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A Dominant Mutation Etiologic for Human Tricho-Dento-Osseous Syndrome Impairs the Ability of DLX3 to Downregulate ΔNp63α

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The homeodomain transcription factors play crucial roles in many developmental processes ranging from organization of the body plan to differentiation of individual tissues. The homeodomain protein Distal-less-3 (DLX3) has an essential role in epidermal stratification and development of ectodermal appendages, placenta and bones. A four-nucleotide deletion in the human DLX3 gene is etiologic for the human hereditary tricho-dento-osseous (TDO) ectodermal dysplasia, a dominant syndrome characterized by abnormalities in hair, nails, teeth, and bones. We have previously demonstrated that DLX3 gene expression induces degradation of ΔNp63α, a specific product of the TP63 gene, a master regulator of multi-layered epithelia. Here we show that the $DLX3^{TDO}$ mutant protein is unable to promote ΔNp63α protein degradation and impairs the expression of cell cycle regulatory proteins and skin differentiation markers. However, we found that in cell expressing equal amounts of mutant and wild-type DLX3, ΔNp63α protein level is efficiently regulated implying that genetic heterozygosity at the DLX3 locus protects TDO patients from developing severe p63-associated skin defects.

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Homeodomain transcription factors play crucial roles in many developmental processes ranging from organization of the body plan to differentiation of individual tissues (Merlo et al., 2000; Morasso and Radoja, 2005). Distal-less-3 (DLX3) is a member of the Dlx vertebrate family of homeodomain transcription factors that comprises six members (Dlx1-6). The human DLX3 gene maps to 17q21.33. It has an essential role in development of the epidermis and ectodermal appendages such as glands, teeth, and hair follicles, all structures involving epithelial–mesenchymal interaction (Hassan et al., 2004; Hwang et al., 2008). DLX3 is broadly expressed in early embryonic ectoderm, as well as in the limb bud and mammary gland and homozygous deletion of the mouse Dlx3 gene results in placental failure leading to embryonic death between days 9.5 and 10 (Morasso et al., 1999).

In skin, DLX3 expression is restricted to the suprabasal layers of epidermis where it is implicated in regulating transcription of late differentiation markers, such as loricrin and profilaggrin. Transgenic expression of Dlx3, in the basal keratinocytes of mice, causes highly abnormal skin with a pronounced reduction in thickness (Morasso et al., 1996). Conversely, Dlx3 conditional knock-out, in epidermis, resulted in epidermal hyperplasia, thickened epidermis, and abnormal hair development (Hwang et al., 2008).

The most common DLX3 gene mutation (c.571_574delGGGG), just downstream of the DLX3 homeodomain, is etiologic for tricho-dento-osseous syndrome (TDO; MIM# 190320). TDO is an autosomal-dominant genetic disorder characterized by kinky hair, brittle nails, enamel hypoplasia, taurodontism, and mild to moderate increased bone density (Price et al., 1998). All these traits are highly penetrant yet clinically variable components of TDO (Price et al., 1998). Recently, another DLX3 gene mutation (c.561_562delCT), inside the DLX3 homeodomain, was reported to be associated with an attenuated TDO phenotype (Wright et al., 2008) or with Amelogenesis Imperfecta with Taurodontism (AIHHT) (Lee et al., 2008).

The c.571_574delGGGG deletion causing TDO syndrome results in a frameshift after the first codon following the homeodomain and thus exhibits an aminoacid sequence that differs from the C-terminal domain in wild-type DLX3. The frameshift also results in the truncation of the protein to 255 amino acids instead of 287 (Duverger et al., 2008). The mutation does not alter the structure of the homeobox domain, the NLS region or the nuclear localization of the protein (Choi et al., 2008; Duverger et al., 2008). The dominant pattern of inheritance may be due to the formation of non-functional complexes involving the truncated DLX3 that acts either through a dominant-negative or a gain of function mechanism (Duverger et al., 2008).

Concerning the transcriptional ability of $DLX3^{TDO}$ alone, it was shown to gain transcriptional activity on the osteocalcin gene promoter (Choi et al., 2008) while it was found defective on the Runx2 promoter (Duverger et al., 2008). Furthermore, when $DLX3^{WT}$ and $DLX3^{TDO}$ were co-expressed they formed a complex that could bind DNA and $DLX3^{TDO}$ exerted a dominant negative effect on $DLX3^{WT}$ transcriptional activity (Duverger et al., 2008).

The DLX3 gene promoter is induced by TAp63α, a specific product of the p63 gene (Radoja et al., 2007). The p63 gene is a master regulatory gene of multi-layered epithelia (Pozzi et al., 2009). P63 null mice show cranial and limb malformations; they also lack stratified skin and all epithelial derivatives (Mills et al., 1999). p63 belongs to the p53 family of transcription factors

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and is expressed as multiple protein isoforms that are classified into two groups designated TA and ΔNp63. Alternative splicing at the 3′ end generates TA and ΔNp63 proteins with different C-termini, denoted α, β, and γ (Yang et al., 1998). In stratified skin, ΔNp63α expression, which supports the proliferative potential of keratinocytes, decreases progressively from the basal to the suprabasal, more differentiated, layers of the epidermis, where TAp63 isoforms appear to be expressed (King et al., 2006). Interestingly, unlike TAp63α, ΔNp63α does not induce DLX3 gene transcription (Lo Iacono et al., 2008).

Mutations of p63 were found to be responsible for several distinct human hereditary syndromes, some associated with ectodermal dysplasia including the split-hand and foot malformation IV (SHFM IV), the ectrodactily ectodermal dysplasia clefting (EEC) and the ankyloblepharon-ectodermal defects-cleft lip/palate (AEC) (Brunner et al., 2002). Recently, we have demonstrated that mutant p63 proteins derived from AEC patients exhibit an impaired ability to transactivate the DLX3 gene promoter thus showing that DLX3 and p63 are involved in a common pathway which is relevant for development of skin, hair, teeth, and other epidermal derivatives (Radoja et al., 2007). Moreover, we have shown that DLX3 triggers proteasome-mediated degradation of ΔNp63α through a Raf 1 kinase-dependent pathway (Di Costanzo et al., 2009).

In this work we explore the effect of the most common TDO mutation on the ability of DLX3 to regulate the ΔNp63α protein level.

Materials and Methods

Plasmids and reagents

Expression plasmids for V5 or FLAG tagged wild-type ($DLX3^{WT}$) and mutant DLX3 ($DLX3^{TDO}$) have been previously described (Duverger et al., 2008).

Cell culture and transfection

Normal human foreskin keratinocytes (HK) were isolated and cultured according to Bitoun et al. (2003). HaCaT and H1299 cells were maintained as already described (Di Costanzo et al., 2009). HK were transiently transfected with increasing amount (2 and 3 μg) of pCI-Neo-DLX3 or pCI-Neo-TDO expression vector using Neon-Transfection system (Invitrogen, Life Technologies. Inc., Milan, Italy), according to manufacturer's instructions.

