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ΔNp63 is an ectodermal gatekeeper of epidermal morphogenesis

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p63, a member of p53 family, has a significant role in the development and maintenance of stratified epithelia. However, a persistent dispute remained over the last decade concerning the interpretation of the severe failure of *p63*-null embryos to develop stratified epithelia. In this study, by investigating both *p63*-deficient strains, we demonstrated that p63-deficient epithelia failed to develop beyond ectodermal stage as they remained a monolayer of non-proliferating cells expressing K8/K18. Importantly, in the absence of p63, corneal-epithelial commitment (which occurs at embryonic day 12.5 of mouse embryogenesis) was hampered 3 weeks before corneal stem cell renewal (that begins at P14). Taken together, these data illustrate the significant role of p63 in epithelial embryogenesis, before and independently of other functions of p63 in adult stem cells regulation. Transcriptome analysis of laser captured-embryonic tissues confirmed the latter hypothesis, demonstrating that a battery of epidermal genes that were activated in wild-type epidermis remained silent in p63-null tissues. Furthermore, we defined a subset of novel *bona fide* p63-induced genes orchestrating first epidermal stratification and a subset of p63-repressed mesodermal-specific genes. These data highlight the earliest recognized action of ΔNp63 in the induction epidermal morphogenesis at E11.5. In the absence of p63, a mesodermal program is activated while epidermal morphogenesis does not initiate.
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At embryonic day 12.5 of mouse embryogenesis, the ectoderm is committed to differentiate into various epithelial lineages. This step is hallmarked by the substitution of the ectodermal cytokeratins (K8/K18) by the epithelial cytokeratins (K5/K14). Consequently, stratification program begins and necessitates proliferation, stem cells regeneration, differentiation and apoptosis. This coordinated process gives rise to pluristratified epithelia within 3–4 days (at E16.5) (Figure 1a).[1–4]

p63 gene, which belongs to the p53 family, is a major epithelial transcription factor with pleiotropic functions including cell adhesion, cell proliferation, apoptosis and senescence.[1–4] The central role of p63 in epithelial development has been strikingly demonstrated by two p63 knockout mouse strains generated by two independent groups.[5,6] Although both mice strains were similarly born lacking limbs, epidermis and skin appendages, Yang *et al.*[6] have reported that *p63*$^{-/-}$ embryonic surface displayed rare clumps formed by superposed (packed) cells, positive for epidermal markers. This observation led the authors to conclude that p63 is necessary for epidermal stem cell renewal. Consistently, the expression levels of ΔNp63 in epidermal stem/progenitor cells correlate with their proliferating capacity.[7]

In contrast, Mills *et al.* showed no expression of epidermal differentiation markers during embryonic skin development,[5] suggesting that p63 is essential for epidermal differentiation/stratification. Accordingly, ectopic expression of TAp63 induced *in vivo* stratification of single-layered lung epithelia[8] and ΔNp63 induced epidermal commitment of embryonic stem cells.[9,10] This major discrepancy resulted in a strong controversy concerning the function of p63 during skin development.[11] It has been suggested that the difference of background strains, namely, 129sv/C57BL/6 (129sv/C)[5] *versus* C57BL/6-BALB/c (C/BALB/c[6]) could explain the apparent phenotype differences.

In this study, we demonstrated that embryonic epithelia of the two p63-knockout mice strains are not exhausted because of a non-regenerative epithelial differentiation, but failed to develop beyond the ectodermal stage. Comparative transcriptome profiling of *in vitro* and *in vivo* samples highlighted that ΔNp63 is an ectodermal-specific gatekeeper controlling positively epidermal specific genes while repressing mesodermal genes.

Results and Discussion

Corneal epithelial differentiation was hampered in p63-null mice, 3 weeks before corneal renewal. Like the epidermis, the corneal epithelium originates from the

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Abbreviations: K, cytokeratin; ES cells, embryonic stem cells; ES-EC, embryonic stem cell-derived ectodermal progenitors
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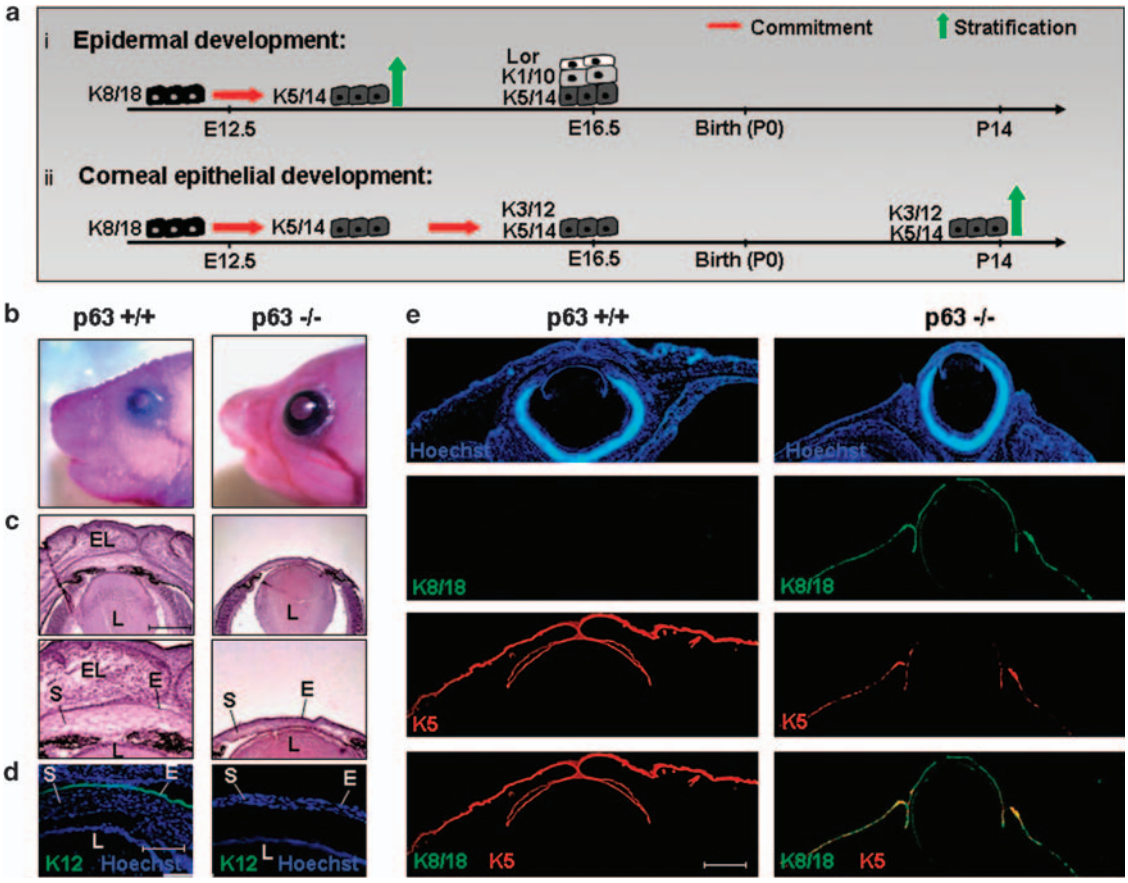

