Published in final edited form as:
J Steroid Biochem Mol Biol. 2023 September ; 232: 106352. doi:10.1016/j.jsbmb.2023.106352.

Vitamin D receptor cross-talk with p63 signaling promotes epidermal cell fate

Yuko Oda[a], **Christian T. Wong**[b,e], **Dennis H. Oh**[b,e], **Mark B. Meyer**[c], **J. Wesley Pike**[d], **Daniel D. Bikle**[a,*]

[a]Departments of Medicine and Endocrinology, United States

[b]Department of Dermatology, University of California San Francisco, United States

[c]Department of Nutritional Sciences, University of Wisconsin-Madison, United States

[d]Department of Biochemistry, University of Wisconsin-Madison, United States

[e]San Francisco VA Health Care system, United States

Abstract

The vitamin D receptor with its ligand 1,25 dihydroxy vitamin D_3 ($1,25D_3$) regulates epidermal stem cell fate, such that VDR removal from *Krt14* expressing keratinocytes delays re-epithelialization of epidermis after wound injury in mice. In this study we deleted *Vdr* from Lrig1 expressing stem cells in the isthmus of the hair follicle then used lineage tracing to evaluate the impact on re-epithelialization following injury. We showed that *Vdr* deletion from these cells prevents their migration to and regeneration of the interfollicular epidermis without impairing their ability to repopulate the sebaceous gland. To pursue the molecular basis for these effects of VDR, we performed genome wide transcriptional analysis of keratinocytes from *Vdr* cKO and control littermate mice. Ingenuity Pathway analysis (IPA) pointed us to the TP53 family including p63 as a partner with VDR, a transcriptional factor that is essential for proliferation and differentiation of epidermal keratinocytes. Epigenetic studies on epidermal keratinocytes derived from interfollicular epidermis showed that VDR is colocalized with p63 within the specific regulatory region of MED1 containing super-enhancers of epidermal fate driven transcription factor genes such as *Fos* and *Jun*. Gene ontology analysis further implicated that Vdr and p63 associated genomic regions regulate genes involving stem cell fate and epidermal differentiation. To demonstrate the functional interaction between VDR and p63, we evaluated the response to $1,25(OH)_2D_3$ of keratinocytes lacking p63 and noted a reduction in epidermal cell fate determining transcription factors such as *Fos, Jun*. We conclude that VDR is required for the epidermal stem cell fate orientation towards interfollicular epidermis. We propose that this role of VDR involves cross-talk with the epidermal master regulator p63 through super-enhancer mediated epigenetic dynamics.

*Correspondence to: Endocrine Research 111N, Veterans Affairs Medical Center San Francisco, University of California San Francisco, 1700 Owens Street, San Francisco, CA 94158, United States. daniel.bikle@ucsf.edu (D.D. Bikle).

Author statement

We are resubmitting an extensively revised manuscript newly titled to reflect the essence of the revised manuscript. Major revised sections are marked with underlining, although the numerous smaller changes are not as most of the manuscript has been modified to some degree.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jsbmb.2023.106352.

Keywords

Vitamin D; Vitamin D receptor; P63; Epigenetics; Keratinocytes, wounding; Cell fate

1. Introduction

Chronic skin wounds are estimated to affect 6.5 million patients in the US at a cost of over $25 billion [1]. A number of studies have noted an association between poor wound healing and vitamin D deficiency [2–5]. We have focused on the role of vitamin D and calcium signaling in the epidermis in the control of stem cell (SC) activation and function during the initial response to wounding of the skin. Adult SCs residing in regenerative tissues like the epidermis and hair follicles (HF) play essential roles in the maintenance of those tissues. Understanding the mechanisms controlling adult SC is one of the fundamental goals in the field of skin biology. The skin provides an excellent model system for the study of adult SC in tissue regeneration. Skin epithelia are derived from the ectoderm and differentiate into the interfollicular epidermis (IFE), sebaceous gland (SG) and HF during the embryonic developmental process. After birth, adult SC residing in the basal layer of the epidermis (eSC), junctional zone/ infundibulum (jSC), isthmus (iSC) and bulge (bSC) regions of the HF are responsible for the regeneration of the IFE, SG and the cycling portion of the HF, respectively [6–9]. In the IFE, this regeneration is continuous to produce transient amplifying cells (TAC), which leave the basal layer and differentiate producing proteins such as keratin 1 (Krt1), Krt10, involucrin, filaggrin and loricrin. Cells from the junctional zone/infundibulum in the distal portion of the HF closest to the epidermis contribute to this process. In the isthmus iSC provide cells for the maintenance of the SG. In contrast, the proximal portion of the HF is cyclic, with activation initiated with signals between the bSC and dermal papilla [10–12]. These SC have distinctive markers in their separate niches (eg. Lgr5 for bSC, Lgr 6 for iSC, Lrig1 for jSC) but as discussed below these distinctive markers are lost after wounding [13]. When the skin is wounded the progeny of SC from all regions of the HF and IFE contribute at least initially [6,9], although to variable extent.

We have shown that IFE and HF SC express high levels of VDR [14,15]. Moreover, we have found that SC loss, decreased activation, and delayed differentiation and migration of progeny after wounding are present in the *Vdr* cKO mouse [15]. *Vdr* removal also leads to abnormal utricle structures in which epidermal makers and lipids accumulate, further supporting a role for VDR in epidermal cell fates. Our previous lineage tracing studies demonstrated that *Vdr* deletion prevents *Krt14* labeled keratinocytes to regenerate interfollicular epidermis after skin wounding, although Krt14 is not specific for stem cells [15]. Therefore, in this study we labeled Lrig1^{+} iSC and traced their cell fate after wound injury. Although we identified a specific role of VDR for stem cells to commit to interfollicular epidermis, the molecular basis for the function is not well understood.

P63 is known as a master regulator to control epidermal proliferation and differentiation in skin. P63 induces VDR by direct binding to the *VDR* promoter [16]. Like *Vdr*, deletion of *p63* blocks epidermal differentiation [17]. Previous studies with ChIP-seq determined the binding of p63 to enhancers and super-enhancers (SE) for ongoing transcription during

keratinocyte differentiation [18,19]. One potential partnering transcription factor to p63 in these super enhancers was VDR. These sites were enriched in super-enhancers considered critical for cell fate determination. These large SEs mediate transcriptional regulatory machinery essential for gene expression of fate determining genes [20,21], that differ from typical enhancers in size and ability to induce transcription. Dense incorporation of key transcription factors together in the Mediator complex, including MED1, promote gene transcription by linking enhancers to the RNA polymerase complex at transcriptional start sites (TSS) in gene promoter sites. Gene activation is accompanied with elevated histone acetylation (H3K27ac) [20], and the enhancer domains are separated by DNA insulator CTCF [22]. In addition, a previous study showed binding profiles of p63 to enhancers and super-enhancers in keratinocytes [19]. Therefore, we examined the ability of VDR and p63 to promote epidermal cell fate by utilizing super-enhancer based epigenetic machinery.