HaCaT keratinocytes were grown until 80% confluence (corresponding to day 0 = t0), then differentiation was induced for up to 5 days in DMEM w/o serum containing 2 mM calcium.

HaCaT cells were transiently transfected with increasing amount (3 and 4 μg) of pCI-Neo-V5-DLX3 or pCI-Neo-TDO expression vector using LipofectAMINE 2000 reagent (Invitrogen, Life Technologies. Inc.). When necessary, the total amount of transfected DNA was kept constant using an empty expression vector. At 24 h post-transfection, cells were lysed and total protein quantified with the BioRad protein assay. Twenty micrograms of extracts was resolved by sodium dodecyl sulfate (SDS)–PAGE. To detect Raf1 and TDO interaction in HaCaT cells, 1.0×10^6 cells were plated in 100 mm dishes and transfected with 4 μg of pCI-Neo-DLX3 or pCI-Neo-TDO expression vector. To detect interaction between TDO and DLX3, 1.0×10^6 cells were plated in 100 mm dishes and transiently transfected with equal amount (5 μg for plate) of pCI-Neo-TDO or pcDNA 3.1 FLAG-DLX3 alone or pCI-Neo-TDO and pcDNA 3.1 FLAG-DLX3 in a 1–1 ratio (2.5 μg of DLX3 and 2.5 μg of TDO expression plasmid). To generate HaCaT stable clones expressing $DLX3^{TDO}$, 7×10^5 cells were plated in 100 mm dishes and transfected with 5 μg of empty vector, pCI-Neo-V5-DLX3 or pCI-Neo-V5-TDO plasmid. Twenty-four hours after transfection, each 100 mm plate was split (1:20) and cells were selected by adding G418 (100 μg/ml) to the cell culture medium. After 3 weeks, HaCaT stable clones were established only from $DLX3^{TDO}$ transfected cells. Isolated clones were picked up and expanded, and their $DLX3^{TDO}$ gene expression was determined by western blot and specific immunodetection using antibodies against the V5 epitope. The #HC3TDO stable clone (1.0×10^6 cells) was transiently transfected, in 100 mm dishes, with 5 μg of pcDNA3-FLAG-DLX3 using Neon-Transfection system (Invitrogen, Life Technologies, Inc.). Extracts were prepared and subjected to co-immunoprecipitation.

Western blot analysis and co-immunoprecipitation

At 24 h after transfection cells were lysed in 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl, 0.5% NP-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.5% sodium deoxycholate, and protease inhibitors. Cell lysates were incubated on ice for 30 min and the extracts were centrifuged at 13,000 rpm for 10 min to remove cell debris. Protein concentrations were determined by the BioRad protein assay. After the addition of 2× loading buffer (2% SDS, 30% glycerol, 300 mM β-mercaptoethanol, 100 mM Tris–HCl pH 6.8), the samples were incubated at 95°C for 5 min and resolved by SDS–PAGE. Proteins were transferred to a PVDF (Millipore, Mila, Italy) and probed with the appropriate antibodies. For immunoprecipitation in HaCaT keratinocytes, 1.0×10^6 cells were seeded in 100 mm dishes and transfected with plasmids as indicated (see legend to figure). Cells were harvested 24 h post-transfection and cell lysates were prepared as described above. One microgram of whole cell extracts was precleared with 30 μl of protein A-agarose (50% slurry; Roche, Manheim, Germany) and then incubated overnight at 4°C with the following antibodies: 3 μg of anti-V5 (AbD Serotec, Oxford, UK), 3 μg anti-Flag (M2 clone, Sigma-Aldrich, Germany). Immunocomplexes were collected by incubation with 30 μl of protein A-agarose (Roche) at 4°C for 4 h. The beads were washed with Co-IP buffer (50 mM Tris–HCl pH 7.5; 150 mM NaCl; 5 mM EDTA; 0.5% NP-40; 10% glycerol), resuspended in 2× loading buffer (Sigma) and loaded into an SDS–10% polyacrylamide gel. Proteins were then transferred onto a PVDF membrane (Millipore) and probed with the indicated primary antibodies. Proteins were visualized with an enhanced chemiluminescence detection system (Amersham). Images were taken with CHEMIDOC (Biorad) and analyzed with the QuantityONE software.

Antibodies

p63 (4A4), actin (I-19), citokeratin 1 (4D12B3), citokeratin 10 (VIK-10) sc-51581, anti-14-3-3σ (stratifin) sc-7681, anti-Raf1 (C-12) sc-133, anti-DLX3 (C-20) sc-18143, and anti-p21WAF (C19) sc-397 were from Santa Cruz, Biotechnologies Inc., Germany. Anti-FLAG (M2 clone) from Sigma; anti-V5 (AbD Serotec); anti-cyclin D1 (2922), Phospho-c-Raf (Ser338) (56A6), Phospho-Akt (Ser473) (9271S), and Phospho-c-Raf (Ser259) (9421S) were from Cell Signaling Technology, Denver.

Luciferase assay

For luciferase assays, 200 ng of reporter DNA (ΔNp63-3Kb promoter or p21/WAF1 promoter luciferase construct) and different amounts of each of the indicated effectors (see legend to figure) were transfected along with 40 ng of pRL (Renilla Luciferase) as internal transfection control (Dual Reporter Luciferase Assay, Promega, Milan, Italy). Cells were lysed after 24 h and luciferase activity determined.

λ-Phosphatase (λ-PPase) treatment and immunoblotting

Total cell lysates prepared as duplicate samples were incubated with or without 4 U of λ-phosphatase (New England Biolabs, Frankfurt, Germany) for 1 h at 30°C as suggested by the manufacturer. Extracts were analyzed by SDS–PAGE using a high resolving 8% gel (37.5:1 acryl/bis acrylamide).

PCR analyses

HaCaT cells were transfected with DLX3^{WT} or DLX3^{TDO} constructs (0.5, 1, and 1.5 μg). For PCR analysis total RNA from HaCaT cells was isolated using the RNA Mini Extraction Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Total RNA (1 μg) was used to generate reverse transcribed cDNA using SuperScript III (Invitrogen, Life Technologies, Inc.). Real Time PCR was performed with a 7500 RT-PCR Thermo Cycler (Applied Biosystem, Monza, Italy) using SYBR GREEN Master Mix (Applied Biosystem). All samples were done in triplicate. Human hypoxanthine phosphoribosyl transferase (HPRT) was used for normalization. The results were expressed with the value relative to HPRT (set at 1) for each mRNA sample.

