Homeobox gene *Dlx3* is regulated by p63 during ectoderm development: relevance in the pathogenesis of ectodermal dysplasias

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Ectodermal dysplasias (EDs) are a group of human pathological conditions characterized by anomalies in organs derived from epithelial-mesenchymal interactions during development. Dlx3 and p63 act as part of the transcriptional regulatory pathways relevant in ectoderm derivatives, and autosomal mutations in either of these genes are associated with human EDs. However, the functional relationship between both proteins is unknown. Here, we demonstrate that *Dlx3* is a downstream target of p63. Moreover, we show that transcription of *Dlx3* is abrogated by mutations in the sterile α-motif (SAM) domain of p63 that are associated with ankyloblepharon-ectodermal dysplasia-clefting (AEC) dysplasias, but not by mutations found in ectrodactyly-ectodermal dysplasia-cleft lip/palate (EEC), Limb-mammary syndrome (LMS) and split hand-foot malformation (SHFM) dysplasias. Our results unravel aspects of the transcriptional cascade of events that contribute to ectoderm development and pathogenesis associated with *p63* mutations.

KEY WORDS: *Dlx3*, *p63*, Transcription, Ectodermal dysplasias, Mouse development

INTRODUCTION

During embryonic development and organ formation, a series of signals between epithelial cells and the underlying mesenchymal cells are the basis for the formation of a variety of appendages and/or organs (Pispa and Thesleff, 2003). Anomalies in epithelial-mesenchymal-derived organs are characteristics of human pathological conditions defined as ectodermal dysplasias (EDs) (Priolo and Lagana, 2001).

Mutations in *DLX3* and *p63*, among other genes, have been directly linked with EDs. The Dlx and p63 families of transcriptional effectors are essential for the development of the epidermis and/or embryonic appendages (Panganiban and Rubenstein, 2002; Merlo et al., 2003; Morasso and Radoja, 2005; Koster and Roop, 2004). *Dlx3* expression has been detected in the hair follicle, tooth, limb bud, branchial arches, labyrinthe layer of the placenta, osteoblasts and epidermis (Morasso et al., 1995; Morasso et al., 1999; Hassan et al., 2004). Here, we present evidence that *Dlx3* is regulated by p63 as part of a transcriptional regulatory pathway relevant to specific EDs.

p63 regulates multiple signaling pathways, such as the bone morphogenetic protein (BMP) and fibroblast growth factor (FGF) pathways (Laurikkala et al., 2006; Barbieri and Pietenpol, 2006). Transgenic and knockout (KO) mouse models indicate that p63 has essential roles in the development and maintenance of the stratified epidermis (Yang et al., 1999; Mills et al., 1999; Koster et al., 2004; Koster and Roop, 2004). The *p63* gene is transcribed from two distinct promoters, giving rise to proteins that either contain (TAp63) or lack (ΔNp63) the amino terminal transactivating domain. The TA and ΔN isoforms both possess the DNA-binding and oligomerization domains, and, by alternative splicing at the 3′ end, produce isoforms with different C-termini, termed alpha (α), beta (β) and gamma (γ) (Yang et al., 1998). The α isoforms contain a sterile α motif (SAM) – a domain with reputed importance in protein-protein interactions (Qiao and Bowie, 2005). p63 isoforms act as transcriptional activators and/or repressors (Ghioni et al., 2002; King et al., 2003; Wu et al., 2005), and bind to two or more tandem repeats of RRRCWWGYYY, but preferentially activate the RRRCGTGYYY sequence (Osada et al., 2005).