P63 has two known isoforms from alternative promoters. Transcriptionally active p63 (TAp63) includes all 14 exons, whereas the ΔNp63 isoform starts in an intron downstream of exon 3. Though ΔNp63 lacks the TA domain (exons 1–3), it retains the ability to bind to DNA and induce genes [23]. TAp63 and ΔNp63 bind to the same DNA element, and in the current study these isoforms will not be distinguished. That said, it is the ΔNp63 isoform that is most highly expressed in the basal layer of the epidermis and is critical for keratinocyte differentiation [24]. In the current study we show that keratinocytes lacking p63 have a blunted response to $1,25(OH)_2D_3$ ($1,25D_3$) indicating the importance of the p63/VDR interaction.

In the current study we first demonstrate that VDR is required for injury induced cell fate conversion towards interfollicular epidermis using lineage tracing. Through epigenetic approaches, we then explored molecular foundations for VDR to drive epidermal fate through superenhancer associated cross-talk with the epidermal master regulator p63.

2. Materials and methods

2.1. Animals

The floxed *Vdr* mice (*Vdr*tm1Pcn Δex3-flox/flox) (C57BL6) were a gift from S. Kato Japan. *Krt1*4-Cre (Tg(*Krt14*-cre^{amc}) (C57BL6) mice and mice expressing the Rosa tomato (TdT) reporter gene (Gt(ROSA) 26Sortm9(CAG-tdTomato)Hze) were from the Jackson Laboratory. Tamoxifen inducible *Lrig1*-CreERT2 mice were a gift from Dr. Kim B. Jensen. All experiments were approved by the Institutional Animal Care and Ethics Committee at the San Francisco VA Medical Center.

2.2. Lineage tracing

The red fluorescent reporter gene Rosa TdTomato (TdT) was introduced into mice expressing tamoxifen (Tam) inducible *Lrig1*-CreERT2 with (cKO) or without (controls) the floxed *Vdr* gene. First, the triple transgenic mice expressing floxed *Vdr, Lrig1*-CreERT2 and TdT were prepared, so that red TdT is expressed upon *Lrig1* driven tamoxifen inducible Cre recombination. The *Vdr* gene was deleted from TdT labeled cells, and TdT expression was activated by single intraperitoneal injection of low dose tamoxifen 10 ug/d body weight

(Sigma) dissolved in corn oil at postnatal day 21 (P21). Next day, a full-thickness skin biopsy punch wound (3 mm) was made in back skin, then the skin was allowed to heal until wounds were completely closed at 30 or 35 days. The skin biopsies at the 0 day and 30 (35) days post wounding time points were harvested and frozen sections were prepared to monitor mobility of red fluorescent cells using a Zeiss fluorescent microscope, in which the images were processed with Zen pro software. The red area in IFE and SG were manually evaluated in 8–10 different fields for control and cKO mice (2 each). Statistical significance was evaluated by the Student t-test.

2.3. Gene expression profiling microarray analysis

Gene expression profiles were analyzed using an Illumina beads chip based gene array (Mouse Ref-8 v2.0 Ambion) in the UCLA Neuroscience Genomics Core. The data were normalized by Genome Studio (Illumina), and analyzed by using pathway software IPA (Ingenuity Systems). The array data were submitted to a public data base (GEO/NCBI/NIH http://www.ncbi.nlm.nih.gov/geo). Data for *Vdr* cKO keratinocytes vs CON keratinocytes are available as an accession number GSM1679838 and GSM1679839 under super-series GSE68729.

2.4. Chromatin immunoprecipitation

Chromatin IP was performed using the LowCell# ChIP kit (Diagenode) for H3K27ac, and by using the iDeal ChIP-kit for transcription factors (Diagenode) for VDR, P63, MED1 and CTCF according to the manufacturer's protocol with some modifications as described.

Pre-confluent keratinocytes were treated with vehicle (EtOH) or $1,25D_3$ (1×10^{-7}M) for 4 h. They were cross-linked by 1 % paraformaldehyde for 8 min for H3K27ac and for 15 min for MED1/CTCF/VDR/p63/CTCF then quenched with 0.125 M glycine. Whole cell lysates (H3K27ac) or purified chromatin (MED1/CTCF/VDR/p63/CTCF) were sonicated by Covaris S2200 ultrasonicator (Covaris, Inc.). The shearing conditions were optimized to obtain the DNA fragments with an average length of 300 ± 50 bp, the size of which was verified by an Agilent 2100 Bioanalyzer (Agilent Technologies). Sheared chromatin was immunoprecipitated with Protein A-coated magnetic beads (Diagenode) preincubated with antibodies: 5 mg of VDR (Santa Cruz C20), 3 μg of p63 (Cell Signaling, D2K8X), 3 μg of antibody against H3K27ac (ab4729, Abcam), 1 μg CTCF antibody (Diagenode) and 4.5 μg MED1 antibody (Bethyl). Experiments were carried out in triplicates, and a chromatin input sample was used as a reference to subtract background for peak calling. Complexes were washed, eluted from the beads with washing buffer and crosslinks were reversed by incubation at 65 °C for 4 h. Immunoprecipitated DNA along with genomic DNA (Input) were purified using IPure v2 kit (Diagenode). IP efficiency was confirmed by qPCR using primers provided in the kits.

2.5. ChIP-seq library preparation, DNA sequencing and bioinformatic analyses

DNA Sequencing libraries were generated using the Accel-NGS 2S Plus DNA library kits (Swift Sciences) and amplified by PCR for 11 cycles. To remove high MW smear in the library, right side size selection was conducted by using SPRI beads (Beckman Coulter). The library was quantified by Agilent 2100 Bioanalyzer with the High sensitivity DNA assay.

They were sequenced on an Illumina HiSeq 4000 (UCSF Center of advanced technology) in a single strand 50 bp run (combining 8 libraries per lane).

Sequencing reads were mapped to the human genome hg19 using Bowtie2 with standard parameters only allowing uniquely aligned reads as previously described [25–29]. Peaks were called against input using MACS2 for VDR, P63, H3K27ac, MED1, and CTCF using both broad Peak and gapped Peak output [30]. Motif analysis (de novo and known) was performed using HOMER software [31]. All data were displayed using UCSC genome browser. Enhancers were stitched and super-enhancers were defined using ROSE code as described [20]. ROSE was run with stitching distance of 12.5 kb. Dense enhancers were then assigned to the RefSeq genes whose TSS was the nearest to the center of the identified dense enhancers. ChIP-seq data for VDR, p63, MED1, H3K27ac, and CTCF in vehicle and $1,25D_3$ treated keratinocytes are available in accession numbers GSE154221 and GSExxx.

