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Regulation of *Dlx5* and *Dlx6* gene expression by p63 is involved in EEC and SHFM congenital limb defects

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The congenital malformation Split Hand-Foot Malformation (SHFM, or ectrodactyly) is characterized by a medial cleft of hands and feet, and missing central fingers. Five genetically distinct forms are known in humans; the most common (type-I) is linked to deletions of *DSS1* and the *distalless*-related homeogenes *DLX5* and *DLX6*. As *Dlx5;Dlx6* double-knockout mice show a SHFM-like phenotype, the human orthologs are believed to be the disease genes. SHFM-IV and Ectrodactyly-Ectodermal dysplasia-Cleft lip (EEC) are caused by mutations in *p63*, an ectoderm-specific p53-related transcription factor. The similarity in the limb phenotype of different forms of SHFM may underlie the existence of a regulatory cascade involving the disease genes. Here, we show that p63 and Dlx proteins colocalize in the nuclei of the apical ectodermal ridge (AER). In homozygous $p63^{-}$ (null) and $p63^{EEC}$ (R279H) mutant limbs, the AER fails to stratify and the expression of four Dlx genes is strongly reduced; interestingly, the $p63^{+/EEC}$ and $p63^{+/-}$ hindlimbs, which develop normally and have a normally stratified AER, show reduced Dlx gene expression. The $p63^{+/EEC}$ mutation combined with an incomplete loss of *Dlx5* and *Dlx6* alleles leads to severe limb phenotypes, which are not observed in mice with either mutation alone. In vitro, ΔNp63α induces transcription from the *Dlx5* and *Dlx6* promoters, an activity abolished by EEC and SHFM-IV mutations, but not by Ankyloblepharon-Ectodermal defects-Cleft lip/palate (AEC) mutations. ChIP analysis shows that p63 is directly associated with the *Dlx5* and *Dlx6* promoters. Thus, our data strongly implicate *p63* and the *Dlx5-Dlx6* locus in a pathway relevant in the aetio-pathogenesis of SHFM.

KEY WORDS: Dlx, p63, Ectrodactyly, Limb development, Transcription regulation

INTRODUCTION

Congenital limb reduction defects occur in approximately 1:2000 live births, among which the anomalies of the central ray constitute an important subgroup (Buss, 1994). The malformation Split-Hand/Foot (SHFM, MIM 183600), also known as ectrodactyly, affects the distal portion of the upper and lower limbs, and is characterized by a deep medial cleft and missing central fingers (Sifakis et al., 2001). Genetically, SHFM comprises both isolated and hereditary forms, linked to five distinct loci (types I-V).

For SHFM-II (MIM 313350) and SHFM-V (MIM 606708), the disease genes have not been identified. SHFM-III (MIM 600095) is associated with genomic alterations on chromosome 10q24-q25 (deMollerat et al., 2003), which results in a complex rearrangement around the Dactylyn locus, possibly associated with gene inactivation. Dactylyn is also involved in a complex rearrangement/duplication in *dactylaplasia* (*dac*) mutant mice, which exhibit ectrodactyly-like limb defects (Chai, 1981; Johnson et al., 1995; Crackower et al., 1998). In spite of these findings, no demonstration that Dactylyn is the disease gene for SHFM-III, or in the *dac* mice, has been provided.

SHFM-I, the most common form, is associated with deletions of variable extent on chromosome 7q21. The minimal common deletion includes *DSS1* and the *distalless*-related homeogenes *DLX5* and *DLX6* (Simeone et al., 1994; Scherer et al., 1994a; Scherer et al., 1994b; Crackower et al., 1996). The double knock-out (DKO) of *Dlx5* and *Dlx6* in the mouse leads to ectrodactyly (Robledo et al., 2002; Merlo et al., 2002; Merlo et al., 2003), implicating the human orthologs *DLX5* and *DLX6* in this pathology. Mutations in the *DLX5-DLX6* locus have not been found, therefore the molecular alteration remains unknown; however, a 'position effect' mutagenic mechanism for SHFM-I has been proposed (Scherer et al., 2003). Dlx genes code for six *distalless*-related homeodomain transcription factors (Dlx1-Dlx6) that play key roles in the development and morphogenesis of the head and limb skeleton (Merlo et al., 2000; Merlo et al., 2003; Panganiban and Rubenstein, 2002). Expression of *Dlx5* and *Dlx6* has been detected in the apical ectodermal ridge (AER) of the embryonic limb buds, in the pharyngeal arches, in the osteoblasts of developing bones and in interneurons of the basal forebrain (Simeone et al., 1994; Acampora et al., 1999; Levi et al., 2003). In spite of known functions of *distalless* for the development of insect appendages, little is known about the molecular regulation of Dlx genes in mammalian limbs. Defining the upstream regulation of the *Dlx5-Dlx6* locus during limb development might help to clarify the molecular basis of SHFM.

SHFM-IV (MIM 605289) is caused by mutations in *p63*, a gene coding for a transcription factor homologous to p53 and p73 (Ianakiev et al., 2000; vanBokhoven and Brunner, 2002; Berdon-Zapata et al., 2004). In 50 unrelated SHFM patients, five mutations in *p63* were found, suggesting that these may account for about 10% of sporadic SHFM (vanBokhoven et al., 2001; vanBokhoven et al., 2002). Mutations of *p63* are also responsible for other autosomal, dominantly inherited human syndromes, including Ectrodactyly-Ectodermal dysplasia-Cleft lip (EEC), Limb-Mammary Syndrome

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(LMS) and Ankyloblepharon-Ectodermal defects-Cleft lip/palate (AEC) (Celli et al., 1999; vanBokhoven et al., 2001; Rinne et al., 2006; Rinne et al., 2007). *p63*-null mice show severe defects affecting their skin, limbs, craniofacial skeleton, and they lack teeth, hair and mammary glands. In $p63^{-/-}$ newborn animals, the hindlimbs (HLs) are absent, whereas the forelimbs (FLs) are severely truncated in the distal segments (Mills et al., 1999; Yang et al., 1999).

p63 is transcribed as two classes (*TA* and *ΔN*) of mRNAs. The use of alternative promoters drives the transcription of either TAp63 proteins, comprising a p53-related N-terminal transactivation (TA) domain, a DNA-binding (DB) and an oligomerization (OD) domain, or ΔNp63 proteins, lacking the TA domain. Additional TA domains have been identified that account for the transcriptional activities of the ΔN isoforms (Dohn et al., 2001; Ghioni et al., 2002; Laurikkala et al., 2006). Three alternative splicing routes at the 3′-end generate TAp63 and ΔNp63 proteins with different C-termini, denoted α, β and γ (vanBokhoven and Brunner, 2002). A Sterile-Alpha-Motif (SAM) and a Transcription-Inhibitory-Domain (TID) are present only in the α-isoforms (Serber et al., 2002; Qiao and Bowie, 2005).

The limb defects of $p63^{-/-}$ mice have been associated with a failure of AER formation and the loss of expression of key morphogens. *p63* is expressed in several ectoderm-derived tissues: it is essential for the initiation of the epithelial stratification program during embryonic development (Koster et al., 2004; Barbieri and Pietenpol, 2006; Laurikkala et al., 2006) and to maintain the proliferation potential of epithelial stem cells (Senoo et al., 2007). The AER is a stratified embryonic epithelium, therefore it seems logical that p63 might control its function and maintenance via regulation of AER-restricted target genes (Koster and Roop, 2004; Koster et al., 2007), such as the Dlx genes.

Here, we provide evidence that *p63* is genetically upstream of Dlx genes in the AER, a region critical for normal limb development. We identify ΔNp63α as the main regulatory isoform, able to induce transcription of the *Dlx5* and *Dlx6* promoters, whereas EEC and SHFM-IV mutations impair this activity. In vivo, combining the $p63^{+/EEC}$ mutation with the incomplete loss of *Dlx5-Dlx6* results in aggravated limb defects. These data indicate that alteration of the *p63*-Dlx pathway is the most likely molecular basis of SHFM-I and SHFM-IV.

MATERIALS AND METHODS

Mouse strains

Dlx5 and *Dlx5;Dlx6* embryos were genotyped as described (Acampora et al., 1999; Merlo et al., 2002). The *Brdm2* mice carry a null allele, also denominated $p63^{-}$ (Mills et al., 1999), and were genotyped by RT-PCR on embryonic RNA, with primers annealing to the C terminus of the α isoform mRNA and amplifying a 450 bp fragment. Primers were:

Fwd p63, 5′-CCAGATGATGAGCTGCTGTACC-3′; and

Rev p63, 5′-TAGTCCAGGCATGATGAG-3′.

Mice carrying the $p63^{R279H}$ ($p63^{EEC}$) mutation (an EEC mutation in humans) were generated by A. Mills (Cold Spring Harbor Laboratory, USA; E.G. and A.A.M., unpublished). The mutation abrogates a *Bsa*HI restriction site. The genotype was determined by PCR amplification with primers flanking codon 279, followed by digestion with *Bsa*HI to distinguish the two alleles. Primers were:

Fwd Exon 7, 5′-TCTGGAGCGCAGAGCAAAAGCC-3′; and

Rev Exon 7, 5′-AGAATTCTGCTTGGTCCTTGGCC-3′.