Mutations in the *p63* gene have been associated with EDs that include ectrodactyly-ectodermal dysplasia-cleft lip/palate (EEC), limb-mammary syndrome (LMS), split hand-foot malformation (SHFM) and ankyloblepharon-ectodermal dysplasia-clefting (AEC) syndrome. There is a correlation between the position of the mutation and the observed abnormal phenotype (van Bokhoven et al., 2001; McGrath et al., 2001; van Bokhoven and Brunner, 2002). Mutations in the *DLX3* gene are linked to tricho-dento-osseous (TDO) syndrome, which, like AEC, is characterized by defects in the development of hair, teeth and bone, and by absence of overt limb malformations (Price et al., 1998).
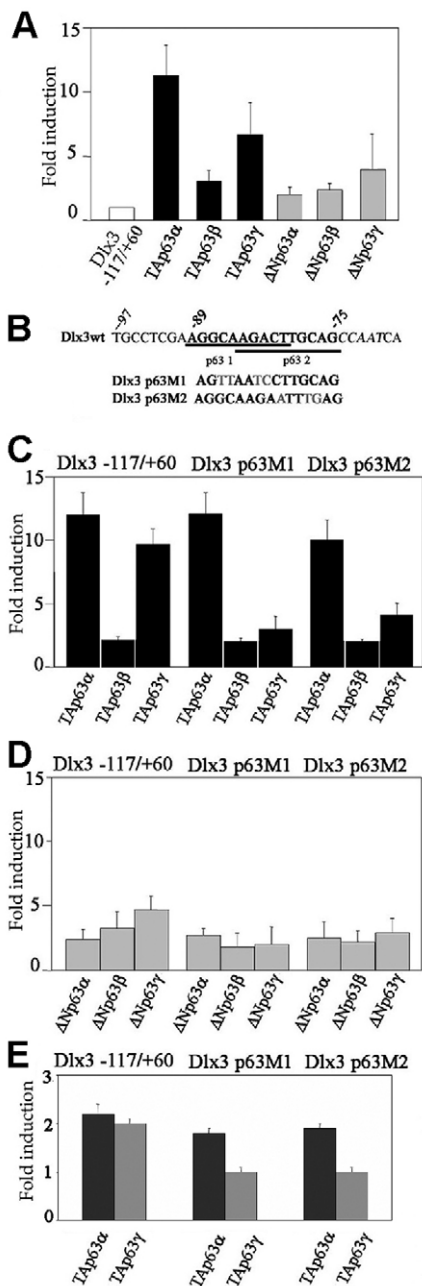
Partial-overlapping mRNA expression and phenotypes of specific human malformations caused by molecular lesions in either *p63* or *DLX3* suggest that these genes are components of common signaling pathways during embryonic development. Here, we show that p63 is able to bind and transactivate *Dlx3* both in vitro and in vivo. Mutant p63 proteins derived from AEC patients exhibit an impaired ability to transactivate *Dlx3*, indicating that the misregulation of the *DLX3* gene is involved in the pathogenesis of human syndromes associated to AEC.

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Fig. 1. Transcriptional regulation of the Dlx3 promoter by p63. (**A**) Fold-induction increase, compared with wild type, of Dlx3$_{-117/+60}$ co-transfected with vectors expressing the different p63 isoforms. (**B**) Sequence of the mouse *Dlx3*-promoter region containing the two overlapping p63-binding sites (p63 site1 and p63 site2; underlined) and CCAAT box (italics). The mutated Dlx3 sequences are shown in gray. (**C-D**) Dlx3$_{-117/+60}$, Dlx3p63M1 and Dlx3p63M2 constructs were co-transfected with vectors expressing the TAp63 (C) and ΔNp63 (D) isoforms, with activity shown relative to the basic activity of Dlx3$_{-117/+60}$. Transient transfection experiments were performed using mouse keratinocytes (C-D) and Saos-2 cells (**E**). Basal activity of the reporter was set to 1. Each histogram bar represents the mean of three independent transfection duplicates. Standard deviations are indicated. Dlx3 p63M1, mutated at p63-binding-site 1; Dlx3p63M2, mutated at p63-binding-site 2.

newborn foreskin and cultured in K-SFM (Invitrogen). The human osteosarcoma Saos-2 cell line was maintained in RPMI 1640 and 10% FCS. The immortalized human keratinocyte HaCaT and H1299 non-small-lung-carcinoma cell lines were grown in DMEM and 10% FBS.

Transient transfection
Transient transfections of keratinocytes were performed with FuGENE6 (Roche) in a 1:3 ratio. PRL-SV40 vector was used as an internal control. Luciferase activity was measured 24-36 hours after transfection using the Dual-Luciferase Reporter Assay System (Promega). Transient transfections of Saos-2 and H1299 cells were performed according to Calabrò et al. (Calabrò et al., 2002). CAT reaction was performed 48 hours after transfection using 90 μg of cell extract. β-Gal was used to normalize for transfection efficiency.

EMSA analysis
Nuclear extracts and EMSA analysis were carried out according to Park and Morasso (Park and Morasso, 1999) using the Dlx3 p63site1+2 and non-specific competitor AP-2 binding site.

Chromatin immunoprecipitation assay
Chromatin immunoprecipitation (ChIP) was performed with chromatin from mouse keratinocytes and H1299 cells transfected with TAp63α according to Caretti et al. (Caretti et al., 2004) using no antibody, IgG or p63 antibody (Santa Cruz, H137 and 4A4). Real-time PCR was performed using the Mx3000P System (Stratagene) and SyberGreen MasterMix (Applied Biosystems) with independent DNA samples and the following oligonucleotides: for mouse, Dlx3(F) 5′-GAGAAAGCGCGAGCGT-GTTTTGCC-3′ and Dlx3(R) 5′-CCGGCTGTCGGTCAGTCGCTGCGT-3′; for human, DLX3(F) 5′-AGAGAGGCGGAAGAGACGAG-3′ and DLX3(R) 5′-GAGGAGGGGAGGAGAGAAGGA-3′; and for JAG2(F) 5′-CAAGTGGTGAACAAGGGAGACT-3′ and JAG2(R) 5′-ACTG-CTGCCTTCTGGAAACTC-3′. Data are presented as fold differences relative to input and values are obtained by IgG with the formula $2^{[(CtIgG-CtInput)-(CtAb-CtInput)]}$, where Ct is threshold cycles, IgG is normal rabbit IgG, Ab is specific antibody and Input is input genomic DNA. ACHR amplification was performed as a control for H1299 transfected with TAp63α using ACHR(F) 5′-TGCCTCGGGTGAACTAAGATG-3′ and ACHR(R) 5′-GCCTCATTCGTCTTGGGAACT-3′.

