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p63 regulates multiple signalling pathways required for ectodermal organogenesis and differentiation

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Heterozygous germline mutations in *p63*, a transcription factor of the p53 family, result in abnormal morphogenesis of the skin and its associated structures, including hair follicles and teeth. In mice lacking *p63*, all ectodermal organs fail to develop, and stratification of the epidermis is absent. We show that the ectodermal placodes that mark early tooth and hair follicle morphogenesis do not form in *p63*-deficient embryos, although the multilayered dental lamina that precedes tooth placode formation develops normally. The N-terminally truncated isoform of p63 (ΔNp63) was expressed at high levels in embryonic ectoderm at all stages of tooth and hair development, and it was already dominant over the transactivating TAp63 isoform prior to epidermal stratification. *Bmp7*, *Fgfr2b*, *Jag1* and *Notch1* transcripts were co-expressed with ΔNp63 in wild-type embryos, but were not detectable in the ectoderm of *p63* mutants. In addition, β-catenin and *Edar* transcripts were significantly reduced in skin ectoderm. We also demonstrate that BMP2, BMP7 and FGF10 are potent inducers of *p63* in cultured tissue explants. Hence, we suggest that p63 regulates the morphogenesis of surface ectoderm and its derivatives via multiple signalling pathways.

KEY WORDS: BMP7, FGF10, FGFR2b, Notch1, β-Catenin

INTRODUCTION

p63 is a homologue of the tumour suppressor p53. The p53 family of transcription factors consists of three members: p53, p63 and p73 (Mills, 2005). p63 possesses at least two promoters that direct the expression of two fundamentally different classes of protein, namely one which contains an N-terminal transactivation (TA) domain and one which lacks this domain, the N-terminally truncated (ΔN) isoform (Yang et al., 1998). Extensive alternative splicing is seen at the 3′ end of p63 transcripts resulting in three different C termini: α, β and γ. Exogenously expressed p63 can bind to consensus p53 target sequences, and can activate and repress the promoters of several p53 responsive genes. It is generally assumed that ΔNp63 isoforms are repressor molecules against TAp63 and p53. However, the physiological targets, either induced or repressed by p63, are largely unknown (Levrero et al., 2000; Westfall and Pietenpol, 2004).

Despite the high similarity in their transcriptional activities, members of the p53 family seem to play mostly distinct functions in tumour suppression and development (Melino et al., 2003). Heterozygous germline mutations in *p63* result in a plethora of human syndromes involving defective development of the limbs, and/or ectodermal dysplasia characterised by defects in skin and its associated structures (van Bokhoven and McKeon, 2002). Mice lacking all p63 isoforms die at birth and show severe developmental abnormalities, including limb truncations, and defects in the epidermis and its appendages (Mills et al., 1999; Yang et al., 1999). The surface epithelium is thin, lacks stratification, and does not express markers of epithelial differentiation. The epithelial phenotype has been interpreted to result from either a lack of commitment of the immature ectoderm to epidermal lineages (Mills et al., 1999), or a lack of proliferative potential of the p63-deficient epidermal stem cells (Yang et al., 1999). Ectodermal organs such as hairs, whiskers, teeth and several glands, including mammary, salivary and lacrimal glands, are lacking in *p63*-deficient mice (Mills et al., 1999; Yang et al., 1999).

A common theme in the development of ectodermal organs is that their morphogenesis is regulated by a complex series of reciprocal interactions between epithelial and mesenchymal tissues. Tooth morphogenesis is governed by interactions between the oral ectoderm and neural crest-derived mesenchyme. Development starts from the dental lamina, which forms as a stripe of thickened epithelium at the site of the future dental arch. The initiation of both molars and incisors becomes morphologically visible in the mouse at embryonic day 11 (E11) when the dental lamina epithelium thickens locally. The dental placodes form from this epithelium at E12. The placodal epithelium buds into the mesenchyme during E13, and subsequent epithelial growth and folding determine the shape of the tooth crown (Jernvall and Thesleff, 2000). The program of hair follicle morphogenesis is similar to that of tooth morphogenesis; the onset of both of these morphogenetic processes is heralded by the formation of the ectodermal placode (Pispa and Thesleff, 2003). The primary pelage hair placodes are visible at E14 in the dorsal back skin; these will form the guard hairs. The placodes bud into the mesenchyme and undergo morphogenesis in co-operation with the mesenchymal dermal papilla (Millar, 2002).

The molecular mechanisms regulating the development of distinct epithelial organs are shared to a great extent (Pispa and Thesleff, 2003). Many genes required for placode initiation have been identified in studies of genetically modified mice. Inhibition of the WNT pathway by overexpression of the WNT inhibitor DKK1 caused developmental arrest prior to the placode stage in all ectodermal organs analysed (Andl et al., 2002). The ectodermal organ phenotype is similar also in *Msx1/Msx2* double-mutant mice and indicates a role of BMP signalling in placode formation (Bei and Maas, 1998). The formation of tooth placodes is inhibited in double mutants of *Dlx1/Dlx2* and *Gli2/Gli3* (Thomas et al., 1997; Hardcastle et al., 1998), highlighting the importance of FGF and

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SHH signalling for placode formation. Furthermore, signalling by EDA via the EDAR receptor is required for the placodes of guard hairs to be initiated (Thesleff and Mikkola, 2002a), and stimulation of this specific TNF signal pathway stimulates placode formation in several ectodermal organs (Mustonen et al., 2004). Placode development also requires an intricate control of cell adhesion (Jamora et al., 2003). Thus, formation of the ectodermal placode requires the coordination of multiple signalling pathways. However, precisely how these diverse pathways are integrated is currently unknown.

In this study, we have examined the function of *p63* in tooth and hair development. We show that it is required for the formation of individual dental and hair placodes, but not for the specification of the dental field. Intriguingly, the truncated *ΔNp63* isoform was the main isoform expressed at all stages of development. Our results indicate that *Bmp7*, *Fgfr2b*, jagged 1 (*Jag1*) and *Notch1* lie downstream of p63, whereas BMP2, BMP7 and FGF10 were potent inducers of p63 expression in cultured tissue explants. We conclude that ΔNp63 integrates multiple signalling pathways required for the formation of tooth and hair placodes.

MATERIALS AND METHODS

Animals

The generation, genotyping and analysis of the *p63* and *Fgfr2b* mutant mice have been described earlier (Mills et al., 1999; De Moerlooze et al., 2000). Mice were mated overnight, and the day of formation of a vaginal plug was taken as embryonic day 0.

Histology

The embryonic and postnatal tissues for histology, radioactive in situ hybridisation and immunostaining were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; 4°C; overnight), dehydrated, embedded in paraffin wax, and serially sectioned at 7 μm. Sections for normal histology were stained with Haematoxylin and Eosin.

Organ cultures

First mandibular molar tooth germs were dissected from E11-E14 mouse embryos and cultured with protein-releasing beads as previously described (Laurikkala et al., 2001; Laurikkala et al., 2002). The recombinant proteins were: activin A, FGF4, FGF8, FGF10 and SHH (50 ng/μl; all from R&D Systems, Abingdon, UK); BMP2 (75 ng/μl, a kind gift from J. Wozney, Genetics Institute); BMP7 (100 ng/μl, kind gift from Creative Biomolecules, USA); EGF (25ng/μl; Boehringer Mannheim, Germany); TGFβ1 (10 ng/μl; R&D Systems) and bovine serum albumin (BSA; 1 μg/μl; Sigma). The NIH3T3 cell line expressing WNT6 (Kettunen et al., 2000) was a kind gift from Seppo Vainio.