Preparation of tissue samples

All experiments involving the use of animals were approved by the Institutional Animal Care Committee and by the Ministry of Health. Pregnant mice were sacrificed between 10.5 and 12.5 days of pregnancy, embryos were collected by caesarean section. For histochemistry and section in situ hybridization, limbs were dissected, fixed in 4% paraformaldehyde (PFA) overnight, rinsed in PBS at 4°C, cryoprotected with 20% sucrose, embedded in OCT and sectioned at 11 μm. For whole-mount hybridization, PFA-fixed embryos were stored in methanol at –20°C. Skeletal staining was done according to published procedures (Wallin et al., 1994). For RT-PCR or RealTime analyses, limbs were dissected in PBS, transferred in RNAlater (Ambion) and stored at –20°C until extraction.

Immunohistochemistry and in situ hybridization

Antibodies used were: mouse monoclonal anti-p63 (4A4, Santa Cruz, 1:100), rabbit anti-distalless (from Dr G. Boekhoff-Falk, 1:100) and rabbit anti-E-cadherin (C20820, Translucent Laboratory, 1:200). Secondary antibodies were: anti-mouse-Cy2 and anti-rabbit-Cy3 (Jackson ImmunoResearch, 1:200). Confocal micrographs were obtained using the sequential frame-scanning mode, followed by stacking and digital merging.

In situ hybridization was carried out with digoxigenin (DIG)-labeled antisense murine RNA probes corresponding to the complete coding sequence of *Dlx5* and *Dlx2*, and to a fragment of *Dlx6* comprising exons III and IV. For each probe, two normal and two mutant specimens were examined. Section and whole-mount hybridization was done according to published procedures (Levi et al., 2006).

RT-PCR, RealTime PCR and western blot analyses

FL and HL buds (eight for each genotype) were dissected, genotyped and pooled in TriPure (Roche) for RNA extraction, as indicated by the supplier. One μg of RNA was reverse-transcribed using SuperScript II (Invitrogen). RealTime quantitative PCR (qPCR) was performed with LightCycler and FastStart DNA MasterPLUS SYBR-Green I (Roche). Five μl of diluted cDNA were used in each reaction, standard curves were determined using wild-type cDNA with four calibration points. Samples were tested in duplicates. Specificity and absence of primer dimers was controlled by denaturation curves. *GAPDH* mRNA was used for normalization, results were calculated using LightCycler Software 3.5.3. For primers for RT-PCR and RealTime qPCR, see Tables 1 and 2.

Western blots were performed with anti-p63 H129 and 4A4 antibodies (Santa Cruz Biotech), as described (Beretta et al., 2005). Total proteins were extracted in 2% SDS, 30% glycerol, 300 μM β-mercaptoethanol, 100 mM Tris-HCl (pH 6.8) from pools of (at least eight) HLs and FLs of E10.5 and E12.5 normal embryos.

Chromatin immunoprecipitation (ChIP) assay

ChIP was performed on sheared genomic DNA from cultured mouse keratinocytes and from H1299-tet-on ΔNp63α, using 2 μg of the 4A4 antibody, as described (Ceribelli et al., 2006; LoIacono et al., 2006). IP

Table 1. Primers for RT-PCR

Gene	Orientation	Sequence (5′ to 3′)
Mouse		
GAPDH	S	TGTCAGCAATGCATCCTGCA
GAPDH	AS	TGTATGCAGGGATGATGTTC
ΔNp63	S	GTTGTACCTGGAAAACAATGCTCAG
TAp63	S	GTGGATGAACCTTCCGAAAA
p63α	AS	TGATACGCTGCTGCTTGTTGCG
p63β	AS	CTTGCCAAATCCTGACAATGCTGC
p63γ	AS	CTCCACAAGCTCATTCCTGAAGC
Human		
GAPDH	S	TCACCAGGGCTGCTTTTAAC
GAPDH	AS	TGGAAGATGGTGATGGGATT
DLX1	S	TCCAGCCCCTACATCAGTTCG
DLX1	AS	CTGGAACCTCCGGTTCAAAGC
DLX2	S	GCACATGGGTTCCTACCAGT
DLX2	AS	ACTTTCTTTGGCTTCCCGTT
DLX5	S	CTACAACCGCGTCCCAAG
DLX5	AS	CACCTGTGTTTGTGTCAATCC
DLX6	S	CCTCGGACCATTTATTCCAG
DLX6	AS	TTGTTCTGAAACCATATCTTCACC

AS, antisense; S, sense.

Table 2. Primers for RealTime qPCR (*Dlx* and other SHFM mRNAs in mouse limbs)

Gene	Orientation	Sequence (5′ to 3′)
GAPDH	S	TGTCAGCAATGCATCCTGCA
GAPDH	AS	TGTATGCAGGGATGATGTTC
Dlx1	S	TCCGAGAAGAGTACGGTGGT
Dlx1	AS	ACTTGGAGCGTTTGTTCTGG
Dlx2	S	TGGGCTCCTACCAGTACCAC
Dlx2	AS	TGGTTTCCGGACTTTCTTTG
Dlx5	S	CTGGCCGCTTTACAGAGAAG
Dlx5	AS	CTGGTGACTGTGGCGAGTTA
Dlx6	S	AGAGCGCTTATTCTGAAACCAT
Dlx6	AS	CTCAATACCTGGCCCTTCC
Dlx6-antisense	S	GGGTGACCACTCCTCTGTGT
Dlx6-antisense	AS	CTAAGGAAGCAGCCAGTTCG
Evf2	S	GCTTCAAATTCCTACTGTGA
Evf2	AS	TTTCCCCCTTCCTACTGTGA
Dactylyn	S	GTTGTGGGCACTTCTCACCT
Dactylyn	AS	GCAGTGTAGAGGGGGACTCA
ΔNp63	S	ATGTTGTACCTGGAAAACAATG
ΔNp63	AS	GATGGAGAGAGGGCATCAAA
TAp63	S	AGACAAGCGAGTTCCTCAGC
TAp63	AS	TGCGGATACAATCCATGCTA
Perp	S	CCCAGATGCTTGTTTTCCTG
Perp	AS	ACCAGGGAGATGATCTGGAAT

AS, antisense; S, sense.

material was analysed by PCR using primers designed to amplify three fragments of approximately 200 bp of the mouse *Dlx5* promoter, two fragments of the *Dlx6* promoter, and one region of the *IκKα* and *ΔNp63* promoters, as positive control (see Table 3).

DNA constructs and plasmids

A *Sma*I-*Hin*dIII 1120-bp murine genomic fragment corresponding to part of the *Dlx5* promoter and 5′ UTR was used. A *Nco*I-*Sac*I 2000-bp fragment comprising the proximal *Dlx6* promoter, the 5′ UTR and part of the first exon was excised and treated with *Bal31* exonuclease to remove ORF sequences. A 1740-bp fragment was identified that retained the promoter/5′ UTR and terminated 11-bp upstream of the start codon. Both fragments were inserted into pGL3-Basic Luciferase Vector (Promega). Deletions in the *Dlx5* and *Dlx6* promoters were generated by restriction digestion. Wild-type and mutant *p63* cDNAs have been previously described (Rossi et al., 2006), except for the R279H mutation, which was obtained by site-directed mutagenesis and sequence verified.

Table 3. Oligonucleotides for ChIP-PCR analyses

Gene	Orientation	Sequence (5′ to 3′)
Dlx5 –2000	S	TTTAGAGCAGACCGCAATCA
Dlx5 –2000	AS	CCTACTTGCAGCCTTGGTGT
Dlx5 R1	S	AGCTTCCAAGTAGTCGAGG
Dlx5 R1	AS	CAGTTGGACACGCAGCAAGC
Dlx5 R2	S	GTGTTGCTCTGCTCCCGAAG
Dlx5 R2	AS	GGGCGGTGGCTGTTCTTCTA
Dlx6 R1	S	GCTTCTAAGCACAGGCAACC
Dlx6 R1	AS	TACCTTCCGCAGTTCCAATC
Dlx6 R2	S	AGGCCCCGCTCACTATATCT
Dlx6 R2	AS	TGCAGCCACCTTAGCTCTTT
Iκkα	S	CTCAAGTCGGAGAACAAGG
Iκkα	AS	CTCGAATCCTACCACACTCG
ΔNp63	S	AAGTTTTACCTGTCTGTCTCCTGGG
ΔNp63	AS	ATGCTGCTGTCCTTTCTTCTG

AS, antisense; S, sense.