Figure 1 Analysis of corneal development of WT and p63$^{-/-}$ mice. (**a**) Schematic representation of epidermal (i) and corneal-epithelial (ii) development. The commitment of the ectoderm into epidermal (**a**(i)) and corneal (**a**(ii)) lineages occurs at embryonic day 12.5 of mouse embryogenesis and is hallmarked by the substitution of cytokeratins K8/K18 by K5/K14. Epidermal stratification directly follows and pluristratified epidermis is present within few days, hallmarked by typical markers, namely, K5/K14, K1/K10 and Loricrin (**a**(i)). However, the cornea remains static non-proliferative mono layer for 3 weeks until corneal stratification begins 2 weeks after birth (P14) on eyelids opening (**a**(ii)). The heads of WT (*p63*$^{+/+}$) and the *p63*-deficient (*p63*$^{-/-}$) mice (of C/BALB/c strain) were pictured (**b**) or processed for hematoxilin and eosin staining (**c**) or immunofluorescent staining for the indicated markers (**d** and **e**) at E18.5. Higher magnification of H&E staining is shown in the lower panels (**c**). Each panel in **e** contains three channels of the same field and a merge of the green and red costaining. Similar results were found in 129sv/C strain (Supplementary Figure S1). The dashed line indicates the dermal-epidermal junction. Scale bar for **c** and **e** is 500 μm, and for **d** is 250 μm. EL, eyelids; L, lens S, stroma; E, corneal epithelium

ectoderm through similar molecular steps as illustrated in Figure 1a. In contrast to all other epithelia, corneal commitment and corneal stratification are chronologically separated events as corneal stratification begins 2 weeks after birth (~P14).[12] During this static period (embryonic day 12.5 of mouse embryogenesis–P14), this epithelium remains mono/bi-layer without significant proliferation as corneal regeneration is not required yet (Figure 1a). Therefore, by studying the developing cornea at E14.5–18.5, we addressed the role of p63 in the commitment of the ectoderm into corneal epithelial lineage, independently of stratification/self-renewal events.

At E18.5, the corneal epithelium of *p63*-null mice (C/BALB/c strain) was continuous and appeared normal (Figure 1b and c), but failed to express corneal differentiation marker, K12 (Figure 1d), corneal progenitor markers, K5 (Figure 1e) and K14 (not shown), and remained positive for ectodermal markers, K8/K18 (Figure 1e), indicating that *p63*-null epithelia could not develop beyond ectodermal stage. This failure could not be attributed by a failure in corneal stratification or by loss of corneal stem cells renewal. Similar results were found in 129sv/C strain of *p63*-deficient mice (Supplementary Figure S1).

Initiation of eyelids formation appeared normal in *p63*-null mice as the structures of protruding ridges and eyelid grooves were similar to wild type (WT) at E14. However, *p63*-deficient eyelids did not fuse and remained open after birth (Figure 1b, c and e). Interestingly, mice lacking *FGFR2* which is a p63 target gene (Candi *et al*.[13] and Figure 7c) also display similar 'open eyelids at birth' phenotype.[14] This phenotype was found in 129sv/C strain of *p63*-deficient mice as well (Supplementary Figure S1).

p63-null ectoderm failed to initiate embryonic stratification. Mosaic images of mouse embryonic heads from the two independent *p63*-deficient mouse strains[5,6] are shown here to simultaneously visualize various epithelia (Figure 2). Other epidermal regions that were examined displayed similar results (not shown).

At E18.5, WT epidermis appeared pluristratified (Figure 3a) and showed strong expression of K5 (Figure 2a) and K14 (not shown). In line with previous reports,[6,7] a low and punctuated