In situ hybridisation and immunohistochemistry

Radioactive in situ hybridisation was carried out as described earlier (Wilkinson and Green 1990). Probes were labelled with ^{35}S-UTP (Amersham) and exposure time was 10-14 days. Whole-mount in situ hybridisation was performed as described earlier (Raatikainen-Ahokas et al., 2000), by using the InSituPro Robot (Intavis AG, Germany). The digoxigenin-labelled probes were detected with BM Purple AP Substrate Precipitating Solution (Boehringer Mannheim Gmbh, Germany). The *p63* probe (pan-p63) has been described (Mills et al., 1999); the *Fgf20* probe was a kind gift from Dr N. Itoh (Kyoto University). The probe detecting a 324 bp fragment of murine *Pvrl1* (nucleotides 58-382, GenBank Accession Number AF 297665) was made by cloning the PCR fragment into the pCRII-TOPO vector (Invitrogen).

Probes specific for the transactivating (TA) and N-terminally truncated (ΔN) isoforms of p63 were made by cloning a 269 bp PCR fragment of murine *TAp63* (nucleotides 35-303, GenBank Accession Number AF 075434) and a 154 bp PCR fragment from *ΔNp63* cDNA (nucleotides 20-173, EST BB 649754) into pCRII-TOPO (Invitrogen). Details for other probes, all previously described, are available upon request. Immunostaining was performed as described earlier (Laurikkala et al., 2002). The primary antibodies used were anti-p63 (4A4, 1:500; NeoMarkers) and anti-ΔNp63 (sc-8609, 1:100, Santa Cruz Biotechnology).

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed as described previously (Alberts et al., 1998). Back skin was dissected from E13 NMRI mouse embryos in Dulbecco's PBS. For the separation of skin epithelium from mesenchyme, the explants were incubated in 0.75% pancreatin (Gibco), 2.25% trypsin (Difco) for 25 minutes at room temperature. The epithelium was further dissociated by trypsin-EDTA solution for 30 minutes at room temperature. The cells were allowed to recover in organ culture medium for 5 minutes at room temperature, fixed with 1% formaldehyde for 15 minutes at room temperature, lysed (10 mM EDTA, 50 mM Tris, 1% SDS), sonicated five times for 10 seconds, and diluted 10-fold (1.2 mM EDTA, 16.7 mM Tris, 167 mM NaCl, 1.1% Triton X-100, 0.01% SDS) with a cocktail of proteinase inhibitors (Complete mini, Roche). After preclearing treatment, cell extracts were incubated with p63 antibody (4A4; NeoMarkers) overnight at 4°C followed by precipitation with ProteinA sepharose. Washing and elution of the immune complexes, as well as precipitation of DNA, were performed as described previously (Braunstein et al., 1993). The presence of the putative p63 target sites of the *Bmp7*, *Notch1* and *p21* genes in the immune complexes were detected by PCR using primers amplifying the following genomic regions (a detailed description of primers is available upon request): Bmp7 gene, –1797 to –1586, as a control –209 to –90; Notch1, –4949 to –4765, –3402 to –3232, +13,151 to +13,324, as a control –194 and –25; p21, –2875 to –2715. Positions of PCR fragments correspond to NCBI Mouse genome Build 33.1.

Quantitative RT-PCR

For real-time RT-PCR, back skin from wild-type E13 embryos was dissected. In order to get corresponding samples from E9 embryos, head as well as the most posterior part of the embryo were cut out, all internal organs were excised, and the remaining trunk skin (including somites) was used for the analysis. E7 (n=5) and E8 (n=5) whole embryos, or tissues from individual E9 (n=4) or E13 embryos (n=6), were placed straight into 350 μl lysis buffer of the RNeasy mini kit (Qiagen) containing 1% β-mercaptoethanol. Total RNA was isolated as specified by the manufacturer and quantified using UV spectroscopy. RNA (100 ng) was reverse transcribed using random hexamers (Promega) and Superscript II (Invitrogen), according to the manufacturer's instructions. Quantitative PCR was carried out using the 2×SYBR-green PCR master mix (Applied Biosystems) and Applied Biosystems' default PCR conditions for the ABI 7000. Amplification was performed with the following primers: TAp63 forward, 5′-GTGGATGAACCTTCCGAAAA-3′; ΔNp63 forward, 5′-CAAAACCCTGGAAGCAGAAA-3′; and reverse for both isoforms, 5′-GAGGAGCCGTTCTGAATCTG-3′. This resulted in 158 and 159 bp products specific for the TAp63 and ΔNp63 isoforms, respectively. PCR products were run on a 2% agarose gel to verify the absence of non-specific reaction products and primer dimers. Gene expression was quantified by comparing the sample data against a dilution series of plasmids containing the corresponding cDNA fragments of the TAp63 and ΔNp63 isoforms. Data were analysed using Applied Biosystems' Prism SDS software and normalised against *Hprt*.

Western blotting

The open reading frames of the α, β and γ isoforms of ΔNp63 were cloned into the pcDNA3 vector (Invitrogen). An optimised Kozak sequence (ACCACC**ATG**) was tailored at the 5′ end of each construct. NIH3T3 cells were transfected 24 hours prior use with ΔNp63 constructs or with empty vector using Lipofectamine 2000 (Invitrogen), and directly lysed in Laemmli sample buffer. Dissected skin from E13 and E14 *p63*$^{-/-}$ mouse embryos or control littermates (combined +/– and +/+) was homogenised in 2% SDS in PBS by boiling, and by using a syringe and needle. Protein (20 μg) was separated in 10% SDS-PAGE, transferred onto a Hybond-C-extra membrane (Amersham) and probed with a p63 antibody (4A4, 1:1000, NeoMarkers), detecting all p63 isoforms; finally, blots were developed by enhanced chemiluminesence (ECL, Amersham).

RESULTS

Expression of *p63* during tooth and skin development

We examined the expression of *p63* in developing teeth of wild-type embryos from E10 to postnatal day 3 (P3), and in dorsal back skin from E11 to birth, using in situ hybridisation with a probe recognizing all *p63* isoforms (hereafter called *pan-p63*) (Fig. 1). *Pan-p63* transcripts were expressed at E10 throughout the simple epithelium covering the mandibular arch (Fig. 1A). During the initiation (E11), bud (E12) and cap (E15) stages of mandibular molar development, the *pan-p63* hybridisation signal was seen throughout the dental epithelium, and it extended into the oral epithelium (Fig. 1B-D). At the bell stage (E17), *pan-p63* expression was intense in the outer enamel epithelium, whereas the intensity of expression was reduced in the inner enamel epithelium and stellate reticulum (Fig. 1E). At E14, transcripts were abundant in the epithelium of palatal shelves (Fig. 1F).

In the back skin, *pan-p63* transcripts were detected throughout the simple ectoderm in E11-E13 embryos (Fig. 1G). At E15, expression was intense in the ectoderm, except for in the most superficial cell layers (Fig. 1H). At E17, *pan-p63* staining was seen in the basal epithelial cells and in the maturing stage 3-4 hair follicles of guard hairs, as well as in the initiated epithelial placodes of the awl hairs (Fig. 1I). Expression was also detected in the eye, in tongue mesenchyme, and in some nerves (data not shown).

Because little is known about the spatiotemporal expression of the different *p63* isoforms during embryogenesis, we next analysed the expression of *ΔNp63* and *TAp63* isoforms using probes specific to the different 5′ ends of these transcripts. A similar distribution of p63 in the surface epithelium, hair follicles and teeth was revealed by the ΔNp63 probe as by the *pan-p63* probe (Figs 1, 2). *ΔNp63* transcripts were seen throughout the simple oral epithelium and in the dental lamina at E10 (data not shown), and in all dental epithelia from E11 onwards (Fig. 2D-G); they were lost from the inner enamel epithelium when it differentiated into ameloblasts (Fig. 2I). No expression was seen in developing teeth and hairs with the *TAp63*-specific probe (Fig. 2A-C,H). *TAp63* transcripts were detected in some mesenchymal structures of the tongue at E14 and E15, as well as in the eye and in some nerves, in patterns similar to those seen with the pan-*p63* probe (data not shown).