Cell culture and transfection

Primary mouse keratinocytes were isolated from newborn mice and cultured in Keratinocyte Basal Medium (Clonetics, San Diego CA) with EGF (10 ng/ml). The human U2OS osteosarcoma and H1299 lung carcinoma cell lines were grown in DMEM with 10% FBS. The procedure and expression vectors for luciferase assay have been previously described (Beretta et al., 2005). H1299 cells stably transfected with a tet-on ΔNp63α expression plasmid were induced with Doxycyclin, as described (LoIacono et al., 2006).

RESULTS

Dlx and p63 proteins colocalize in the AER

Dlx and p63 proteins need to be coexpressed in the AER cells to be part of a regulatory cascade. We first determined that *Dlx5* and *Dlx6* mRNA are expressed in the AER of normal FLs and HLs by in situ hybridization (Fig. 1A-D′). We then carried out double p63/Dlx immunostaining on sections from wild-type E10.5 limbs. Dlx proteins were detected with an antibody raised against insect Dll, which recognizes all mammalian Dlx proteins (Panganiban et al.,

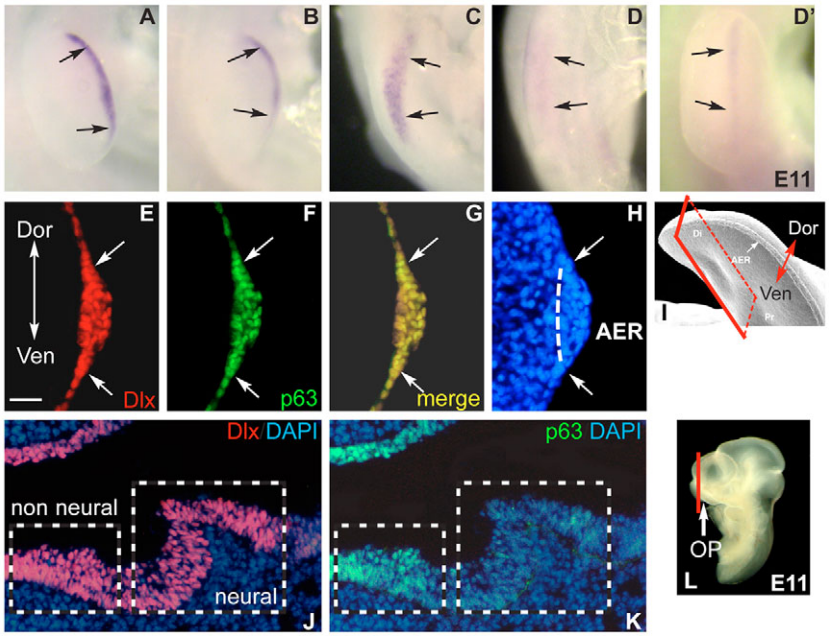

Fig. 1. p63 and Dlx proteins in the embryonic limbs. (**A-D**) Whole-mount in situ hybridization on wild-type FLs (A,B) and HLs (C,D) of E10 embryos for *Dlx5* (A,C) and *Dlx6* (B,D). (**D′**) Hybridization for *Dlx6* on wild-type HLs of E11 embryos. Lateral views are shown, the signal is indicated with black arrows. Anterior is to the top. (**E-H**) Double immunostaining for pan-Dlx (red) and p63 (green) on sections of E10.5 HLs. The merged image is shown in G. (H) Nuclei were counterstained with DAPI. Dashed line indicates the border between the AER and the limb mesenchyme. Section plane and orientation is indicated (**I**). (**J**,**K**) Double immunostaining for pan-Dlx and p63 on sections of the frontonasal region of E11 embryos, counterstained with DAPI. The olfactory placode is shown, dashed boxes indicate the neural and non-neural regions. Colocalization of p63 and Dlx proteins occurs in the non-neural epithelium. Section plane is indicated (**L**). Dor, dorsal; Ven, ventral; Di, distal; Pr, proximal; OP, olfactory placode. Scale bar: 25 μm.

1995). We observed colocalization in nearly all nuclei of the AER of both the FLs and the HLs (Fig. 1E-H). Colocalization was also observed in distal regions of the dorsal limb ectoderm, where *Dlx3* is present (Radoja et al., 2007). p63 and Dlx proteins do not always colocalize: in the olfactory placodes, Dlx and p63 are coexpressed in nuclei of the non-neural region but not in the neural region, where only Dlx proteins are present (Fig. 1J,K).

Dlx and *p63* expression are dynamically co-expressed

There are contrasting reports on the expression of the *TAp63* and *ΔNp63* mRNAs in the developing ectoderm (Nylander et al., 2002; Koster et al., 2004; Laurikkala et al., 2006). Furthermore, the dynamic expression of *p63* and Dlx genes during limb development has not been addressed. We decided to quantify, by RealTime qPCR, the levels of *TAp63* and *ΔNp63* mRNAs, and to compare them with those of *Dlx1*, *Dlx2*, *Dlx5* and *Dlx6* mRNAs in embryonic FLs and HLs at different ages (E10.5-E12.5). Primers were designed to amplify either all *TA* or all *ΔN* mRNA isoforms. The abundance of *TAp63* and *ΔNp63* mRNAs always increased from E10.5 to E12.5 (4- and 2.5-fold, respectively; Fig. 2A). However, the *TA* cDNAs were detected at a higher cycle number (CP=29) than the *ΔN* cDNAs (CP=21), indicating that the *ΔN* mRNAs are more abundant than the *TA*. A 4- and a 7-fold increase in *Dlx5* expression was observed, respectively, in FLs and HLs; in the same samples, a 4- and 3-fold increase in *Dlx6* expression was observed, whereas *Dlx1* and *Dlx2* expression increased modestly (Fig. 2A).

To clarify which p63 isoforms are predominantly expressed, we applied semi-quantitative RT-PCR using primers that can distinguish each isoform, to examine *p63* expression in embryonic FLs and HLs at different ages (E10.5-E12.5). The *TAp63α* mRNA was detected at 38 cycles, but not at 29 cycles. On the contrary, the *ΔNp63α* mRNAs was easily detected at 29 cycles and the reaction had reached a plateau before 38 cycles. The *TAp63β* and *ΔNp63β* mRNAs were both detected only after 38 cycles (Fig. 2B). The *TAp63γ* and *ΔNp63γ* mRNAs were both detected at 29 cycles. In general, the expression of p63 isoforms increased from E10.5 to E12.5, and expression in the HL was generally lower than in the FL.

Finally, we carried out western blot analysis on extracts from wild-type embryonic limbs collected at E10.5-E12.5. ΔNp63α expression was detected in the HaCaT cell extracts and in the limb samples (Fig. 2C). The abundance of ΔNp63α protein increased sharply from E10.5 to E12.5 in both FLs (Fig. 2C) and HLs (data not shown), consistent with the mRNA expression data. By contrast, TAp63α isoforms could not be detected.

These data indicate that the ΔNp63 isoforms are the predominant isoforms expressed in the embryonic limbs at the time of AER function and maintenance (E10.5-E12.5), and that the increase of Dlx gene expression parallels that of *p63* at these developmental stages.

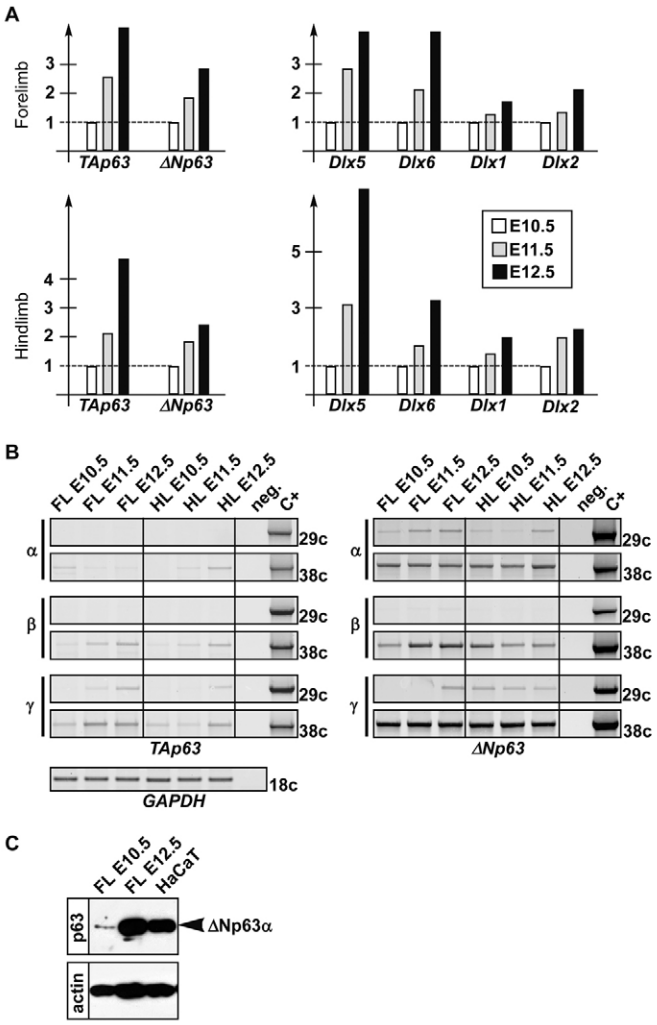

Fig. 2. Expression of *p63* and Dlx genes in embryonic limbs. (**A**) Expression of *TAp63* and *ΔNp63* (left), and of *Dlx1*, *Dlx2*, *Dlx5* and *Dlx6* (right) in FLs (top) and HLs (bottom), collected at E10.5 (white bars), E11.5 (grey bars) and E12.5 (black bars). Data are normalized against *GAPDH*. The E10.5 abundance is set at 1. (**B**) RT-PCR semiquantitative analysis of each *TAp63* (α, β, γ; left) and *ΔNp63* (α, β, γ; right) isoform mRNAs in FLs and HLs at the indicated embryonic ages. For each mRNA, 29 or 38 amplification cycles are shown (indicated on the right). *GAPDH* mRNA is used as a reference. As a positive control (C+), the cloned cDNA of each isoform was used (right-most lanes). (**C**) Western blot analysis on total protein extracts from HaCaT cells (expressing ΔNp63α) and FLs collected at E10.5 and E12.5, showing the ΔNp63α protein (black arrowhead). Expression sharply increases between E10.5 and E12.5. β-actin is used for control.