2.6. Keratinocyte cultures

Primary human keratinocytes (NHK) were isolated from neonatal foreskin and maintained in defined Keratinocyte Serum Free Basal Medium (K-SFM) (Thermo Fisher) supplemented with the growth factors and 0.07 mM $CaCl_2$ (Thermo Fisher). Keratinocytes in third or fourth passages were treated with 0.1 % EtOH (vehicle) or $1,25D_3$ (1×10^{-7} M) for 4 h and used for ChIP-seq studies. The immortalized keratinocyte cell line N/TERT prepared by overexpression of telomerase subunit TERT (the gift of J. Rheinwald [32]) was maintained as previously described [33]. The cells were transduced 4:1 by an HIV-1-based lentivirus that stably expresses short-hairpin RNAs (shRNA) encoding non-targeting sequences (shNTS) or sequences that target the DNA binding domain common in all p63 isoforms (shp63), in which the shRNAs were incorporated into the lentivirus using commercial kits (ViraPower Lentiviral Expression System, Invitrogen, Carlsbad, CA). The shRNA transcript targeting p63 is:5′GAG CUG AGC CGU GAA UUC AAC GAG GUU CAA GAG ACC UCG UUG AAU UCA CGG CUC AGC UCU U3′. These cells were maintained in the supplemented K-SFM with 0.07 mM $CaCl_2$ and 10 mg/mL blasticidin that provides selection pressure to maintain shRNA transfection. Control NTS-N/TERT and p63 targeted shp63 N/TERT cells were then treated with $1,25D_3$ (1×10^{-8} M) overnight (the blasticidin was removed) and used for RT-PCR gene expression analysis.

2.6.1. Reproducibility and statistical analysis—All the experiments using *Vdr* cKO and littermate control mice (n = 3) were repeated with at least two litters, and reproducibility was confirmed. The experiments using immortalized keratinocytes (TERT and shp63) were conducted in three independent cultures and reproducibility was confirmed. Statistical significance was calculated using software integrated methods or two-tailed unpaired Student's t-test. If not differently noted, differences with a *p*-value of less than 0.05 were considered as statistically significant.

Other methods are described in Supplemental information.

3. Results and discussion

3.1. Deletion of VDR impairs the proliferation and migration of Lrig1 expressing isthmus stem cells towards the interfollicular epidermis during wound re-epithelialization

We determined the role of VDR in regulating the ability of epidermal stem cells to respond to wounding. As proof of concept we used a mouse in which *Lrig1* expressing isthmus stem cells (iSC) were labeled by a fluorescent marker (tomato) at the same time the *Vdr* was deleted from those cells, and the effect of *Vdr* deletion was investigated by lineage tracing experiments as described [6,11]. Mice lacking *Vdr* were generated by mating tamoxifen (Tam) inducible *Lrig1*CreERT2 mice expressing Rosa TdTomato (TdT) (red fluorescence) with floxed *Vdr* mice (cKO). They were compared to control mice (CON) with *Lrig1*CreERT2 and RosaTdT but lacking floxed *Vdr*. A single injection of low dose Tam was given to make clonal labeling of Lrig1 iSC to facilitate the ability to follow the progeny into the wound site. After labeling, a full-thickness skin biopsy (5 mm) was taken from the back skin. As expected, only a few isthmus stem cells are labeled in either CON or *Vdr* cKO 2 day after Tam injection in the biopsy samples as shown by clonal fluorescent labeling (Fig. 1a arrows in upper panels). After the wounds were completely closed (30–35 days after wound excision), numerous labeled isthmus SC were found in the interfollicular epidermis as well as SG in the CON mice (Fig. 1a arrows in lower left panel). In contrast, in the cKO skin there were fewer labeled cells in the IFE, but SG labeling was equivalent to controls (Fig. 1a lower right panel cKO), in which the location of iSC and SG and IFE is shown (Fig. 1b upper diagram). These results indicate decreased ability of these *Vdr* depleted iSC cells to participate in the regeneration of the IFE. The results were quantitated by counting the number of red cells per IFE and SG on multiple sections and normalized by CON (Fig. 1b, graph n = 4, $P < 0.05$).

3.2. Deletion of Vdr affects genes involved in keratinocyte proliferation and differentiation through p53/63 signaling pathways

Our lineage tracing studies showed the cell fate changes in Lrig1 expressing iSC cells, although the overall wound healing rate was unchanged, as only a portion of these cells had their *Vdr* deleted. To identify a role of VDR through gene expression profiling, we utilized our previously established mouse model in which *Vdr* was deleted in keratinocytes with Krt14 cre [15] as these mice showed a decrease in rate of wound healing.

The transgenic mice with floxed *Vdr* in combination with the *Krt14* driven Cre transgene were compared to control littermates that have floxed *Vdr* but no cre (CON). As Vdr deletion affects hair cycling in skin, we isolated keratinocytes from the skin at 3 mo (n = 3) of age when the skin is at resting telogen in both *Vdr* cKO and CON so that we eliminate hair cycling effects on gene expression. We also used mice maintained on a low calcium diet that our previous studies showed delays in wound re-epithelialization [34]. The RNA was isolated and the gene expression profiles were analyzed using an Illumina beads-based microarray including 25,600 annotated transcripts and 19,100 genes as described [35] (data are available in GSE68727 under super-series GSE68729). In addition to decreased hair differentiation genes and hair follicle stem cell markers in *Vdr* cKO, IPA (Ingenuity Pathway Analysis) of whole transcriptomic data from *Vdr* cKO keratinocytes

revealed abnormalities in organismal injury, cellular proliferation and movement in *Vdr* cKO (Table 1 upper panel). Furthermore, IPA analysis also identified members of the p53 family (Tp63/53/73) as top upstream inducers of that were down-regulated by *Vdr* deletion (Table 1 lower panel) implying that VDR exerts at least some of its functions in combination with Tp53/63 signaling. Together with our in vivo observations in *Vdr* cKO of delayed wound re-epithelialization, these results led us to investigate the underlying epigenetic and transcriptional mechanisms by which VDR promotes epidermal proliferation and differentiation in collaboration with p63.

3.3. VDR is co-localized with p63 in chromatin regulatory regions for epidermal fate driving transcription factors Fos, Jun and Sox7.

To further explore the interaction between VDR and the p53/p63 family at genomic DNA levels, we performed chromatin immunoprecipitation and sequencing (ChIP-seq) analysis, and determined the genes associated with VDR/p53/p63 regulation that are relevant to the response of stem cells to wounding with respect to proliferation and differentiation. We performed VDR and p63 ChIP-seq of the cultured keratinocytes that were treated with either vehicle or $1,25D_3$ (1×10^{-7} M). The cells were treated for 4 h to identify the primary response to $1,25D_3$. ChIP-seq was also performed with MED1 to identify the super-enhancers that are critical to control cell fate genes [20], H3K27ac to mark actively transcribed regions and super-enhancers (SE) [20] because it is known that histone acetylation (H3K27ac) generally co-localizes with Mediator such as Med1 at SEs and acts as a major inducer of gene expression by increasing chromatin accessibility [20,36], and CTCF to define boundaries of chromatin loops to insulate functionally distinct enhancers and SEs [22]. We found a number of VDR binding peaks accumulating close to the transcription start site (TSS), but the majority were found in the distal intergenic regions, in which a number of VDR binding peaks were within super-enhancers that are marked by MED1 binding. The VDR peaks were proximate within 100 bp to p63 binding peaks in 99 sites in the absence of $1,25D_3$, but the number increased to 799 sites after $1,25D_3$ administration as shown in a Venn Diagram (Fig. 2a). To explore biological function of VDR/p63/53 peaks, we conducted gene ontology (GO) analysis of genes associated with the 799 sites. As can be seen in Fig. 2b, these genes were found to represent regulators of wound healing, stem cell differentiation, adherens junction formation, and response to calcium ion that are major factors in epidermal differentiation (Fig. 2b pink highlight). The associated genes in these biological processes are shown in Supplemental Table SI.