For ΔNp63α and p21WAF expression, the following oligonucleotides were used:

ΔNp63α (sense)	5′CCA CAG TAC ACG AAC CTG GGG
ΔNp63α (antisense)	5′CCG GGT AAT CTG TGT TGG AG
p21WAF (sense)	5′AAGACCATGTGGACCTGTCA
p21WAF (antisense)	5′GGCTTCCTCTTGGAGAAGAT

HPRT gene was amplified using the following primers:
(F) 5′ CCT GCT GGA TTA CAT TAA AGC
(R) 5′ CTT CGT GGG GTC CTT TTC

The amplification sequence consisted of 30 cycles of 94°C/1′, 55°C/1′, 72°C/1′. PCR products were resolved by 2% agarose electrophoresis. RT-PCR amplification results were analyzed by Quantity One software (Biorad).

Results

DLX3^{TDO} is unable to induce ΔNp63α degradation

We have previously shown that DLX3 expression induces degradation of ΔNp63α protein, in human and mouse keratinocytes (Di Costanzo et al., 2009). Since TDO patients exhibit ectodermal dysplasia characterized by hair, nail, and tooth defects, we explored the possibility that the TDO mutation could, in some way, affect the ability of DLX3 to down-regulate ΔNp63α protein levels. To address this question, we compared the effect of enforced expression of wild-type (DLX3^{WT}) or mutant DLX3 (DLX3^{TDO}) on ΔNp63α protein level, in human keratinocytes. Briefly, we transfected both human embryonic (NHEK) and spontaneously immortalized (HaCaT) keratinocytes either with DLX3^{WT} or DLX3^{TDO} expression plasmids and evaluated the level of endogenous ΔNp63α by immunoblot. In agreement with our previous findings (Di Costanzo et al., 2009), DLX3^{WT} efficiently reduced ΔNp63α protein level. Conversely, the expression level of ΔNp63α was unaffected by DLX3^{TDO} (Fig. 1A,B). Therefore, we examined the possibility that DLX3^{TDO} protein might enhance ΔNp63α gene transcription thus resulting in higher levels of ΔNp63α in cells expressing DLX3^{TDO}, compared to those expressing the wild-type protein. The ability of DLX3^{TDO} to transactivate ΔNp63α gene transcription was first analyzed by reporter assays. The ΔNp63α promoter-luciferase construct was co-transfected with increasing amount

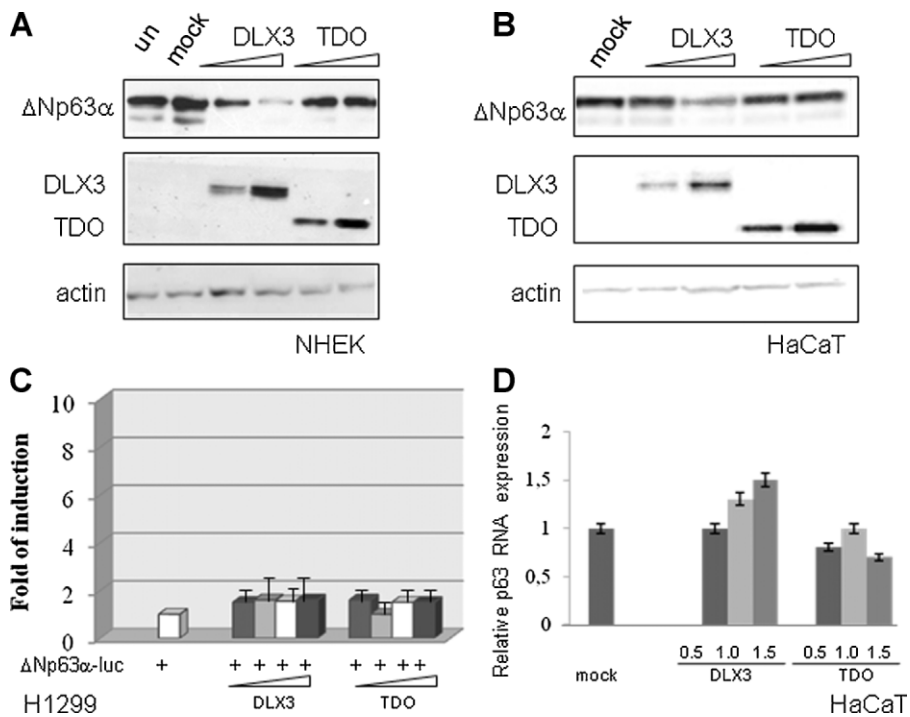

Fig. 1. DLX3^{TDO} mutant was unable to induce ΔNp63α degradation. NHEK (A) or spontaneously immortalized (HaCaT) keratinocytes (B) were transiently transfected with an empty vector or increasing amount (3 and 4 μg) of pCI-V5-DLX3 or pCI-V5-TDO encoding plasmids. At 24 h after transfection cells were lysed and 20 μg protein extracts were fractionated by SDS–PAGE and analyzed by Western blot. ΔNp63α expression level was revealed by immunoblot with antibodies directed against p63 (4A4) while V5-DLX3^{WT} and V5-DLX3^{TDO} expression levels were evaluated with antibodies against the V5 tag. Actin was used as loading control. Images were acquired with CHEMIDOC (Biorad) and analyzed with the Quantity-ONE software. C: H1299 cells were transiently transfected with ΔNp63α promoter luciferase-construct alone or with increasing amount (0.3, 0.6, 0.8, 1 μg) of DLX3^{WT} or DLX3^{TDO} expression plasmids. Luciferase activity was assayed 24 h after transfection. Each histogram bar represents the mean of at least three independent biological replicates after normalization to Renilla luciferase activity to correct for transfection efficiency. The activity of the sample transfected with the reporter alone was set to one. D: Quantitative RT-PCR expression analysis of endogenous ΔNp63α was performed on total RNA from HaCaT keratinocytes transiently transfected with increasing amounts (from 0.5 to 1.5 μg) of DLX3^{WT} or DLX3^{TDO} encoding vectors. The histogram shows the fold change of expression levels in experimental samples relative to samples transfected with 1.5 μg of empty plasmid. Each histogram bar represents the mean of three biological replicates while error bars show the standard deviation of ΔC_T values obtained for three replicates of each sample type.

of DLX3^{WT} or DLX3^{TDO} expression plasmid into the p53/p63-null H1299 cell line, to avoid interference by endogenous p53 or p63. As shown in Figure 1C, the luciferase activity of the ΔNp63α-luciferase construct was not significantly increased in the presence of DLX3^{WT} or DLX3^{TDO} when compared to the baseline in absence of proteins. QRT-PCR assays, performed on total RNA from mock, DLX3^{WT} or DLX3^{TDO}-transfected HaCaT keratinocytes, confirmed that DLX3^{TDO} expression does not increase the abundance of ΔNp63α transcript (Fig. 1D).

It is well known that primary proliferating keratinocytes exhibit high levels of ΔNp63α which is down-regulated during differentiation (Parsa et al., 1999; Bamberger et al., 2002) while the upregulation of the cyclin-dependent kinase inhibitor (CDKI) p21WAF is required for initial commitment of keratinocytes to differentiate (Wong et al., 2010).