ΔNp63 activates the *Dlx5* and *Dlx6* promoters in vitro

To test the possibility that p63 may regulate *Dlx5* and *Dlx6* expression, we isolated genomic fragments comprising the proximal promoters of murine *Dlx5* and *Dlx6*, of 1150 and 1740 bp, respectively, to generate the m*Dlx5-luc* and m*Dlx6-luc* reporter vectors. These vectors were co-transfected with plasmids expressing *TA*- and *ΔNp63* cDNA isoforms into U2OS cells; U2OS cells do not normally express p63. The TAp63 isoforms showed little (*Dlx6*) or no (*Dlx5*) activity. Conversely, a 7- and 10-fold activation of *Dlx5*- and *Dlx6*-dependent transcription, respectively, was observed with ΔNp63α co-transfection (Fig. 3A). ΔNp63β showed modest activity (4- and 3-fold, respectively), and ΔNp63γ was slightly more active on the *Dlx6* promoter (7-fold) than the *Dlx5* promoter (4-fold; Fig. 3A). We repeated these experiments in the HaCaT keratinocyte cell line, which expresses *ΔNp63α* endogenously: similar results were obtained, although the overall fold-activation was lower than in U2OS cells (data not shown).

We then examined whether *p63* mutations associated with SHFM-IV (K193E and K194E), EEC (R279H and C306R) or AEC (L518F) affect the capacity of p63 to activate the *Dlx5* and *Dlx6* promoters. Except for L518F, these mutations fall within the DNA-binding domain of p63 and should result in the loss of DNA binding

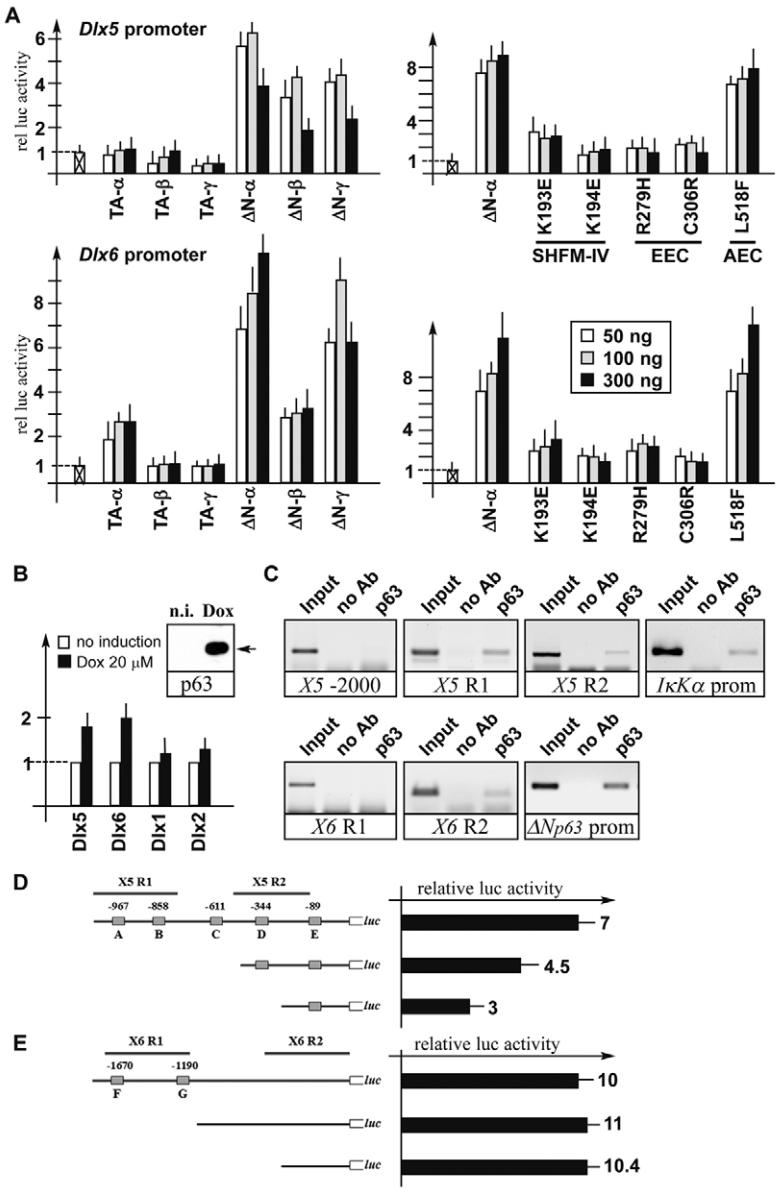

Fig. 3. Regulation of the *Dlx5* and *Dlx6* promoters by p63. (**A**) Left histograms: co-transfection of *mDlx5luc* and m*Dlx6luc* reporters (300 ng) with 50 (white bars), 100 (grey bars) or 300 ng (black bars) of *TAp63* and *ΔNp63* expression plasmids. Data are reported as fold of activation relative to basal expression (reporter vector alone, set=1). Right histograms: mutated ΔNp63α proteins EEC R279H and C306R, and SHFM-IV K193E and K194E, show reduced activity on the *Dlx5* and *Dlx6* promoters. The AEC L518F mutation did not affect p63 activity. Standard deviation is indicated. (**B**) Expression of *DLX1, DLX2, DLX5* and *DLX6* analysed by RT-PCR in H1299 cells induced to express *ΔNp63α*. White bars, no induction; black bars, induced cells (20 μM Dox). p63 induction was confirmed by western blot. (**C**) p63 is bound in vivo to the *Dlx5* and *DLX6* promoters. Specific enrichment was detected in two regions of the *Dlx5* promoter, R1 (–1200/–800) and R2 (–500/–100), and in one region of the *DLX6* promoter, R2 (–500/–100). Input is shown on the left. Amplification of the *IκKa* and of the *ΔNp63* promoters is shown as a positive control. (**D**,**E**) Deletions of the *mDlx5luc* (D) and *mDlx6luc* (E) promoters. The position of the predicted p53 sites and the *X5*-R1, *X5*-R2, *X6*-R1 and *X6*-R2 regions are indicated. Luciferase activity is shown on the right.

(Celli et al., 1999). The SHFM and EEC mutant p63 proteins showed a strongly reduced transactivation potential on both Dlx promoters (Fig. 3A), whereas AEC mutant *p63* behaved as wild type. Importantly, AEC patients do not exhibit limb abnormalities.

To verify whether *ΔNp63α* expression could activate transcription from the endogenous DLX genes, we used H1299 cells stably transfected with a Doxycyclin-inducible *ΔNp63α* expression vector. Induction of *ΔNp63α* resulted in a 1.8- and 2-fold increased expression of *DLX5* and *DLX6*, respectively. By contrast, the expression of *DLX1* and *DLX2* was minimally increased (Fig. 3B).

These data suggest that ΔNp63 positively regulates the transcription of both isolated *Dlx5* and *Dlx6* promoters, and of the endogenous *DLX5* and *DLX6* genes, and that EEC and SHFM-IV mutations abolish this capacity.

p63 binds to the *Dlx5* and *Dlx6* promoter in vivo

We searched the murine and human *Dlx5* and *Dlx6* promoters and conserved intergenic sequences (Zerucha et al., 2000) for p53 consensus binding sites, using the PatSearch prediction algorithm (Grillo et al., 2003; Osada et al., 2005; Sbisà et al., 2007). No p53 sites were found in the intergenic enhancer sequences. p53 binding sites were predicted in the promoter regions of m*Dlx5* (–967, –858, –611, –344 and –89 from the start site, designated A, B, C, D and E, respectively) and m*Dlx6* (–1670 and –1190 from the start site, designated F and G, respectively; see Fig. S1 in the supplementary material).

