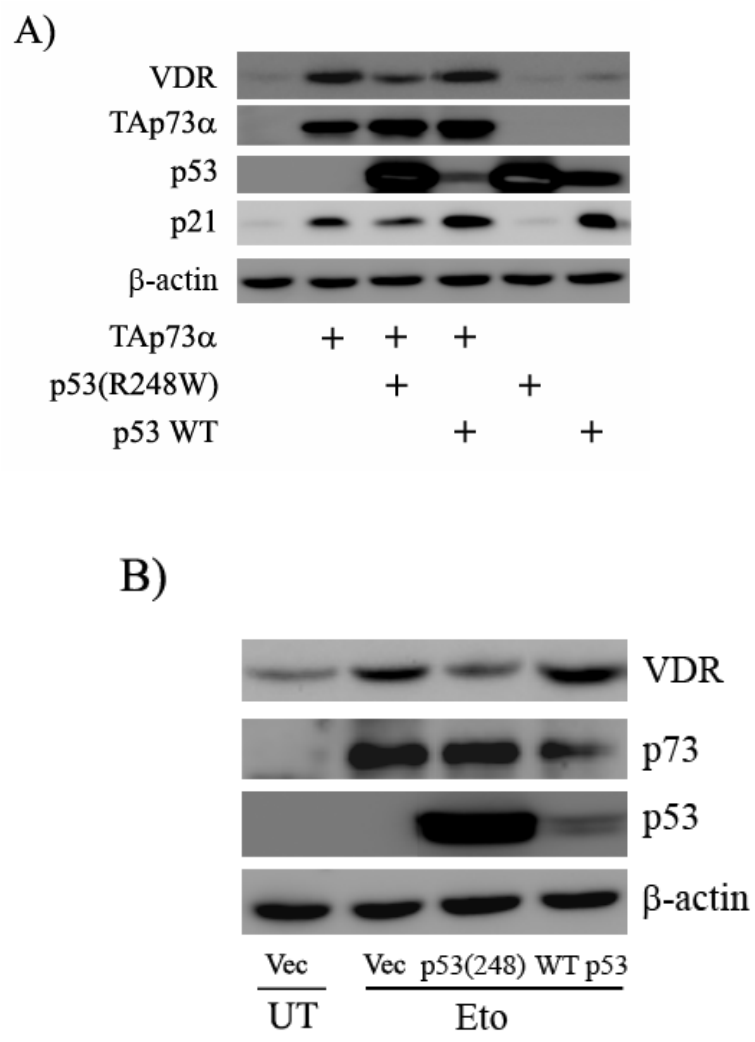
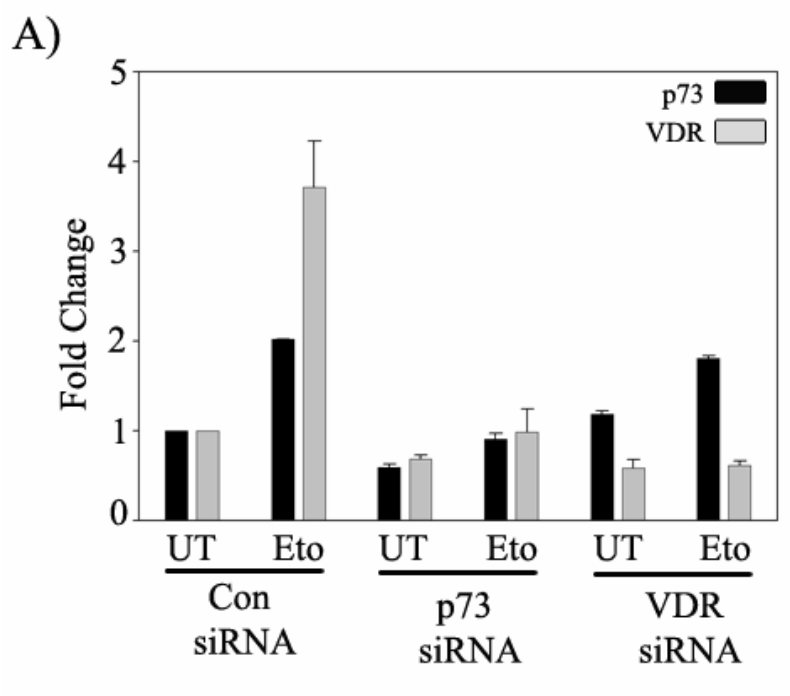
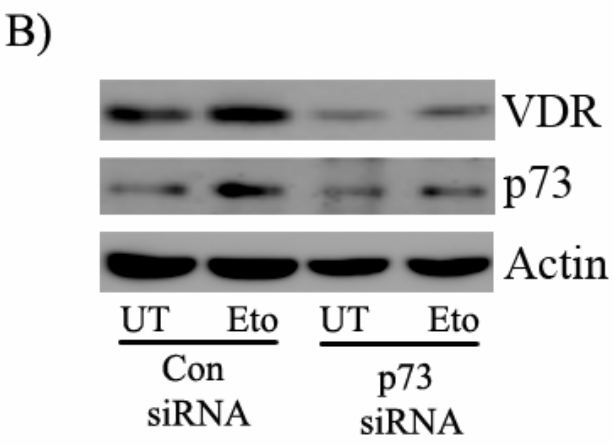

A)
VDR
TAp73α
p53
p21
β-actin
TAp73α + + +
p53(R248W) + +
p53 WT + +
B)
VDR
p73
p53
β-actin
Vec
Vec p53(248) WT p53
UT
Eto

Figure 33. p73 is required for DNA damage induced expression of VDR. H1299 cells were transfected with control siRNA, VDR siRNA, or p73 siRNA and after 24 hr post transfections cells were trypsinised and re-plated onto 6 well plates. After cells adhered properly in wells, cells were treated with 8 μM etoposide or left untreated for 24 hrs. A) TaqMan real time PCR was performed to detect the mRNA levels of p73 and VDR. Y-axis represents the fold change in transcript levels of VDR and p73 compared to control siRNA transfected cells with control vehicle (UT).B) Immunoblot analysis was performed to detect the endogenous VDR and p73 protein levels. p73 protein levels were detected by performing immunoprecipitation with monoclonal anti-p73 antibody and immunoblotted with polyclonal anti-p73 antibody.


A)
Fold Change
5
4
3
2
1
0
p73
VDR
UT
Eto
UT
Eto
UT
Eto
Con siRNA
p73 siRNA
VDR siRNA


B)
VDR
p73
Actin
UT
Eto
UT
Eto
Con siRNA
p73 siRNA

VDR induction by ectopic TAp73α was determined. As shown in Figure 32A**,** p53 (R248W) mutant modestly down regulated TAp73α mediated induction of VDR. Consistent with previous observation that wild type p53 cannot inhibit p73 mediated transcriptional activity (Di Como et al., 1999), wild type p53 by itself did not affect TAp73α mediated induction of VDR (Figure 32A). Further, blocking the endogenous TAp73 transcriptional activity by p53 (R248W) mutant but not wild type p53 significantly down regulated the etoposide induced VDR protein expression levels (Figure 32B). To unequivocally demonstrate that p73 is essential for VDR expression upon DNA damage, VDR induction upon DNA damage was assessed in presence and absence of p73. As shown in Figure 33, in control siRNA transfected cells, compared with untreated cells, etoposide treated cells showed a significant induction in both p73 and VDR transcript and protein levels. However, in presence of p73 siRNA a significant inhibition of both basal and etoposide induced transcript and protein levels of p73 were observed. Concomitant decreases in elevated levels of VDR with etoposide were also observed in the presence of p73 siRNA, suggesting the requirement of p73 for DNA damage induced VDR expression (Figure 33). Overall, results from these studies demonstrate a novel p73 dependant induction of VDR in p53 null cell lines.

K. DNA damage induced VDR expression sensitizes the cells to vitamin D treatment through p73.

Given that vitamin D exerts its transcriptional activity through VDR, endogenous p73 must be essential for vitamin D-mediated transcriptional activity. In addition, p73 mediated induction of VDR upon DNA damage should further enhance the vitamin D-mediated transcriptional activity. To address this, studies were conducted to test whether