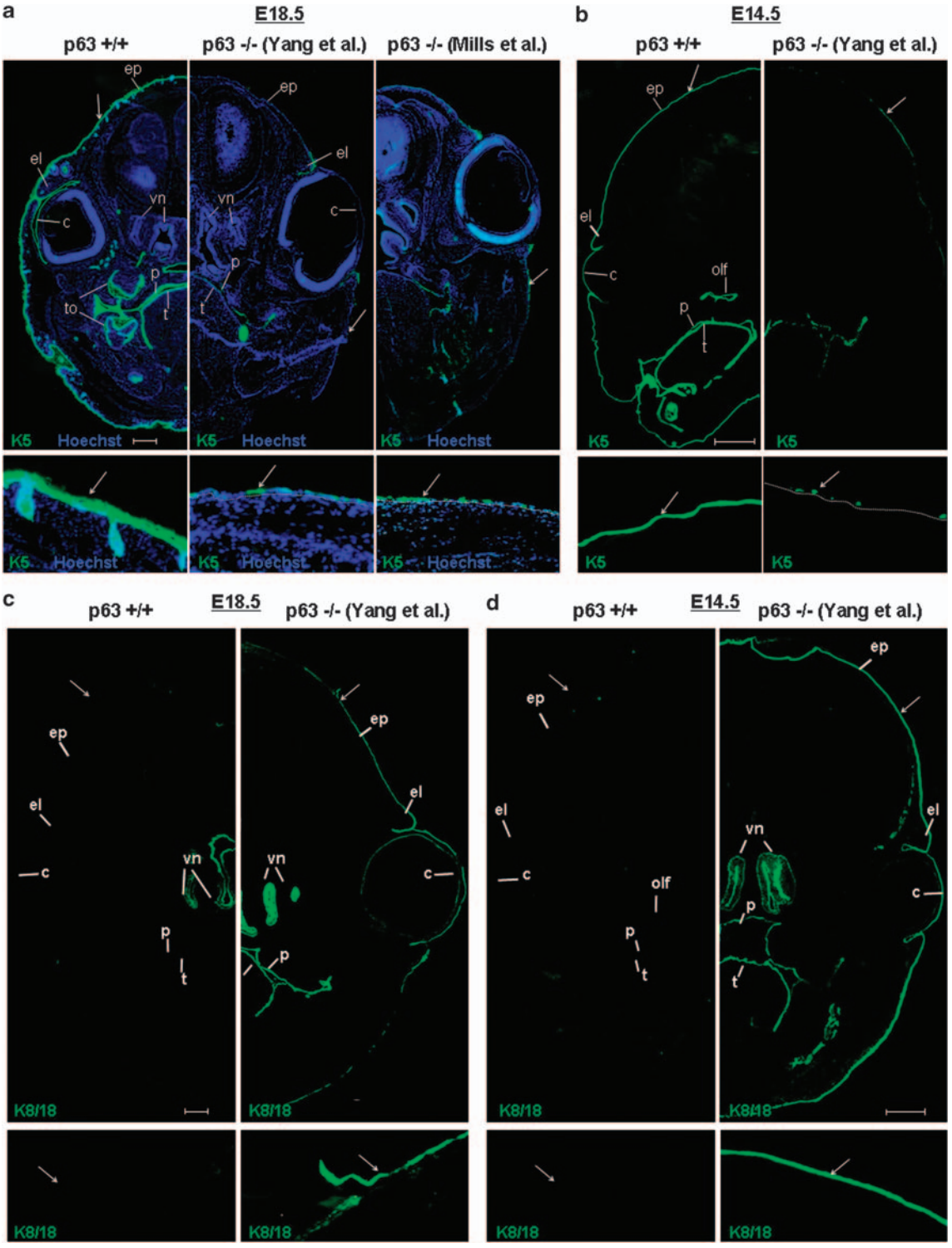

Figure 2 Mosaic images of K5 and K8/K18 immunostaining at E18.5 and E14.5. Transversal head sections of WT ($p63^{+/+}$) and the indicated *p63*-deficient ($p63^{-/-}$) mice strains were prepared for immunofluorescent staining at embryonic day 18.5 (E18.5) (**a** and **c**) or E14.5 (**b** and **d**) using anti K5 antibodies (**a** and **b**) or K8K18 antibodies (**c** and **d**). The specific *p63*-deficient strains are indicated in brackets. Lower panels are enlarged regions of the epidermis indicated by the arrows in the upper panels. ep, epidermis; t, tongue; p, palate; el, eyelids; c, cornea; olf, olfactory; vn, vumeronasal. Scale bars for **a** and **b** are 500 μm. Comparable observations were found in both $p63^{-/-}$ strains (129sv and C/BALB/c strains)

staining of K5 was found at E18.5 in the *p63*-null epidermis (C/BALB/c strain, Yang *et al*.) that appeared to be lost (Figure 2a). Surprisingly, however, we found similar staining in 129sv/C strain (Mills *et al*.) of *p63*-deficient mice (Figure 2a), in contrast to the original report.[5] As the levels of K5 signal were relatively low in p63−/− epidermal cells as compared