We first focused on Chip-seq profiles of the *Fos* and *Jun* genes because they are the major cell fate determining transcription factors to control keratinocyte proliferation and differentiation. In addition, *Fos* is directly regulated by the $1,25D_3$ /VDR through epigenetic mechanisms as shown in our earlier study [26]. Here, we found that the VDR binding peaks were colocalized with p63 in epidermal keratinocytes when induced by $1,25D_3$ administration. These peaks are located at – 24 kb and – 57 kb upstream of the *Fos* promoter within the SE (Fig. 3a red bar) that is marked by broad peaks of MED1 spanning a long DNA stretch (68 kb). The MED1 peaks are higher in $1,25D_3$ treated cells. These peaks are also actively transcribed as mapped by H3K27ac and located within one DNA loop not separated by CTCF (Fig. 3a vehicle, and $1,25D_3$ treated cells are shown by

yellow and blue peaks respectively, and overlap peaks are shown as green). Moreover, these VDR and p63 binding sites are just upstream of the TCF site (Fig. 3b) supporting previous observations regarding the interaction between VDR and β-catenin signaling in the regulation of keratinocyte differentiation [37,38]. We also found VDR/p63 overlapped peaks at – 65 kp and – 149 kb upstream of the promoter of the *Jun* gene (Fig S2 peak allocations are the same as Fig. 3a) that functions with *Fos* as an AP-1 factor to drive epidermal differentiation. These peaks were located within the large SE spanning over 222 kb DNA (red bar) in the H3K27ac marked region. The downstream part of the large SE was separated by 3 CTCF sites indicating functional diversity of the SE (Fig S2).

These data together demonstrated that VDR is colocalized with p63 within the SEs, suggesting their functional role in cell fate decision as described in a previous study demonstrating that incorporation of key transcription factors in SEs is associated with high level transcription of cell fate genes [20].

In Table 2, we list the binding motifs of VDR and p63 that were further enriched in the SEs in a $1,25D_3$ dependent manner suggesting the importance of VDR/p63 interactions in regulating cell fate determination as would be important during epidermal differentiation.

3.4. P63 is required for $1,25D_3$ induction of epidermal fate transcription factors

Next, we evaluated the functional relationship between VDR and p63. Previous studies showed the mutual regulation of VDR with partner transcription factors as seen in the case of β-catenin (TCF) to regulate epidermal differentiation [38]. Previous studies showed their regulation at expression levels. VDR is required for p63 expression as Vdr silencing decreased basal expression of an isoform of p63 (ΔNp63a) in primary keratinocytes [40]. However, ΔNp63a does not affect VDR expression, although another isoform does [16].

We then examined the functional requirement of p63 for VDR function by measuring ligand induced VDR transactivation. As VDR is required for epidermal differentiation we utilized epidermal keratinocyte cell lines derived from human IFE. These cells were immortalized by overexpression of the telomerase subunit TERT. The expression of p63 was decreased by a lentivirus carrying a short hairpin RNA targeting both isoforms of p63 (DNA binding domain) or a non-targeted shRNA (TERT) (Fig. 4a). These cells were then treated with $1,25D_3$ (1×10^{-8} M overnight), and their responses were evaluated by measuring gene expression through RT-qPCR. As p63 is important for cell proliferation, RNA recovery was moderately decreased in shp63 cells compared to control, suggesting lower cellularity (86.3 $\pm$7.2 %, $p < 0.05$) in shp63 cells. However, we compensated for the decrease by using the same amount of RNA in each RT, and evaluated relative expression compared to control gene *L19* by qPCR, then calculated percentile expression compared to control TERT cells. When P63 was knocked down, vitamin D induction rate was substantially decreased in the transcription factors, *FOS, JUN* and *SOX7* but basal expressions were not affected (Fig. 4b upper panel). These effects are specific as the known VDR regulated innate immune gene *CAMP* was not affected (Fig. 4b lower panel). We also found high peaks of VDR and p63 in the *Sox7* gene within MED1 labeled SE, that are associated with H3K27ac within one DNA loop (CTCF)(Fig S2b). Sox7 regulates cell fate of mesoderm lineages [39] and is expressed in keratinocytes (our data), but its function in skin is not known. These results suggest that

VDR drives normal epidermal cell fate by regulating these transcription factors by crosstalk with p63 signaling.

Here, we propose a new mode of VDR action in which VDR regulates cell fate transcription factors through cross-talk with p63 at epigenetic levels. VDR and p63 are colocalized in super-enhancers to control critical genes that promote epidermal fate. As p63, in particular the ΔNp63 isoform, is a key regulator of keratinocyte differentiation and TAp63 is induced in wound injury, our data suggest that p63 and VDR cross-talk regulate this process enabling the change of cell fate towards interfollicular epidermal lineage.

In conclusion, we have demonstrated that VDR is required for the epidermal differentiation towards interfollicular epidermis that is essential during wound induced re-epithelialization. These processes involve at least partially the interaction of VDR with the epidermal master regulator p63.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

We thank Ms Sun Hee Kim Wong for her assistance in maintaining the mice and technical assistance. This work was supported by the NIH grant R01 AR050023 (DDB), DOD grant CA110338 (DDB), VA Merit I01 BX003814–01 (DDB).

Data availability

Data will be made available on request.

References

[1]. Sen CK, et al. , Human skin wounds: a major and snowballing threat to public health and the economy, Wound Repair Regen.: Off. Publ. Wound Heal. Soc. Eur. Tissue Repair Soc 17 (2009) 763–771, 10.1111/j.1524-475X.2009.00543.X.

[2]. Burkievcz CJ, et al. , Vitamin D deficiency in patients with chronic venous ulcers, Rev. do Col. Bras. De. Cir 39 (2012) 60–63.

[3]. Zubair M, Malik A, Meerza D, Ahmad J, 25-Hydroxyvitamin D [25(OH)D] levels and diabetic foot ulcer: is there any relationship? Diabetes Metab. Syndr 7 (2013) 148–153, 10.1016/j.dsx.2013.06.008. [PubMed: 23953180]

[4]. Smart H, AlGhareeb AM, Smart SA, 25-hydroxyvitamin D deficiency: impacting deep-wound infection and poor healing outcomes in patients with diabetes, Adv. Ski. Wound Care 32 (2019) 321–328, 10.1097/01.ASW.0000559614.90819.45.