To determine whether p63 binds to the *Dlx5* and *Dlx6* promoters, we carried out ChIP analysis for *Dlx5* on genomic DNA from mouse keratinocytes, in which both *ΔNp63* and *Dlx5* are expressed (Morasso et al., 1999), and for *DLX6* on H1299 cells induced to express *ΔNp63α* (LoIacono et al., 2006). PCR primers were designed to amplify genomic fragments from the *Dlx5* and *DLX6* promoters (Fig. 3C). p63-specific enrichment was observed in two regions of the *Dlx5* promoter, roughly corresponding to –1200/–800 and –500/–100 (Fig. 3C,D), named *X5*-R1 and *X5*-R2, respectively. Importantly, the X5-R1 and *X5*-R2 regions comprise, respectively, the A and B and the D and E predicted p53-binding sites. As a control, PCR amplification of an upstream region (X5-2000) yielded

no enrichment (Fig. 3C). p63-specific enrichment was observed in one region of the *DLX6* promoter, named *X6*-R2 and corresponding to –500/–100 (Fig. 3C,E), but not in the region named *X6*-R1, comprising the F and G predicted p53-binding sites. As a positive control, ChIP assays were performed on fragments of the *IκKα* and *ΔNp63* promoters (Candi et al., 2006; Lanza et al., 2006) (Fig. 3C). In summary, p63 is physically associated to both *Dlx5* and *Dlx6* promoters in vivo.

We generated two deleted versions of the *Dlx5* promoter, one removing the A+B+C p53-binding sites, the second removing the A+B+C+D sites. Co-transfection of these reporter constructs with *ΔNp63α* showed that *Dlx5* promoter activation was progressively reduced in the deleted versions, compared with m*Dlx5-luc*, but it was not abolished (Fig. 3D). This result suggests that both of the regions of the *Dlx5* promoter (*X5*-R1 and *X5*-R2) to which p63 binds play a role in mediating the observed p63-dependent transcriptional activation. We also generated two deleted versions of the *Dlx6* promoter: one removing the F+G p53-binding sites and the second removing an additional 600-bp of sequences. Surprisingly, transactivation of these promoter fragments by *ΔNp63α* was similar to the full-length fragment (Fig. 3E). These results suggest that the *Dlx6* promoter is not activated by p63 through the predicted p53-binding sites, but is possibly activated through interaction with other transcription factors (Koutsondis et al., 2005; Testoni and Mantovani, 2006).

Dlx expression is reduced in *p63* mutant limbs

To determine whether the inactivation or mutation of *p63* result in altered Dlx gene expression in vivo, we used the $p63^{EEC}$ mouse strain, in which the EEC mutation R279H (Celli et al., 1999) was introduced into the mouse germline by homologous recombination. $p63^{EEC/EEC}$ mice show severe truncations of the FLs, absence of the HLs, ectodermal dysplasia and craniofacial anomalies, similar to those of $p63^{-/-}$ mice (see Fig. 6 and next paragraph). The limb buds of $p63^{EEC/EEC}$ E10.5 embryos are reduced in size and have a pointed shape (Fig. 4A,A′), and the AER appears unstratified; $p63^{+/EEC}$ mice have normal limbs and a stratified AER (Fig. 4B-E). A mild ectrodactyly is rarely observed

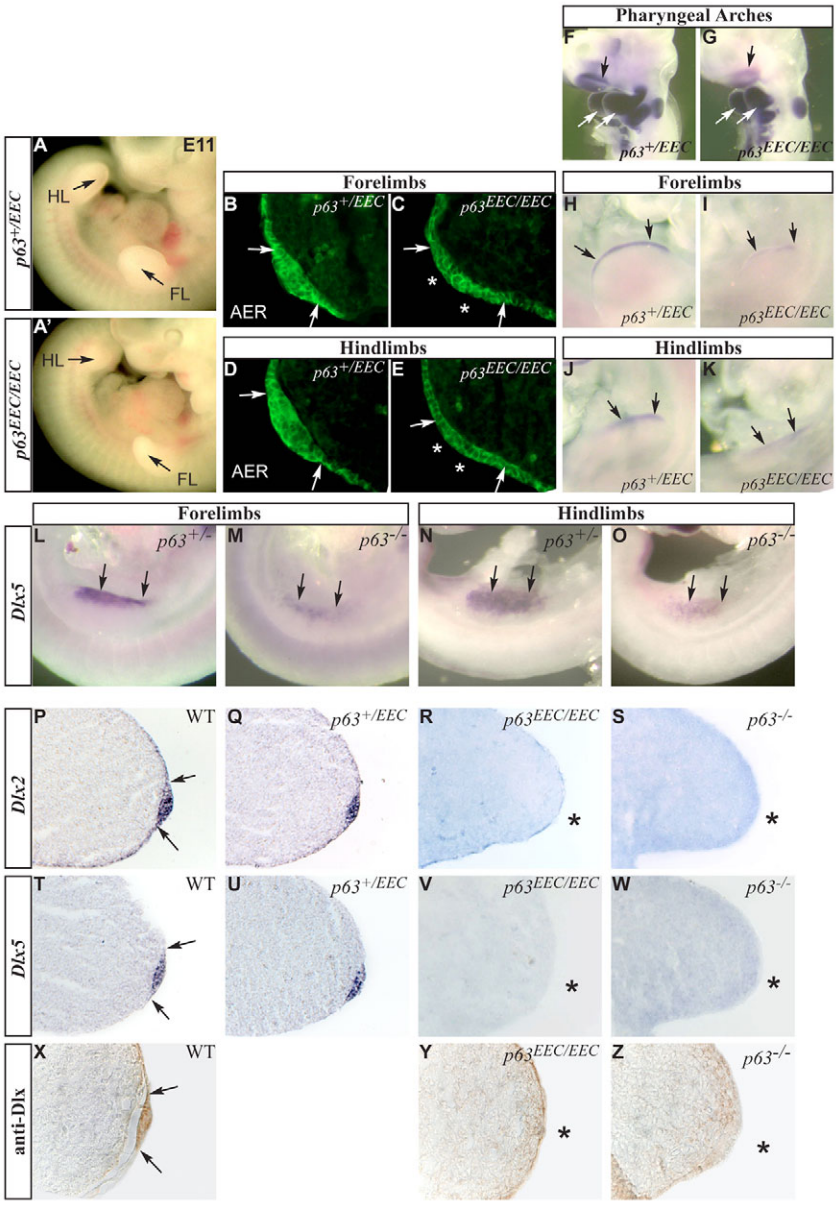

Fig. 4. Expression of Dlx genes in *p63* mutant limbs. (**A**,**A′**) Lateral view of $p63^{+/EEC}$ (A) and $p63^{EEC/EEC}$ (A′) E11 embryos. Note the dysmorphology of both FLs and HLs in the homozygous embryo (black arrows). (**B-E**) Immunostaining for E-cadherin on sections of $p63^{+/EEC}$ (B,D) and $p63^{EEC/EEC}$ (C,E) E10.5 FL (B,C) and HL (D,E), to examine stratification of the AER (white arrows). Section plane as in Fig. 1. Scale bar: 25 μm. White arrows indicate the dorsal and ventral border of the AER. (**F-K**) Whole-mount in situ hybridization for *Dlx5* on $p63^{+/EEC}$ (F,H,J) and $p63^{EEC/EEC}$ (G,I,K) E10 embryos, showing the pharyngeal arches (F,G), the FLs (H,I) and the HLs (J,K). Signal is reduced in the AER of $p63^{EEC/EEC}$ limbs (black arrows in H-K), but not in the $p63^{EEC/EEC}$ pharyngeal arches (white arrows in F,G). (**L-O**) Whole-mount in situ hybridization for *Dlx5* on FLs (L,M) and HLs (N,O) of E9.5 $p63^{+/-}$ (L,N) and $p63^{-/-}$ (M,O) embryos, in lateral view. The signal on the pre-AER is indicated (black arrows). Anterior is to the right. (**P-W**) In situ hybridization for *Dlx2* (P-S) and *Dlx5* (T-W) on sections of WT, $p63^{+/EEC}$; $p63^{EEC/EEC}$ and $p63^{-/-}$ E10.5 HLs. The AER is indicated with black arrows. Asterisks indicate absence of hybridization signal. (**X-Z**) Immunostaining for pan-Dlx on sections of wild-type, $p63^{EEC/EEC}$ and $p63^{-/-}$ E10.5 HLs.

(1/40) in $p63^{+/EEC}$ limbs (see Fig. 6C,N). Other phenotypes of $p63^{+/EEC}$ mice (skin, palate, genitalia) will be reported separately (E.G. and A.A.M., unpublished).