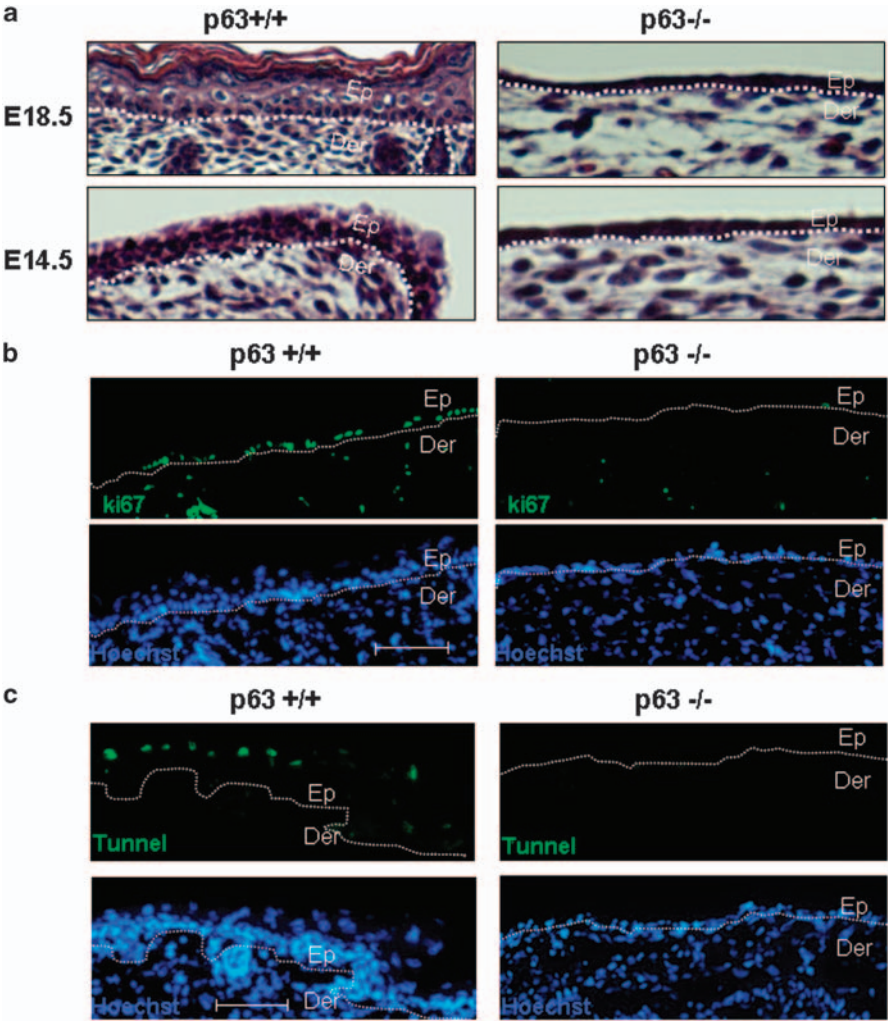

Figure 3 Examination of proliferation and apoptosis during first epidermal stratification. (**a**) Paraffin sections of the indicated embryonic skin (of C/BALB/c strain) that were prepared at E18.5 and E14.5 were used for hematoxilin and eosin staining. Tissue sections of embryonic skin (of C/BALB/c strain at E15.5) were subjected to immunostaining using ki67 antibody (**b**) or alternatively used for Tunel assay (**c**). The dashed line indicates the dermal-epidermal junction. Der, dermis; Ep, epidermis. Scale bars for **b** and **c** are 100 μm

with wild-type epidermal cells (Figure 2a), it is possible that they were not detected by Mills *et al*. because of albeit lower assay sensitivity. To clarify whether this feature illustrates the disappearance of embryonic epidermis by lack of its self-renewal ability in absence of *p63*, we systematically examined earlier stages (E11.5–E16.5) when epidermal morphogenesis starts. At E11.5, the ectoderm of both genotypes remained a monolayered tissue positive for K8/K18 (shown in Figure 7a) but not K5/K14 (not shown) while, at E14.5, WT epidermis was composed of 2–4 layers of cells (Figure 3a) positive for K5 (Figure 2b) and K14 (not shown). In *p63*-deficient mice, whatever the strain or stage, we have never detected any region of stratified epidermis but rather a continuum of a single layer (Figure 3a), rarely punctuated by K5 staining (Figure 2b).

Next, we stained several epithelia at E14.5–18.5 for ectodermal markers (Figure 2c and d). In contrast to WT epidermis that lost K8/K18 expression already at E14.5, *p63*-null epidermis abnormally maintained K8/K18 expression (Figure 2c and d). Similar results were obtained using additional K18-specific antibodies (not shown). The apparent tissue detachment (Figure 2c, lower panel) was probably due to the reduced expression of p63-dependent adhesion molecules in *p63*-deficient epithelia.[15]

Embryonic stratification begins at approximately embryonic day 12.5 of mouse embryogenesis and requires significant proliferation. This stage requires p63-dependent proliferation as only pluristratified WT epidermal cells expressed ki67, while $p63^{-/-}$ epidermis remained non-proliferative (Figure 3b). The loss of *p63*-null epidermis that initially developed but failed to be maintained, as suggested previously,[7] would have required massive apoptosis during first stratification cycle. As illustrated in Figure 3c, there was no significant apoptosis in *p63*-deficient 'epidermis' at E14.5–E16.5 whereas apoptotic cells were found mainly in the suprabasal layers of WT epidermis, as expected. Only in very late embryogenesis (E18.5–birth), apoptotic cells were found in p63-deficient epidermis[16] and, therefore, are not linked with the loss of epidermis at much earlier stages. Altogether, the first cycle of proliferation/stratification is definitively not occurring in *p63*-null mice, whatever the strain was (not shown).