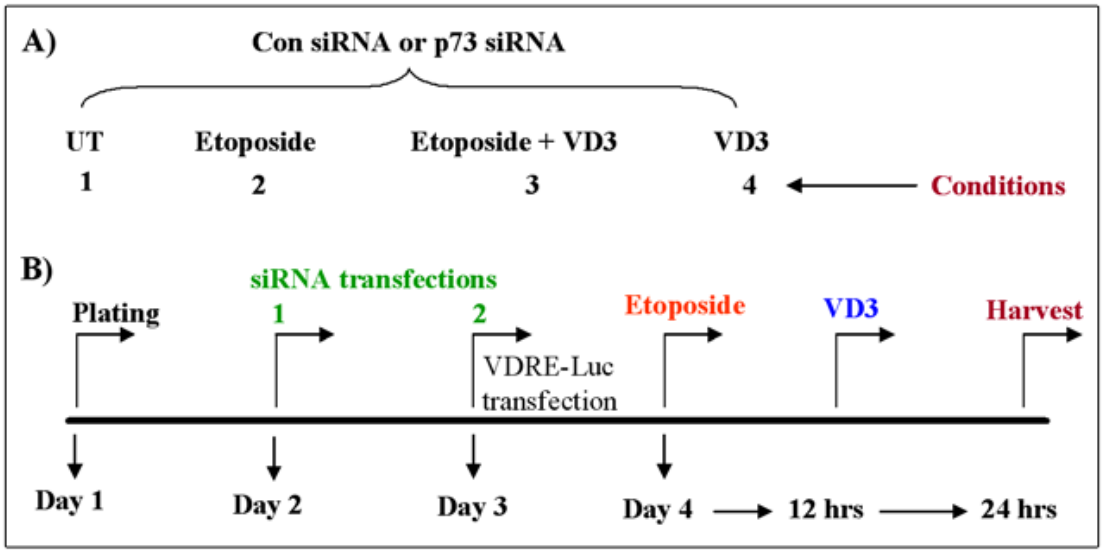
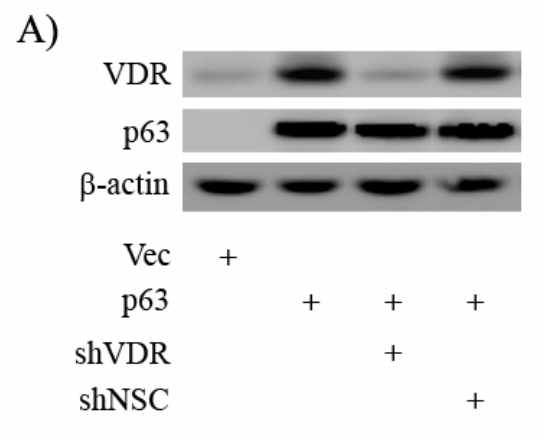
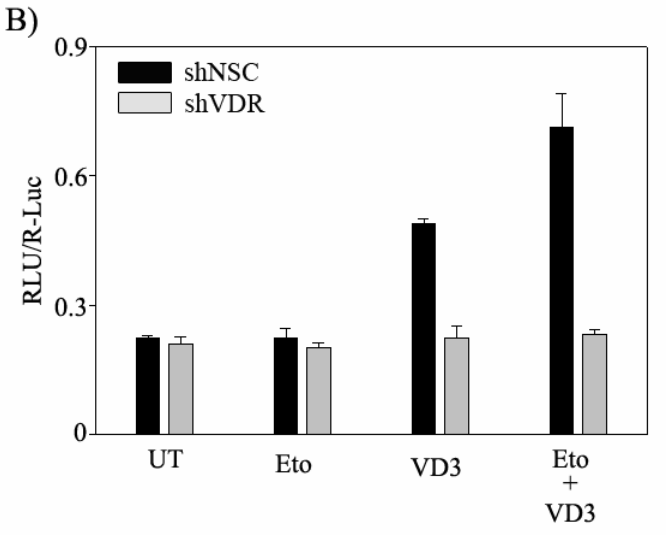
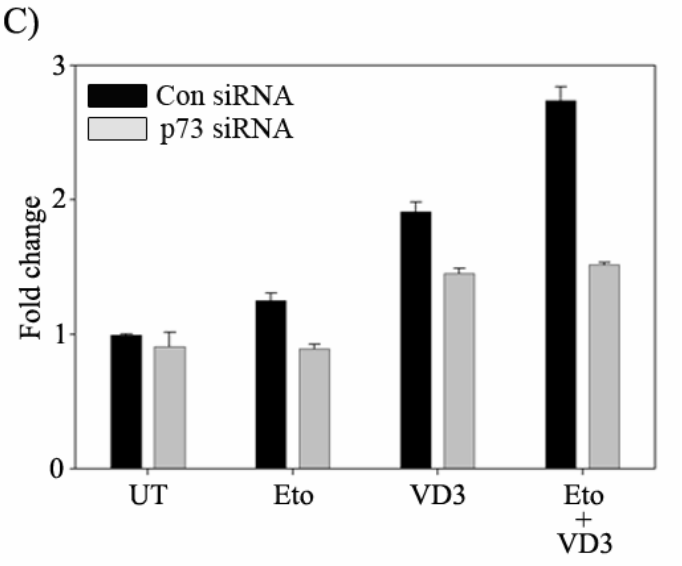


Figure 34. Schematic representation of experimental design for Figure 35. Flowchart representing the experimental design to determine the effect of p73 silencing on vitamin D mediated transcriptional activity in presence and absence of DNA damage.

Figure 35. p73 is required for DNA damage mediated enhancement of vitamin D transcriptional activity. A) H1299 cells were transfected with control vector or p63 alone or along with shVDR or shNSC as indicated. At 24 hr post transfection cells were harvested for whole cell lysates and subjected to immunoblot analysis to detect VDR, p63 and β-actin using specific antibodies. B) H1299 cells were plated on 12 well plates and co-transfected with VDRE-Luc reporter along with either shNSC or shVDR as indicated. Next day cells were either treated with 8 μM etoposide or left untreated as indicated. After 12 hr of post treatment cells were treated with 100 nM VD as indicated. At 24 hr post VD treatment cells were subjected to dual luciferase assay. Y-axis represents the ratio of firefly luciferase units to renilla luciferase units. C) H1299 cells were transfected with control siRNA or p73 siRNA and after two rounds of transfections with siRNA cells were re-plated onto 24 well plates and reverse transfections were performed with VDRE-Luc reporter. Next day cells were either treated with 8 μM etoposide or left untreated as indicated. Subsequently cells were treated with 100 nM VD as indicated. At 24 hr post VD treatment cells were subjected to dual luciferase assay as mentioned earlier. Y-axis represents fold change relative to control siRNA transfected cells with control vehicle (UT).


A)
VDR
p63
β-actin
Vec
p63
shVDR
shNSC
+
+
+
+
+


B)
shNSC
shVDR
RLU/R-Luc
0.9
0.6
0.3
0
UT
Eto
VD3
Eto
+
VD3

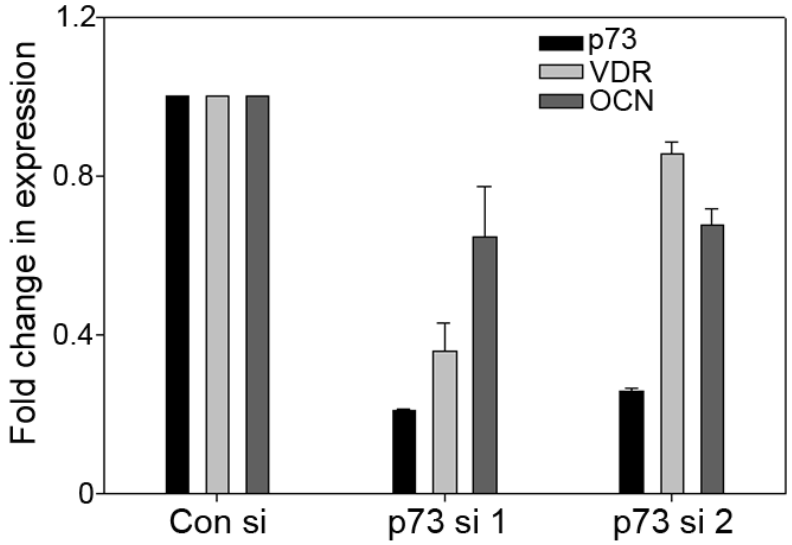

C)
Con siRNA
p73 siRNA
Fold change
3
2
1
0
UT
Eto
VD3
Eto
+
VD3

elevated levels of VDR observed upon DNA damage enhances the vitamin D-mediated transcriptional activity using short hairpin VDR construct (shVDR). A vitamin D responsive reporter, VDRE-Luc which bears two VDR responsive elements upstream of luciferase gene was used to monitor the vitamin D-mediated transcriptional activity. Silencing of VDR was achieved by using a short hairpin RNA (shRNA) specific to VDR (shVDR) and non-silencing control shRNA (shNSC) was used as a negative control. To confirm the effective silencing of VDR by shVDR, H1299 cells were transfected with expression plasmid encoding TAp63γ along with shVDR or non-silencing control. The increase in VDR levels induced by TAp63γ were abolished by shVDR but not by shNSC, confirming the specificity of the shVDR (Figure 35A). H1299 cells were co-transfected with VDRE-Luc reporter along with either shNSC or shVDR and subsequently cells were treated with etoposide, followed by 24 hr incubation in vitamin D. After 24 hrs, cells were harvested and VDRE-Luc activity was measured. Compared with control vehicle (UT), a significant increase in VDRE-Luc activity was observed with vitamin D treatment (Figure 35B). As expected, etoposide enhanced the increase in vitamin D-mediated VDRE-Luc activity (Figure 35B). The increase in VDRE-Luc activity observed with vitamin D in presence and absence of etoposide was completely abolished in the absence of VDR, indicating that elevated VDR levels upon DNA damage enhance vitamin D mediated transcriptional activity (Figure 35B).

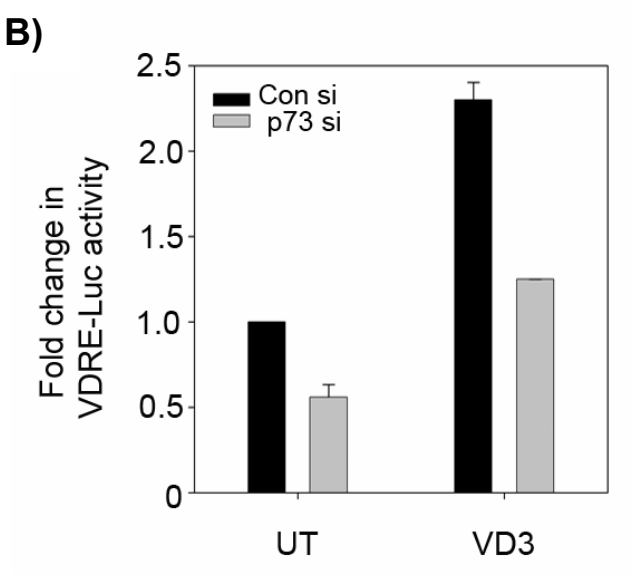
Next, to address whether p73 is required for DNA damage mediated enhancement of vitamin D transcriptional activity, a similar experiment was carried out by silencing p73 in H1299 cells as illustrated in Figure 34. As shown earlier, etoposide significantly enhanced the vitamin D-mediated transcriptional activity, however silencing p73 led to a

Figure 36. Silencing p73 results in reduction in vitamin D mediated transcriptional activity in SaOS2 cells. A) SaOS2 cells, mock transfected or transfected with two rounds of control siRNA or p73 siRNAs and after 48 hrs VDR and p73 transcript levels were determined. Y-axis represents the fold change in transcript levels of VDR, OCN and p73 compared to control siRNA transfected cells. B) SaOS2 cells transfected with either control siRNA or p73 siRNA were transfected with VDRE-Luc construct along with Renilla luciferase construct. Dual luciferase assays were performed to detect the VDRE-Luc activity and Y-axis represents the fold change in VDRE-Luc activity compared to control siRNA transfected cells treated with vehicle.