First, we carried out whole-mount in situ hybridization for *Dlx5* on E10 embryos. In $p63^{EEC/EEC}$ FLs and HLs, the signal was strongly reduced when compared with wild-type or heterozygous limbs (Fig. 4H-K), whereas the signal in the pharyngeal arches, olfactory and otic placodes was only slightly reduced (Fig. 4F,G). To determine whether this signal reduction might be due to failure of AER induction in the first place, we carried out in situ hybridization for *Dlx5* on E9.5 embryos, using *Dlx5* expression as a marker for pre-AER cells (Kimmel et al., 2000; Robledo et al., 2002). *Dlx5* signal was present in both the FLs and the HLs of normal and *p63*-null embryos, although it was reduced in the later (Fig. 4L-O), indicating that the pre-AER is properly induced.

Next, we hybridized sections of the HLs from wild-type, $p63^{+/EEC}$, $p63^{EEC/EEC}$ and $p63^{-/-}$ E10.5 embryos for *Dlx2* and *Dlx5*. The expression of both genes was strongly reduced in the AER of *p63* homozygous mutant limbs (Fig. 4P-W). Immunostaining of sections of E10.5 HLs with anti-Dll antibody revealed a marked reduction of Dlx immunoreactivity in the AER of *p63* homozygous mutant limbs (Fig. 4X-Z).

To quantify Dlx gene expression in *p63* mutant limbs, we applied RealTime qPCR to detect *Dlx1*, *Dlx2*, *Dlx5* and *Dlx6* mRNAs in samples from E10.5 wild-type and $p63^{EEC/EEC}$ limbs. The abundance of *Dlx1*, *Dlx2*, *Dlx5* and *Dlx6* mRNAs was significantly reduced in $p63^{EEC/EEC}$ FLs (–50, –60, –70 and –70%, respectively) and HLs (–75, –75, –80 and –80%, respectively), compared with wild type (Table 4A). A similar reduction in Dlx gene expression was observed in *p63* null embryos at the same age (Table 4A).

We wanted to gain further evidence to exclude that, in *p63* homozygous mutant limbs, Dlx genes (and AER expressed genes) might be downregulated as a consequence of the lack of stratification. We examined the histology of the AER in FLs and HLs from E10.5 $p63^{EEC/EEC}$ and $p63^{+/EEC}$ embryos, by immunostaining for E-cadherin. Although in $p63^{EEC/EEC}$ limbs the AER had lost stratification, in $p63^{+/EEC}$ limbs it appeared normally stratified (Fig. 4B-E). The same has been reported for the *p63* null limbs (Mills et al., 1999; Yang et al., 1999). We examined Dlx gene expression in wild-type and $p63^{+/-}$ and $p63^{+/EEC}$ limbs. In $p63^{+/-}$ and $p63^{+/EEC}$ HLs, *Dlx5* and *Dlx6* mRNAs were reduced (–40% to –50%), relative to in wild type. *Dlx1* and *Dlx2* mRNAs were reduced (–40%) in the $p63^{+/-}$ HL, but were minimally changed (–10%) in the $p63^{+/EEC}$ HL. In the FLs of heterozygous embryos, the expression of Dlx genes was not reduced, in fact it was slightly increased (see Table 4B). Thus, the loss of Dlx gene expression in homozygous *p63* mutant HL is unlikely to be the mere consequence of AER failure. Furthermore, these data indicate that FLs and HLs do not share the same regulation of Dlx gene expression.

To complete the analysis, we compared the expression of Dlx genes with that of *Perp*, a know *p63* target (Ihrie et al., 2005), in normal and *p63* mutant limbs. In $p63^{EEC/EEC}$ FLs and HLs, *Perp* mRNA was reduced, respectively, by –45% and –60%, whereas an intermediate reduction (–30%) was observed in $p63^{+/EEC}$ HLs (Table 4B). In $p63^{-/-}$ FLs and HLs, *Perp* mRNA was reduced, respectively, by –80% and –70%.

To exclude that the entire *Dlx5-Dlx6* genomic region might be silenced in *p63* mutant limbs, we determined the abundance of two RNAs transcribed within the locus, *Dlx6* antisense (as) and *Evf2* ncRNA (Liu et al., 1997; Feng et al., 2006), in wild-type and *p63* mutant limbs. Although *Evf2* was practically undetectable, the expression of *Dlx6as* was decreased in $p63^{-/-}$ FLs and increased in $p63^{EEC/EEC}$ FLs and HLs (2- and 3-fold, respectively, Table 4A). These data suggest that p63 regulates *Dlx5* and *Dlx6* independently of the high-order epigenetic regulations of this locus (Horike et al., 2005).

Finally, we examined the possibility that *p63* expression might be controlled by Dlx. Very similar p63 immunostaining was observed in the AER and ectoderm of E11 *Dlx5;Dlx6* double knock-out

Table 4. Expression of Dlx genes in limbs of *p63* mutant embryos

A

Target mRNA	Dlx1	Dlx2	Dlx5	Dlx6	Dlx6as	Dactylyn	Perp
FL*							
+/+	1†	1	1	1	1	1	1
$p63^{-/-}$	0.6	0.5	0.5	0.3	0.5	1.2	0.2
$p63^{EEC/EEC}$	0.5	0.4	0.3	0.3	2	1.5	0.55
HL*							
+/+	1	1	1	1	1	1	1
$p63^{-/-}$	0.35	0.25	0.25	0.3	1	1.3	0.3
$p63^{EEC/EEC}$	0.25	0.25	0.2	0.2	3	1.3	0.4

B

Target mRNA	Dlx1	Dlx2	Dlx5	Dlx6	Perp
FL					
+/+	1†	1	1	1	1
$p63^{+/-}$	1.2	1.6	1.5	1.3	1.0
$p63^{+/EEC}$	1.2	1.3	2	1.1	1.0
HL					
+/+	1	1	1	1	1
$p63^{+/-}$	0.6	0.6	0.5	0.5	0.5
$p63^{+/EEC}$	0.9	0.9	0.6	0.55	0.7

*FL, forelimb; HL, hindlimb. The genotypes of $p63^{-}$ (Brdm2 strain) and $p63^{EEC}$ (R279H) mutant limbs are indicated.
†Values are normalized to the wild-type (+/+) sample, set=1. An external calibration sample was used, *GADPH* mRNA was determined as a positive control reference. Most determinations were repeated three times.

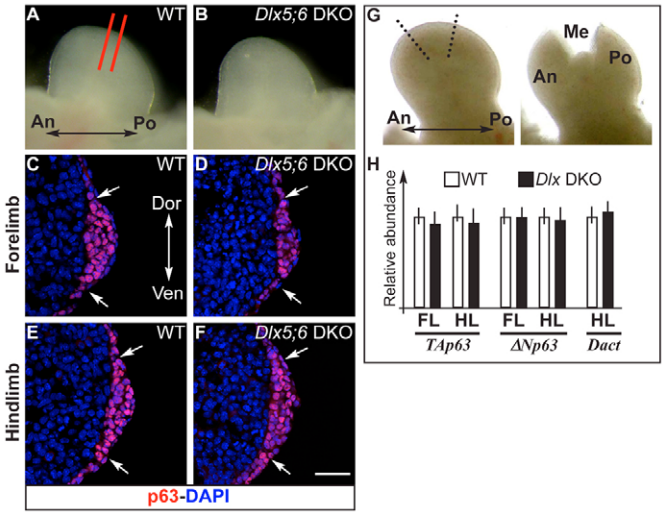

Fig. 5. Expression of *p63* and Dactylyn in *Dlx5;Dlx6* DKO limbs. (**A**,**B**) Morphology of E11 wild-type (A) and *Dlx5;Dlx6* DKO (B) HLs. Section plane is shown in A. (**C-F**) Immunostaining for p63 on sections of normal (C,E) and *Dlx5;Dlx6* DKO (D,F) FLs (top panels) and HLs (bottom panels) at E11. Nuclei were counterstained with DAPI. The AER is indicated with white arrows. The dorsoventral orientation is indicated. (**G**) Dissection of the medial (Me) sector from wild-type and *Dlx5;Dlx6* DKO limbs. (**H**) *TAp63, ΔNp63* and Dactylyn mRNA abundance in the Me sector of wild-type (white bars) and *Dlx5;Dlx6* DKO (black bars) FLs and HLs, by RealTime qPCR. Wild-type samples are set=1. Scale bar: 25 μm.

(DKO) FLs and HLs compared with wild type (Fig. 5C-F). We then isolated RNA from the central wedge of wild-type and Dlx DKO limbs and determined the abundance of *TA* and *ΔNp63* mRNAs by qPCR; we did not observe significant differences compared with wild type (Fig. 5G,H). Thus, Dlx5 and Dlx6 are unlikely to control *p63* expression. We also determined the abundance of Dactylyn mRNA in samples from E11 wild-type, *Dlx5;Dlx6* DKO and $p63^{EEC/EEC}$ limbs by qPCR. We observed a modest increase in Dactylyn expression in *p63* and Dlx mutant limbs, when compared with wild type (Fig. 5H; Table 4A). These data tend to exclude the participation of Dactylyn in Dlx- or *p63*-dependent regulation.