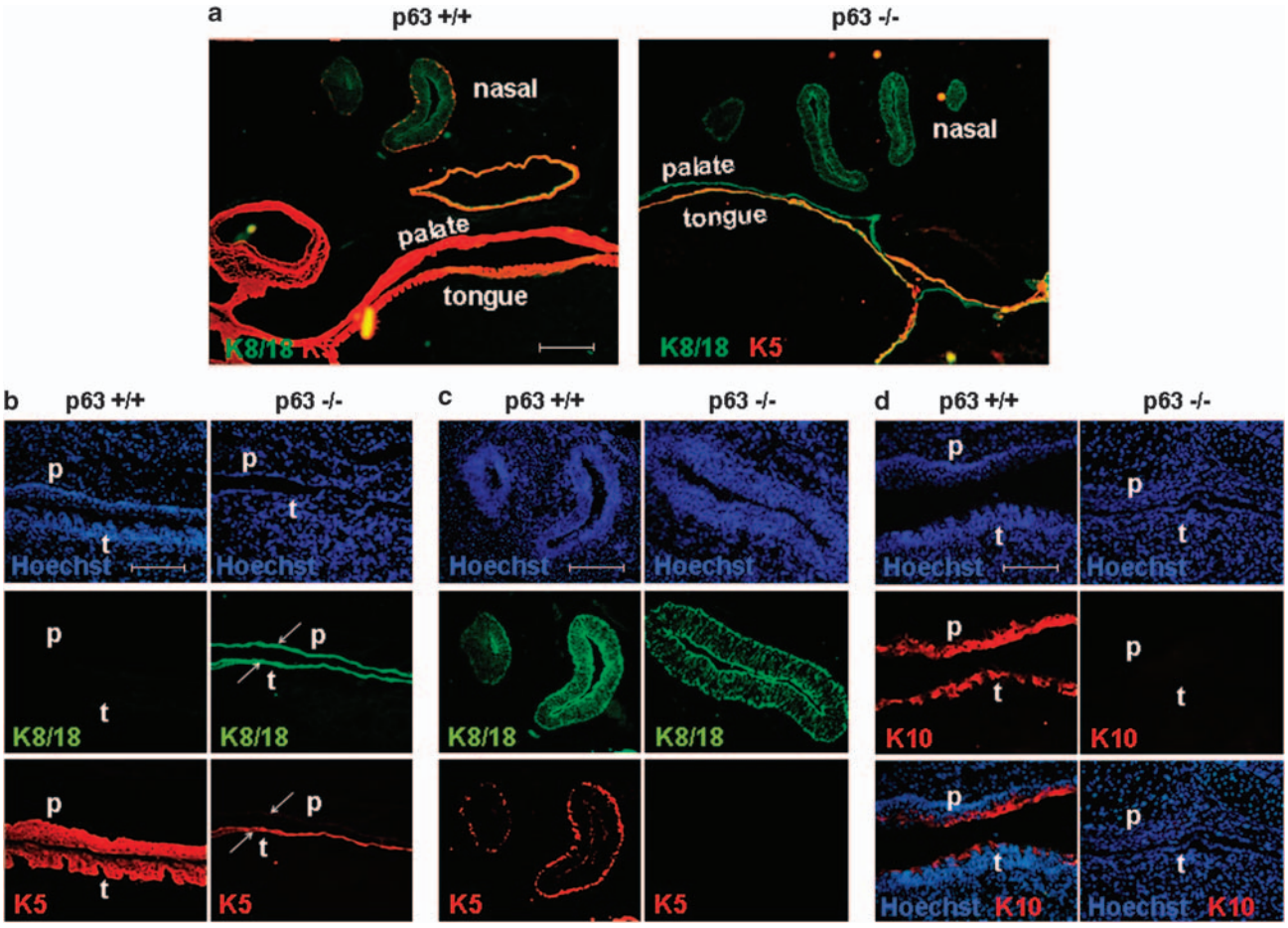

Figure 4 Immunostaining of oral epithelia. Oral tissues (E18.5, C/BALB/c mouse strain) were subjected to costaining of the indicated proteins. Merges shown in **a**, while **b** and **c** are higher magnification of selected regions shown in **a**. Comparable observations were found in 129sv strains (not shown). Scale bar for **a** is 300 and 100 μm for **b–d**. p, palate; t, tongue

Likewise, oral *p63*-null epithelial tissues of both strains of *p63*-deficient mice remained a continuous monolayer of cells, incapable of stratification (Figure 4). Wild-type epithelia of the tongue and palate, and vumeronasal organ expressed epithelial progenitors markers (K5 (Figure 4a–c), and K14 (not shown)) as well as differentiation markers (K10 (Figure 4d)). In contrast, although at a lower level, K5 (and K14, not shown) was uniformly coexpressed with K8/K18 in large regions of p63-deficient tongue (Figure 4a and b), but was absent in large regions of the palate (Figure 4a and b) and of the vumeronasal organ (Figure 4a and c). Nevertheless, in contrast to wild-type, *p63–/–* tongue and palate epithelia were a continuous monolayer of cells uniformly expressing K8/K18 (Figure 4a and b) and completely missing K10 (Figure 4d), integrin β4 and laminin-5 (not shown). This indicates that specific factors that are expressed in the oral epithelia were able to partially allow the expression of K5/K14 but not other markers of basal layer cells or differentiated cells in the absence of p63. Altogether, the abnormal coexpression of K8/K18, the absence of K10, integrin β4, laminin-5 and the lack in stratification indicate that these cells could not commit into oral epithelial lineages.

Computerized analysis of these results (shown in Figures 1, 2 and 4) showed that various types of $p63^{-/-}$-epithelial lineages (of the cornea, eyelid, tongue and palate) entirely maintained the expression of ectodermal phenotype at E18.5 (Figure 5a). Patches of $p63^{-/-}$ cells that expressed K5 (shown in Figure 2a and b) and K14 (not shown) were substantially rare at E14.5 or E18.5 (quantified in Figure 5b). Furthermore, early differentiation marker K10 or corneal differentiation marker K12 were infrequent or completely absent, respectively (Figure 5c). We thus concluded that *p63*-deficient epithelia fail to develop beyond the ectodermal stage.

Rare 'differentiated' p63-deficient cells abnormally coexpressed K8/K18 but not integrin β4 or laminin-5. We next questioned the epidermal nature of the rare $K5^{+}$ cells found infrequently in *p63*-null embryos (Figure 2). E18.5 embryos (of C/BALB/c strain) were costained with K5 and K8/K18 (Figure 6a), K10 and K8/K18 (Figure 6b) or K5 and integrin β4 antibodies (Figure 6c). Although few cells were found to express K5 or K10 in $p63^{-/-}$ embryonic tissues, they were never stratified but remained single-layer primitive epithelia, coexpressing K8/K18. Moreover, we never detected two canonical keratinocyte markers, namely integrin-β4 (Figure 6c and Supplementary Figure S2A) and laminin-5 (Supplementary Figure S2B), in such K5-positive *p63*-deficient cells. Similar results were found in 129sv/C strain of