Accordingly, in differentiating HaCaT keratinocytes, we observed down-regulation of ΔNp63α in parallel with the enhancement of p21WAF and DLX3 expression levels (Fig. 2A). Since ΔNp63α was shown to bind to the p21WAF1 gene promoter resulting in transcriptional repression (Westfall et al., 2003), we reasoned that down-regulation of ΔNp63α induced by DLX3, would cause de-repression of p21WAF gene transcription. According to this notion, enforced expression of DLX3, in HaCaT cells, resulted in an increase of the p21WAF protein level, as assessed by western blot assay (Fig. 2B). Unexpectedly, we observed a similar increase of p21WAF following DLX3^{TDO} enforced expression but without reduction of ΔNp63α abundance (Fig. 2B). Therefore, we transiently transfected DLX3^{WT} or DLX3^{TDO} expression vectors into H1299 cells along with a PGL3 plasmid carrying the human p21WAF promoter. Luciferase assays showed that mutant DLX3^{TDO} gained the ability to induce the p21/WAF promoter (Fig. 2C). Next, we performed Real-Time PCR on total RNA from HaCaT keratinocytes. As shown in Figure 2D, we found an increase of p21WAF specific transcript both in DLX3^{WT} and DLX3^{TDO} transfected cells.

It was reported that differentiation of human keratinocytes is impaired by expression of ΔNp63α (Parsa et al., 1999; Bamberger et al., 2002). Therefore, we analyzed the expression of p21/WAF and specific skin differentiation markers, such as CK1, CK10, and stratifin (14-3-3σ by immunoblot in HaCaT keratinocytes transfected with DLX3^{WT} or DLX3^{TDO} expression vectors). As shown in Figure 3A, DLX3^{WT} enforced expression caused induction of 14-3-σ tratifin, p21/WAF, and the suprabasal keratins CK1 and CK10, along with the expected reduction of ΔNp63α. Interestingly, DLX3^{TDO} expressing cells showed induction of p21/WAF while stratifin, CK1, CK10 as

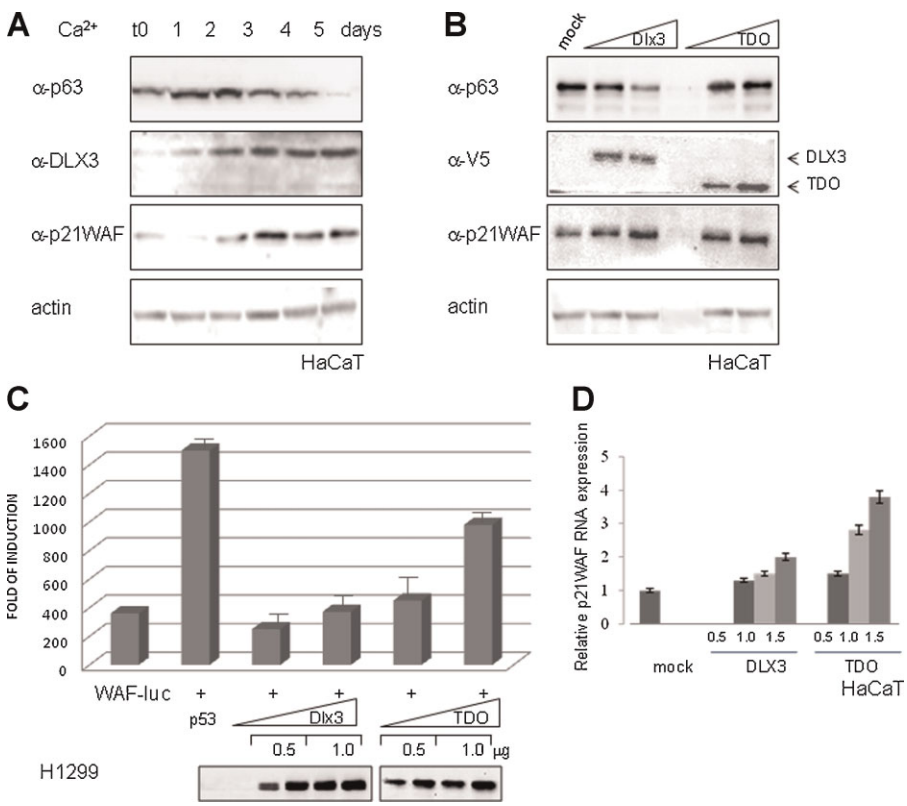

Fig. 2. p21-WAF1 up-regulation in HaCaT cells expressing DLX3^{WT} or DLX3^{TDO} proteins (A). HaCaT cells were induced to differentiate with 2 mM Ca^{2+} and extracts were subjected to immunoblot with anti-p63 (4A4), DLX3, and p21WAF antibodies. Actin was used as loading control. B: HaCaT cells were transfected with increasing amount (3 and 4 μg) of DLX3^{WT} or DLX3^{TDO} plasmids. Twenty-four hours after transfection 20 μg protein extracts were subjected to immunoblot and revealed with antibodies against p63, p21WAF1, and the V5 tag. Actin was used as loading control. C: H1299 cells were transfected with pGL3-p21WAF1 promoter alone, with a 1 μg of pcDNA3-p53 plasmid as positive control or increasing amount (0.5 and 1 μg) of DLX3^{WT} or DLX3^{TDO} expression plasmids. Luciferase activity was assayed 24 h after transfection. Each histogram bar represents the mean of at least three independent transfections after normalization to Renilla luciferase activity. D: Quantitative RT-PCR expression analysis of endogenous p21WAF was performed on total RNA from HaCaT keratinocytes transiently transfected with increasing amounts (from 0.5 to 1.5 μg) of DLX3^{WT} or DLX3^{TDO} encoding vectors. The histogram shows the fold change of expression levels in experimental samples relative to samples transfected with 1.5 μg of empty plasmid (mock). Each histogram bar represents the mean of three biological replicates while error bars show the standard deviation of ΔC_T values obtained for three replicates of each sample type.