Immunohistochemical analysis with antibodies recognising either all p63 isoforms (Fig. 2J-L) or the ΔNp63 isoforms (see Fig. S1 in the supplementary material) confirmed that ΔNp63 is the major p63 splice variant expressed during embryonic tooth and hair formation. Protein and mRNA expression patterns corresponded well with each other. Western blot analysis preformed on wild-type E13 and E14 skin samples revealed that the alpha isoform of p63 is the predominant one expressed during the initiation of hair development and epidermal stratification; although a faint band corresponding to the gamma isoform was also discernible (Fig. 2M).

To further verify our in situ hybridisation and immunohistochemical results, we analysed E13 skin samples by quantitative RT-PCR (Fig. 2N). Only one percent of all p63 represented the TA isoform, confirming our in situ hybridisation results. We also analysed E7 and E8 whole embryos, as well as E9 skin samples by RT-PCR. Intriguingly, p63 was not expressed at E7, whereas at E8 and E9 only ΔNp63 was detected (Fig. 2N).

p63 is required for the formation of both tooth and hair placodes

The original phenotypic analyses of *p63*$^{-/-}$ mice revealed a lack of tooth and hair development, but the pathogenesis and exact stage of arrest were not studied (Mills et al., 1999; Yang et al., 1999). In order to characterise this defect more thoroughly, we examined back skin sections and serial frontal sections of the heads and jaws of *p63* mutants and their wild-type littermates between E10 and E17. No morphological difference was noted in surface ectoderm at E10 (not shown). At E11, the dental lamina, which forms a horse shoe-shaped stripe of multilayered epithelium in the lower jaw, was morphologically normal in *p63* mutant embryos, and was increased in thickness in the molar and incisor regions (Fig. 3A,B). At E12, dental placodes had formed in the wild-type dental lamina at the sites of incisors and molars, and at E13 they had progressed into tooth buds surrounded by condensed mesenchyme (Fig. 3C,E). In *p63*-deficient embryos, development did not advance from the dental lamina stage. Dental placodes were absent in E12 and E13 embryos (Fig. 3D,F), and the thickened dental lamina epithelium appeared to regress in *p63*$^{-/-}$ embryos during later stages of development (Fig. 3G,H; and data not shown). Only occasionally (2/15), was a rudimentary bud-like structure seen in a mutant embryo (data not shown).

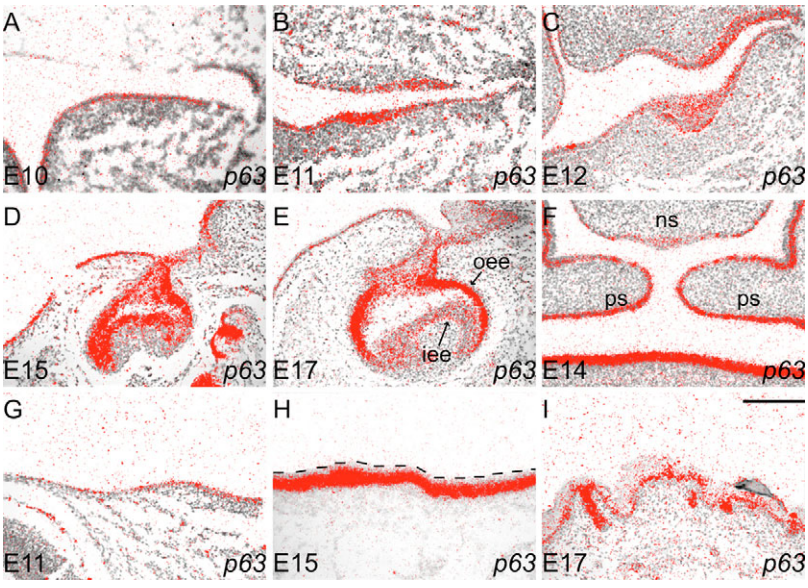

Fig. 1. Localisation of *p63* mRNA in frontal sections of embryonic mouse heads and in developing skin. (**A**) Prior to morphological tooth formation (E10), *p63* transcripts were seen throughout the simple epithelium. (**B-D**) During the initiation (E11), placode (E12) and cap (E15) stages, *p63* expression continued in all oral and dental epithelia. (**E**) At the bell stage, *p63* expression was particularly intense in the outer enamel epithelium and weaker in the inner enamel epithelium. (**F**) At E14, transcripts were intense in the epithelium of palatal shelves. (**G**) *p63* transcripts were present in simple surface ectoderm at E11. (**H**) At E15, *p63* expression continued in the basal epithelial cell layer, whereas it was downregulated in the most superficial cells. Dashed line indicates the epidermal surface. (**I**) At E17, *p63* expression continued in the basal epithelial cells and it was intense in the stage 1-4 hair follicles. iee, inner enamel epithelium; ns, nasal septum; oee, outer enamel epithelium; ps, palatal shelf. Scale bar: 200 μm.