[5]. Burkiewicz CJ, et al. , Vitamin D and skin repair: a prospective, double-blind and placebo controlled study in the healing of leg ulcers, Rev. Col. Bras. Cir 39 (2012) 401–407, 10.1590/s0100-69912012000500011. [PubMed: 23174792]

[6]. Mascre G, et al. , Distinct contribution of stem and progenitor cells to epidermal maintenance, Nature 489 (2012) 257–262, 10.1038/nature11393. [PubMed: 22940863]

[7]. Lim X, et al. , Interfollicular epidermal stem cells self-renew via autocrine Wnt signaling, Science 342 (2013) 1226–1230, 10.1126/science.1239730. [PubMed: 24311688]

[8]. Jensen KB, et al. , Lrig1 expression defines a distinct multipotent stem cell population in mammalian epidermis, Cell Stem Cell 4 (2009) 427–439, 10.1016/j.stem.2009.04.014. [PubMed: 19427292]

[9]. Plikus MV, et al. , Epithelial stem cells and implications for wound repair, Semin. Cell Dev. Biol 23 (2012) 946–953, 10.1016/j.semcdb.2012.10.001. [PubMed: 23085626]

[10]. Page ME, Lombard P, Ng F, Gottgens B, Jensen KB, The epidermis comprises autonomous compartments maintained by distinct stem cell populations, Cell Stem Cell 13 (2013) 471–482, 10.1016/j.stem.2013.07.010. [PubMed: 23954751]

[11]. Alcolea MP, Jones PH, Lineage analysis of epidermal stem cells, Cold Spring Harb. Perspect. Med 4 (2014), a015206 10.1101/cshperspect.a015206. [PubMed: 24384814]

[12]. Solanas G, Benitah SA, Regenerating the skin: a task for the heterogeneous stem cell pool and surrounding niche, Nat. Rev. Mol. Cell Biol. 14 (2013) 737–748, 10.1038/nrm3675. [PubMed: 24064540]

[13]. Joost S, et al. , Single-cell transcriptomics of traced epidermal and hair follicle stem cells reveals rapid adaptations during wound healing, Cell Rep. 25 (2018) 585–597, 10.1016/j.celrep.2018.09.059. [PubMed: 30332640]

[14]. Oda Y, et al. , Combined deletion of the vitamin d receptor and calcium-sensing receptor delays wound re-epithelialization, Endocrinology 158 (2017) 1929–1938, 10.1210/en.2017-00061. [PubMed: 28368538]

[15]. Oda Y, et al. , Vitamin D receptor is required for proliferation, migration, and differentiation of epidermal stem cells and progeny during cutaneous wound repair, J. Investig. Dermatol 138 (2018) 2423–2431, 10.1016/j.jid.2018.04.033. [PubMed: 29787748]

[16]. Kommagani R, Caserta TM, Kadakia MP, Identification of vitamin D receptor as a target of p63, Oncogene 25 (2006) 3745–3751, 10.1038/sj.one.1209412. [PubMed: 16462763]

[17]. Truong AB, Kretz M, Ridky TW, Kimmel R, Khavari PA, p63 regulates proliferation and differentiation of developmentally mature keratinocytes, Genes Dev. 20 (2006) 3185–3197, 10.1101/gad.1463206. [PubMed: 17114587]

[18]. Kouwenhoven EN, et al. , Transcription factor p63 bookmarks and regulates dynamic enhancers during epidermal differentiation, EMBO Rep. 16 (2015) 863–878, 10.15252/embr.201439941. [PubMed: 26034101]

[19]. Sethi I, Gluck C, Zhou H, Buck MJ, Sinha S, Evolutionary re-wiring of p63 and the epigenomic regulatory landscape in keratinocytes and its potential implications on species-specific gene expression and phenotypes, Nucleic Acids Res. 45 (2017) 8208–8224, 10.1093/nar/gkx416. [PubMed: 28505376]

[20]. Whyte WA, et al. , Master transcription factors and mediator establish superenhancers at key cell identity genes, Cell 153 (2013) 307–319, 10.1016/j.cell.2013.03.035. [PubMed: 23582322]

[21]. Adam RC, et al. , Pioneer factors govern super-enhancer dynamics in stem cell plasticity and lineage choice, Nature 521 (2015) 366–370, 10.1038/nature14289. [PubMed: 25799994]

[22]. Vietri Rudan M, et al. , Comparative Hi-C reveals that CTCF underlies evolution of chromosomal domain architecture, Cell Rep. 10 (2015) 1297–1309, 10.1016/j.celrep.2015.02.004. [PubMed: 25732821]

[23]. Fisher ML, Balinth S, Mills AA, p63-related signaling at a glance, J. Cell Sci 133 (2020) 10.1242/jcs.228015.

[24]. Koster MI, Roop DR, Mechanisms regulating epithelial stratification, Annu Rev. Cell Dev. Biol 23 (2007) 93–113, 10.1146/annurev.cellbio.23.090506.123357. [PubMed: 17489688]

[25]. Meyer MB, Benkusky NA, Pike JW, The RUNX2 cistrome in osteoblasts: characterization, down-regulation following differentiation, and relationship to gene expression, J. Biol. Chem 289 (2014) 16016–16031, 10.1074/jbc.M114.552216. [PubMed: 24764292]

[26]. Meyer MB, Benkusky NA, Pike JW, 1,25-Dihydroxyvitaniin D3 induced histone profiles guide discovery of VDR action sites, J. Steroid Biochem. Mol. Biol 144 (Pt A) (2014) 19–21, 10.1016/j.jsbmb.2013.09.005. [PubMed: 24041718]

[27]. Meyer MB, Goetsch PD, Pike JW, VDR/RXR and TCF4/beta-catenin cistromes in colonic cells of colorectal tumor origin: impact on c-FOS and c-MYC gene expression, Mol. Endocrinol 26 (2012) 37–51, 10.1210/me.2011-1109. [PubMed: 22108803]

[28]. Langmead B, Trapnell C, Pop M, Salzberg SL, Ultrafast and memory-efficient alignment of short DNA sequences to the human genome, Genome Biol. 10 (2009) R25, 10.1186/gb-2009-10-3-r25. [PubMed: 19261174]

[29]. Meyer MB, et al. , A kidney-specific genetic control module in mice governs endocrine regulation of the cytochrome P450 gene Cyp27bl essential for vitamin D3 activation, J. Biol. Chem 292 (2017) 17541–17558, 10.1074/jbc.M117.806901. [PubMed: 28808057]

[30]. Zhang Y, et al. , Model-based analysis of ChIP-Seq (MACS, Genome Biol. 9 (2008) R137, 10.1186/gb-2008-9-9-r137. [PubMed: 18798982]