A)


1.2
0.8
0.4
0
Fold change in expression
p73
VDR
OCN
Con si
p73 si 1
p73 si 2

B)


2.5
2.0
1.5
1.0
0.5
0
Fold change in VDRE-Luc activity
Con si
p73 si
UT
VD3

dramatic reduction in VDRE-Luc activity that was observed with etoposide and vitamin D together (Figure 35C). In addition, depletion of p73 also resulted in reduced basal vitamin D-mediated transcriptional activity (Figure 35C). These results clearly demonstrate the requirement of p73 for vitamin D-mediated transcriptional activity in presence and absence of DNA damage.

L. p73 is required for vitamin D mediated osteoblastic differentiation.

To investigate the role of p73 in vitamin D mediated differentiation, we first tested whether endogenous p73 is essential for the basal VDR expression of SaOS2 cells, a well studied model system for vitamin D mediated osteoblastic differentiation. SaOS2 cells were transfected with either control siRNA or two different p73 siRNA (siRNA p73_1 or siRNA p73_2). At 48 hr post transfections, real time PCR was performed to determine the transcript levels of p73 and VDR. Since vitamin D promotes the terminal differentiation, the detection of terminal osteoblastic differentiation marker OCN was used as a control for VDR down stream target. Compared to control siRNA transfected cells, cells transfected with siRNA p73_1 showed a considerable reduction in both p73 and VDR transcript levels (Figure 36A). However, compared to control siRNA transfected cells, cells transfected with siRNA p73_2 showed a modest reduction in VDR transcript levels. In addition, a substantial down regulation in OCN transcript levels was also observed with both p73 siRNA_1 and p73 siRNA_2. Although both siRNA's against p73 reduced the basal p73 expression levels, siRNA p73 _1 was more potent than siRNA p73_2, and thus p73 siRNA_1 was employed for subsequent studies. Consistent with the reduction in VDR transcript levels, compared to control siRNA transfected cells, a significant reduction in VDR protein expression was observed in p73 siRNA transfected

Figure 37. Effect of vitamin D on p73 expression in SaOS2 cells. SaOS2 cells were treated with either vehicle (UT) or increasing concentrations of vitamin (VD) as indicated. At 48 hr post-treatment, total RNA was extracted and TaqMan based real time PCR was performed to detect the transcript levels of p73 and OPN and OCN. Y-axis represents the fold change in the transcript levels compared to vehicle treated cells (UT).

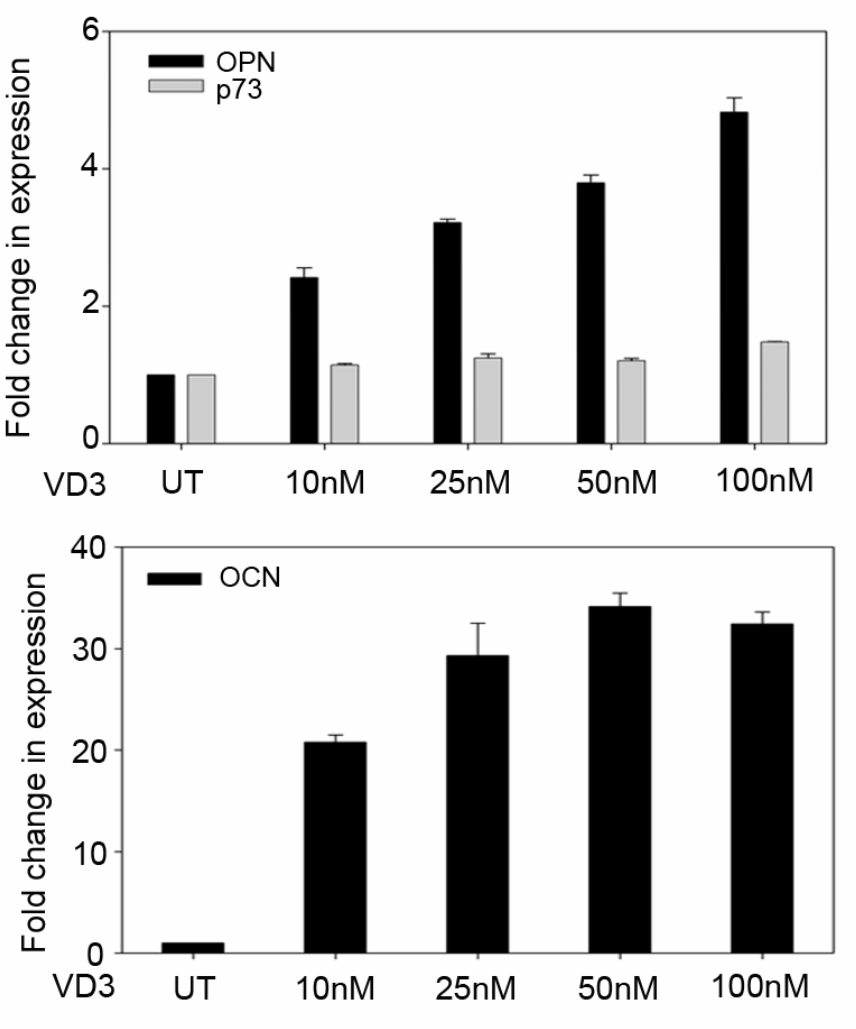
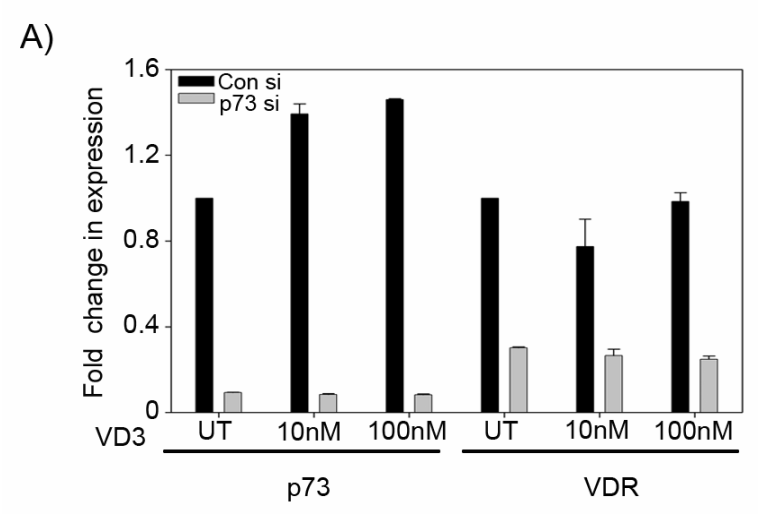
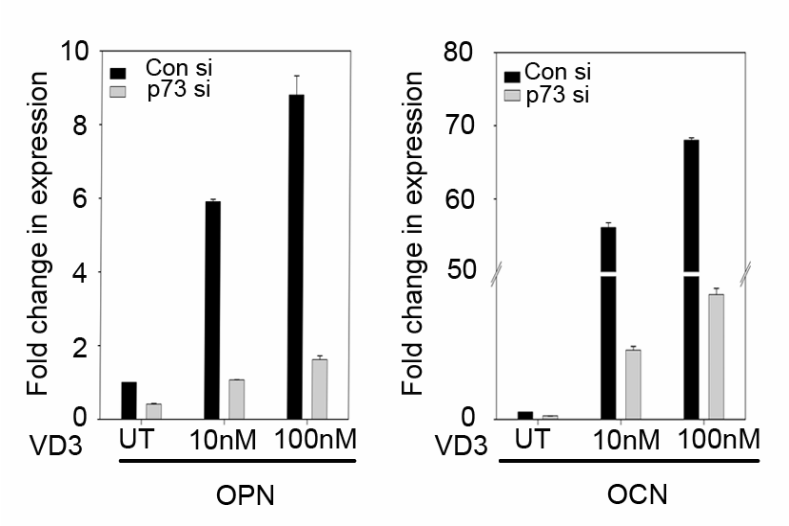
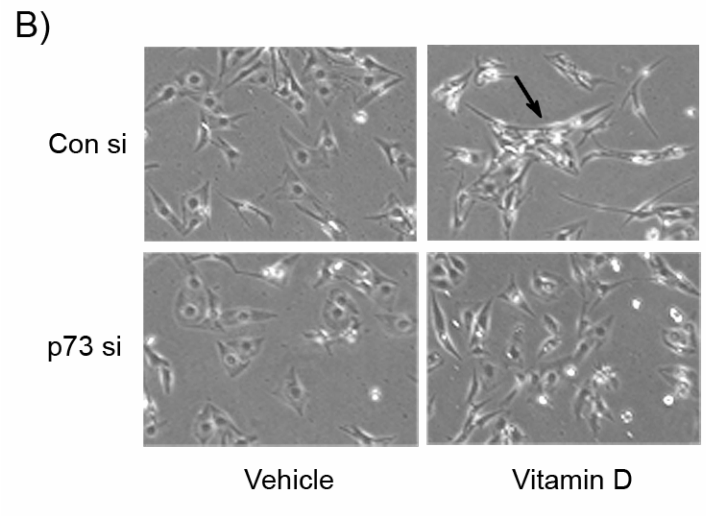

Fold change in expression
6
4
2
0
OPN
p73
VD3
UT
10nM
25nM
50nM
100nM
Fold change in expression
40
30
20
10
0
OCN
VD3
UT
10nM
25nM
50nM
100nM

Figure 38. p73 is required for vitamin D mediated differentiation of SaOS2 cells. A) SaOS2 cells transfected with control siRNA or p73 siRNA were harvested and re-plated onto 6 well plates. Next day, cells were treated for 48 hr with vehicle or 10 nM or 100 nM VD and total RNA was extracted and transcript levels of p73, VDR, OPN and OCN were determined. Y-axis represents the fold change in transcript levels compared to control siRNA transfected cells with vehicle treatment (UT). B) SaOS2 cells transfected with control siRNA or p73 siRNA were split and re-plated onto 6 wells. Next day, cells were treated with vehicle or 10 nM VD as indicated and at 48 hr post VD treatment morphological changes in cells were observed by phase contrast microscopy.