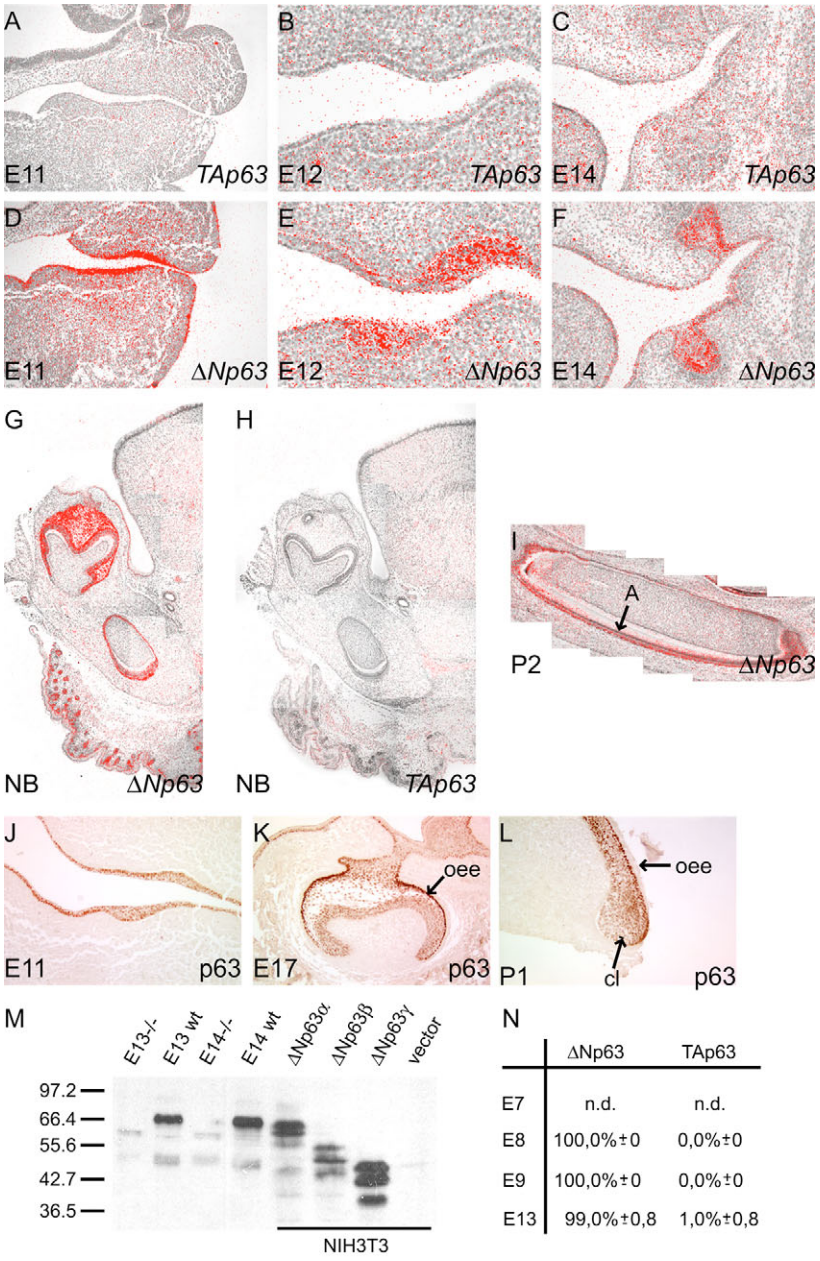

Fig. 2. Expression of *TAp63* and *ΔNp63* transcripts, and of p63 protein. (**A-C**) No *TAp63* mRNA was seen in surface ectoderm or tooth germ epithelia at the initiation (E11), placode (E12) or cap stages of molar tooth development (E14). (**D-G**) Intense expression of *ΔNp63* was seen in the oral and dental epithelium, as well as in hair follicles, in similar patterns to those observed with the *p63* probe that detects all isoforms (see also Fig. 1). (**H**) Expression of the *TAp63* isoform was not detected in the epithelia of the newborn (NB) mouse. (**I**) In P2 incisors, *ΔNp63* expression was intense in the enamel organ epithelium contacting the ameloblasts, but ameloblasts did not express *ΔNp63*. (**J**) Immunohistochemical analysis showed p63 protein expression throughout the oral and dental epithelia at E11. (**K**) Expression was particularly intense in the outer enamel epithelium in the molar at bell stage. (**L**) In the P1 incisor, p63 protein expression was most intense in the outer enamel epithelium. (**M**) Western blot analysis of p63 isoforms in E13 and E14 skin samples of $p63^{-/-}$ and wild-type (combined +/– and +/+) embryos. NIH3T3 cells transfected with ΔNp63α, ΔNp63β or ΔNp63γ were used as control samples. The major band detected in wild-type tissues corresponds to ΔNp63α; TAp63α typically migrates substantially more slowly in SDS-PAGE. (**N**) Quantification of the expression of ΔNp63 and TAp63 isoforms in E7 and E8 whole embryos, and in E9 and E13 skin by real-time PCR. Trace amounts of the TAp63 isoform were detected at E13. A, ameloblasts; cl, cervical loop; oee, outer enamel epithelium; n.d., not detectable.

Our analysis of skin sections confirmed the previously described phenotypical features of surface ectoderm development in *p63* mutant embryos (Mills et al., 1999; Yang et al., 1999). The placodes of guard and awl hairs, which are seen in the wild-type mouse embryo, were not detected in *p63*-deficient embryos (Fig. 3I,J).

To confirm the presence of the dental lamina in the mutants, we analysed the expression of two dental lamina markers *Pitx2* and *Shh* in E11-E12 embryonic lower jaws by whole-mount in situ hybridisation (Mucchielli et al., 1997; Keränen et al., 1999). *Pitx2* and *Shh* transcripts were co-expressed in the dental lamina both in wild-type and *p63* mutant jaws at E11 (data not shown). At E12, *Pitx2* and *Shh* expression became restricted to the incisor and molar placodes, and their expression was downregulated in the diastema region between the incisors and molars in wild-type mandibles; interestingly, however, their expression remained continuous in the mutant dental lamina (Fig. 4A-D).

The placodes of guard hairs become morphologically evident in the back skin at E14 and they can be visualised by the punctuate expression of several marker genes, such as β-catenin and *Edar* (Huelsken et al., 2001; Laurikkala et al., 2002). Whole-mount in situ hybridisation analysis of *p63* mutant embryos at E14 showed that both β-catenin and *Edar* were absent (Fig. 4E-H). In conclusion, these results show that, in the absence of p63, tooth development arrests at the dental lamina stage and hair follicle development is not initiated, suggesting that p63 is required in the ectoderm for the formation of both the tooth and hair placodes.

Search for downstream targets of p63 by in situ hybridisation

We studied the expression of 29 potential downstream target genes of p63 by in situ hybridisation analysis. The expression patterns of the following genes are shown: *Shh*, *Pitx2*, *Fgf8*, *Fgf9*, *Fgfr2b*, *Bmp4*, *Bmp7*, *Msx1*, activin βA, *Notch1*, *Notch2*, *Notch3*, *Jag1*,

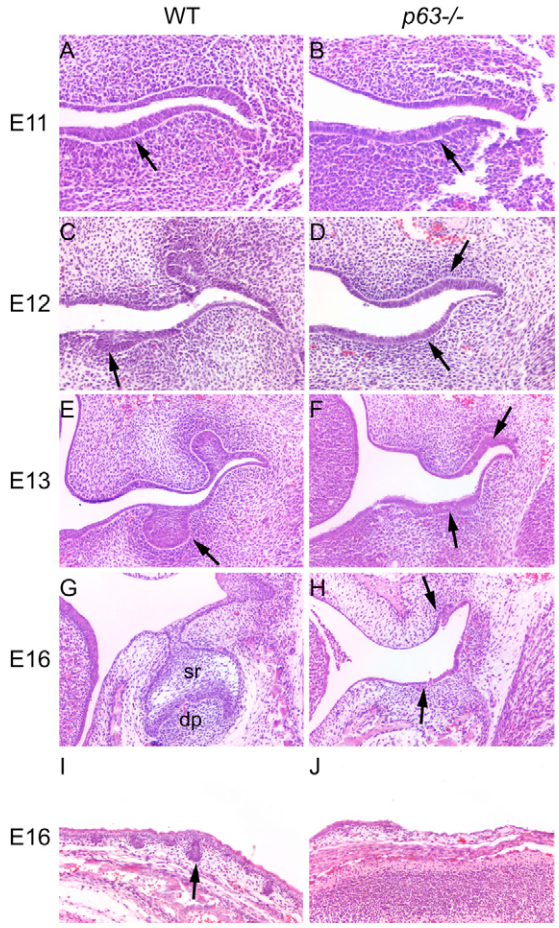

Fig. 3. Comparison of molar tooth and hair follicle development between wild-type and *p63* mutant embryos. (**A**,**B**) At E11, no difference could be detected between wild-type and *p63*$^{-/-}$ tissues during the initiation of tooth development (arrows). (**C-F**) At E12 and E13, dental placodes and tooth buds (arrows) had formed in wild-type embryos, whereas in the mutant littermates development had arrested at the stage of thickened dental epithelium (arrows). (**G**,**H**) At E16, wild-type molars had advanced to the bell stage, whereas in *p63* mutants the epithelial thickening had degenerated. (**I**) The first wave of hair follicles (guard; arrow) had developed to stage 3-4, the placodes of the second wave awl follicles had appeared in wild-type skin at E16.5 and the epidermis had increased in thickness. (**J**) *p63*$^{-/-}$ epidermis lacked stratification and hair follicles. sr, stellate reticulum; dp, dental papilla.

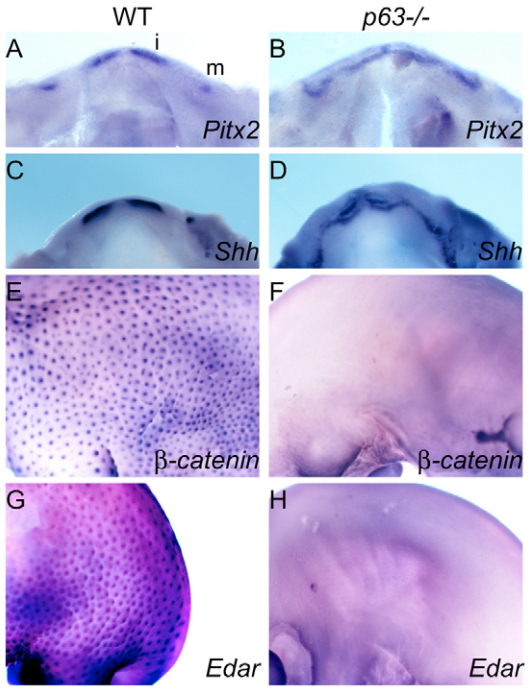

Fig. 4. Tooth and hair placodes do not form in *p63* null mutants. (**A**,**C**) The placodes of incisors and molars are visualised by *Pitx2* and *Shh* expression at E12 in the wild-type mandible. (**B**,**D**) In mutant mandibles, *Pitx2* and *Shh* remained continuous in the dental lamina, indicating the absence of placodes. (**E**,**G**) β-catenin and *Edar* are early markers of hair placodes in wild-type skin at E14. (**F**,**H**) In *p63* null skin, β-catenin and *Edar* expression was absent. i, incisor; m, molar placode.