Real-time PCR
For analysis of the expression of *p63* in mouse keratinocytes and embryonic tissues, the following oligonucleotides were used: TAp63(F) 5′-AGA-CAAGCGAGTTCCTCAGC-3′, TAp63(R) 5′-TGCGGATACAATCC-ATGCTA-3′, ΔNp63(F) 5′-ATGTTGTACCTGGAAAACAATG-3′, ΔNp63(R) 5′-GATGGAGAGAGGGCATCAAA-3′. These oligonucleotides are designed in regions of the mRNA common to all isoforms (α, β and γ), and do not distinguish between these variants.

For *Dlx3* expression in mouse keratinocytes, the following oligonucleotides were used: Dlx3(F) 5′-ATTACAGCGCTCCTCAGCAT-3′ and Dlx3(R) 5′-GCCTATAGGATCCCCCGTAG-3′. In embryonic

MATERIALS AND METHODS
DNA constructs
The –117 to +60 DNA fragment of the *Dlx3* promoter (Park and Morasso, 1999) was inserted into the pGL3-Basic and pCAT-Basic vectors (Promega). Mutations in the p63-binding sites of the *Dlx3* promoter were obtained using the ExSite Mutagenesis kit (Stratagene). The coding sequences for *ΔNp63α*, *ΔNp63β*, and *ΔNp63γ* were cloned into pBK-CMV (Stratagene). The *TAp63α*, *TAp63β* and *TAp63γ* constructs were a gift from E. Candi (University of Rome 'Tor Vergata', Rome, Italy) and G. Melino (University of Rome 'Tor Vergata', Rome, Italy). The p63 mutants *L518F*, *L518V* and *Q540L* (AEC); *E639X* (SHFM); *FS525* (EEC); and *G76W* and *ΔAA* (LMS) were kindly provided by H. van Bokhoven (Radboud University, Nijmegan, The Netherlands).

Cell culture
Primary mouse keratinocytes were grown according to Park and Morasso (Park and Morasso, 1999). Normal human epidermal keratinocytes [NHEK; a gift of M. Simon (SUNY, Stony Brook, NY, USA)] were derived from

tissues: Dlx3(F) 5′-CGTTTCCAGAAAGCCCGTA-3′ and Dlx3(R) 5′-CGTGGAATGGGAAGATGTGT-3′. For normalization: GAPDH(F) 5′-TGTCAGCAATGCATCCTGCA-3′ and GADPH(R) 5′-TGTATGC-AGGGATGATGTTC-3′.

For the analysis of *p63* and *Dlx3* mRNA levels in embryonic tissues, E10.5, E11.5 and E12.5 anterior or posterior limb buds from wild-type embryos were dissected, pooled in TRIZOL (Roche) and extracted, as indicated by the manufacturer. Real-time PCR was performed with a LightCycler (Roche) using FastStart DNA MasterPLUS SYBR-Green I (Roche). Standard curves were performed using wild-type cDNA with four calibration points: TQ; 1:3; 1:9 and 1:27. All samples were done in duplicates and the analysis was repeated twice. GAPDH was used for normalization, calculated using LightCycler Software 3.5.3. The results are expressed with the value relative to E10.5 (set at 1) for each mRNA.

Immunohistochemistry and whole-mount in situ hybridization
Immunohistochemistry was performed on 11 μm cryostatic sections of E10.5 embryonic forelimbs. Sections were blocked with 10% goat serum in PBS for 40 minutes at room temperature. Antibodies used were: mouse monoclonal anti-p63 (4A4, 1:100, Santa Cruz) and rabbit anti-distal-less [pan-anti-Dlx, 1:100; kindly provided by G. Boekhoff-Falk (University of Wisconsin Medical School, WI, USA)]. As secondary antibody, anti-mouse-Cy2, anti-rabbit-Cy3 (1:100; Jackson Immuno-Research) and Envision anti-rabbit HRP (Dako) were used. Fluorescence micrographs were taken by confocal microscopy.

Whole-mount in situ hybridization was performed according to Acampora et al. (Acampora et al., 1999) on E10.5 *p63* KO embryos [Brdm2 line of *p63* KO kindly provided by D. Roop (Baylor College of Medicine, Houston, USA)] using a *Dlx3* probe (Morasso et al., 1995).