Combined $p63^{EEC}$ and *Dlx5;Dlx6* mutations result in aggravated limb phenotypes

We sought in vivo evidence that *p63*-Dlx gene regulation plays a role in the onset of the SHFM-related limb phenotypes. Because the expression of *Dlx5* and *Dlx6* is reduced in $p63^{+/EEC}$ HLs, the combination of a heterozygous $p63^{EEC}$ mutation with an incomplete loss of *Dlx5-Dlx6* alleles might result in a further reduced level of Dlx gene expression and the appearance of HL phenotypes.

Dlx5;Dlx6 DKO mice show ectrodactyly of the HL (Fig. 6T), but neither the $Dlx5^{-/-}$ nor the $Dlx5^{+/-};Dlx6^{+/-}$ mice display limb defects (Fig. 6F,R). In mice with the genotype $Dlx5^{-/-};Dlx6^{+/-}$ the HLs are normal (Fig. 6G,S), although a deviation of central phalanges has rarely (1:30) been observed. We crossbred $p63^{+/EEC}$ mice with $Dlx5^{+/-}$ and with $Dlx5^{+/-};Dlx6^{+/-}$ mice, to obtain animals doubly ($p63^{EEC};Dlx5$) or triply ($p63^{EEC};Dlx5;Dlx6$) heterozygous. None of these mice showed limb abnormalities (0/8 and 0/9, respectively). We then crossbred $p63^{+/EEC};Dlx5^{+/-}$ mice with $Dlx5^{+/-};Dlx6^{+/-}$ mice, to obtain mice with the genotype $p63^{+/EEC};Dlx5^{-/-};Dlx6^{+/-}$, in which three out of four alleles of the *Dlx5-Dlx6* cluster are deleted. At birth, these mice (3/3) showed severe ectrodactyly (in one case monodactyly) of their HLs, with two to four missing fingers, syndactyly, and dysmorphologies of the most proximal segments of the central phalanges (Fig. 6U,V). These defects were never observed with any of the two mutations ($p63^{+/EEC}$ or $Dlx5^{-/-};Dlx6^{+/-}$) individually. $p63^{+/EEC};Dlx5^{-/-};Dlx6^{+/-}$ mice also showed anomalies of their FLs (two out of three), comprising missing posterior fingers and syndactyly (Fig. 6K). Importantly, FL defects were never observed in our strain of *Dlx5;Dlx6* DKO mice (Merlo et al., 2002) (Fig. 6) or in $p63^{+/-}$ ones, although a mild deviation of the central phalanges was rarely (1:40) observed in $p63^{+/EEC}$ mice. In summary, the HL defects in $p63^{+/EEC};Dlx5^{-/-};Dlx6^{+/-}$ animals represent a significant aggravation and point to a developmental function for *p63*-Dlx regulation, in vivo.

DISCUSSION

SHFM-like limb malformations have a similar clinical appearance but are genetically heterogeneous; at least five loci have been identified in the hereditary forms and additional SHFM loci might exist to account for the sporadic cases. For type I and IV, the transcription factors *DLX5*, *DLX6* and *p63*, respectively, are the disease genes, although mutations in the *DLX5-DLX6* locus have not been found. For type III, the F-box/WD40 gene Dactylyn has been proposed (Sidow et al., 1999). The corresponding murine models $p63^{-}$, $p63^{EEC}$ (SHFM-IV), *Dlx5;Dlx6* DKO (SHFM-I) and *dactylaplasia* (SHFM-III) show defects in limb development of varying severity. The existence of these phenotypic similarities suggested the possibility that the corresponding disease genes might participate in a regulatory cascade. Here we show that: (1) p63 colocalizes with Dlx proteins in the embryonic AER and is associated with the *Dlx5* and *Dlx6* promoters in vivo; (2) ΔNp63α, the predominant p63 isoform expressed in the developing limbs, can activate *Dlx5* and *Dlx6* transcription; (3) EEC and SHFM, but not AEC, mutations nearly abrogate the transcriptional activity of *Dlx5* and *Dlx6* promoters; (4) the expression of *Dlx5* and *Dlx6* is reduced in $p63^{-}$ and $p63^{EEC}$ heterozygous and homozygous limbs; and (5) heterozygous $p63^{EEC}$ mutations combined with the incomplete loss of *Dlx5* and *Dlx6* alleles results in aggravated limb phenotypes. These observations indicate that p63 lies genetically upstream of the Dlx genes during limb development.

The involvement of *p63*, *Dlx5* and *Dlx6* in SHFM-related limb phenotype is well established, although their molecular functions in the maintenance of the AER are incompletely known. On the contrary, the disease genes responsible for SHFM-III and SHFM-V are still unknown. SHFM-V has been associated with deletions on chromosome 2q24-q31, a large cytogenetic region proximal to, and perhaps including, *DLX1* and *DLX2* (Boles et al., 1995; DelCampo et al., 1999; Maas et al., 2000). More recently, Goodman and co-authors (Goodman et al., 2002) proposed that the SHFM-V locus is located in the interval between *EVX2* (2q31-q32) and marker D2S294, 5 Mb centromeric to *EVX2*. This raises the possibility that SHFM-V and other digit anomalies may be caused by haploinsufficiency of the 5′ *HOXD*, *EVX2* or *DLX1* and *DLX2* genes. In *p63* heterozygous and homozygous limbs, expression of *Dlx1* and *Dlx2* is reduced, but overall to a lower extent than *Dlx5* and *Dlx6*. Thus, our findings are compatible with a role for the *DLX1-DLX2* locus in SHFM-V, although they do not actually demonstrate this. The single inactivation of *Dlx1* or *Dlx2* in mice does not result in limb phenotypes; however, a possible role of these two genes will be clarified by examining the *Dlx1;Dlx2* DKO model.

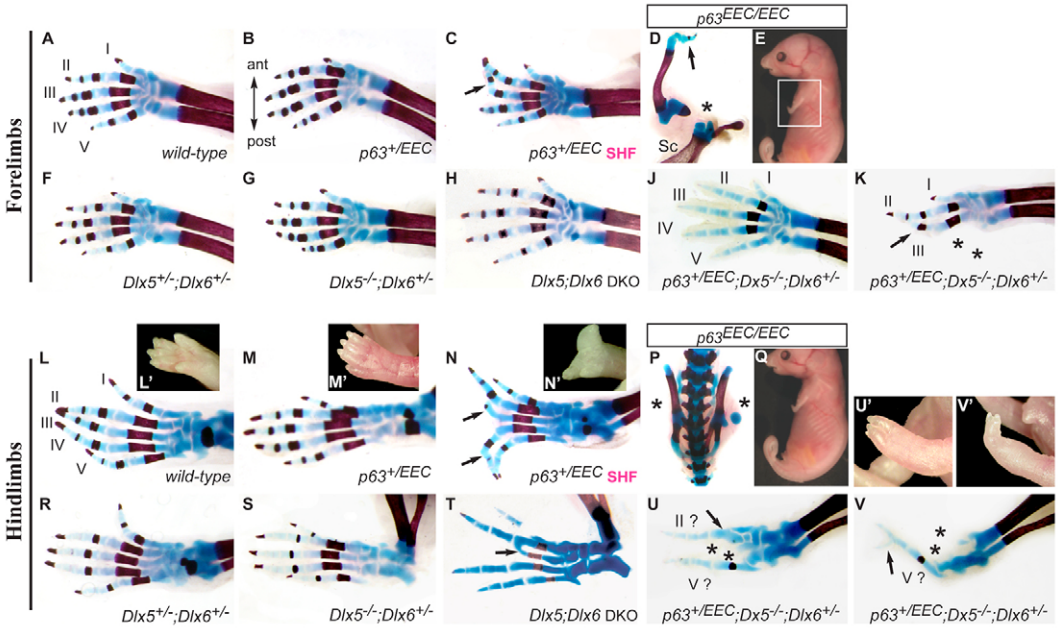

Fig. 6. Limb phenotypes of *p63^{EEC}*/Dlx combined mutant mice. Skeletal staining of neonatal FL (**A-K**) and HL (**L-V**). Wild type is shown in A and L. For the *p63$^{+/EEC}$* limbs, both normal (common phenotype, B,M) and mild ectrodactyly (rare phenotype, C,N) are shown. *Dlx5;Dlx6* DKO limbs are shown in H,T. Note that the defects observed in *p63$^{+/EEC}$* (rare) and *Dlx5;Dlx6* DKO mice only affect the distal-most portion of the central phalanges. (F,G,R,S) FL and HL of *Dlx5$^{+/-}$;Dlx6$^{+/-}$* and *Dlx5$^{-/-}$;Dlx6$^{+/-}$* mice. (J,K,U,V) FL and HL of *p63$^{+/EEC}$;Dlx5$^{-/-}$;Dlx6$^{+/-}$* mice. Note the severe HL defects: missing fingers (black asterisks), bent and fused distal phalanges (black arrows), and altered proximal elements (U,V). Note the defects in the FLs, observed in two out of three cases (K), whereas one was normal (J). FLs and HLs of *p63$^{EEC/EEC}$* animals are also shown for comparison (D,E and P,Q). Digit numbers are indicated (A,L).