Jag2, *Wnt3a*, *Wnt6*, *Wnt10b*, β-catenin, *Lef1*, *Edar*, *Eda*, *Tnfrsf19*, *Pvrl1* and *Ptc1* (Figs 5, 6; see also Figs S2, S3 in the supplementary material). Expression patterns are not shown for *Egfr*, *Hes1*, lunatic fringe, *Msx2* and *Pax9*. We compared serial tissue sections from the heads of E10-E12 embryos. The sections included the molar area, as well as surface ectoderm of the head and neck. In addition, sections from E12-E14 back skin were examined. We focused on genes that have been associated with the initiation and morphogenesis of teeth and hairs.

During tooth development, FGF and FGFR genes are expressed both in the epithelium and mesenchyme, and they regulate tooth development at all stages (Thesleff and Mikkola, 2002b). *Fgfr2b* is expressed in the epithelial cells of developing teeth (E11-P4) (Kettunen et al., 1998), and we saw expression in wild-type embryos throughout the oral epithelium, including the tooth at E11-E12 (Fig. 5A,C). Interestingly, *p63* mutant epithelium completely lacked *Fgfr2b* expression (Fig. 5B,D). Similarly, *Fgfr2b* transcripts were completely missing from *p63*-deficient epidermis at E12 (Fig. 5E,F). FGF3, FGF7 and FGF10 use exclusively FGFR2b, and are expressed in developing teeth and hair follicles (Chuong, 1998; Millar, 2002). Hence, our results suggest that signalling by these FGFs is impaired in *p63* null embryos.

Fgf8 and *Fgf9* are necessary for early tooth morphogenesis (Trumpp et al., 1999). It was reported previously that *Fgf8* transcripts were severely downregulated/absent in the *p63* mutant limb buds, which fail to develop further (Mills et al., 1999; Yang et al., 1999). However, we detected similar expression domains of *Fgf8* (Fig. 5G,H), as well as *Fgf9*, in the branchial arch ectoderm (E10) and in the dental lamina (E11-E12) in wild-type embryos and *p63* mutants (see Fig. S2 in the supplementary material).

Several BMPs show developmentally regulated expression patterns during murine tooth morphogenesis (Åberg et al., 1997). *Bmp7* is co-expressed with *p63* in the oral epithelium during the formation of dental lamina and early tooth buds (Fig. 5I; data not shown). Interestingly, *Bmp7* transcripts were not detected in the oral epithelium of *p63*-deficient embryos (Fig. 5J). Accordingly, *Bmp7* transcripts were also completely absent from the back skin epithelium of *p63* mutants at E14 (Fig. 5K,L). No apparent changes were detected in the expression of two mesenchymal TGFβ superfamily members, *Bmp4* and activin βA (see Fig. S2 in the supplementary material).

β-Catenin, the key mediator of the WNT signals, was expressed similarly in the oral epithelium of wild type and *p63* mutants at E12 (Fig. 5M,N). Interestingly, it was significantly downregulated in the

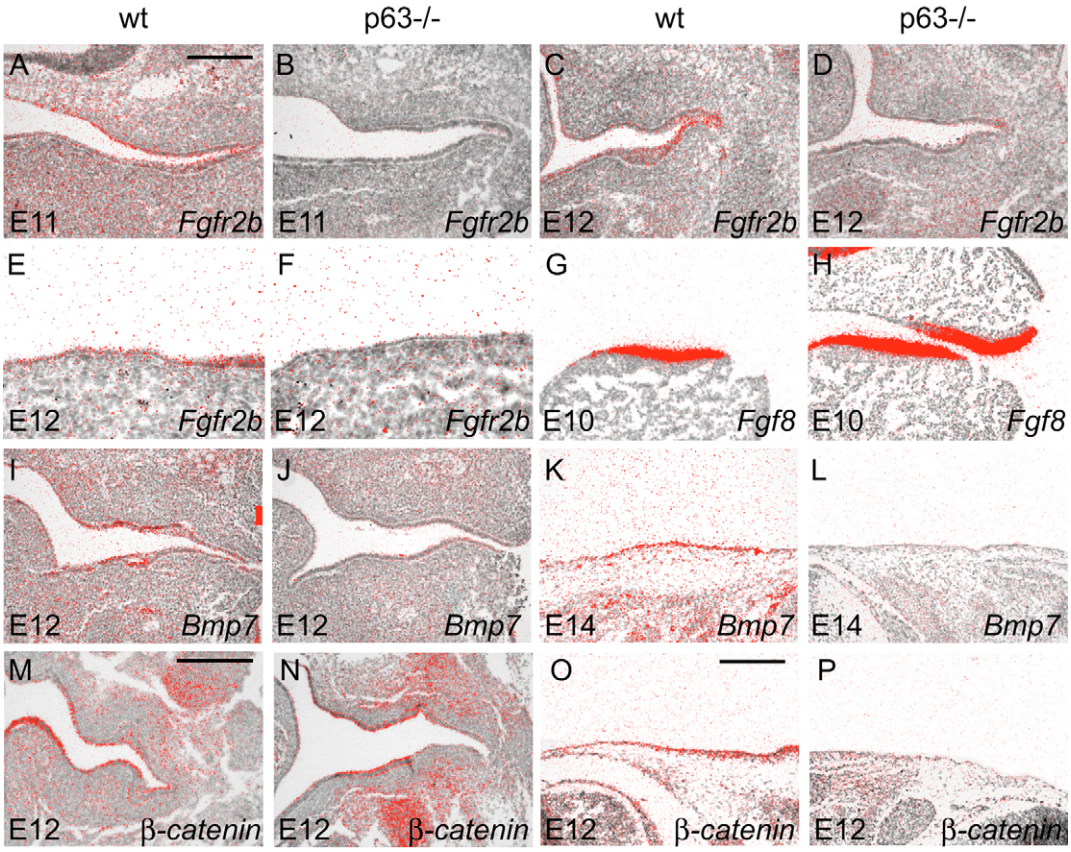

Fig. 5. Expression of *Fgfr2b*, *Fgf8*, *Bmp7* and β-catenin in wild-type and *p63* mutant embryos. (**A**-**D**) *Fgfr2b* transcripts were seen throughout simple oral epithelium and thickened dental epithelium (E11) and dental placode (E12) in wild-type embryos, but were absent in the *p63* mutant littermate. (**E**,**F**) *Fgfr2b* transcripts were absent in skin epithelium of *p63* mutants. (**G**,**H**) *Fgf8* expression was detected in the area of tooth development both in wild-type and in mutant embryos prior to morphological tooth formation (E10). (**I**,**J**) *Bmp7* transcripts were absent in *p63* null oral epithelium. (**K**,**L**) At E14, *Bmp7* was expressed throughout surface ectoderm in a wild-type embryo, whereas expression was completely absent in a *p63* mutant littermate. (**M**,**N**) β-catenin was intensely expressed both in wild-type and mutant oral epithelium. (**O**,**P**) β-catenin was downregulated in skin epithelium in the *p63* null embryo. Scale bars: in A, 200 μm for A-L; in M, 500 μm for M,N; in O, 200 μm for O,P.

surface epithelium of *p63* mutants, although some transcripts were occasionally observed (Fig. 5O,P). We also analysed the expression of WNT ligands WNT3, WNT6 and WNT10b in developing teeth. *Wnt3* and *Wnt6* transcripts were similarly expressed throughout oral and dental epithelia in wild-type and *p63*$^{-/-}$ embryos (see Fig. S3 in the supplementary material). *Wnt10b* was expressed throughout the epithelium at E11 and was localised to the tooth placode at E12 in wild-type embryo. By contrast, in *p63* null embryos, the *Wnt10b* hybridisation signal remained continuous throughout the dental and oral epithelia (see Fig. S3 in the supplementary material).