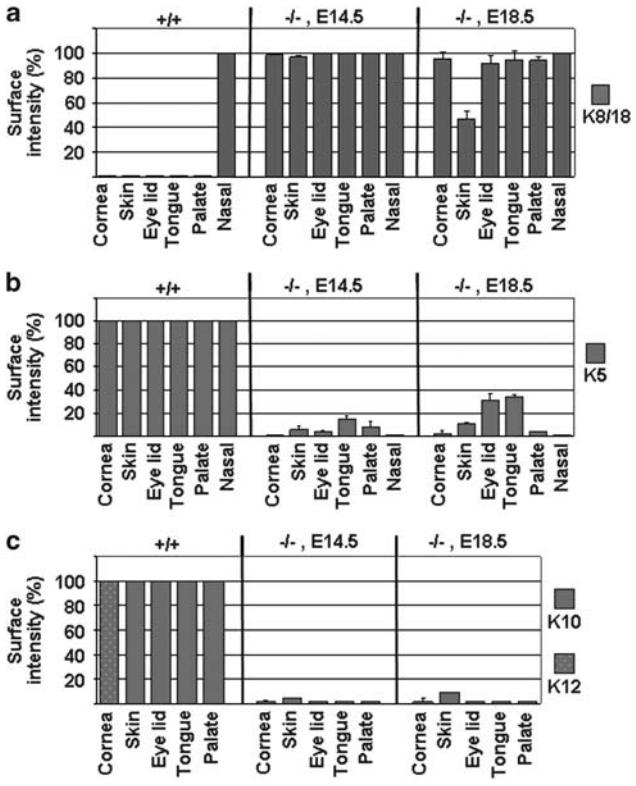

Figure 5 Quantification of the immunofluorescent staining. Computerized analysis of the immunostaining shown in Figures 1–3. The surface-intensity values of immunofluorescence staining were determined using computerized analysis as described in Materials and Methods. The relative expression was normalized as a percentage of values recorded for WT (**b** and **c**) or *p63*$^{-/-}$ (**a**)

p63-deficient mice (Supplementary Figure S3). Altogether, these *p63*-null 'differentiated' cells are not true keratinocytes but are intermediate hybrids blocked between ectodermal and epidermal phenotypes. These hybrid cells were still detected after birth (not shown).

In agreement, Khavari's group has demonstrated that absence of ΔNp63 in adult keratinocytes activated *de novo* K8/K18 expression and abolished stratification.[17] A recent study has generated a considerable degree of confusion in the field as the authors suggested that the Brdm2 (129sv/C strain) of *p63*-deficient mice are still producing a truncated p63 transcript while displaying, at E15.5, patchy regions consisting of pluristratified epidermis.[18] For the present study, we have used – side by side – the original Brdm2 mice distributed by JAX along with the C/BALB/c p63-deficient strain. As described above, our data ruled out the formation, even temporally, of any pluristratified epidermis. Therefore, the observations of Wolff *et al*. most probably represent a new strain that was driven by spontaneous genetic reversion from the original Brdm2 (129sv/C) strain. Indeed, as shown in Supplementary Figure S4, we confirmed that p63 transcript is not expressed in p63-deficient Brdm2 (129sv/C) strain.

A network of epidermal genes was activated in wild-type but remained silent in p63-null ectodermal tissue. To corroborate our observation that epidermal differentiation is abortive in *p63*-null mice and to dissect the molecular mechanism of p63 in early epidermal morphogenesis, we performed transcriptome analysis at early stages of first epidermal stratification. Epidermal tissues were isolated by laser capture microdissection at E11.5 (before epidermal commitment of the ectoderm (Figure 7a)) and at E14.5 (at early time of stratification (Figure 7b)). Careful attention has been made to limit the laser capture to the epithelial layers while avoiding contamination with dermal tissues. Accordingly, there was no detection of various dermal markers (e.g., nidogen, Dermo-1, Msx-1 and fibronectin) in our samples (not shown). The top-rank 51 genes that were upregulated in WT epidermis at E14.5 as compared with WT tissue at E11.5 are shown in Figure 7c. Interestingly, gene annotation (GO) analysis of these 51 upregulated genes using DAVID algorithm[19] showed that 'epithelial differentiation' and 'epithelial development' are on the top of the significant GO terms followed by many other development-related terms (Table 1). Among these upregulated genes, we found an array of 24 known epidermal genes (Figure 7c, annotated in red) involved in epidermal gap junctions, tight junctions or adhesion (GJB6, GJA1, COL18A1, COL17A1, LGALS7, LGALS3, EMP1, IQGAP and PKP4), known epidermal stem/progenitor markers (e.g., K14, K17, LMO4, K15 and FGF18), genes that are linked with epidermal stratification or differentiation (K10, KRTDAP, DMKN, S100A11, LOR and FGFR2) and genes whose mutations result in ectodermal dysplasia (GJB6) and epidermolysis bullosa (COL17A1) syndromes. The reliability of the transcriptome results was confirmed by qPCR analysis as shown in Figure 8a. In agreement with the array data (Figure 7), the expression levels of K14, K10 and Loricrin increased at E14.5 only in wild-type tissues while they remained low in p63-null samples (Figure 7a). In addition, overexpression of ΔNp63 in ES-derived ectodermal progenitor (ES-EC) cells induced K14 expression but did not affect K10 and Loricrin expression (Figure 8a). Furthermore, these data were confirmed at the protein level by immunofluorescent staining (Figure 8b). Again, the epidermis of wild-type mice was immunoreactive for K14, Loricrin and Galectin7 while the p63-null epidermis was not (Figure 8b), altogether confirming the array data.

Importantly, in contrast to the marked increase in the expression of various epidermal genes in WT epidermis at E14.5, the expression of these genes in *p63*-null 'epidermis' remained silent (Figure 7c). The average fold increase in the expression of those 24 epidermal genes at E14.5 was 2.7 for wild-type and 1.1 for *p63*-null tissue (Figure 7e), clearly confirming that *p63*-deficient tissues could not develop beyond the ectodermal stage.

Embryonic signature of early ΔNp63-induced epidermal commitment. ΔNp63 expression induces epidermal commitment of ES-EC.[9,10] We thus analyzed the global transcriptome profiling following ectopic expression of ΔNp63 in ES-EC cells (Figure 7c, right panel). As culture conditions did not allow stratification of ES-EC transfectants, the list of differentially regulated genes was considered as a defined subset of ΔNp63-responsive genes that are changed before and not as a consequence of stratification. Interestingly, 18 genes that were activated between E11.5 and E14.5 in