RT-PCR
Total RNA from human Saos-2 and HaCaT cells was prepared with TRIZOL (Roche). For reverse transcription (RT)-PCR, 3-4 μg of total RNA were reverse-transcribed using SuperScript II (Invitrogen). The following oligonucleotides were used: Dlx3(F) 5′-ACCTACGGAGCCTCCTACCG-3′, Dlx3(R) 5′-ACTCAGGTTCTGTGCGTGAT-3′, p63α(F) 5′-GTCT-CCATCTTCATATGGTAAC-3′, p63α(R) 5′-CACACTGACTGTAGAG-GCA-3′, p63β(F) 5′-GTCTCCATCTTCATATGGTAAC-3′, p63β(R) 5′-CTTGCCAAATCCTGACAATGCTGC-3′, p63γ(F) 5′-GAGGATAG-CATCAGAAAACAGCAAG-3′ and p63γ(R) 5′-CTCCACAAGCTCAT-TCCTGAAGC-3′. For normalization: Cyclophillin(F) 5′-ATCACCA-TTGCTGACTGTGG-3′, Cyclophillin(R) 5′-ACTCTGCAATCCAGC-TAGGC-3′, GAPDH(F) 5′-GTCTCCATCTTCATATGGTAA-3′ and GAPDH(R) 5′-CCACAGTCCATGCCATCACT-3′ were used.

RESULTS AND DISCUSSION
The existence of malformations caused by either *p63* or *DLX3* gene mutations that translate to partially overlapping phenotypes suggests that these genes are transcriptional effectors in common signaling cascades regulating epidermal development. The severity of the phenotype in p63-null mice suggests that it is a crucial upstream regulator of these signaling pathways (Mills et al., 1999; Yang et al., 1999). Detailed analysis of the *Dlx3* proximal promoter region revealed a sequence with two p63-like overlapping binding sites immediately upstream of the CCAAT box, located from –89 to –80 bp (site 1) and from –84 to –75 bp (site 2) of the transcriptional start site (Park and Morasso, 1999). Because the expression patterns of *p63* and *Dlx3* overlap throughout embryonic development (Morasso and Radoja, 2005), we proceeded to test the ability of different p63 isoforms to transactivate the *Dlx3* promoter. The Dlx3$_{-117/+60}$ construct, which contains the two overlapping sites, was transiently transfected into primary mouse keratinocytes in either the absence or presence of expression vectors encoding TAp63α, TAp63β, TAp63γ, ΔNp63α, ΔNp63β or ΔNp63γ. The TA isoforms activated the Dlx3$_{-117/+60}$ promoter at a magnitude of twelve-, three- and seven-
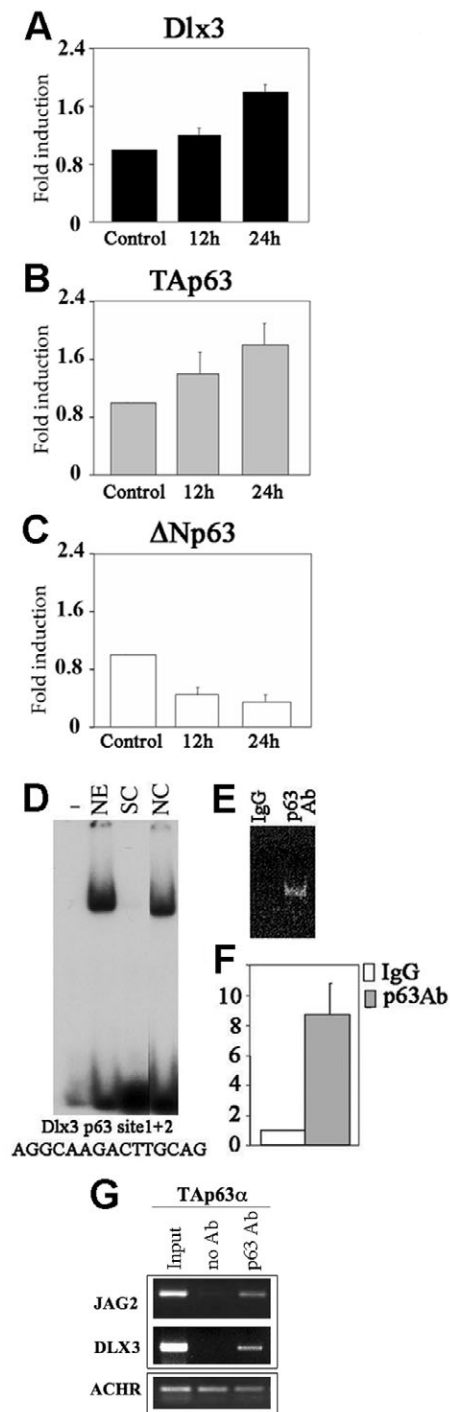