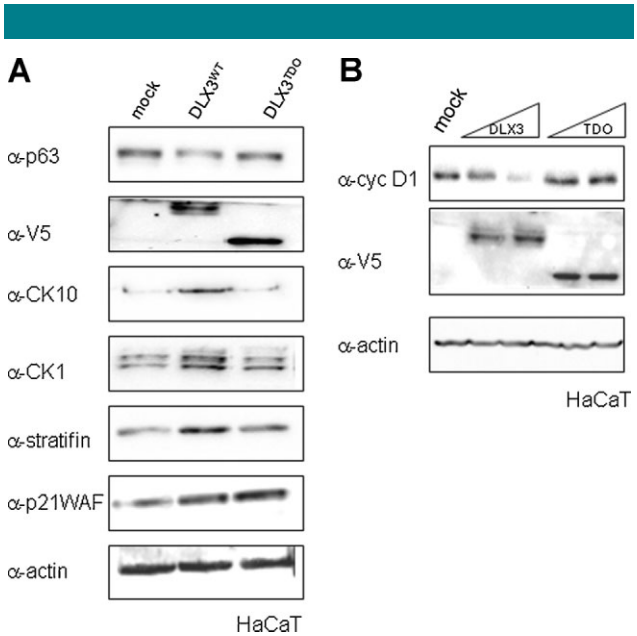

Fig. 3. DLX3^{TDO} impairs the expression of skin differentiation markers. A: HaCaT cells were trasfected with an empty plasmid (mock) or with a fixed amount (3 μg) of DLX3^{WT} or DLX3^{TDO} expression plasmids. Filters were probed with antibodies against p63 (4A4), V5, 14.3.3σ, CK1, CK10, and p21WAF. Actin was used as loading control. B: HaCaT cells were transiently transfected with an empty vector (mock) or increasing amount (3 and 4 μg) of DLX3^{WT} and DLX3^{TDO} constructs. Twenty-four hours after transfection total lysate (20 μg) was immunoblotted with anti-cyclin D1 and anti-V5 antibodies. Actin was used as loading control.

well as ΔNp63α protein levels were almost undistinguishable from those observed in mock transfected cells thus suggesting that DLX3^{TDO} expression impairs the expression of skin differentiation markers (Fig. 3A).

It was previously demonstrated that during differentiation of HaCaT keratinocytes, persistence/enforced expression of cyclin D1, rather than p21WAF up-regulation, inhibits the expression of skin differentiation markers and that down-regulation of cyclin D1 is crucial for permanent cell cycle exit and keratinocyte terminal differentiation (Nishi et al., 2009). Therefore, we monitored the expression levels of cyclin D1, in total extracts from DLX3^{WT} or DLX3^{TDO} transfected cells. Remarkably, DLX3^{WT}, but not DLX3^{TDO}, caused a dramatic reduction of cyclin D1 (Fig. 3B).

DLX3^{TDO} is unable to interact and induce Raf1 phosphorylation at Ser338

We have previously shown that DLX3 interacts with Raf1 and causes enhancement of Raf1 phosphorylation at Ser338. Both events were found to be necessary for phosphorylation-dependent ΔNp63α degradation induced by DLX3 (Di Costanzo et al., 2009). We analyzed whether impaired degradation of ΔNp63α following mutant DLX3^{TDO} expression, could be ascribed to the inability of the DLX3^{TDO} protein to interact with Raf1 and/or promote Raf1 phosphorylation. To address this point, we transfected DLX3^{TDO} in HaCaT cells to examine the phosphorylation status of Raf1 with two different phospho-specific Raf1 antibodies. The first antibody was directed against phospho-Ser338 (pSer338) which is the target of Raf1 self-phosphorylation in response to mitogens (Zang et al., 2008), while the latter was against phospho-Ser259 (pSer259), a phosphoacceptor site specifically targeted by Akt kinase activation (Diaz et al., 1997). As expected, transient expression of DLX3^{WT}, in HaCaT cells, increased the phosphorylation status of Raf1 at Ser338 while mutant DLX3^{TDO} seemed even to reduce Raf1 phosphorylation (Fig. 4A). Neither DLX3^{WT} nor DLX3^{TDO} significantly affected the phosphorylation status of Akt kinase and Raf1 at Ser259 (Fig. 4A). We also examined if Raf1 expression was modulated by wild-type or mutant DLX3 at the transcription level by Real Time PCR and found no significant differences (Fig. 4B). Next, we hypothesized that impaired phosphorylation of Raf1 at Ser338 was due to the inability of DLX3^{TDO} homodimers to interact with and activate Raf1 kinase autophosphorylation. To address this point we transiently expressed, in HaCaT cells, DLX3^{WT} or DLX3^{TDO}, as V5-Tag fusion proteins and immunoprecipitated them using antibodies against the V5 epitope. As expected endogenous Raf1 was efficiently co-immunoprecipitated in cells expressing V5-DLX3^{WT}, while in cells expressing V5-DLX3^{TDO} it was barely detectable, thus suggesting that the TDO mutation impaired the ability of DLX3 to interact with Raf1 (Fig. 4C).

Moreover, on conventional SDS–PAGE, DLX3^{WT} but not DLX3^{TDO} had a smeared appearance (see Fig. 1A,B). To check if this was due to DLX3 protein phosphorylation we employed λ-phosphatase assay on extracts from HaCaT cells expressing DLX3^{WT} or DLX3^{TDO}. The extracts were then analyzed by high-definition SDS–PAGE and specific immunoblot (see Materials and Methods Section). Following this treatment the electrophoretic band corresponding to DLX3^{WT} showed a different electrophoretic mobility and appeared unique and sharp, similar to that of DLX3^{TDO} which was completely unaffected by λ-phosphatase treatment (Fig. 4D). This observation suggests that, in human keratinocytes, DLX3^{WT}, but not DLX3^{TDO}, is a phosphoprotein.

DLX3^{WT}/DLX3^{TDO} heterodimers are able to interact with Raf1 and promote p63 degradation

Although DLX3^{TDO} protein is defective in controlling the level of ΔNp63α protein in keratinocytes, TDO patients rarely show overt skin abnormalities (Hart et al., 1997). We wanted to mimic the in vivo situation of TDO patients expressing both the wild-type and mutant protein. Since it was already reported that DLX3^{WT} and DLX3^{TDO}, when co-expressed, are able to form heterodimeric complexes that can bind DNA (Duverger et al., 2008) we checked for presence of DLX3^{WT}/DLX3^{TDO} heterodimers in HaCaT cell lysates transfected with both proteins. Briefly, HaCaT keratinocytes were transfected with expression vectors for DLX3^{WT} tagged with a Flag epitope and DLX3^{TDO} tagged with a V5 epitope. Protein complexes were immunoprecipitated with anti-Flag antibodies to immunoprecipitate DLX3 and analyzed for the presence of DLX3^{TDO} using antibodies against the V5 epitope. As shown in Figure 5A, DLX3^{TDO} protein was present in DLX3^{WT} immunocomplexes.

Next, we investigated whether DLX3^{WT}/DLX3^{TDO} heterodimers were able to promote Raf1 phosphorylation and ΔNp63α degradation. Therefore, we transfected equal amount of DLX3^{WT} and DLX3^{TDO} plasmids in HaCaT cells and checked by immunoblot the level of ΔNp63α protein. As shown in Figure 5B, co-expression of equivalent amounts of DLX3^{WT} and DLX3^{TDO} protein, in HaCaT cells, resulted in efficient ΔNp63α degradation and enhancement of the phosphorylation status of Raf1 at Ser338.