Notch signalling has been linked with epidermal cell differentiation, and with tooth and hair morphogenesis (Rangarajan et al., 2001; Mitsiadis et al., 1995; Fuchs and Segre, 2000). During the early stages of tooth development (E11-E12), *Notch1*, *Notch2* and *Notch3* were co-expressed in wild-type embryos in the suprabasal cells of oral and dental epithelium, but the basal cells were devoid of transcripts (Fig. 6A,C,E,G). *Notch1* expression was not detected in the mutant epithelium at E11-E12, whereas *Notch2* and *Notch3* transcripts were expressed normally (Fig. 6B,D,F,H). The Notch ligands *Jag1* and *Jag2* were co-expressed with *Notch1* and *Notch2* in wild-type dental epithelium at E11-E12 (Fig. 6I,K). Mutant embryos lacked epithelial *Jag1*, and the mesenchymal expression of *Jag1* was significantly downregulated (Fig. 6J). *Notch1* and *Jag1* were intensely expressed in dorsal back skin ectoderm in E12 and E14 wild-type embryos (Fig. 6M,O; data not shown), but their transcripts were generally absent in the mutants (Fig. 6N,P), although we occasionally detected *Notch1* and *Jag1* expression in some epithelial cells (data not shown). Recently, *Jag1* and *Jag2* were shown to be upregulated by ectopic expression of p63 and p73 in several human cancer cell lines (Sasaki et al., 2002). However, we found normal *Jag2* expression in *p63* mutants (Fig. 6L).

Mutations in the genes encoding the TNF receptor EDAR and its ligand EDA cause hypohidrotic ectodermal dysplasia (Thesleff and Mikkola, 2002a). *Edar* is expressed throughout the branchial arch ectoderm in E10 wild-type embryos, and becomes upregulated in the thickened dental epithelium at E11 and in the dental placode at E12 (Fig. 6Q; data not shown) (Tucker et al., 2000; Laurikkala et al., 2001). In *p63* mutants, *Edar* was expressed as in wild-type mice at E10 (data not shown), but we did not notice localised upregulation at E11 that occurs in wild-type embryos (Fig. 6R). *Edar* was also expressed at reduced levels in mutant epidermis at E12 (Fig. 6S,T). Expression of *Tnfrsf19* (also called *Taj* or *Troy*), which encodes a TNF receptor homologous to EDAR, was unaffected in the *p63* mutant dental epithelium (see Fig. S3 in the supplementary material). Likewise, expression of *Eda*, which colocalises with *p63*

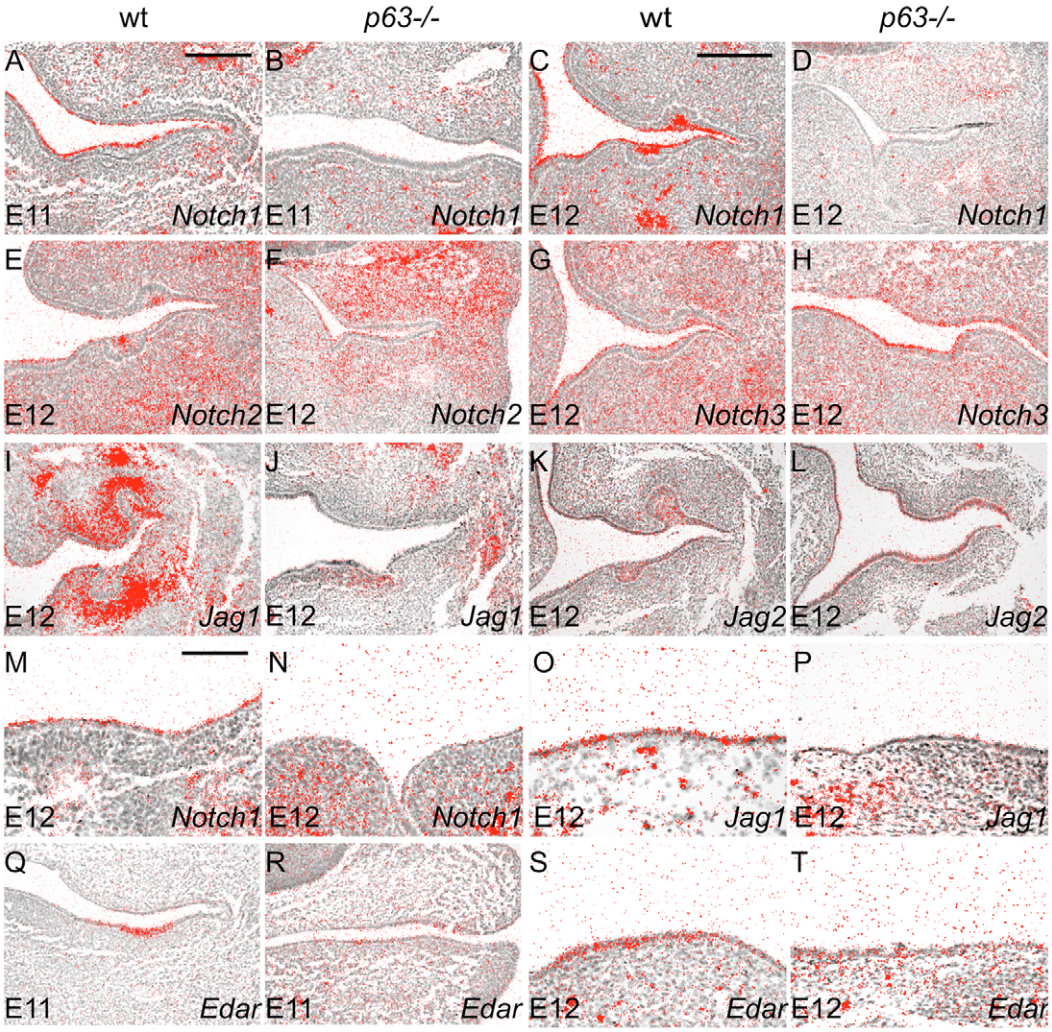

Fig. 6. Expression of NOTCH pathway genes and *Edar* in wild-type and *p63* mutant embryos. (**A**,**C**,**E**,**G**) *Notch1*, *Notch2* and *Notch3* were co-expressed in dental and oral epithelium in wild-type suprabasal cells. (**B**,**D**) *Notch1* transcripts were absent in *p63* null epithelium at E11 and E12. (**F**,**H**) *Notch2* and *Notch3* showed normal expression in the *p63* mutants. (**I**) At E12, *Jag1* was intensely expressed in suprabasal cells in the epithelium and in the mesenchyme surrounding the tooth bud in wild-type embryo. (**J**) The *p63* mutant epithelium was devoid of *Jag1* transcripts but faint expression was seen in the mesenchyme. (**K**,**L**) *Jag2* was expressed in the oral epithelium in both wild-type and mutant embryos. (**M**,**N**) In E12 skin epithelium, intense *Notch1* expression was seen in the wild-type embryo, whereas transcripts were not detected in the mutant littermate. (**O**,**P**) *Jag1* was not observed in *p63*-deficient skin epithelium. (**Q**-**T**) The intensity of *Edar* expression was weaker in the oral, dental and skin epithelium of p63 mutants than in wild-type embryos. Scale bars: in A, 200 μm for A,B; in C, 500 μm for C-L; in M, 200 μm for M-T.

in the early ectoderm (Laurikkala et al., 2001; Laurikkala et al., 2002), was normal in $p63^{-/-}$ embryos at E10-E12 (Fig. S3 in the supplementary material).