A)
Con si
p73 si
Fold change in expression
1.6
1.2
0.8
0.4
0
VD3
UT
10nM
100nM
UT
10nM
100nM
p73
VDR

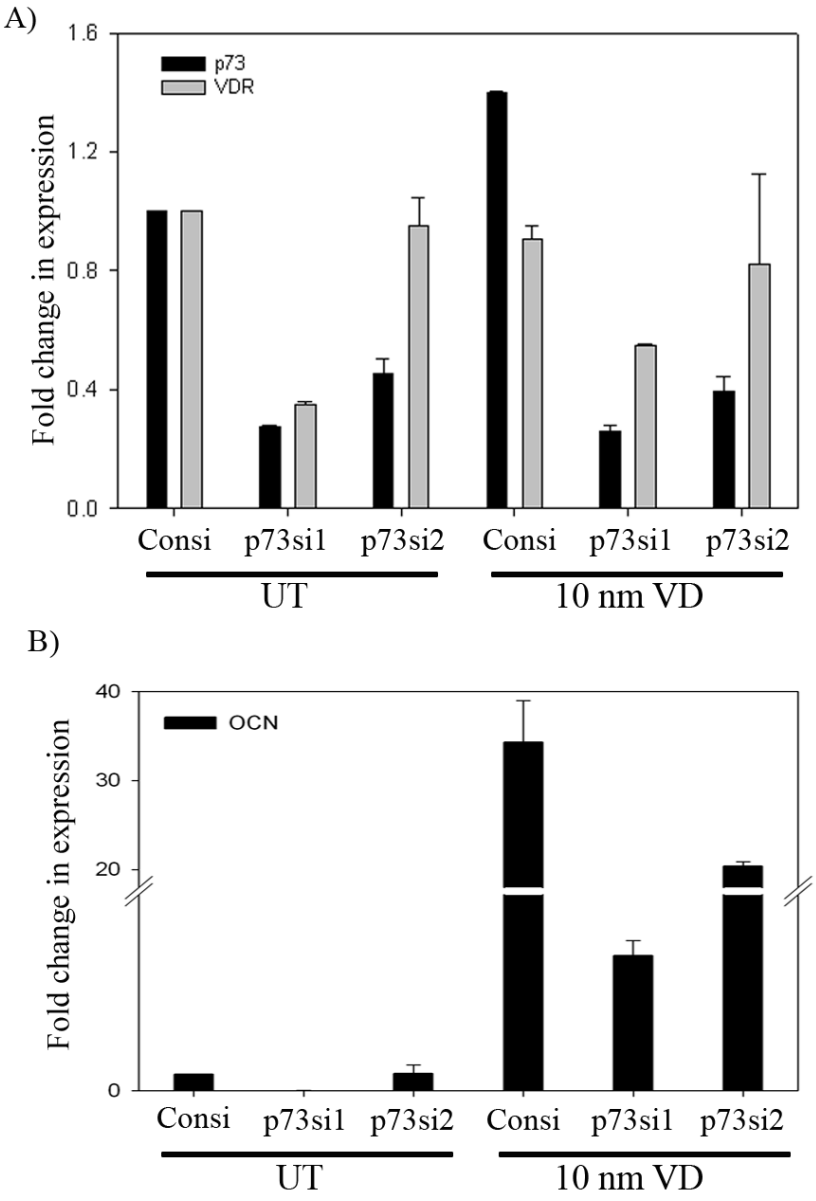

Con si
p73 si
Fold change in expression
10
8
6
4
2
0
VD3
UT
10nM
100nM
OPN
Con si
p73 si
Fold change in expression
80
70
60
50
0
VD3
UT
10nM
100nM
OCN


B)
Con si
p73 si
Vehicle
Vitamin D

cells in immunofluorescence (IF) assay (data not shown). Since, vitamin D mediated genomic actions are exclusively dependant on its receptor VDR, the effect of p73 silencing on vitamin D mediated transcriptional activity in SaOS2 cells was determined. This was determined by monitoring the vitamin D mediated increase in VDRE-Luc reporter activity in presence and absence of p73. As expected, a significant increase in the VDRE-Luc activity was observed in cells transfected with control siRNA and treated with vitamin D (Figure 36B). However, silencing p73 lead to a significant reduction in vitamin D mediated VDRE-Luc activity (Figure 36B).

Since vitamin D and VDR primarily promote osteoblastic differentiation and p73 knockdown also led to a decrease in basal VDR expression levels in osteosarcoma cells, next the effect of p73 silencing on vitamin D mediated osteoblastic differentiation was investigated. To investigate this, studies were conducted first to determine whether vitamin D by itself has any effect on endogenous p73 expression in SaOS2 cells. SaOS2 cells were treated with increasing concentrations of vitamin D (10 nM, 25 nM, 50 nM, and 100 nM) for 48 hours, and the transcript levels of p73, OCN and OPN were examined. Since vitamin D mediated osteoblastic differentiation is associated with the induction of OPN and OCN expression, detection of OCN and OPN transcript levels were used as a positive control for vitamin D mediated genomic effect. As expected, a dose dependant induction in both OCN and OPN transcript levels were observed with increasing concentrations of vitamin D (Figure 37). However, vitamin D had no effect on p73 transcript levels at any of the tested doses, indicating that p73 is not regulated by vitamin D in osteosarcoma cells (Figure 37A). Next, to examine whether p73 is essential for vitamin D mediated osteoblastic differentiation, expression of OPN and OCN

Figure 39. Effect of different p73 siRNA on vitamin D mediated induction of OCN. SaOS2 cells transfected with control siRNA or p73 siRNA 1 & 2 were harvested and re-plated onto 6 well plates. Next day, cells were treated with vehicle (UT) or 10 nM VD and total RNA was extracted. After 48 hrs transcript levels of p73 and VDR (A) and OCN (B) were determined. Y-axis represents the fold change in transcript levels compared to control siRNA transfected cells with vehicle treatment.


A)
p73
VDR
Fold change in expression
1.6
1.2
0.8
0.4
0.0
Consi
p73si1
p73si2
Consi
p73si1
p73si2
UT
10 nm VD
B)
OCN
Fold change in expression
40
30
20
0
Consi
p73si1
p73si2
Consi
p73si1
p73si2
UT
10 nm VD

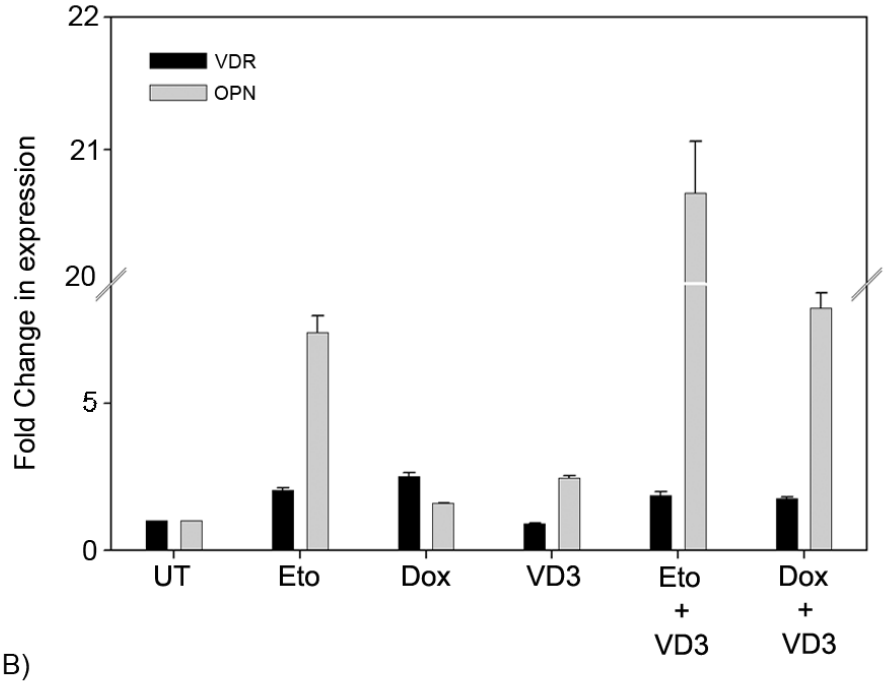
was monitored in SaOS2 cells treated from vitamin D in presence and absence of p73. As expected, p73 knockdown led to a significant decrease in p73 as well as VDR transcript levels (Figure 38A). A significant increase in OCN and OPN expression levels was observed in cells transfected with control siRNA and treated with vitamin D for 48 hrs, indicating the onset of differentiation in SaOS2 cells (Figure 38). p73 silencing however, led to a significant reduction in the elevated expression levels of OCN and OPN that were observed with vitamin D (Figure 38A).