Fig. 2. *p63* and *Dlx3* expression in primary mouse keratinocytes cultured in vitro. *Dlx3* (**A**) and *TAp63* (**B**) mRNAs were induced, and *ΔNp63* (**C**) mRNA was downregulated after 12- and 24-hours of high-[Ca^{+2}] treatment. (**D-F**) p63 binds to the Dlx3 promoter region in vitro and in vivo. (D) EMSA assay performed with a DNA fragment that included the Dlx3 p63-binding site 1 and 2 using nuclear extract from primary keratinocytes (NE), and in the presence of 100 M excess of specific (SC) and nonspecific (NC) competitors. (**E-F**) ChIP analysis on mouse keratinocytes with either control IgG or p63 antibody (p63 Ab) on the region of the *Dlx3* promoter containing the p63-binding sites by regular (E) and real-time (F) PCR. (**G**) ChIP analysis on TAp63α-transfected H1299 cells with no antibody (no Ab) or p63 antibody on DLX3 and JAG2 promoters. ACHR was used as a control.
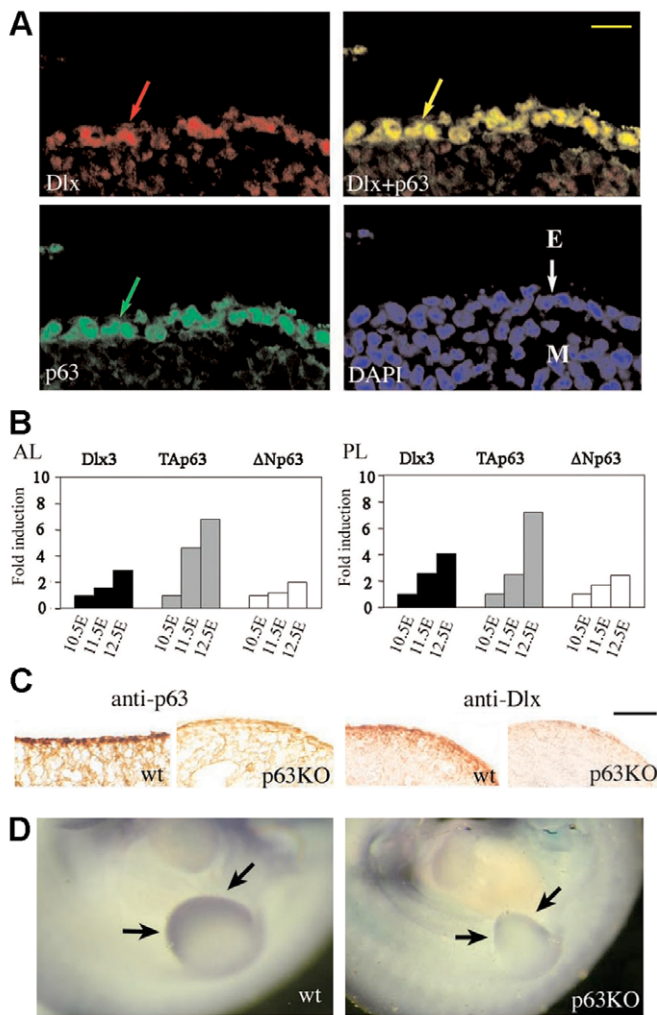
Fig. 3. p63 and Dlx3 colocalize in the embryonic ectoderm, and
***Dlx3* expression is downregulated in p63-KO embryos.** (**A**)
Histochemistry with anti-distal-less (pan-anti-Dlx; red) and p63 (green)
antibodies on the dorsal forelimb ectoderm of E10.5 wild-type embryos
(merge of Dlx and p63 expression is yellow). DAPI staining is also
shown (blue). Arrows indicate the dlx-p63 double-positive nucleus. (**B**)
Relative mRNA abundance, determined by real-time PCR, for *Dlx3*,
TAp63 and *ΔNp63* in the anterior limb (AL) and posterior limb (PL) of
E10.5, E11.5 and E12.5 wild-type embryos. The relative abundance is
expressed as fold-induction relative to the value at E10.5 (set at 1). (**C**)
Histochemistry with anti-p63 and anti-Dlx on E10.5 wild-type and p63-
KO limb-bud ectoderm. (**D**) Whole-mount in situ hybridization on wild-
type and *p63*-KO E10.5 embryos with a *Dlx3* antisense probe. Arrows
indicate limb buds. M, mesoderm; E, ectoderm. Scale bars: 10 μM in A
and 40 μM in C. wt, wild type.

fold, respectively, compared to normal activation, whereas the
exogenous expression of the ΔN isoforms resulted in a two- to four-
fold greater transactivation compared with wild type (Fig. 1A). These
effects are specific for p63 isoforms, because p53 did not transactivate
the *Dlx3* promoter (data not shown).