In conclusion, of the 29 genes analysed, *Bmp7*, *Fgfr2b*, *Jag1* and *Notch1* were completely absent from the epithelium of *p63*-deficient embryos, including the regions of both hair and tooth development. In addition, β-catenin and *Edar* transcripts were downregulated in the surface ectoderm. None of the known marker genes of ectodermal placodes showed localised expression in the ectoderm of *p63* null embryos. Mesenchymal genes such as *Msx1* and *Lef1* (see Fig. S3 in the supplementary material) showed reduced expression, apparently because the stimulatory signals from placodes were lacking.

Our in situ hybridisation analysis indicated that *Bmp7* and *Notch1* lie downstream of p63. To determine whether p63 directly regulates their transcription, we performed chromatin immunoprecipitation (ChIP) assays using freshly isolated E13 epidermal cells. p63 is known to bind to p53-responsive elements (El-Deiry et al., 1992; Sasaki et al., 2002; Westfall et al., 2003), and therefore we tested several putative p53-responsive elements found in the promoter/intron regions of *Bmp7* and *Notch1* genes in ChIP (see Fig. S4 in the supplementary material). The p53 binding site (site 1) in the *p21* gene, which is known to be regulated by p63, was used as a positive control (Westfall et al., 2003). ChIP analysis revealed that one candidate sequence on the *Bmp7* promoter, as well as one out of three candidate sites in the *Notch1* gene, could be amplified from the p63 immune complexes (Fig. S4 in the supplementary material).

p63 expression is induced by BMPs and FGF10

To analyse the genes upstream of *p63*, we studied several signalling molecules for their ability to regulate *p63* expression in cultured whole tooth explants or isolated dental epithelia (E11-E14). The expression of *p63* was maintained in the epithelium even in the absence of the mesenchyme (data not shown). Beads releasing

BMP2 and BMP7 induced the expression of *p63* in the dental epithelium in whole tooth explants at all stages analysed (E11-E14; Fig. 7A,B), as well as in the isolated dental epithelium (data not shown). In addition to BMPs, FGF10 induced the expression of *p63*, whereas FGF4 and FGF8 did not (Fig. 7C-E). Similar induction with BMPs and FGF10 was seen with the *ΔNp63*-specific probe as with the *pan-p63* probe, whereas no induction of the *TAp63* isoform was observed (data not shown). FGF10 signals exclusively via FGFR2b, yet we found intact expression of *p63* in *Fgfr2b* mutant epidermis (Fig. 7K,L), indicating that FGF10 is not necessary for *p63* expression. None of the other signal molecules analysed, including activin A, EGF, SHH, TGFβ1 and WNT6, stimulated p63 expression (Fig. 7F-J; data not shown), and we found intact expression of p63 in *Lef1*$^{-/-}$ embryonic ectoderm (data not shown).

DISCUSSION

The ΔNp63 isoform, but not the TAp63 isoform, of p63 is expressed in epithelial cells during morphogenesis of the epidermis, hair follicles and teeth

Analyses by three independent methods indicated that ΔNp63 isoform is expressed in the simple ectoderm prior to the morphological onset of tooth and hair formation. During subsequent stages, ΔNp63 expression continued in epithelial cells, but was downregulated during the differentiation of keratinocytes and ameloblasts. Transactivating TAp63 isoforms were not detected during early development (E8-E9), and only trace amounts were observed during ectodermal appendage development. These results contradict with those recently reported by Koster et al., who proposed that TAp63 isoforms dominate over ΔNp63 isoforms in the epidermis until E18.5, based on RT-PCR analysis (Koster et al., 2004). The reason for this discrepancy is currently unclear. However, the degree of normalisation of these PCR reactions was not described (Koster et al., 2004). On the basis of our data, we conclude that ΔNp63 is the main isoform expressed in the developing tooth and hair, as well as in the embryonic epidermis. Accordingly, ΔNp63 was the only isoform detected in the developing epidermis in zebrafish, where it is required for proper epidermal and limb development (Lee and Kimelman, 2002; Bakkers et al., 2002).

Our in vitro analysis showed that mesenchymal signals are not needed for the maintenance of *p63* expression. Our results indicate that BMPs may be epithelial signals regulating *ΔNp63* expression in embryonic ectoderm. This is in line with earlier findings showing that the expression of zebrafish *ΔNp63* is activated by Smad4/Smad5-mediated Bmp signalling, and that *ΔNp63* is downregulated in *bmp7* mutant zebrafish embryos (Bakkers et al., 2002). Of the other signalling molecules tested, only FGF10 stimulated *ΔNp63* expression in dental epithelium.

Search for downstream targets: *Fgfr2b*, *Jag1*, *Notch1* and *Bmp7* are downstream of ΔNp63

In our search for downstream targets of p63, we found several genes that were co-expressed with *p63* in wild-type embryos but that were downregulated in *p63*-deficient embryos. We show, for the first time, the absence of *Bmp7*, *Jag1*, *Notch1* and *Fgfr2b*, and the downregulation of *Edar* and β-catenin, in embryos deficient for *p63*. The finding that ΔNp63α, which was initially classified as a non-transactivating molecule (Yang et al., 2002), is the main isoform during the crucial early stages of tooth and hair development is intriguing. However, there are several possibilities as to how these genes could be regulated by ΔNp63. First, there is increasing evidence that ΔNp63 isoforms can act as transcriptional activators and regulate the same genes as the TA isoforms (Ghioni et al., 2002; Wu et al., 2003) in a cell-specific manner (King et al., 2003; Ihrie et al., 2005), possibly via a second transactivating domain present within the first 26 N-terminal amino acids (Dohn et al., 2001). Indeed, our ChIP results suggest that both *Bmp7* and *Notch1* could be direct transcriptional targets of ΔNp63α. However, additional experiments will be required to validate these suggestive data. Previous studies using cells overexpressing TAp63 have also implicated *Jag1* as a direct transcriptional target of p63 (Sasaki et al., 2002; Ross and Kadesch, 2004). Second, ΔNp63 isoforms could regulate the expression of specific repressors (Wu et al., 2003). Third, they could exert their effects via protein-protein interactions (Patturajan et al., 2002). Recently, Fomenkov et al. showed that wild-type ΔNp63α binds to ABBP1, a member of the RNA-processing machinery, promoting the formation of the IIIb splice variant of FGFR2, whereas ΔNp63α harbouring an ectodermal dysplasia-associated mutation failed to do so (Fomenkov et al., 2003). In line with our results, they also noted downregulation of the FGFR2b splice variant in the skin samples of *p63* knockout mice by semi-quantitative RT-PCR. Apparently, a lack of ΔNp63 expression leads to the absence of several, direct or indirect, downstream targets via multiple mechanisms.