In addition, morphological changes in cells observed upon differentiation were also monitored from cells treated with vitamin D in presence and absence of p73. In control siRNA transfected cells, compared to control vehicle a significant number of cells with differentiation morphology features were observed with vitamin D treatment (Figure 38B, marked by an arrow). However, silencing p73 led to a significant reduction in number of cells with differentiation features that were observed with vitamin D treatment (Figure 38B).

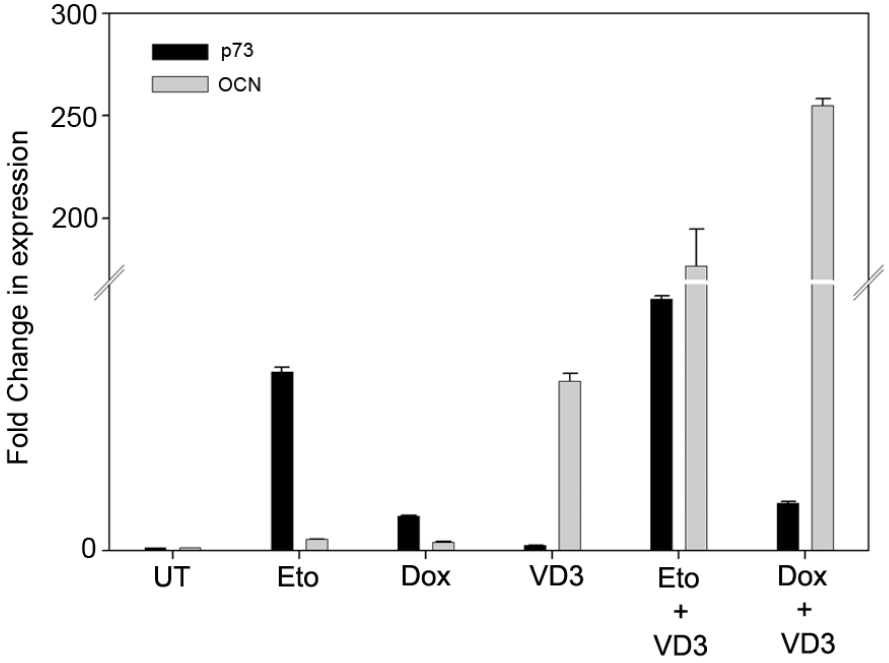
To validate the role of p73 in vitamin D mediated differentiation, next the effect of two different siRNA against p73 on vitamin D mediated differentiation was carried out. Testing two different siRNA against p73 will also address the off target effects of p73 siRNA during vitamin D mediated differentiation. SaOS2 cells were transfected with two different siRNA against p73 and vitamin D mediated osteoblastic differentiation was monitored by detecting the OCN expression. As shown in Figure 39, cells treated with vitamin D showed a increase in OCN transcript levels, however both the siRNA against p73 down regulated the OCN transcript levels that were observed with vitamin D treatment. Since, p73 siRNA_2 was less effective in silencing p73, compared

Figure 40. DNA damage enhances vitamin D mediated differentiation of SaOS2 cells. SaOS2 cells were pretreated for 24 hrs with 4 μM etoposide or 4 μM doxorubicin and subsequently cultured with vehicle or 10 nM VD for an additional 48 hrs. Total RNA was extracted and transcript levels of VDR, OPN (top panel) and p73, OCN (lower panel) were determined by TaqMan based real time PCR. Y-axis represents the fold change in transcript levels compared to vehicle treated (UT) cells.

A)


22
21
20
5
0
VDR
OPN
Fold Change in expression
UT
Eto
Dox
VD3
Eto + VD3
Dox + VD3

B)


300
250
200
0
p73
OCN
Fold Change in expression
UT
Eto
Dox
VD3
Eto + VD3
Dox + VD3

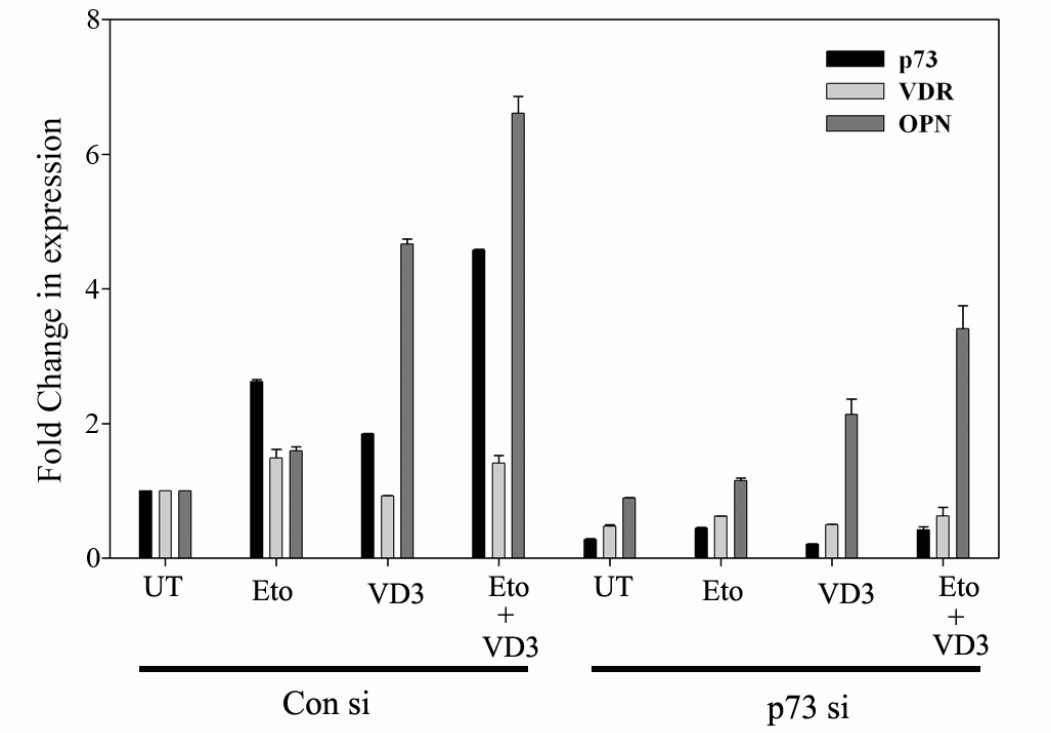
to p73 siRNA_1, a modest decrease on OCN transcript levels was observed with p73 siRNA_2. Together these results clearly indicate that p73 is required for vitamin D mediated differentiation of SaOS2 osteosarcoma cell lines.

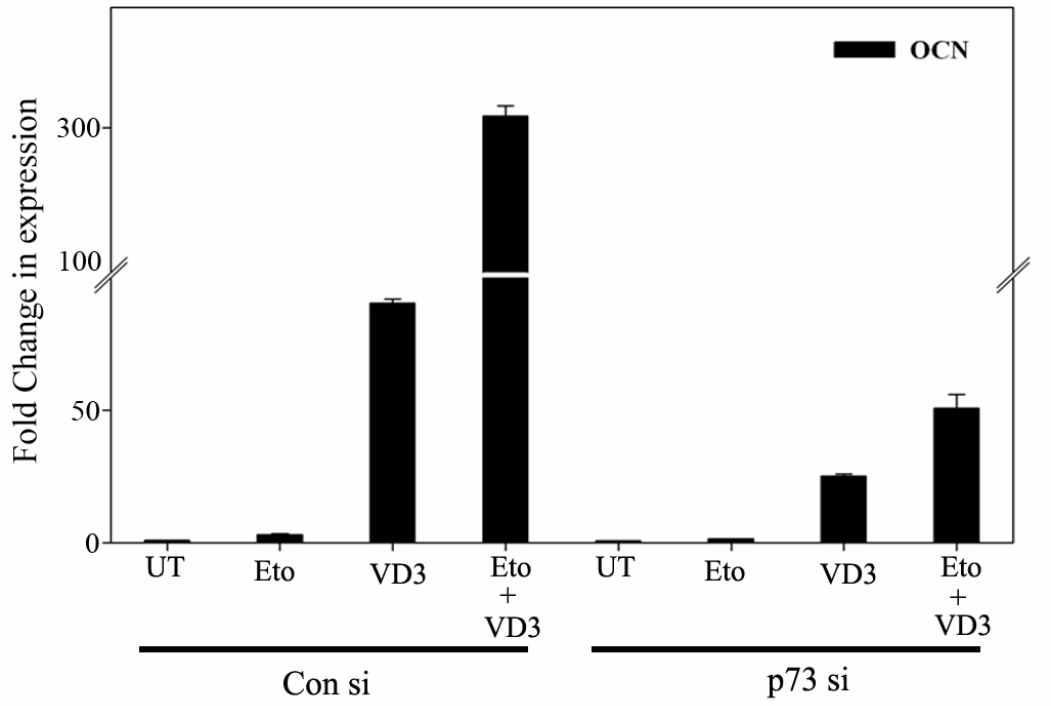
M. DNA damage induced enhancement of vitamin D mediated osteoblastic differentiation requires p73.