Then, we decided to generate stable clones, from HaCaT cells, expressing both DLX3^{WT} and DLX3^{TDO} proteins. Stable transfection of DLX3^{WT} caused a massive cell death making it impossible to isolate DLX3 expressing HaCaT clones. Instead, we could easily isolate clones expressing the DLX3^{TDO} mutant protein (Fig. 6A). Remarkably, stable clones expressing

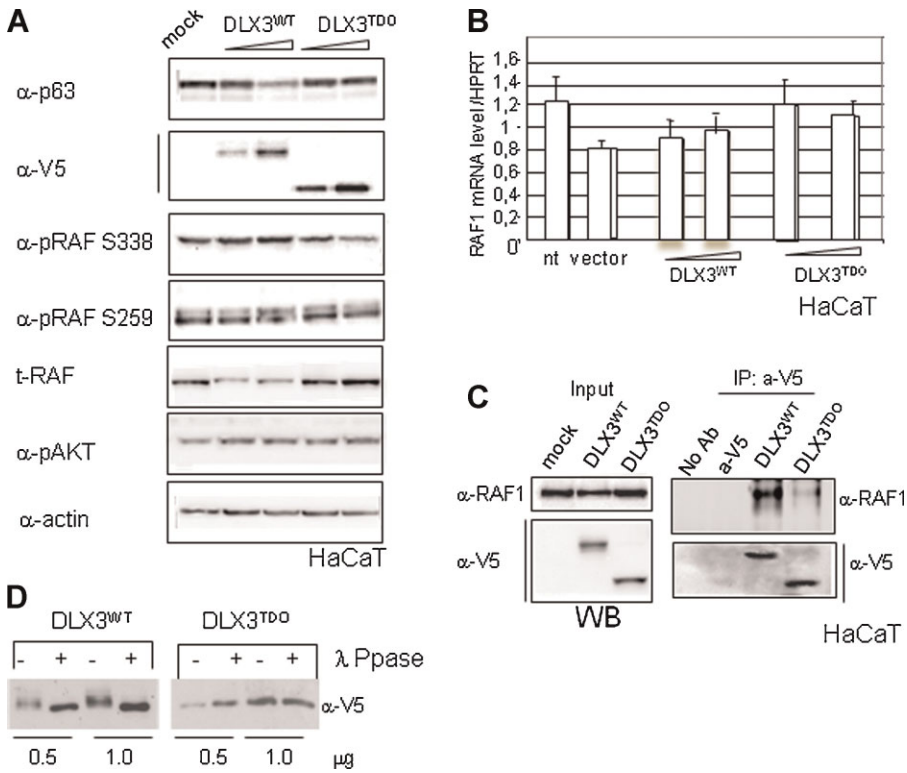

Fig. 4. DLX3^{TDO} protein is unable to interact with Raf1 and/or promote Raf1 phosphorylation. A: HaCaT were transiently transfected with an empty vector (mock) or increasing amount (3 and 4 μg) of DLX3^{WT} or DLX3^{TDO} expression plasmids. Protein extracts were fractionated by SDS–PAGE and analyzed by Western blot with anti p63, anti-V5, anti-pRAFS338, anti-pRAFS259, anti-RAF, and anti-pAKT antibodies. Actin was used as loading control. B: HaCaT cells were mock transfected or transfected with 3 and 4 μg of DLX3^{WT} or DLX3^{TDO} expression vector. Total RNA was prepared from untransfected, mock, DLX3^{WT} or DLX3^{TDO} transfected HaCaT cells at 24 h post-transfection. QRT PCR was performed with Raf1 and HPRT specific oligonucleotides as described in Materials and Methods Section. The experiment was performed twice and samples were done in triplicate. HPRT was used for normalization. The results were expressed with the value relative to HPRT (set at 1) for each mRNA sample. Standard deviations are also shown. C: Western blot of HaCaT cells transiently transfected with 4 μg of V5-DLX3^{WT} and V5-DLX3^{TDO} encoding plasmid. Equal amounts of total protein extracts were subjected to immunoblot and revealed with antibodies against RAF1 and the V5 tag (left part). Equal amounts of extract from mock, V5-DLX3^{WT} or V5-DLX3^{TDO} transfected cells were immunoprecipitated with anti-V5 antibody and the immunocomplexes were blotted and probed with anti-V5 or anti-Raf1 antibodies (right part). D: HaCaT cells were transfected with different amount (0.5 or 1 μg) of V5-DLX3^{WT} or V5-DLX3^{TDO} plasmids as indicated. At 24 h after transfection 10 μg of cell lysates were treated or not with λ protein phosphatase (λ-PPase), analyzed by high resolving SDS–PAGE (see Materials and methods Section) and blotted with antibody to V5.

detectable levels of DLX3^{TDO} exhibited higher levels of ΔNp63α compared to the others showing no DLX3^{TDO} expression (Fig. 6A, compare lanes 3 and 4 with lanes 1 and 2). The #HC3^{TDO} clone, expressing a high level of DLX3^{TDO}, was selected for transient transfection with the expression vectors for Flag tagged DLX3^{WT}. At 48 h from transfection, total cell lysates were prepared and the expression level of ΔNp63α and cyclin D1 were analyzed by immunoblot. As shown in Figure 6B, ΔNp63α abundance was greatly reduced while cyclin D1 was upregulated, thus proving that DLX3^{WT} and DLX3^{TDO} co-expression can rescue the negative control on ΔNp63α. Furthermore, we performed co-immunoprecipitation assay in HC3^{TDO} cells, transiently transfected with the expression vectors for Flag tagged DLX3^{WT} and we found the presence of DLX3^{TDO} in Flag-immunoprecipitated complexes (Fig. 6C), thus confirming that DLX3^{WT} and DLX3^{TDO} form heterodimers when co-expressed in keratinocytes.

Discussion

DLX3 and p63 act as part of a series of regulatory cascades that ultimately lead to the formation of ectoderm-derived organs. DLX3 and p63 gene mutations, in vivo, are very rare and are associated to human ectodermal dysplasia syndromes with variable clinical expression. Our previous studies proved a functional relationship between p63 and DLX3, with DLX3 being induced by TAp63 (Radoja et al., 2007) and responsible for ΔNp63α protein degradation (Di Costanzo et al., 2009). These findings provided a possible mechanistic explanation of how DLX3 can control epithelial differentiation. Here, we report that a naturally occurring mutation in the DLX3 gene, responsible for TDO syndrome affects the ability of DLX3 to down-regulate ΔNp63α protein level (Fig. 1A,B), further strengthening the notion that DLX3 and p63 belong to a common regulatory pathway relevant for the pathogenesis of ectodermal dysplasias. Remarkably, enforced expression of wild-type DLX3, but not mutant DLX3^{TDO} protein, in human keratinocytes, was accompanied by induction of skin specific differentiation markers, such as Keratin 1 (CK1), Keratin 10 (CK10), and stratifin (14-3-3σ) (Fig. 3A).