[31]. Heinz S, et al. , Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities, Mol. Cell 38 (2010) 576–589, 10.1016/j.molcel.2010.05.004. [PubMed: 20513432]

[32]. Dickson MA, et al. , Human keratinocytes that express hTERT and also bypass a p16(INK4a)-enforced mechanism that limits life span become immortal yet retain normal growth and differentiation characteristics, Mol. Cell Biol 20 (2000) 1436–1447, 10.1128/MCB.20.4.1436-1447.2000. [PubMed: 10648628]

[33]. Dickson MA, et al. , Human keratinocytes that express hTERT and also bypass a p16(INK4a)-enforced mechanism that limits life span become immortal yet retain growth and differentiation characteristics, Mol. Cell. Biol 20 (2000) 1436–1447. [PubMed: 10648628]

[34]. Nowak JA, Fuchs E, Isolation and culture of epithelial stem cells, Methods Mol. Biol 482 (2009) 215–232, 10.1007/978-1-59745-060-7_14. [PubMed: 19089359]

[35]. Yoshizaki K, et al. , Ablation of coactivator Med1 switches the cell fate of dental epithelia to that generating hair, PLOS One 9 (2014), e99991 10.1371/journal.pone.0099991. [PubMed: 24949995]

[36]. Hnisz D, et al. , Super-enhancers in the control of cell identity and disease, Cell 155 (2013) 934–947, 10.1016/j.cell.2013.09.053. [PubMed: 24119843]

[37]. Hu L, Bikle DD, Oda Y, Reciprocal role of vitamin D receptor on beta-catenin regulated keratinocyte proliferation and differentiation, J. Steroid Biochem. Mol. Biol 144 (Pt A) (2014) 237–241, 10.1016/j.jsbmb.2013.11.002. [PubMed: 24239508]

[38]. Palmer HG, Anjos-Afonso F, Carmeliet G, Takeda H, Watt FM, The vitamin D receptor is a Wnt effector that controls hair follicle differentiation and specifies tumor type in adult epidermis, PLOS One 3 (2008), e1483. [PubMed: 18213391]

[39]. Doyle MJ, et al. , Sox7 regulates lineage decisions in cardiovascular progenitor cells, Stem Cells Dev. 28 (2019) 1089–1103, 10.1089/scd.2019.0040. [PubMed: 31154937]

[40]. Hill NT, et al. , 1alpha, 25-dihydroxyvitamin D(3) and the vitamin D receptor regulates DeltaNp63alpha levels and keratinocyte proliferation, Cell Death Dis. 6 (2015), e1781 10.1038/cddis.2015.148. [PubMed: 26068789]

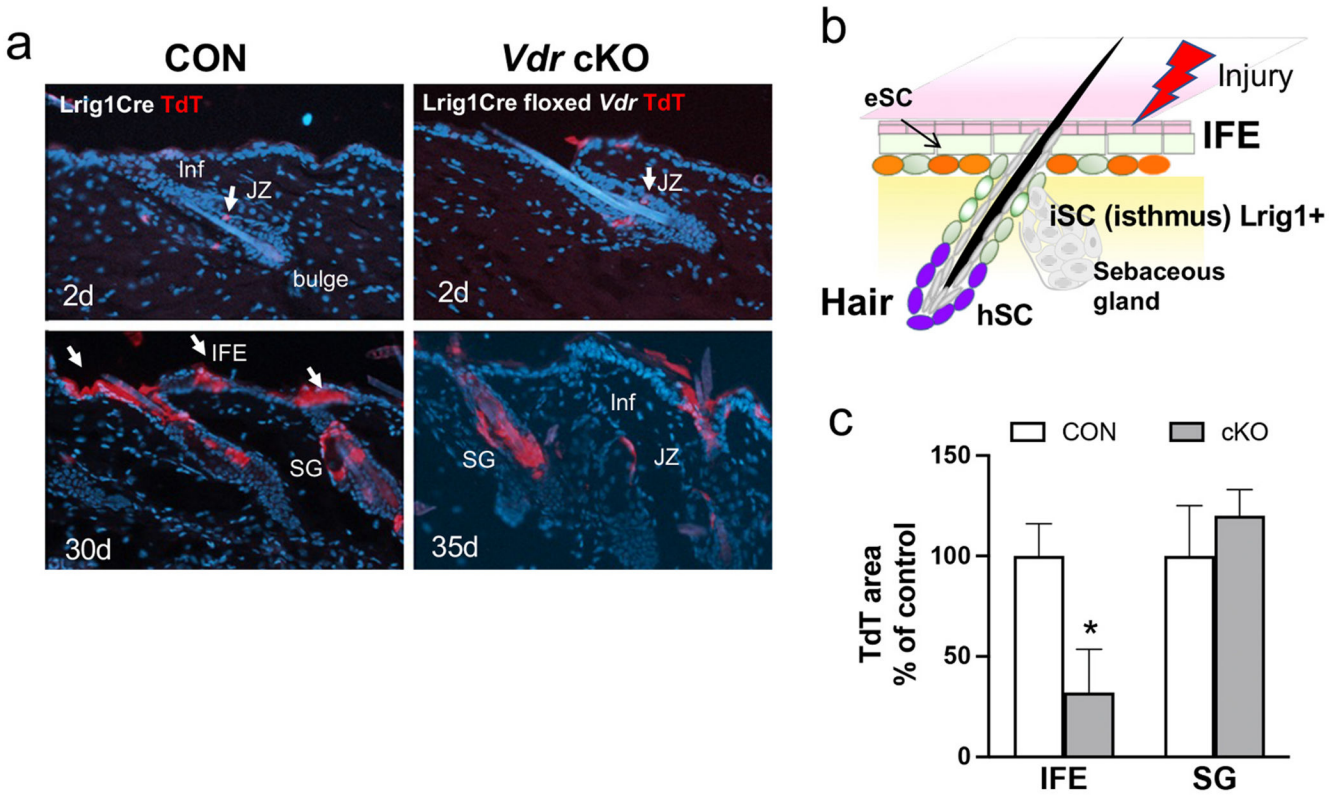

Fig. 1.

Ablation of *Vdr* impairs iSC movement to interfollicular epidermis but not to SG during wound re-epithelialization. **a** Representative images of 2d (upper panels) and 30 or 35 days after skin wounding (lower panel). TdT labeled iSCs after 2 d migrated to the IFE in CON (white triangles, upper panels) but less so in cKO (bottom panels). TdT iSC regenerate SG similarly in CON and cKO. (b) Schematic localization of Lrig1 expressing isthmus stem cells, SG, IFE epidermis in skin. **c** Quantification of the TdT labeled cells in the IFE epidermis and SG was determined in 8–10 different fields for control and cKO mice (2 each). Statistical significance was calculated by t-test (* $P < 0.05$).