In order to test each overlapping site (p63-binding-site 1 and
p63-binding-site 2, Fig. 1B), we compared the activities of
the Dlx3$_{-117/+60}$, Dlx3$_{-117/+60}$ p63M1 (mutated p63 site 1) and
Dlx3$_{-117/+60}$ p63M2 (mutated p63 site 2) constructs (Fig. 1B) by co-
transfection performed in the presence of the TA and ΔNp63
isoforms (Fig. 1C,D). Our results indicate that TAp63α induction of

Dlx3 is mediated through either of the two p63 sites, whereas
TAp63γ required an intact regulatory region. These results are not
cell-type specific, because a similar profile was obtained using Saos-
2 cells (Fig. 1E).

Since *Dlx3* is induced in keratinocytes cultured in 0.12 mM Ca^{2+}
(Park and Morasso, 1999), we compared the endogenous expression
of the *p63* isoforms with *Dlx3* after 12- and 24-hours of 0.12 mM
Ca^{2+} treatment (Fig. 2A-C). The real-time PCR results showed a
correlation between the upregulation of *Dlx3* and *TAp63* and the
downregulation of *ΔNp63* mRNAs associated by Ca^{2+}-induced
differentiation, and are consistent with the recent report by King et
al. (King et al., 2006). The specificity of the PCR products was
corroborated by sequencing (data not shown). *TAp63* proteins are
found in normal adult tissues (Nylander et al., 2002) and during
mouse embryonic development (Koster et al., 2004) (also our own
data). Findings of Koster et al. (Koster et al., 2004) support a role for
TAp63 as a molecular switch for the initiation of epithelial
stratification. Our findings support a working model in which, once
transactivated by *TAp63α*, *Dlx3* will in turn regulate the expression
of terminal differentiation markers (Morasso et al., 1996).

In order to demonstrate direct binding to the p63 region in the *Dlx3*
promoter, we performed EMSA with a fragment comprising –89 to
–75 bp (Dlx3 p63 sites 1 and 2) using nuclear extracts from primary
keratinocytes (NE). A shift was detected (Fig. 2D, lane NE), and the
complexes were competed with a specific competitor (Fig. 2D, lane
SC), but not with a nonspecific DNA competitor (Fig. 2D, lane NC).
We next evaluated whether p63 bound this region of the *Dlx3*
promoter in vivo. In mouse keratinocytes, ChIP experiments were
performed with a p63-specific antibody and analyzed by regular PCR
(Fig. 2E) and real-time PCR (Fig. 2F). The data shows that p63
specifically binds to the *Dlx3* promoter in vivo (eightfold higher than
with IgG control). Moreover, ChIP experiments on TAp63α-
transfected H1299 cells, which are devoid of p63, demonstrated the
direct binding of TAp63α to the Dlx3 promoter (Fig. 2G).

To further explore the relationship of Dlx3 and p63 in vivo, we
analyzed their colocalization by immunofluorescence on E10.5
embryonic forelimb sections with anti-p63 and anti-distal-less
antibodies. The latter reagent recognizes Dlx3 in the limb ectoderm.
p63 and Dlx3 immunoreactivity were found to colocalize in the same
nuclei (Fig. 3A). Comparison of the expression of *Dlx3*, *TAp63* and
ΔNp63 in the limbs at embryonic stages E10.5, E11.5 and E12.5 was
performed by real-time PCR. Between E11.5 and E12.5, the relative
abundance of both *TAp63* and *Dlx3* mRNA increased from three- to
eight-fold relative to their expression at E10.5 (set at 1) in the anterior (AL)
and posterior limbs (PL) (Fig. 3B), whereas expression of *ΔNp63* was
only moderately increased. These results for p63 in the limb ectoderm
of embryos at E10.5-E12.5 are in agreement with reported data
(Koster et al., 2004) and show for the first time that a good correlation
is observed between the expressions of *Dlx3* and *TAp63*.

To provide further evidence that p63 is an upstream regulator of
Dlx3, we studied the effect of p63 ablation on *Dlx3* mRNA and
protein expression in the Brdm2 *p63* KO mice (Mills et al., 1999)
(Fig. 3C-D). As assessed by immunohistochemistry, the abundance
of Dlx3 protein was significantly reduced in *p63*-KO limb ectoderm
(Fig. 3C). Analysis by whole-mount in situ hybridization with a
Dlx3 antisense probe demonstrated that the absence of p63 led to a
downregulation of *Dlx3* (Fig. 3D).