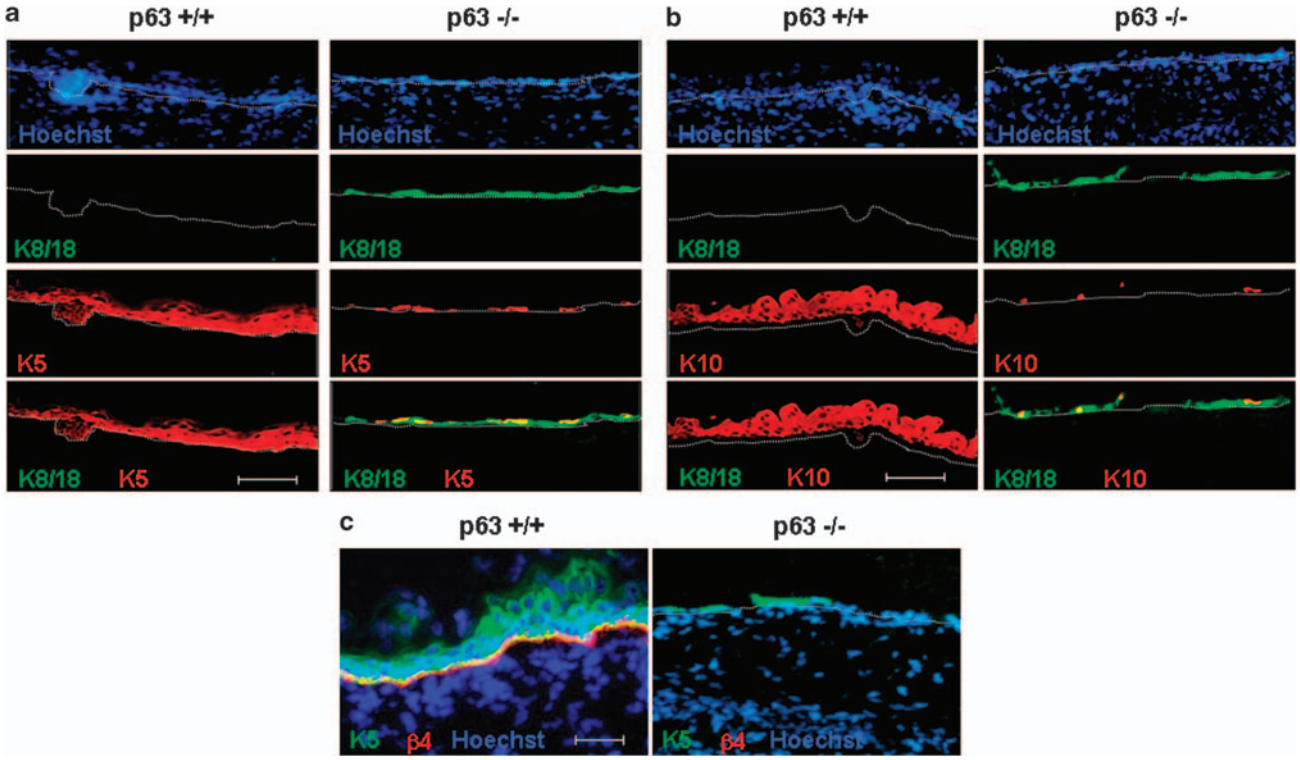

Figure 6 Analysis of epidermal markers in embryonic epidermis. The epidermis of the indicated *p63* genotypes (of C/BALB/c strain) at E18.5 was subjected to immunofluorescent costaining of the indicated proteins (**a**–**c**). Each panel contains three channels of the same field and a merge of the green and red costaining is shown (**a** and **b**). The dashed line indicates the dermal-epidermal junction. Scale bars for **a** and **b** are 100 and 50 μm for **c**. Comparable observations were found in 129sv strain

wild-type embryos *in vivo* were also activated during ES-derived epidermal commitment *in vitro* (see Figure 7c and f (in blue and green)). ΔNp63 transfection induced the expression of genes that are known to be expressed in basal layer of the epidermis (e.g., KRT14, KRT17, LMO4 and KRT15), and also some genes that are linked with keratinocyte differentiation (e.g., KRTDAP, S100A11 and FGFR2), indicating that p63 also induces early keratinocyte differentiation. These genes most probably represent a specific embryonic signature of early ΔNp63-induced epidermal commitment.

A list of new genes that may be involved in early epidermogenesis. In addition, 27 genes are described here for the first time as epidermal factors (see Figure 7c and f). Among them were factors that control adhesion and/or present in epithelial cell membranes (LYPD3, LY6G6C, integral membrane 2B, NME2 and RAB25), linked with different carcinomas (TRIM29, TACSTD2 and TPD52), mitochondrial factors (GLDC and UQCRC1) and 3 unknown 'RIKEN' sequences. Additionally, CETN3, an essential component of the centriole duplication process, was induced in WT and could be linked with proliferation during early stratification. Six of these new epidermal genes (integral membrane 2B, glycolipid transfer protein, TPD52, UQCRC1, LYPD and NPM1) that were enhanced at E14.5 and also induced by ΔNp63 (compare left and right panels of Figure 7c) are, therefore, more likely to be involved in epidermal development.

Identification of potential direct target genes of p63 during epidermal commitment. Next, we examined which of these differentially regulated genes could be under the direct control of p63. We recently identified genes that are directly bound by p63 in human primary keratinocytes (Kouwenhoven *et al*., in press) and, intriguingly, ~55% (28/51) of the genes that were upregulated at E14.5 contained p63-binding sites (Figure 7c, annotated in blue). Among those p63-bound genes, we found nine genes that were also induced by ΔNp63 transfection (see numbers in brackets in Figure 7f), strongly suggesting that these genes are *bona fide* p63-target genes, involved at this developmental step. Some of those are known epidermal genes that were not yet identified as p63-target genes (e.g., KRT17, KRT15, COL18A1 and PKP4) while the integral membrane 2B and the glycolipid transfer protein were not yet related to skin biology.