We have previously demonstrated that Raf1 kinase is a crucial player in DLX3-mediated ΔNp63α protein degradation (Di Costanzo et al., 2009). Apparently, the failure of DLX3^{TDO} to down-regulate ΔNp63α is due to the inability of DLX3 mutant protein to interact with and activate Raf1 kinase, as it does the wild-type protein (Fig. 4A,C).

In vivo evidence shows that ectopic expression of DLX3 gene causes skin defects. In 1996, it was reported that ectopic

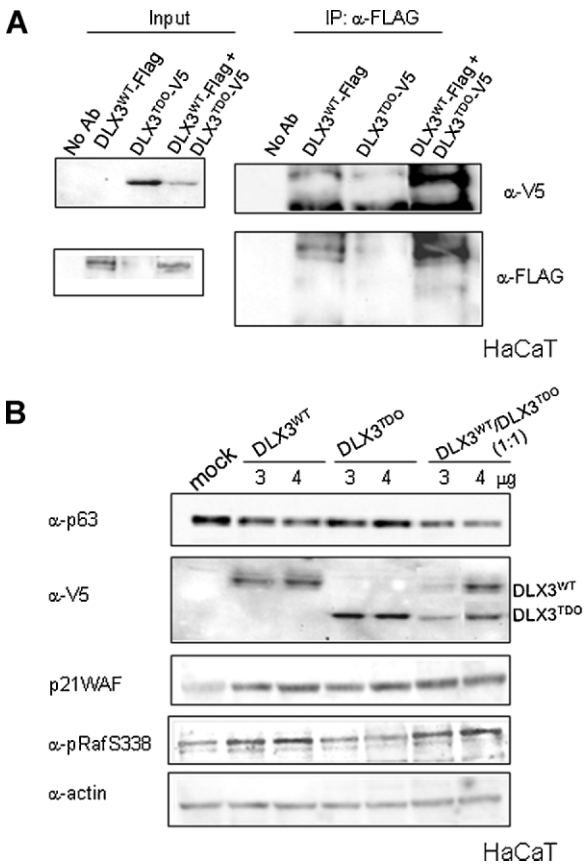

Fig. 5. DLX3^{WT}/DLX3^{TDO} heterodimers interacts with RAF1 and promotes ΔNp63α protein degradation. A: Extracts from HaCaT cells transiently transfected with an empty vector or a fixed amount (4 μg) of DLX3^{WT}, DLX3^{TDO}, or DLX3^{WT}/DLX3^{TDO} (in a ratio of 1:1) were immunoprecipitated (IP) with an anti-FLAG antibody and analyzed by WB with antibodies for FLAG and V5 tags. The input part contains 5% of the material used in the IPs. B: HaCaT cells were transiently transfected with an empty vector or increasing amounts (as indicated) of DLX3^{WT}, DLX3^{TDO}, or DLX3^{WT}/DLX3^{TDO} expression plasmids (in a 1:1 ratio). Total cell lysates were immunoblotted with antibodies against p63, V5, pRAFS338, and p21WAF1. Actin was used as loading control.

expression of the DLX3 gene, under the keratin K5 promoter, in the basal layer of the skin of transgenic mice, resulted in a pronounced reduction in skin thickness reflecting suppression of basal cell proliferation. A general decrease in the number of suprabasal layers and ectopic synthesis of filaggrin and loricrin in lower strata of epidermis was also observed (Morasso et al., 1996). Conversely, epidermal specific ablation of DLX3, in conditional knock-out mice, resulted in complete alopecia and hyperplastic epidermis with an increase in the number of proliferative cells and thickening of the entire stratified epithelium (Hwang et al., 2008). Taken together, these observations indicate that DLX3 expression in basal cells transforms them into more differentiated keratinocytes while the lack of a DLX3-regulated differentiation signal leads to hyperproliferative epidermis. Unfortunately, homozygous DLX3 knock-out mice are embryonic lethal, thus hampering the possibility to examine the effects of a complete loss of DLX3 in adult skin, while DLX3 heterozygous mice did not present any abnormalities thereby eliminating haplo-insufficiency as a probable cause of DLX3-associated ectodermal dysplasia (Morasso et al., 1999; Yang et al., 1999).

Our data show that both DLX3^{WT} and DLX3^{TDO} enforced expression up-regulated p21WAF (Figs. 3A and 5B). However, unlike the wild-type protein, DLX3^{TDO} was unable to down-regulate cyclin D1 (Fig. 3B). This observation is particularly relevant since it is known that, in anchorage deprived keratinocytes, induced to differentiate with calcium, cyclin D1 downregulation more than p21WAF induction, is crucial for permanent cell cycle exit and terminal differentiation (Nishi et al., 2009). Remarkably, HaCaT cells expressing TDO proliferate more than control cells and express higher level of cyclin D1 (Fig. 3B and data not shown). Furthermore, we failed to isolate HaCaT stable clones expressing DLX3 supporting the hypothesis that wild-type DLX3, but not DLX3^{TDO}, promotes irreversible cell cycle exit. Remarkably, we found that, not only ΔNp63α but also cyclin D1 downregulation was rescued in stable clones expressing both wild-type and mutant DLX3^{TDO} (Fig. 6B).

Since it is known that p21WAF and 14.3.3σ promoters are repressed by ΔNp63α association (Westfall et al., 2003), reduction of ΔNp63α level by DLX3, in HaCaT cells, was expected to result in an increase of p21WAF expression. Intriguingly, we found that DLX3^{TDO} was able to induce p21WAF, notwithstanding the presence of ΔNp63α expression (Figs. 2B and 3A). We can postulate that (1) either induction of p21WAF is completely independent from ΔNp63α or (2) DLX3^{TDO} has gained transcriptional activity on the p21WAF promoter. Comparison between wild-type and mutant DLX3 activity on the p21WAF promoter by reporter assays, in H1299 cells bearing no ΔNp63α, supports the last hypothesis (Fig. 2C). Moreover, our phosphatase assay indicated that, in cultured HaCaT keratinocytes DLX3^{WT}, but not DLX3^{TDO}, is a phosphoprotein. It is known that DLX3 is a target of protein kinase C (PKC), we may hypothesize that the structure of the TDO protein might impair phosphorylation of the protein (Park et al., 2001) by PKC.

The role of ΔNp63α in keratinocyte differentiation is still controversial. For instance enforced expression of ΔNp63α in primary murine keratinocytes was reported to counteract growth arrest and block expression of maturation-specific proteins keratin 10, loricrin, and filaggrin (King et al., 2003). However, retinoic acid treated keratinocytes express early differentiation markers in presence of sustained expression of ΔNp63α (Bamberger et al., 2002). Furthermore, transgenic mice overexpressing ΔNp63α under the control of the loricrin promoter show unaltered differentiation of keratinocytes (Liefer et al., 2000). All these findings suggest that ΔNp63α plays a role in skin homeostasis but argue against ΔNp63α down-regulation being the driving force for differentiation. Abnormal persistence of ΔNp63α allows keratinocytes to proceed through the early step of differentiation preventing processes, such as p53 or TAp73δ-mediated transcription, that are specific for terminal differentiation in suprabasal layers of the epidermis (De Laurenzi et al., 2000).