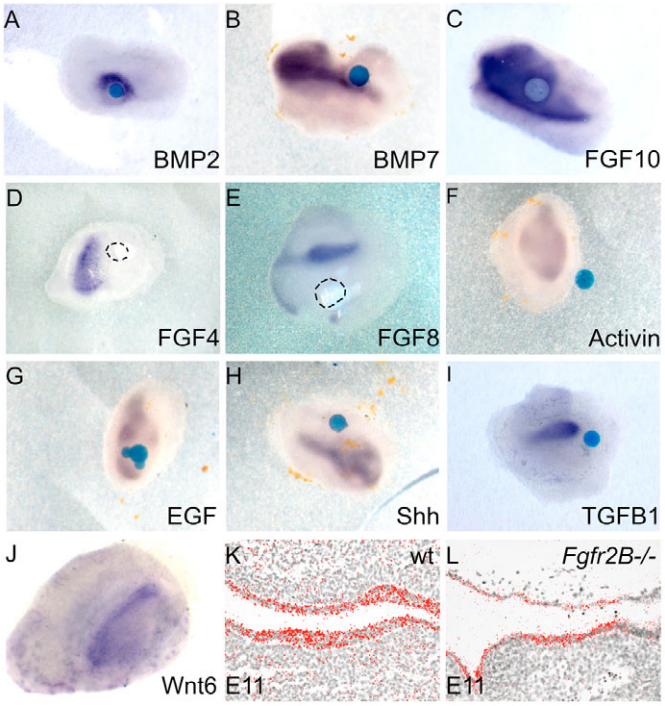

Fig. 7. Induction of *p63* expression by BMP2, BMP7 and FGF10 in dental epithelium in E13 tooth explants, as analysed by whole-mount in situ hybridisation. Proteins were introduced with beads (A-I) or transfected cells (J) on explants and cultured for 24 hours. Note the endogenous expression of *p63* in epithelium in all explants. (**A**,**B**) BMP2 and BMP7 induced *p63* expression. (**C**) FGF10 induced p63 expression. (**D**-**J**) None of the other signals tested (FGF4, FGF8, Activin, EGF, SHH, TGFβ1, WNT6) affected *p63* expression. (**K**,**L**) Expression of *p63* does not require FGFR2b. *p63* transcripts were observed throughout ectoderm, in a similar pattern, in both wild-type and *Fgfr2b* null mutant embryos at E11.

p63 is required for the formation of both tooth and hair placodes

Although tooth morphogenesis failed in the *p63* mutants, the early patterning events of dental development apparently occurred normally, as evidenced by unaffected epithelial expression of *Fgf8* and *Fgf9*, and the typical expression pattern of *Bmp4* in the buccal mesenchyme under the epithelial thickening of molar teeth at E11 (see Fig. S2 in the supplementary material) (Vainio et al., 1993). Also, the dental lamina, a stripe of thickened epithelium prefiguring the dental arches in the upper and lower jaws, formed in its normal location in p63 mutants, but dental placodes did not form. Hence, although the epithelial stratification failed in the p63-deficient surface epidermis, the multilayered epithelial thickenings were apparently normal in the oral ectoderm. This contrasts to the limb phenotype of $p63^{-/-}$ embryos, where no morphologically distinct apical ectodermal ridge can be seen (Mills et al., 1999; Yang et al., 1999). In embryonic skin, we did not detect localised expression of any of the marker genes of the hair placode, indicating that hair development had already failed at the initiation stage.

The severity of the dental and hair phenotype of *p63* mutants most likely results from the abrogation of multiple signalling pathways. The importance of β-catenin for hair development was highlighted by its conditional ablation in the epidermis, which resulted in an early block of hair placode formation that apparently was caused by the inability of the epithelium to respond to WNT signals (Huelsken et al., 2001). Mutations in both *p63* and *EDAR* cause ectodermal dysplasia syndromes in humans, with quite similar hair and tooth phenotypes (Kere and Elomaa, 2002; van Bokhoven and McKeon, 2002), and primary hair placodes do not form in mice lacking the requisite components of the EDAR signalling pathway (Thesleff and Mikkola, 2002a). A role for the Notch pathway in tooth morphogenesis is suggested by the dynamic expression of several Notch receptors and ligands during tooth development (Mitsiadis et al., 1995; Mustonen et al., 2002). However, the phenotype of mice with conditional ablation of γ-secretase, an obligate activator of all Notch receptors, suggests that the Notch pathway is dispensable for the initiation of hair development (Pan et al., 2004). The expression of *Bmp7* was also completely downregulated in oral and skin ectoderm of *p63* mutants. Because both teeth and hair form in *Bmp7* mutant embryos (Dudley et al., 1995; Luo et al., 1995), BMP7 is conceivably redundant with other BMPs, perhaps BMP2, in the regulation of ectodermal organogenesis.

Finally, the absence of *Fgfr2b*, and therefore of FGF3, FGF7 and FGF10 signalling, in p63-deficient ectoderm may play a prominent role in the pathogenesis of p63 mutant phenotype. A link between FGFR2b and p63 is supported by the similar mouse knockout phenotypes, although the phenotype in ectodermal organs is less severe in the *Fgfr2b* mutants (Mills et al., 1999; Yang et al., 1999; De Moerlooze et al., 2000; Petiot et al., 2003). Tooth development of $Fgfr2b^{-/-}$ mice is arrested at the bud stage and hair development shows a reduced number of hair placodes. Taken together, our findings that p63 regulates many genes in different signalling pathways involved in placode initiation conceivably explain the more universal inhibition of placode formation in the *p63* mouse mutants, as compared with most mutants where placodes are affected by the inactivation of genes in one pathway only (Hardcastle et al., 1998; Satokata and Maas, 1994; Laurikkala et al., 2002; van Genderen et al., 1994).

P63 and regulation of epidermal development

The surface epithelium of *p63* null embryos is thin, lacks stratification (Yang et al., 1999; Mills et al., 1999), and cultured keratinocytes from these embryos express K18, a marker of all simple epithelia, including the epidermis prior to stratification (Koster et al., 2004). However, our results indicate that molecular differentiation of the p63-deficient epidermis partially took place, as *Notch2*, *Notch3* and *Jag2*, as well as *Pvrl1*, which encodes the cell adhesion molecule, nectin 1, that is essential for proper development of the ectoderm (see Fig. S3 in the supplementary material), showed normal expression in the suprabasal cells, with concomitant downregulation in the basal cells.

To date, few p63 target genes involved in epithelial maturation have been identified, and the cause of the failed ectodermal development of *p63* null mice has been a matter of debate (McKeon, 2004). Absence of a cohort of genes found in this report may, at least partially, explain the epidermal phenotype of *p63* mutants. The epidermis of *Fgfr2b* null embryos is reminiscent of that of *p63* mutants in that it is abnormally thin (De Moerlooze et al., 2000; Petiot et al., 2003). It appears that p63 regulates epidermal proliferation (at least) via FGFR2b, as a reduction in keratinocyte proliferation was reported to cause the hypoplastic epidermis in *Fgfr2b* null embryos. In addition, the proliferative capacity of *p63*-deficient embryonic epidermis may further be reduced by p21, which is a target of direct transcriptional repression by ΔNp63α (Westfall et al., 2003). However, all of the usual epidermal cell lineages are found in *Fgfr2b*-deficient epidermis, indicating that other targets genes of p63 are involved in triggering differentiation.

Notch signalling has been implicated in epidermal stratification and keratinocyte differentiation (Rangarajan et al., 2001; Nickoloff et al., 2002; Okuyama et al., 2004). Application of JAG1 to an epidermal equivalent model system induces epidermal maturation (Nickoloff et al., 2002), and ectopic expression of activated NOTCH1 in cultured keratinocytes causes a substantial induction of early differentiation markers (Rangarajan et al., 2001). Our observations suggest that NOTCH1 and JAG1 may be important downstream mediators of p63 during epidermal maturation in vivo, although, apparently, other target genes such as *Perp* are also involved (Ihrie et al., 2005).

The relative contribution of individual p63 isoforms during ectodermal differentiation and organogenesis is still far from understood. Recently, a model was put forth that suggests that TAp63 isoforms are the first p63 isoforms to be expressed during embryonic development, and that they are necessary for the commitment to stratification while simultaneously blocking the differentiation programme. Therefore, a shift towards ΔNp63 isoforms during later stages would be required to counterbalance the activity of TAp63, thereby allowing cells to respond to terminal differentiation cues (Koster et al., 2004; Koster and Roop, 2004). Our results, however, appear to contradict this model, as they indicate that ΔNp63 isoforms dominate throughout the development of the epidermis and its appendages. We propose that, during embryonic development, ΔNp63 isoforms have an independent role as transcriptional regulators and do not merely act as inhibitors towards transactivating molecules of the p53 family. The clarification of this issue must wait for the generation of isoform-specific knockouts. In conclusion, we suggest that ΔNp63 isoforms, most notably ΔNp63α, are crucial for the development of the ectoderm and its appendages.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/8/1553/DC1

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