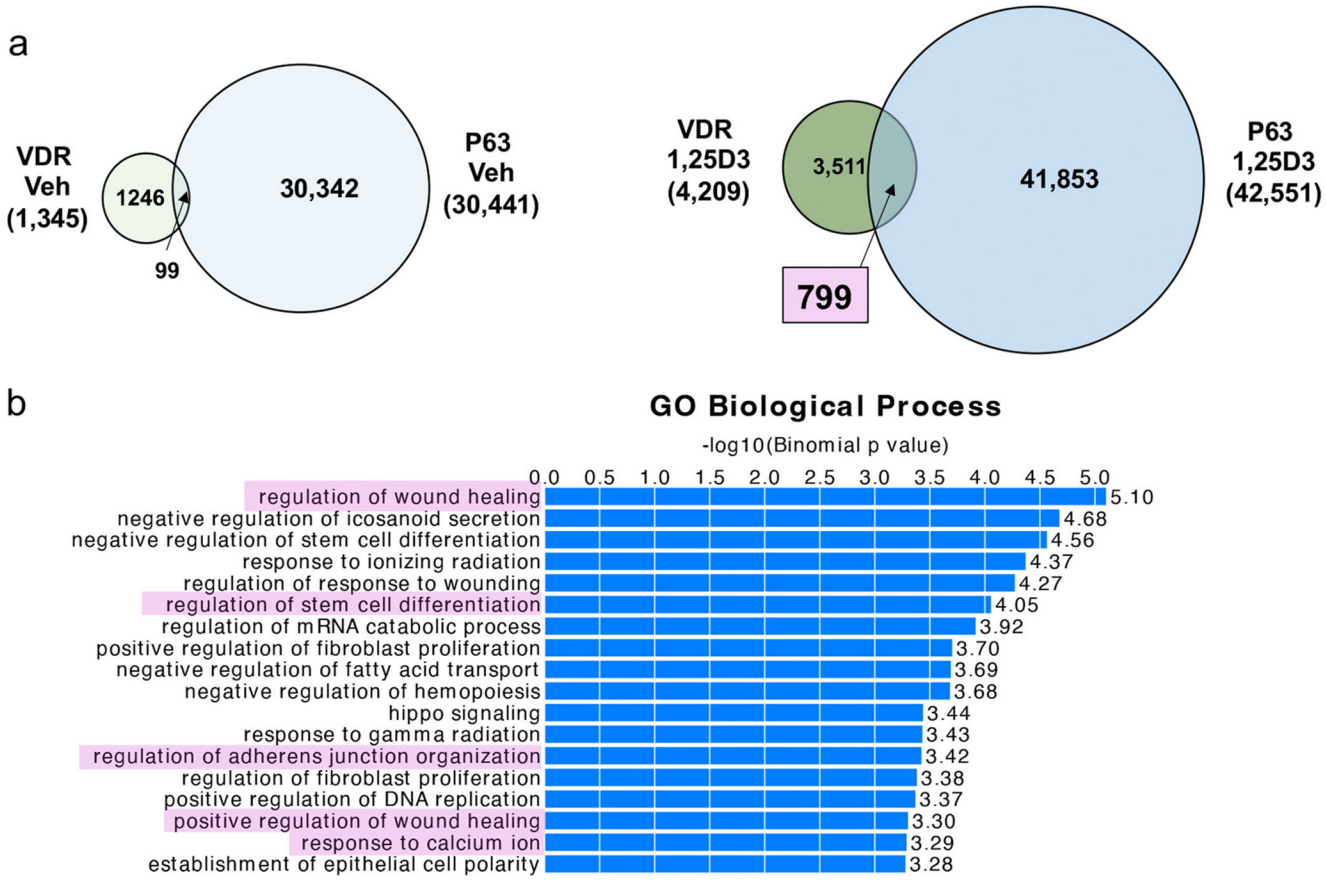

Fig. 2.

a Venn diagram shows the number of VDR and p63 peaks in the absence (vehicle control, left panel) and presence of 1,25D_3 (right) in primary keratinocytes. The increased number of 799 VDR/p63 overlap sites is shown by a pink box. b Gene Ontology (GO) analysis performed on the annotated set of genes from the 799 VDR/p63 overlapped peaks. Top biological processes related to skin wound healing are pink-highlighted, in which GO terms and p-values are shown. The associated genes are listed in Supplemental Table S1.

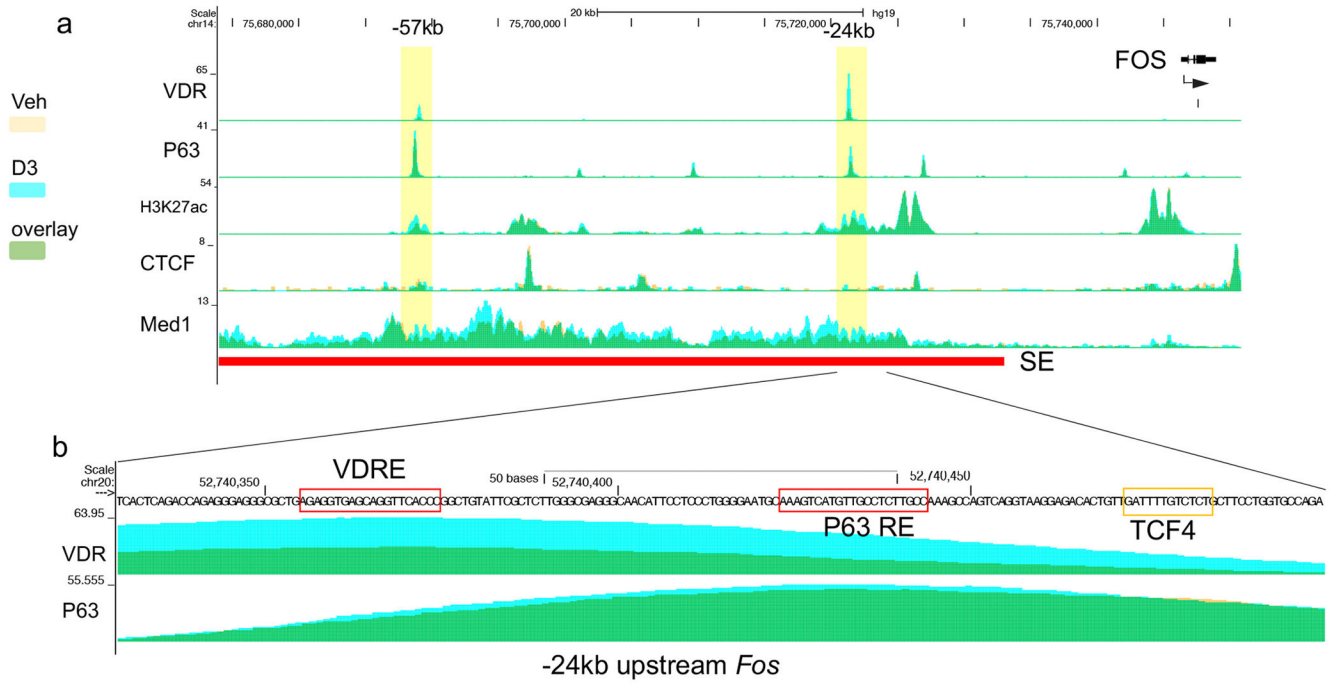

Fig. 3.

a ChIP-seq profiles for keratinocytes treated with vehicle (yellow) or 1,25D_3 (blue) and their overlap are shown by green. The VDR, p63, CTCF, and Med1 peaks for the *FOS* gene, in which the colocalization of VDR/p63 peaks was marked by yellow highlights, and location of Med1 enriched super-enhancers identified by Rose software was marked by a red bar. b The locations of binding motifs for VDR, p63 and TCF4 are shown at – 24 kb upstream region of the *FOS* gene locus.