To test whether p73 mediated induction of VDR expression upon DNA damage enhances the vitamin D mediated differentiation of SaOS2 cells, the effect of DNA damage on vitamin D mediated induction of OPN and OCN was determined. SaOS2 cells were pretreated with etoposide or doxorubicin for 24 hr and then were cultured in vitamin D for 2 days. Compared with untreated cells, cells treated with etoposide or doxorubicin showed a significant induction in both VDR and p73 transcript levels (Figure 40, top panel). As expected, a significant increase in transcript levels of both OPN and OCN were observed in cells treated with vitamin D alone (Figure 40, lower panel). Interestingly, cells pre-treated with etoposide or doxorubicin and cultured in vitamin D for 2 days showed a synergistic increase in both OPN and OCN expression levels that were observed with vitamin D treatment alone (Figure 40, lower panel). These results suggest that DNA damage induced expression of p73 is responsible for the synergistic increase in vitamin D mediated differentiation.

Further, to confirm that p73 is essential for DNA damage-mediated enhancement in vitamin D mediated differentiation, the transcript levels of OPN and OCN were examined in presence and absence of p73 in cells pre-treated with etoposide and subsequently with vitamin D. As expected, p73 silencing led to a reduction of VDR expression levels in cells treated with etoposide alone or in combination with vitamin D,

Figure 41. DNA damage enhances vitamin D mediated osteoblastic differentiation through p73. SaOS2 cells were transfected with either control siRNA or p73 siRNA as indicated and treated with 4 μM etoposide or left untreated as indicated. After 24 hrs cells were either cultured in control vehicle or 10 nM VD for 48 hrs. Transcript levels of VDR, p73, OPN (top panel) and OCN (lower panel) were determined and Y-axis represents the fold change in transcript levels compared to control siRNA transfected cells treated with vehicle.


Fold Change in expression
8
6
4
2
0
p73
VDR
OPN
UT
Eto
VD3
Eto + VD3
UT
Eto
VD3
Eto + VD3
Con si
p73 si


Fold Change in expression
300
100
50
0
OCN
UT
Eto
VD3
Eto + VD3
UT
Eto
VD3
Eto + VD3
Con si
p73 si

indicating that p73 is essential for induction of VDR upon DNA damage in these cells (Figure 41). Additionally, silencing p73 also led to a significant decrease in OPN and OCN expression levels in cells treated with vitamin D alone or in combination with etoposide (Figure 41). In particular, silencing p73 led to a remarkable decrease in OCN expression levels in cells treated with vitamin D alone or in combination with etoposide (Figure 41). Together, these results clearly suggest that induction of VDR expression by p73 upon DNA damage enhances vitamin D mediated differentiation of SaOS2 cells.

IV. DISCUSSION

A. Significance of VDR regulation by p63

Human cancers of epithelial origin comprise eighty percent of solid tumors and are preserved through specific subpopulation of stem cells (Visvader and Lindeman, 2008). The molecular or cellular processes responsible for the origin of epithelial cancers are yet to be identified. Particularly, the underlying signaling pathways associated with hyper proliferative nature of epithelial cancer stem cells are vastly understudied. In contrast to cancers, the epithelial morphogenesis program is associated with coordinated regulation of various signaling networks. Therefore, understanding the critical genetic paradigms associated with the origin of epithelial tissues will give significant insight on the de-regulated cellular factors associated with the initiation of epithelial cancers. The initiation and execution of epithelial morphogenesis program has been shown to be entirely dependent on transcription factor p63 (Koster et al., 2004). In accordance with the role of p63 in epithelial stratification program, p63 null mice lack the tissues of epithelial origin. Further, p63 has been shown to determine the differentiation of mullerian duct epithelium and loss of p63 expression was shown to result in single layered uterine epithelia (Kurita et al., 2004).

These studies implicated a critical role for p63 in epithelial tissue formation, thus identifying the underlying p63 regulated genetic pathways are subject of intense research.

Primarily two different approaches were carried out in identifying such p63 target genes; 1) p63 deficient cells were overexpressed with specific isoforms of p63 and altered gene expression profiles were determined using cDNA profiling assays; 2) endogenous p63 expression was silenced in p63 containing cells and the gene expression profiles were monitored. Each approach has its own advantages and drawbacks, however considerable number of p63 regulated genes were determined using these two approaches (Trink et al., 2007). Similarly, our lab performed a gene expression analysis by over expressing TAp63γ isoform in H1299 cells (data not shown). VDR was one of the several hundred of genes that were up regulated by TAp63γ in H1299 cells. Nuclear receptor family member VDR is a natural receptor for steroid hormone vitamin D. The classical function of vitamin D is to maintain the calcium and mineral homeostasis during the bone re-absorption process. Apart from its classical functions, vitamin D also plays a vital role in epidermal differentiation, cell cycle arrest and in immune responses.

A substantial induction in VDR expression at protein and transcript levels was observed with ectopic TAp63γ in H1299 and HeLa cell (Figure 6). H1299 and HeLa cell lines lack a functional p53 protein and ectopic p53 had no effect on VDR expression in these cell lines, suggesting that the upregulation of VDR by p63 is independent of p53 (Figure 6). Several studies were carried out to understand the co-operation and regulation between p53 and p63 proteins (Harmes et al., 2003). Although a direct interaction was not observed between p63 or p73 and p53, both p63 and p73 were shown to be required for the activation of apoptosis related gene promoters by p53 (Davison et al., 1999; Flores et al., 2002). These observations suggest that p53 family members can modulate each other's ability to regulate target genes through indirect mechanisms. Interestingly, p53

showed no effect on TAp63γ mediated induction of VDR at protein or transcript level (Figure 8), indicating that p53 does not have an effect on VDR expression directly or indirectly.

To understand the functional similarities between p63 and p53, studies were carried out to understand the effect of upstream regulators of p53 on p63 (Bergamaschi et al., 2004; Bernassola et al., 2005; MacPartlin et al., 2005). While some proteins exerted the similar effect on p63 and p53, others showed a differential effect on p63 and p53. For example, $p14^{ARF}$ was shown to activate p53 transcriptional activity by sequestering MDM2 a negative regulator of p53, $p14^{ARF}$ however was shown to inhibit the transcriptional activity of p63 (Calabro et al., 2004; Honda and Yasuda, 1999). In transient transfection studies, co-transfection of $p14^{ARF}$ with TAp63γ resulted in substantial down regulation of p63 mediated induction of VDR (Figure 11 & 12). These observations highlight VDR as a bona fide target of p63 and additional studies are required to decipher the effect of other p53 co-activators on p63 mediated induction of VDR.

In general, p53 family members activate target genes by binding to canonical p53 responsive RRRCWWGYYY tandem repeats (Osada et al., 2005). While p53 and p63 bind to response elements with RRRC(A/T)(A/T)GYYY repeats, response elements consisting of RRRCGTGYYY tandem repeats were specifically activated by p63 (Osada et al., 2005; Osada et al., 2007). Reports also suggest that sequence differences in the response elements might govern the target gene specificity of p53 and p63 proteins (Osada et al., 2006; Perez et al., 2007). A significant increase in full length VDR promoter activity was observed with ectopic TAp63γ but not with p53 (Figure 7). In

addition, deletion of 750 bp fragment from 5' end of VDR promoter did not abolish p63 mediated transactivation of VDR (Figure 14). Interestingly, *in silico* based analysis predicted several RRRCGTGYYY tandem repeats within the -635 to -581 region of VDR promoter (Figure 14 lower panel). A significant increase in -635/-581 region containing reporter activity was also observed with ectopic TAp63γ, suggesting the presence of a potential p63 specific responsive element within VDR promoter (Figure 14). Results from ChIP analysis clearly demonstrated the binding of ectopic TAp63γ but not p53 on VDR promoter *in vivo* (Figure 13). Moreover, the -635/-581 region of VDR promoter was amplified from the p63 immunoprecipitated DNA in the ChIP analysis. These observations clearly suggest that p63 activates VDR by binding to a p63 specific responsive element within the VDR promoter.

Recently adenoviral based gene transfer studies were carried to test the efficacy of p53 family members in promoting the growth suppression (Ganjavi et al., 2006; Ishida et al., 2000; Sasaki et al., 2001). Particularly, adenoviral based transfer of TAp63γ and TAp73β were shown to promote apoptosis and cell cycle arrest in multiple cancer cell lines (Das et al., 2005; Kunisaki et al., 2006). Administration of adenoviral-TAp63γ into SaOS2-xenografts has been shown to promote the tumor growth retardation much higher than that of p53 (Oshima et al., 2007). However, *in vitro* studies showed a similar level of apoptosis induction by both p53 and TAp63γ in SaOS2 osteosarcoma cells (Oshima et al., 2007). These reports clearly suggest that p63 promotes the growth inhibition of osteosarcoma through multiple cellular processes. Results from this study showed a significant induction in differentiation of SaOS2 cells with over expression of TAp63 (Figure 15). Additionally, in SaOS2 cells ectopic p63 up-regulated the transcript levels of

VDR and osteoblastic differentiation markers OPN, OCN and RUNX2 (Figure 16). Interestingly, osteoblastic differentiation markers OPN, OCN and RUNX2 are shown to be the downstream targets of VDR (Bikle, 1992; Koszewski et al., 1996; Noda et al., 1990). In contrast, both p53 and p63γ (R279H) mutant failed to induce VDR expression and differentiation of SaOS2 cells (Figure 14 & 15), suggesting that activation of VDR is essential for the effective differentiation of SaOS2 cells. These results indicate that TAp63γ promotes the growth inhibition of SaOS2 cells by promoting the differentiation through VDR. Vitamin D and its analogues are shown to promote the differentiation of SaOS2 cell through VDR (Wu et al., 2007). Interestingly, results from this study showed that ectopic p63 sensitizes the SaOS2 cells to the vitamin D mediated differentiation (Figure 17**)**. Therefore, adenoviral based gene therapy of p63 in combination with vitamin D may be considered for an effective therapeutic strategy against osteosarcoma.