We next studied the clinical relevance of p63-mediated regulation
of *Dlx3* expression. We examined the transcriptional activity of the
Dlx3 promoter in the presence of p63 mutants causative of human
AEC, EEC, SHFM or LMS (Fig. 4A) in the H1299 cell line. The *p63*
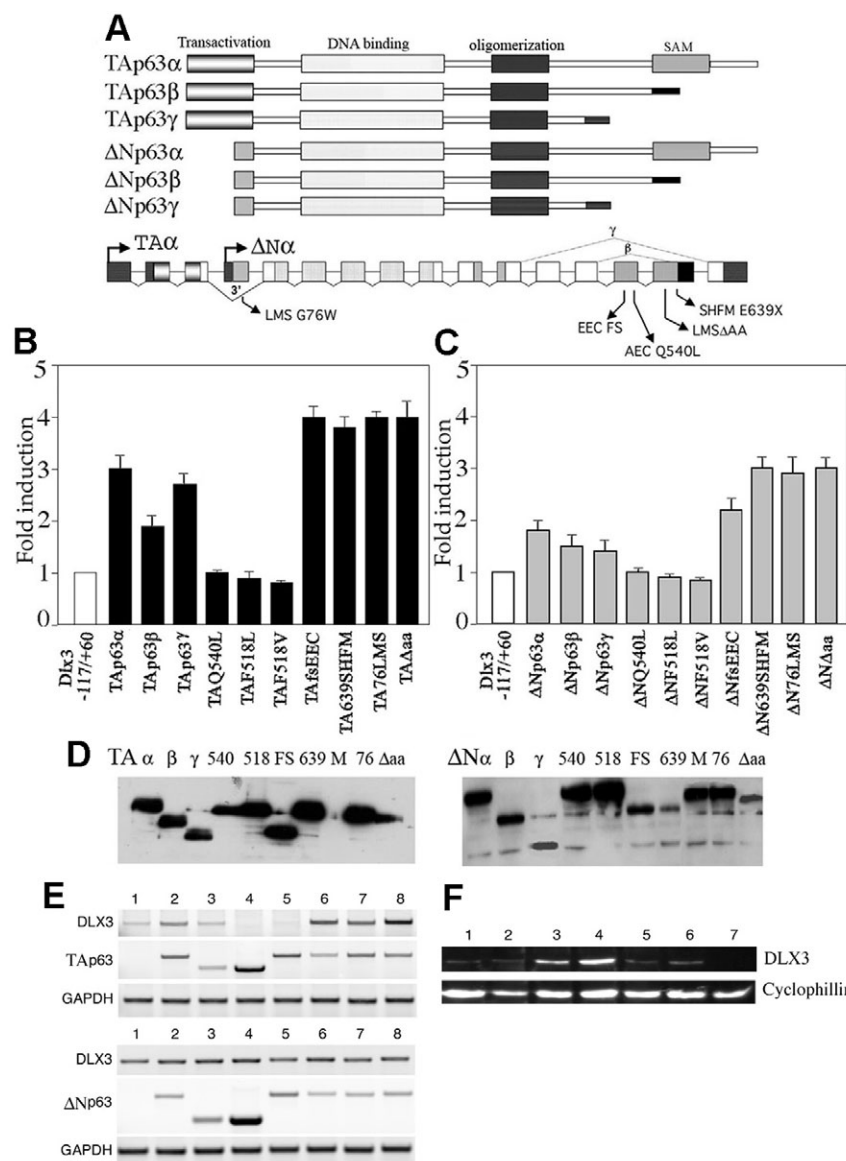AEC mutants – L518V, L518F and Q540L – are all point

Dlx3 and p63 in ectoderm development

Fig. 4. Differential ability of p63-mutant proteins to transactivate Dlx3. (**A**) Schematic representation of p63 transcripts and mutations. (**B-C**) Transcriptional regulation of the *Dlx3* promoter by TAp63 (B) and ΔNp63 (C) wild-type and mutant proteins. H1299 cells were co-transfected with the *Dlx3* reporter plasmid and expression vectors for *TAp63-* and *ΔNp63*-mutant isoforms. The basal activity of the reporter was set to 1. Each histogram bar represents the mean of three independent transfections. Standard deviations are indicated. (**D**) Proteins were corroborated by western blot analysis with anti-p63 4A4 antibody (Santa Cruz). (**E**) The level of endogenous *Dlx3* mRNA upon transfection with p63 mutants in Saos-2 cells. Top panel, lanes: 1, mock; 2, TAp63α; 3, TAp63β; 4, TAp63γ; 5, TAp63F518V-AEC; 6, TAp63FS-EEC; 7, TAp63E639X-SHFM; and 8, TAp63ΔAA-LMS. Bottom panel, lanes: 1, mock; 2, ΔNp63α; 3, ΔNp63β; 4, ΔNp63γ; 5, ΔNp63F518V-AEC; 6, ΔNp63FS-EEC; 7, ΔNp63E639X-SHFM; and 8, ΔNp63ΔAA-LMS. GAPDH was used for normalization. (**F**) The level of endogenous *Dlx3* mRNA upon transfection with TAp63α and TAp63γ in HaCaT cells. Lanes: 1, mock; 2, TAp63α 0.5 μg; 3, TAp63α 2 μg; 4, TAp63α 4 μg; 5, TAp63γ 0.5 μg; 6, TAp63γ 2 μg; 7, TAp63γ 4 μg. Cyclophillin was used for normalization.

