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 p63EEC (R279H) mutant limbs, the AER fails to stratify and the expression of four Dlx genes is strongly reduced; interestingly, the p63EEC and p63null mutations combine 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.
(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 ∆N) of mRNAs. The use of alternative promoters drives the transcription of either TA/p63 proteins, comprising a p53-related N-terminal transactivation (TA) domain, a DNA-binding (DB) and an oligomerization (OD) domain, or ∆N/p63 proteins, lacking the TA domain. Additional TA domains have been identified that account for the transcripational activities of the ∆N isoforms (Dohn et al., 2001; Ghioni et al., 2002; Laurikkala et al., 2006). Three alternative splicing routes at the 3′-end generate TA/p63 and ∆N/p63 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 ∆N/p63α 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+/ECT 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′-CCACGATGATGAGCTGCTGTACC-3′; and
Rev p63, 5′-TAGCCAGGGCATGAGGAG-3′.

Mice carrying the p63R279H mutation (an EEC mutation in p63) (Mills et al., 1999), and were genotyped by RT-PCR on 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 cDNA 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 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 ∆N/p63α, using 2 μg of the 4A4 antibody, as described (Ceribelli et al., 2006; Lolacono et al., 2006). IP

**Immunohistochemistry and in situ hybridization**

Antibodies were used: 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 cDNA 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.

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**Table 1. Primers for RT-PCR**

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<td>TA/p63</td>
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AS, antisense; S, sense.
Table 2. Primers for RealTime qPCR (Dlx and other SHFM mRNAs in mouse limbs)

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<td>Dlx6</td>
<td>S</td>
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AS, antisense; S, sense.

Table 3. Oligonucleotides for ChIP-PCR analyses

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<td>Dlx5 –2000</td>
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<tr>
<td>Dlx5 R1</td>
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<tr>
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</tr>
<tr>
<td>Dlx6 R1</td>
<td>AS</td>
<td>TACCCTGGCGACTGCTTCAACT</td>
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<tr>
<td>Dlx6 R2</td>
<td>S</td>
<td>AGGGCGCGCTCCTATATCT</td>
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<tr>
<td>Dlx6 R2</td>
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<td>TGGACCCACCATGGCCTTT</td>
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<td>Igkα</td>
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<tr>
<td>Np63</td>
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<td>ATGCGTGCTGCTTCTTCTCTC</td>
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</table>

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 Igkα and ΔNp63 promoters, as positive control (see Table 3).

DNA constructs and plasmids

A Smal-HindIII 1120-bp murine genomic fragment corresponding to part of the Dlx5 promoter and 5’ UTR was used. A Ncol-SaeI 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 UTR and part of the first exon were excised and treated with 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.

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 expression vectors for luciferase assay have been previously described (Beretta et al., 2005). H1299 cells stably transfected with a tet-on expression plasmid were induced with Doxycyclin, as described (Lolaco 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., 1999). A double immunostaining approach for pan-Dlx (red) and p63 (green) on sections of E10.5 limbs. The merged image is shown in G. 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 at 29 cycles and the reaction had reached a plateau before 38 cycles. The TAp63α and ΔNp63α 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).

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.

**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 mDlx5-luc and mDlx6-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...
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 p63 expression could activate transcription from the endogenous DLX genes, we used H1299 cells stably transfected with a Doxycyclin-inducible p63 expression vector. Induction of p63 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 p63 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; Sbisa et al., 2007). No p53 sites were found in the intergenic enhancer sequences. p53 binding sites were predicted in the promoter regions of mDlx5 (–967, –858, –611, –344 and –89 from the start site, designated A, B, C, D and E, respectively) and mDlx6 (–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 p63 and Dlx5 are expressed (Morasso et al., 1999), and for DLX6 on H1299 cells induced to express p63 (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, 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 IkKα and of the Δp63 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 Δp63α expression could activate transcription from the endogenous DLX genes, we used H1299 cells stably transfected with a Doxycyclin-inducible Δp63α expression vector. Induction of Δp63α 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 Δp63 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.
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 IkKea 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 mDlx5-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 p63EEC mouse strain, in which the EEC mutation R279H (Celli et al., 1999) was introduced into the mouse germline by homologous recombination. p63EEC/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 p63EEC/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.
samples from E10.5 wild-type and RealTime qPCR to detect p63
The expression of both genes was strongly reduced in the AER of reduction of Dlx immunoreactivity in the AER of sections of E10.5 HLs with anti-Dll antibody revealed a marked p63EEC/EEC
p63EEC/EEC
p63+/EEC
p63–/–
†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.

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 p63EEC/EEC limbs. The abundance of Dlx1, Dlx2, Dlx5 and Dlx6 mRNAs was significantly reduced in p63EEC/EEC FLs (–50, –60, –70 and –70%, respectively) and HLs (–75, –75, –80 and –80%, respectively), compared with wild type (Table 4A).

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

<table>
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<tr>
<th>Target mRNA</th>
<th>Dlx1</th>
<th>Dlx2</th>
<th>Dlx5</th>
<th>Dlx6</th>
<th>Dlx6as</th>
<th>Dactyllyn</th>
<th>Perp</th>
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<tr>
<td>FL*</td>
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<tr>
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<tr>
<td>HL*</td>
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To complete the analysis, we compared the expression of Dlx genes with that of Perp, a known p63 target (Ihrie et al., 2005), in normal and p63 mutant limbs. In p63EEC/EEC FLs and HLs, Perp mRNA was reduced, respectively, by –45% and –60%, whereas an intermediate reduction (~30%) was observed in p63–/– FLs and HLs (Table 4B). In p63EEC/EEC 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 p63EEC/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
We then crossbred 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+/– animals, although one further reduction of the central phalanges was rarely (1:40) observed in p63EEC mice. In summary, the HL defects in p63EEC;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−/−, p63EEC (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) Dlp63α, 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 p63EEC heterozygous and homozygous limbs; and (5) heterozygous p63EEC 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 (2q31-3q2) 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.
Dactyl 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 Dactyl expression has been documented in lymphocytes of SHFM-III patients (Basel et al., 2003), and in dac+/– limbs. Of note, in the SHFM-III and dac+/– rearranged locus, one normal copy of Dactyl is retained; therefore doubts can be raised as to whether Dactyl is the SHFM-III disease gene (deMollerat et al., 2003; Lyle et al., 2006). Our data indicate that the expression of Dactyl is unchanged in Dlx mutant limbs and is minimally increased in p63 mutant limbs; thus, it is unlikely that Dactyl lies genetically downstream of p63 or the Dlx5-Dlx6 locus. SHFM-III has an alternative explanation: three AER-expressed genes Fgf8, Nfkb2 and Lbx1 are located in the Dactyl chromosomal neighborhood. 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 Nfkb2 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 bimagic 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 ΔNp63 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; Laurikka 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+ECR 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+ECR mutation behaves differently in FLs and HLs. In the Dlx- and the p63-mutant mice, as well as in the combined p63+ECR/Dlx mutant ones, HL defects are more prominent and more severe than those of the FLs. FL defects might therefore result from different molecular mis-regulations. In the compound p63−/−ECR;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 Fgfl8, 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 (Ihrle et al., 2005), is also downregulated in p63−/−ECR 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. Ikka, 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). Ikka is also required for keratinocyte differentiation through its kinase-independent (nuclear) activity. During embryonic development, Ikka 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, Ikka−/− null mice show distal limb defects (Sil et al., 2004). The genetic and functional relationship between p63, Dlx genes, Perp and Ikka 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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