The role of p63 in epithelial tissue development, in particular in stratified epithelial morphogenesis is well established (Candi et al., 2007). The proliferative potential of epithelial stem cells in stratified epithelia is dependent on p63 (Senoo et al., 2007). The predominant isoforms of p63 observed during development are TAp63α and ΔNp63α, however the functional relevance of other p63 isoforms still needs to be addressed (Koster et al., 2004). Therefore, generation of isoform specific p63 knock-out mice and deciphering the isoform specific target genes will give better insight on the functional significance of each isoform in development. Vitamin D receptor has also been implicated in epidermal differentiation. The ablation of VDR leads to development of alopecia due to the defects in hair follicle regeneration (Xie et al., 2002), and reduced expression of epidermal differentiation markers including involucrin and loricrin (Bikle

et al., 2002; Hawker et al., 2007). Results from this study demonstrated that all the isoforms of p63 induce the VDR expression (Figure 18 & 19). Recent genetic complementation studies suggested that ΔNp63α isoform by itself does not complement epidermal defects in *p63*-deficient mice, indicating the functional relevance of other p63 isoforms in development (Candi et al., 2006). Thus it is intriguing to test whether VDR is a vital component of both TAp63 and ΔNp63 associated functions during development.

The predominant p63 isoform, ΔNp63α is expressed in basal layers of stratified epithelia and has been shown to be overexpressed in multiple cancers. ΔNp63α isoform is considered to antagonize the functions of p53 and TAp63 by forming hetero-tetramers or by competitive binding to specific DNA sequences (Yang et al., 1998). In support of this notion, several studies have shown the down regulation of TAp63 mediated transactivation by ΔNp63α (Barbieri et al., 2005; Petitjean et al., 2008). Interestingly, in co-transfection studies, while ΔNp63α down regulated the TAp63 γ mediated induction of p21 and IGFBP-3, ΔNp63α showed no effect on the induction of VDR by TAp63γ (Figure 20). This result argues against the assumed dominant negative function for ΔNp63α towards all the targets that are regulated by TAp63. Although ΔNp63 lacks the full length N-terminal TA domain, ΔNp63α isoform has been shown to activate target genes transcriptionally (Helton et al., 2006; Wu et al., 2005). Results from transient transfection studies clearly showed the induction of VDR expression by ΔNp63α isoform at protein and transcript levels (Figure 18 & 19). Additionally, a significant dose dependant increase in VDR-Luc activity with increasing concentrations of ΔNp63α was observed, suggesting a direct transcriptional activation of VDR by ΔNp63α isoform (Figure 21A). Results from ChIP assay on A431 cells clearly showed the occupancy of

ΔNp63α protein on the VDR promoter (Figure 21B). Furthermore, silencing endogenous ΔNp63α but not ΔNp73α in A431 cells led to complete abolishment in endogenous VDR expression (Figure 22). These results strongly suggest VDR as a direct transcriptional target of ΔNp63α.

The ability of cancer cells to invade is an essential component of metastasis and depends upon multiple factors. Recently, down regulation of p63 has been shown to increase the cell migration and invasiveness of multiple cancer cells including A431 (Barbieri et al., 2006; Higashikawa et al., 2007). The active form of vitamin D has also been shown to inhibit the invasiveness of prostate cancer cells (Tokar and Webber, 2005). Results from our studies indeed showed that down regulation of both ΔNp63α and VDR results in increased cell migration and invasion (Figure 23). Additionally, ΔNp63α mediated re-expression of VDR in A431 cells abrogated the increase in invasiveness observed with down regulation of p63 or VDR (Figure 24B). Thus, findings from this study illustrate a novel role for ΔNp63α in ligand independent function of VDR.

Zinc finger transcription factor SNAIL has been shown to promote invasion and metastasis by repressing multiple proteins including E-cadherin and ΔNp63α (Barrallo-Gimeno and Nieto, 2005; Higashikawa et al., 2007). Interestingly, a direct transcriptional activation of E-cadherin by vitamin D has also been shown to promote growth arrest in colon cancer cells (Palmer et al., 2001). Finally, VDR has been shown to be repressed by SNAIL and a negative correlation between SNAIL and VDR expression has been reported in colon cancer cell line (Palmer et al., 2004; Pena et al., 2005). Thus, it is likely that at least in A431 cells, SNAIL represses ΔNp63α-VDR-E-cadherin axis to promote

invasiveness of cancer cells. A recent report indicated that induction of ΔNp63α protein levels by vitamin D is associated with the protection of transformed keratinocytes from irradiation mediated stress (Langberg et al., 2009). Interestingly, in A431 cells down regulation of VDR resulted in down regulation of endogenous ΔNp63α protein levels (Figure 23D). Therefore, it is possible that a feedback regulation of p63 by VDR or vice versa might play a vital role in normal functioning of keratinocytes.

Several reports implicated a functional role for ligand independent VDR in epidermal development (Ellison et al., 2007). Vitamin D receptor has been shown to be essential for normal keratinocyte stem cell function and severe epidermal defects were observed in *vdr*-deficient mice (Cianferotti et al., 2007; Xie et al., 2002). However, the factors involved in the molecular regulation of VDR during epithelial morphogenesis are poorly studied. We observed a marked down regulation of VDR expression levels in p63 deficient MEF's and both p63 and VDR were co-localized in basal layers of epidermis in newborn wildtype mice (Figure 25 and data not shown). In contrast to wildtype mice, p63 deficient mice lack well formed skin and a single layer of cells can be observed. Histochemical analysis on singe layered p63 deficient mice showed the complete abolishment in VDR expression (data obtained from our collaborator Dr. Satrajit Sinha).

Although p63 mutations are shown to be responsible for several human developmental diseases, the precise molecular mechanisms associated with p63 mutants are yet to be identified. Interestingly, we observed a differential effect of naturally occurring p63 mutants on VDR expression (Figure 26). Furthermore, p63γ (R279H) mutant downregulated the WT TAp63γ mediated induction of VDR (Figure 9 & 10). These findings suggest that impairment in VDR regulation by p63 mutants might

contribute to the pathophysiology of the diseases. Altogether these results suggest that the expression of VDR during embryonic development is dependant on p63 expression. However, additional studies are required to understand the precise role of VDR in p63 mediated functions, in particular in epithelial morphogenesis. In conclusion, results from this part of dissertation sheds light on the cross talk between two transcription factors ΔNp63α and VDR, which might be playing a critical role in invasion and epidermal differentiation.

B. Significance of VDR regulation by p73

Identification of new transcriptional networks of p53 family members has given significant insight on the functional diversities of each member (Deyoung and Ellisen, 2007). However, understanding the functional cross talk between each member on basis of their binding to common and unique target genes will be crucial in deciphering their role in tumorigenesis. Recently, Maruyama et al (2006) demonstrated VDR as common target of p53 family (Maruyama et al., 2006). In contrast, results from our earlier studies demonstrated VDR as specific target of p63 (Kommagani et al., 2006). The possible explanation for this discrepancy could be the cell line specificity or the amount of p53 employed for the transient transfection studies. The stability of p53 protein is regulated by a negative feedback process through its canonical target MDM2 (Bose and Ghosh, 2007). Since the transcriptional activity of p53 protein depends on its stability, the dose dependent effect of p53 on VDR expression was carried out in p53 null cells. As expected, expression of VDR was not affected by p53 at any of the tested doses (Figure 27). In addition, elevation of p21 expression by p53 at all the tested doses ruled out the possibility of nonfunctional ectopic p53 (Figure 27). Nonetheless, additional in depth

functional analysis was required to determine the variability in the outcomes of these two groups.

Although ectopic p53 was unable to induce VDR expression, it does not rule out the possibility of VDR regulation by activated p53 upon DNA damage. To test this, we carried out in depth analysis of VDR expression upon DNA damage in multiple cancer cell lines. Induction of VDR protein expression levels upon DNA damage was observed in HeLa , isogenic colon cancer cell lines HCT116 (p53 null) and HCT116 (p53 positive) as well as in SaOS2 (p53 null) and U2OS (p53 positive) cells (data not shown), suggesting a possible p53 independent activation of VDR upon DNA damage. Additionally, in p53 deficient H1299 and SaOS2 cells a significant induction in VDR transcript levels was also observed (Figure 28), which suggest that the elevated levels of VDR observed upon DNA damage are due to the transcriptional activation of VDR rather than the protein stabilization.

Human cancers display genetic alterations of key components involved in DNA damage response signaling pathways, which leads to metastasis and invasiveness (Lengauer et al., 1998). Although, p53 has been shown to be inactivated in 50% human cancers, several other genes are shown to substitute the p53 functions in multiple cancers including p73 (Deyoung and Ellisen, 2007; Levine, 1997). Transcription factor p73 has been shown to be induced upon DNA damage in several different cancer cell lines and shown to be critical for chemosensitivity of these cells (Chen et al., 2001; Kang et al., 2002). Interestingly, a strong correlation between p73 and VDR expression at transcript and protein levels upon DNA damage was observed in H1299 and SaOS2 cells (Figure 29). Monitoring the expression of p73 and VDR at multiple time points demonstrated that

p73 precedes VDR induction upon DNA damage (Figure 29). As seen with p63, a significant induction in VDR expression at transcript and protein levels was also observed with multiple isoforms of p73 (Figure 30). Additionally, silencing endogenous p73 led to reduction in endogenous VDR levels and endogenous p73 did bind to the VDR promoter in vivo, indicating VDR as a direct transcriptional target of p73 (Figure 31). These results clearly suggest a potential role for p73 in DNA damage induced VDR expression.