substitutions within the SAM domain, present only in *TAp63α* and *ΔNp63α*. The *p63αFS* mutant contains a mutation found in EEC that generates a frameshift at amino acid 525, which leads to a premature stop. The E639X mutation, in exon 14, was isolated in a SHFM patient, whereas the 2-bp deletion, in exon 14 (ΔAA), was present in one family with LMS. The G76W mutation, in exon 3, was isolated from a LMS patient and affects all p63 isoforms. Co-transfection experiments were performed with expression vectors encoding *TAp63* and *ΔNp63* mutants. As shown in Fig. 4B and 4C, the AEC mutants failed to yield a significant level of reporter-gene expression despite having intact amino terminal and DNA-binding domains. These results point to a crucial role of the C-terminus of p63α on the regulation of *Dlx3* transcription. The LMS-derived mutants G76W and ΔAA; as well as the *FS*EEC mutant, SHFM-derived E639X TA and ΔN proteins; showed a similar mode of regulation of *Dlx3*-promoter activity compared with the corresponding wild-type proteins (Fig. 4B,C). A similar profile, albeit with a lower amount of induction, was obtained upon transfection in HaCaT cells (data not shown). The level of expression of the mutant proteins was corroborated by immunoblot analysis with anti-p63 antibody (Fig. 4D).

The hampered ability of the AEC mutants, as well as the partial overlapping phenotypes of specific malformations caused by to *p63* (i.e. AEC) or *Dlx3* (i.e. TDO) gene mutations, suggest that *Dlx3* misregulation is involved in aspects of the pathogenesis of AEC. AEC is characterized by ectodermal dysplasia, ankyloblepharon and cleft lip with cleft palate, and by the lack of limb involvement. The absence of limb defects in AEC may reflect the possibility that a putative role of Dlx3 in the limb is compensated for by other Dlx proteins (Panganiban and Rubenstein, 2002; Morasso and Radoja, 2005).

To determine the modulation of endogenous *Dlx3* by p63, we used Saos-2 cells to express wild-type and mutant p63 (Fig. 4E). Interestingly, TAp63α increased *Dlx3* expression (Fig. 4E, lane 2), and this effect was shared, although to different extents, with the EEC, SHFM and LMS mutants (Fig. 4E; lanes 6, 7 and 8). TAp63β did not alter *Dlx3* levels (Fig. 4E, lane 3). Remarkably, an AEC mutant (Fig. 4E, lane 5) and the TAp63γ isoform abolished *Dlx3* expression. Transfections of ΔNp63 isoforms, both wild-type and mutant, had no significant effect on *Dlx3* transcription (Fig. 4E, bottom panel). TAp63α and TAp63γ are potent transactivators (Barbieri and Pietenpol, 2006) and, in our in vitro studies, both isoforms were able to transactivate *Dlx3* at similar levels. However,

whereas TAp63α induction was mediated through either of the two p63 sites in the *Dlx3* promoter, TAp63γ required an intact regulatory region. Surprisingly, a slightly different outcome was obtained for the endogenous *Dlx3* regulation, where upregulation of *Dlx3* was detected with TAp63α and a complete downregulation was found when expressing TAp63γ in Soas-2 cells. To determine if these results could be attributed to cell context, the experiments were also performed in HaCaT cells. In these cells, overexpression of TAp63α showed upregulation of endogenous Dlx3, whereas TAp63γ once again caused a complete downregulation of Dlx3 expression (Fig. 4F). These differences might be attributed to the specific p63RE-CCAAT-box chromatin architecture. An important feature of the *Dlx3* promoter is that the overlapping p63-binding sites are in close proximity to CCAAT box that binds NF-Y in keratinocytes (Park and Morasso, 1999). NF-Y is a general promoter organizer that pre-sets chromatin structure locally. A recent report shows that p63α regulates the transcription of the *hsp70* gene through interactions with NF-Y (Wu et al., 2005). Although we have not determined the significance of NF-Y and p63 interactions on *Dlx3* transcriptional regulation, it might be proposed that there is a dual role for the overlapping p63-binding sites, and that *Dlx3* will be transcriptionally active or repressed depending on the specific p63 isoform bound to the promoter, on which of the p63 sites is occupied and on interactions with NF-Y CCAAT binding factor.

Dlx3 and p63 both function as part of a complex series of cascades that ultimately lead to the formation of ectoderm-derived organs. Unraveling the function of each protein at specific times of embryonic development will prove to be complex because of the differential expression of the p63 isoforms in distinct tissues (Nylander et al., 2002) and the cross-regulation with other developmentally relevant signaling pathways [i.e. FGF, BMP, and Notch (Laurikkala et al., 2006; Nguyen et al., 2006)]. The characterization of p63 target genes promises to improve our knowledge of the signaling cascades that are directly involved in normal ectodermal development. In summary, our study proves a functional relationship between p63 and Dlx3, with *Dlx3* demonstrated to be a direct target of p63. The findings also provide evidence that the misregulation of *Dlx3* is involved in the pathogenesis of p63 molecular lesions in AEC.

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