Tricho-dento-osseous syndrome is a rare human genetic disease. TDO patients express a wild-type and a dominant DLX3^{TDO} mutant allele. They do not usually show an overt skin phenotype and only two clinical cases have been reported, so far, describing TDO female patients with skin lesions (Lichtenstein et al., 1972; Mayer et al., 2010). Herein, we demonstrate that co-expression of wild-type and mutant DLX3^{TDO} results in the formation of DLX3^{WT}/DLX3^{TDO} heterodimers (Figs. 5A and 6C) that are able to down-regulate ΔNp63α protein level (Figs. 5B and 6B) and promote Raf1 kinase activation (Fig. 5B). Our observations suggest that co-expression of the mutant and wild-type DLX3 allele should, at least in part, protect TDO patients from developing p63-associated skin abnormalities. At present, however, we cannot exclude a negative role for DLX3^{WT}/DLX3^{TDO} heterodimers in the control of ΔNp63α functions during development of

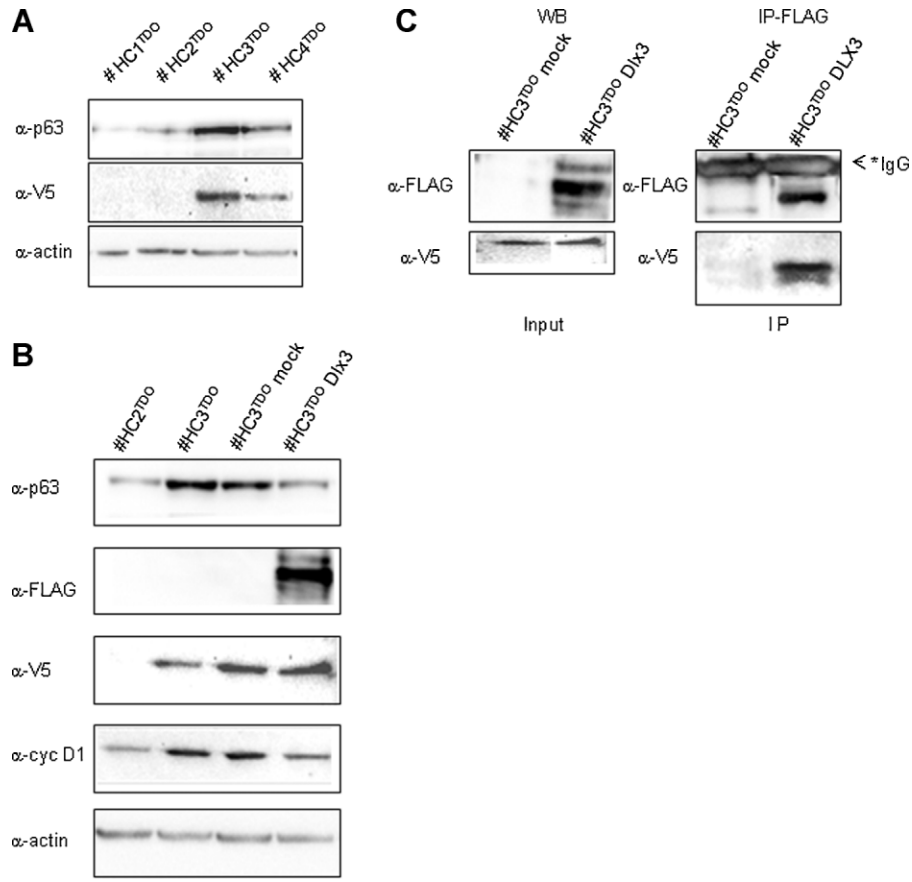

Fig. 6. DLX3^{WT}/DLX3^{TDO} heterodimers can rescue the negative control on ΔNp63α level in HaCaT cells. A: HaCaT cell were transfected with DLX3^{TDO} encoding plasmid, DLX3^{TDO} stable clones (#HCnTDO) were selected for neomycin resistance and isolated. Total cell lysates from 4 randomly selected stable clones were subject to western blot analysis and immunoblotted with anti-V5 and anti-p63 antibodies. Actin was used as loading control. B: HaCaT DLX3^{TDO} (#HC3TDO) stable clone was transiently transfected with an empty vector (#HC3TDOmock) or pcDNA3-DLX3^{WT}-FLAG construct (5 μg). Cells from #HC3^{TDO} clone were transfected with 5 μg of FLAG-DLX3 plasmid or an empty vector (mock). Total extract (30 μg) were fractionated and analyzed by SDS–PAGE along with an equal amount of extracts from untransfected #HC2^{TDO} and #HC3^{TDO} clones. Proteins were immunodetected using antibodies against p63, cyclin D1, the V5 and FLAG epitopes. Actin was used as loading control. C: Western blot of #HC3^{TDO} stable clone transiently transfected with 5 μg of an empty vector (mock) or FLAG-DLX3^{WT} encoding plasmid. Equal amounts of total protein extracts from cells transfected were immunoblotted with anti-FLAG and anti-V5 (left part). Extracts from mock or DLX3^{WT}-FLAG transfected cells were immunoprecipitated with anti-FLAG antibody and the immunocomplexes were blotted and probed with anti-V5 or anti-FLAG antibodies (right part).

other structures derived from epithelial–mesenchymal interaction such as nails, hair, and tooth enamel.

In summary, our data highlight the importance of the p63-DLX3 regulatory circuitry in the control of proliferation and differentiation of epithelial cells. The p63-DLX3 molecular pathway can be essential for the correct maturation and stratification of the adult epidermis as well as for the morphogenesis of other structures requiring epithelial–mesenchymal interaction, such as lip/palate closure and limb bud growth/patterning. Remarkably, altered localization of DLX3 gene expression is associated in retinoic acid-induced forelimb ectrodactyly (Shimizu et al., 2007) and overlap between p63 and DLX3 expression has been found in placenta where major defects are observed in the DLX3−/− embryos (Morasso et al., 1999). Alterations of the p63-DLX3 molecular pathway, either through misregulation of DLX3 gene transcription or different sensitivity of ΔNp63α mutant proteins to DLX3-mediated degradation, might result in p63 hyper or hypo-activity with pathological effects. For instance, we have previously observed that EEC or SHFM-derived TA and ΔNp63α proteins show a different activity on the DLX3 gene promoter compared to the corresponding wild-type proteins (Radoja et al., 2007). It will be of clinical importance to determine, in an in vivo model, how mutant p63 influences the expression of DLX3 and find out if it is correlated with different p63-associated phenotypes.

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