Recently a subset of tumor derived mutants of p53 has been shown to inhibit p73 transcriptional activity through direct physical interaction (Gaiddon et al., 2001; Li and Prives, 2007). Additionally, lack of p73 mediated growth suppression has been reported in cells harboring p53 mutations (Prabhu et al., 1998) and knock down of p53 mutants resulted in enhanced growth suppression of various cancer cell lines (Scian et al., 2004; Vikhanskaya et al., 2007). Ectopic expression of p53 (R248W) mutant resulted in down regulation of VDR expression levels observed upon DNA damage, thus indicating the requirement of p73 for the induction of VDR upon DNA damage (Figure 32). As reported earlier a direct interaction between p73 and p53 (R248W) mutant was observed in immunoprecipitation analysis (data not shown). Furthermore, silencing p73 resulted in significant reduction in VDR expression observed upon DNA damage (Figure 33). Since DNA damage induced VDR expression occurs via p73 and tumor derived mutants inhibits this regulation, one should consider the status of both p73 and mutant p53 when vitamin D mediated growth inhibitions studies are conducted in human cancer cell lines.

It is well documented that vitamin D and its analogues exert their anti proliferative activities through VDR and are extensively used for cancer chemotherapy

(Banwell et al., 2003; Campbell and Adorini, 2006; Ordonez-Moran et al., 2005; Rashid et al., 2001). Since the activity of vitamin D analogues entirely depends on the expression levels of VDR (Palmer et al., 2001), p73 mediated induction of VDR upon DNA damage should further enhance the vitamin D mediated functions. Pre-treatment of cells with etoposide resulted in a substantial increase in vitamin D transcriptional activity (Figure 35B). Further, down regulation of p73 or VDR expression levels resulted in marked decrease in etoposide mediated augmentation of vitamin D transcriptional activity (Figure 35C). These results suggest that induction of VDR by p73 upon DNA damage can sensitize the cells to vitamin D. Additionally, chemotherapeutic agents have been shown to stimulate vitamin D mediated biological functions; particularly etoposide and cisplatin were shown to stimulate vitamin D mediated differentiation and apoptosis (Hershberger et al., 2002; Torres et al., 2000). However, the molecular mechanisms behind this stimulation in vitamin D mediated anti-proliferative activates are poorly studied. Since p73 is essential for the vitamin D transcriptional activity, it is likely that p73 is essential for the stimulation of vitamin D mediated anti-proliferative activates by chemotherapeutic agents. Taken together, the results from this study demonstrate a novel p73 dependent and p53-independent activation of VDR upon DNA damage.

Osteoblasts are the main bone forming cells, which are essential for deposition of bone extracellular matrix. Osteoblasts derive from pluriopotent mesenchymal cells during embryonic development. The characteristic feature of osteoblasts is that they can be cultured in vitro to proliferate and differentiate (Stein et al., 1996). Osteoblastic differentiation is essential for proper bone formation and the osteoblastic differentiation is mediated by multiple factors including VDR (St-Arnaud, 2008). The role of vitamin D

in bone formation and in osteoblastic differentiation is well documented (Yoshizawa et al., 1997). Vitamin D has been shown to promote differentiation of mature osteoblasts through the regulation of osteoblast associated genes including osteocalcin and osteopontin (Matsumoto et al., 1991). Osteosarcomas are the most common type of bone cancers; osteosarcoma cells have characteristics of primary osteoblasts and are widely used for studying osteoblasts (Gill and Bell, 2000).

Vitamin D and vitamin D analogues are in fact shown to promote the differentiation of osteosarcoma cells (Barroga et al., 1999; Matsumoto et al., 1998). Although vitamin D exerts its biological functions on osteoblasts or osteosarcoma cells, the upstream regulation of vitamin D pathway in osteoblasts is still understudied. In this study silencing p73 resulted in a significant down regulation of basal VDR expression in SaOS2 osteosarcoma cells (Figure 36A). The down regulation of p73 in SaOS2 cells also led to the down regulation of vitamin D mediated transcriptional activity (Figure 36B). In addition, down regulation of p73 resulted in significant down regulation in vitamin D mediated induction of osteopontin and osteocalcin and the differentiation of SaOS2 cells (Figure 38). These results clearly suggest a novel upstream regulation of vitamin D signaling pathway by p73 in osteosarcoma cells. Transforming growth factor β (TGF-β) has also shown to play vital role in bone remodeling (Centrella et al., 1991). Particularly TGF-β shown to promote the proliferation of primary osteoblasts and also shown to suppress the vitamin D mediated induction of osteocalcin (Kassem et al., 2000). Interestingly, transcriptional repression of TAp73 by TGF-β has been reported in transformed keratinocytes (Waltermann et al., 2003). Thus, it is likely that TGF-β inhibits the vitamin D mediated differentiation of osteoblasts by repressing p73. Furthermore,

given both VDR and p73 null mice are runted in phenotype; it is interesting to see whether regulation of vitamin D functions by p73 is essential for bone formation during embryonic development (Yang et al., 2000; Yoshizawa et al., 1997).

Vitamin D and its analogues exert anti proliferative actions in different cancer cells through VDR and expression of p73 was reported in majority of human cancer cells (Muller et al., 2006). In light of results from this study, it is intriguing to see whether p73 is essential for VDR expression and subsequently for vitamin D mediated biological functions in all vitamin D responsive human cancer cell lines. Combinatorial use of vitamin D and its analogues with chemotherapeutic agents have shown to be more potent in promoting growth inhibition than using them alone (Hershberger et al., 2002; Moffatt et al., 1999; St-Arnaud, 2008). Clinical trials are currently underway, testing the combinatorial use of vitamin D analogues and chemotherapeutic agents for cancer chemotherapy (Fakih et al., 2007; Trump et al., 2006). Interestingly, a wide range of chemotherapeutic agents have been shown to induce the p73 expression levels in multiple cancer cells (Irwin et al., 2003; Urist et al., 2004). Results from this study demonstrated that regulation of VDR by p73 upon DNA damage can potentiate vitamin D mediated differentiation (Figure 40 & 41). Thus, these findings clearly suggest a novel upstream regulation of the vitamin D signaling pathway by p73 and provide evidence for the requirement of p73 for vitamin D mediated osteoblastic differentiation. Altogether, outcomes from this part of study will aid in designing better strategies for combinatorial use of chemotherapeutic agents with vitamin D.

In conclusion, this dissertation work illustrated the biological significance associated with the regulation of VDR by p53 family members (Figure 42). Biochemical

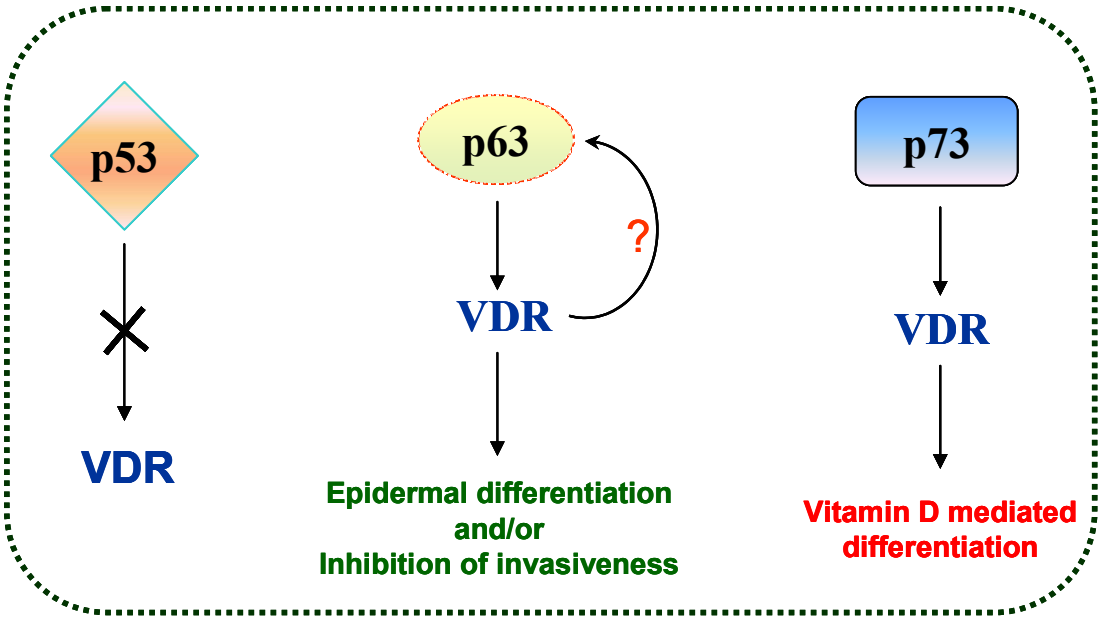


Figure 42. Model illustrating the differential regulation of VDR by p53, p63 and p73. VDR is activated by both p63 and p73 but not p53. Regulation of VDR by p63 is associated with inhibition of invasiveness and possibly in epidermal differentiation. p73 appears to play a role in vitamin D mediated differentiation by regulating VDR.

and molecular studies demonstrated VDR as a direct transcriptional target of both p63 and p73. In contrast, p53 had no effect on VDR directly or indirectly. Functional studies in A431 cells demonstrated that regulation of VDR by p63 is associated with the inhibition of invasiveness. In addition, VDR appears to be regulating the p63 expression in A431 cells, suggesting a possible feedback regulation of p63 through VDR. However, additional functional studies are required to uncover the role of VDR in p63 mediated epidermal differentiation. Furthermore, regulation of VDR by p73 proves to be essential for vitamin D mediated osteoblastic differentiation. Finally DNA damage sensitized the cells to vitamin D mediated differentiation through p73. Taken together, these observations highlight a novel role for p73 in vitamin D functions and will enhance the effects of vitamin D based cancer chemotherapeutics.

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