Dactylyn is the proposed disease gene for SHFM-III (human) and *dac$^{+/-}$* (mouse) (Johnson et al., 1995; Sidow et al., 1999; deMollerat et al., 2003). However, reduced Dactylyn expression has been documented only in lymphocytes of SHFM-III patients (Basel et al., 2003), and not in *dac$^{+/-}$* limbs. Of note, in the SHFM-III and *dac$^{+/-}$* rearranged locus, one normal copy of Dactylyn is retained; therefore doubts can be raised as to whether Dactylyn is the SHFM-III disease gene (deMollerat et al., 2003; Lyle et al., 2006). Our data indicate that the expression of Dactylyn is unchanged in Dlx mutant limbs and is minimally increased in *p63* mutant limbs; thus, it is unlikely that Dactylyn lies genetically downstream of *p63* or the *Dlx5-Dlx6* locus. SHFM-III has an alternative explanation: three AER-expressed genes *Fgf8*, *NFκB2* and *Lbx1* are located in the Dactylyn chromosomal neighbourhood. Of these *Fgf8*, a morphogen essential for limb development (Lewandoski et al., 2000; Sun et al., 2002; Tickle, 2003), is downregulated in both *p63* and Dlx mutant limbs (Mills et al., 1999; Robledo et al., 2002). A downregulation of *Fgf8* (and/or *NFκB2* and *Lbx1*) could explain the limb defects in SHFM-III patients and in *dac$^{+/-}$* mice.

The combined inactivation of *Dlx5* and *Dlx6* leads to a bona fide ectrodactyly, whereas no limb defects have been noted in single *Dlx5* mutants (Acampora et al., 1999). This suggests that *Dlx5* and *Dlx6* may play partially redundant roles in AER function, and that a threshold level of Dlx expression needs to be reached. If this were true, it would be expected that the DKO of other Dlx genes would result in the appearance of SHFM-type limb defects. Indeed *Dlx2;Dlx5* DKO mice show ectrodactyly (Panganiban and Rubenstein, 2002). Limb phenotypes in combined Dlx DKO mice have not been reported as yet, but these data would help to clarify this issue.

The regulation of *Dlx5* and *Dlx6* by p63

Transcriptional regulation within the *Dlx5-Dlx6* bigenic cluster involves at least four mechanisms: (1) tissue-specific enhancers, shared by the two genes in the cluster, and operating at distance, such as the intergenic elements I56i and I56ii and the Mef2c-response element; (2) Mecp2-dependent chromatin looping, possibly linked to partial imprinting of these genes; (3) interaction between *Evf2* ncRNA and Dlx proteins, and (4) cis-acting regulation on the proximal promoter region, which is expected to be specific for each gene (Zerucha et al., 2000; Horike et al., 2005; Feng et al., 2006; Verzi et al., 2007) (this manuscript).

Enhancer-type regulation and chromatin folding (Zerucha et al., 2000; Ghanem et al., 2003; Horike et al., 2005; Feng et al., 2006) are likely to be shared by *Dlx5* and *Dlx6*, and accounts for their nearly identical expression pattern. The highly conserved I56i and I56ii regulatory elements, located in the intergenic region, comprise homeodomain-binding sites to which *Dlx1* and *Dlx2* bind and drive reporter expression in the embryonic forebrain, pharyngeal arches, and limbs (Zerucha et al., 2000; Ghanem et al., 2003). We could not identify any p53-binding element in the I56i and I56ii sequences, and consistently could not detect binding of p63 (L.G., unpublished). Thus, we exclude that p63 might regulate *Dlx5-Dlx6* expression via these intergenic enhancers. *Evf2*-dependent regulation can also be excluded, as *Evf2* ncRNA could not be detected in embryonic limbs.

Our data indicate that p63 regulates *Dlx5* and *Dlx6* transcription, at least in part, by cis-acting regulation at the promoter level. We show that the ΔNp63 isoforms are able to activate transcription of *Dlx5* and *Dlx6*, whereas the TA isoforms are largely inactive. Due to the presence of the TA domain, the TAp63α and -γ isoforms have been considered to be the transcriptionally active forms (Barbieri and Pietenpol, 2006); however, the ΔNp63 isoforms can activate

transcription via alternative activation domains (Ghioni et al., 2002; King et al., 2003; Wu et al., 2005). Considering that ΔNp63α is the predominantly expressed isoform in the AER, the results are consistent with *p63* being genetically upstream of the Dlx genes. By contrast, expression of *Dlx3* in the limb ectoderm is regulated by TAp63 (Radoja et al., 2007), which suggests that individual Dlx genes might be regulated differently by p63 isoforms. It is difficult to determine the exact function of individual p63 isoforms on Dlx gene expression in vivo, as both the deletion and the point mutation introduced into the mouse genome will equally affect both classes. The development of isoform-specific mutant animal models will be essential to resolve this issue.

p63, Dlx and limb development

ΔNp63α is essential to endow stem cells of the stratified epithelia with their proliferation potential and to maintain the stratified organization of some epithelia (Signoretti et al., 2000; Nylander et al., 2002; Koster et al., 2004; Laurikkala et al., 2006; Senoo et al., 2007). The AER is a transitory multi-layered ectoderm acting as a signalling centre essential for distal limb development, digit patterning and morphogenesis (Niswander, 2002; Tickle, 2003). Consistently, the stratified organization of the AER and the expression of morphogenetic molecules are dramatically compromised in *p63* mutant limbs (Mills et al., 1999; Yang et al., 1999). AER stratification is also lost in *Dlx5;Dlx6* DKO limbs (Robledo et al., 2002; Merlo et al., 2002), although restricted to the central AER wedge, and in $dac^{+/-}$ limbs (Seto et al., 1997; Crackower et al., 1998). Thus, it appears that the activity of these three genes is required for maintaining the AER organization. However, the time of onset of this defect in *p63* mutant limbs is earlier, compared with the other models (Mills et al., 1999; Yang et al., 1999). We have considered the possibility that changes in Dlx gene expression are the mere consequence of loss of AER stratification; here, we report that *Dlx5* and *Dlx6*, and to a lesser extent *Dlx1* and *Dlx2*, are downregulated in heterozygous $p63^{-}$ and $p63^{EEC}$ HLs, which display a normally stratified AER. Because *Dlx5* expression is detectable (although reduced) in the pre-AER of early $p63^{-/-}$ embryos, it appears unlikely that Dlx gene expression is altered solely as a consequence of AER failure. Conversely, Dlx gene expression is not reduced, but is rather increased, in the heterozygous *p63* FLs. This suggests that the $p63^{EEC}$ mutation behaves differently in FLs and HLs. In the Dlx- and the *p63*-mutant mice, as well as in the combined $p63^{EEC}$*;Dlx* mutant ones, HL defects are more prominent and more severe that those of the FLs. FL defects might therefore result from different molecular mis-regulations. In the compound $p63^{+/EEC}$*;*$Dlx5^{-/-}$*;*$Dlx6^{+/-}$ mice, FL defects have been observed that affect the distal region but that do not resemble ectrodactyly. The meaning of this is still unclear: Dlx and p63 may converge on the regulation of a common target or may engage in an autoregulatory loop.

ΔNp63 might regulate the expression of a large number of (direct and indirect) genes (Yang et al., 2006), only some of which might be essential for AER function. The expression of *Fgf8*, the key AER-secreted morphogen essential for proximal-distal limb growth, is severely downregulated in *p63* null limbs (Mills et al., 1999). *Perp*, another p63 target gene, which codes for a desmosome-associated protein required for the integrity of stratified epithelia (Ihrie et al., 2005), is also downregulated in $p63^{EEC}$ mutant limbs. However, no limb defects have been reported in *Perp*-null mice, and therefore its role remains uncertain. One possibility is that *Perp* downregulation might contribute to the increased severity of the *p63*-null phenotype when combined with reduced Dlx gene expression. *Ikkα*, a p63 transcriptional target required for epithelia stratification and differentiation, is transactivated directly by TA and ΔNp63, and indirectly by ΔNp63 via Gata3 (Candi et al., 2006; Koster et al., 2007). *Ikkα* is also required for keratinocyte differentiation through its kinase-independent (nuclear) activity. During embryonic development, *Ikkα* acts cell-autonomously in the ectoderm to maintain normal epithelial-mesenchyme interactions, acting via repression of *Fgf8* and other Fgfs. These, in turn, are essential for normal craniofacial and limb development; in fact, *Ikkα*-null mice show distal limb defects (Sil et al., 2004). The genetic and functional relationship between *p63*, Dlx genes, *Perp* and *Ikkα* in maintaining the AER stratification and function is currently unclear, and will need to be addressed by crossbreeding the corresponding mutant mice and examining their limb development.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/7/1377/DC1

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