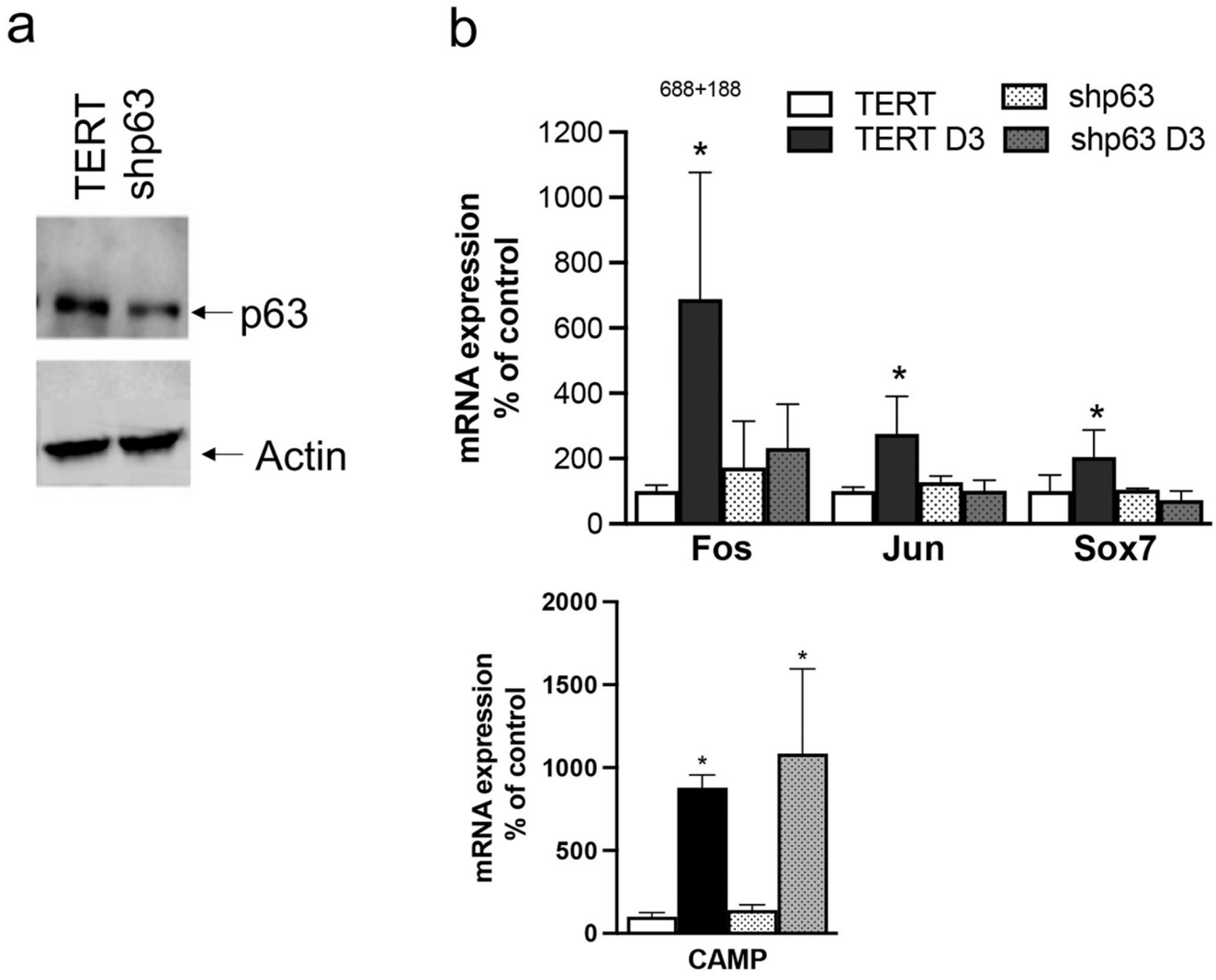

Fig. 4.

Decreased p63 expression affects the $1,25D_3$ response in fate transcription factors of *FOS* and *JUN* in keratinocytes. a Decreased protein expression of p63 in the shp63 cell line compared to the parent line TERT, in which actin was used as a control. b. mRNA expression of *FOS, JUN, SOX7* and *CAMP* in shp63 and TERT keratinocytes treated with vehicle and 1×10^{-8} M $1,25D_3$ overnight. Gene expression is evaluated by qPCR, and fold induction compared to control TERT cells was calculated. Averages of three batches of $1,25D_3$ induction were calculated t-test * <0.05.

Table 1

p53 family as potential upstream regulator for VDR, Cellular functions and upstream regulators affected by VDR deletion in keratinocytes (IPA analysis). Top, Biological processes associated with loss of *Vdr* in *Krt14* expressing keratinocytes as identified by Ingenuity Pathway Analysis (IPA) on microarray gene expression data. Bottom, Upstream regulators responsible for biological processes induced by loss of *Vdr* as identified by IPA on microarray data with statistical significance (p-values). The activation z-scores make predictions about potential regulators by using information about the direction of gene regulation. Here, our data show the that upstream regulator p53 family (P53/63/73) is down-regulated with highest statistical significance (8.86E-28).

Name	**p-value**	**# Molecule**
Organismal Injury and Abnormalities	2.55E-11–1.09E-4	556
Cellular Growth and Proliferation	3.92E-32–8.72E-5	707
Cellular Movement	4.96E-23–7.84E-5	442
Upstream regulator	**Activation z-score**	**p-value**
TP53	**−2.113**	**8.86E-28**
TP63	0.227	1.16E-09
TP73	−1.321	2.97E-06

Table 2

Enriched transcription factor binding motifs (percentile of target sequences) found in super-enhancers identified by MED1 peaks in control keratinocytes compared to $1,25D_3$ treated cells in HOMER analysis for known motif analysis; the ranking number is shown. Raw data and statistical significance of $\log_{10}(p\text{-values})$ are shown in supplemental Table SII. All the ChIP-seq data are the average of triplicates conducted in 3 independent cultures of human keratinocytes that were derived from different individuals.

Transcription Factors	Motif	Veh % (bg)	rank	1,25D3 % (bg)	rank
VDR/DR3		34.01 (1.65)	1	36.1 (2.19)	1
TP63/53		9.68 (2.69)	10	9.09 (2.96)	12