Absence of p63 resulted in the activation of mesodermal genes. A list of 24 genes showed significantly higher expression in p63-deficient tissues (Figure 7d). Intriguingly, the majority of the genes that were activated in *p63*-null ectoderm and not in the WT epidermis (annotated in green) were muscle-specific genes. Recently, De Rosa *et al*. have showed that non-epidermal genes are induced by loss of p63 in adult epidermal cells through Smad-7 pathway,[20] which is required to induce muscle fate.[21] In agreement, ΔNp63 has been shown to repress Xenopus mesoderm fate.[22] Interestingly, Barbieri *et al*.[23] have shown that the loss of

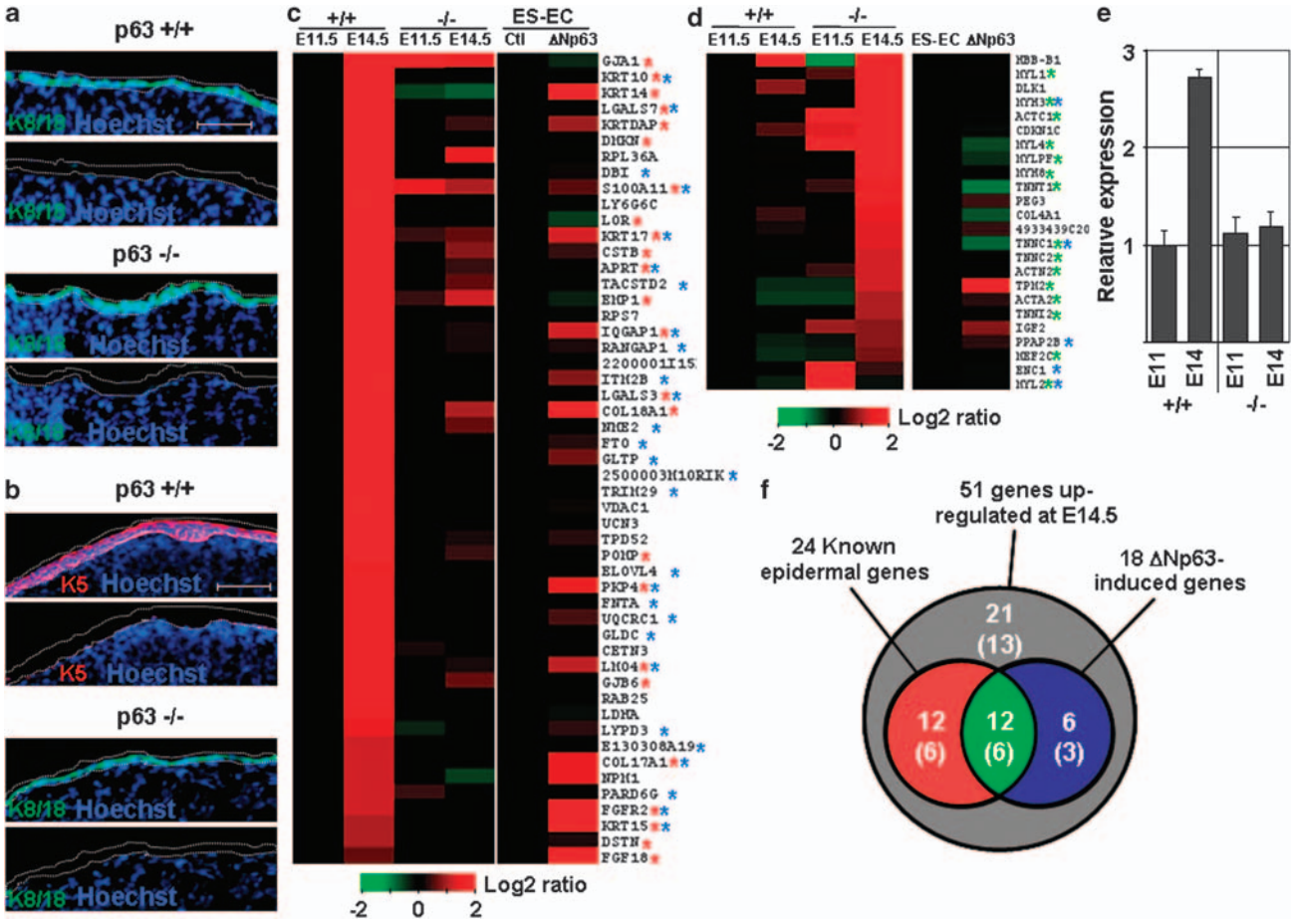

Figure 7 Transcriptome analyses of embryonic epidermal tissues and of ES-derived ectodermal-like cells transfected with ΔNp63. The indicated genotypes (of C/BALB/c strain) at E11.5 (**a**) and E14.5 (**b**) were immunostained as indicated before laser capture microdissection of the epidermis (lower panels are the same fields following epidermal laser capture). Tissues were used for transcriptome analysis (**c** and **d**, left panel). In addition, murine ES-derived ectodermal cells (ES-EC) transfected with empty vector (Ctl) or ΔNp63α (ΔNp63), were subjected to transcriptome analysis (**c** and **d**, right panel). The top-rank 51 genes with highest WT/KO ratio at E14.5 are presented by heat map presentations (**c**, left panel). The top-rank 24 genes with highest KO/WT expression ratio at E14.5 are presented by heat map presentations (**d**, left panels). The corresponding expression in the transcriptome of ES-EC transfectants is presented (**c** and **d**, right panels). The expression (Log2) levels are shown (see color code below). Known epidermal or mesodermal genes are annotated by red or green asterisks in **c** and **d**, respectively. Genes that were found to contain p63-binding site are annotated by blue asterisks (**c** and **d**). The average expression of the 24 epidermal genes is presented in **e**. (**f**) Venn diagram categorizing the 51 genes that were upregulated in WT at E14.5 (gray), among them known epidermal genes (red), ΔNp63-induced genes (blue) or both (green) (**e**). The number of genes that also contained p63-binding site in each group is indicated in brackets (**e**). Scale bars for **a** and **b** are 100 μm

Table 1 Top GO terms of differentially upregulated genes

GO term No.	GO terms	No. of genes	% Of analyzed genes	*P*-value*	Bonferroni
GO:0030855	Epithelial cell differentiation	6	11.8	261E−05	0.01695848
GO:0060429	Epithelial development	7	13.7	1.16E−04	0.73536236
GO:0009888	Tissue development	9	17.7	4.04E−04	0.23265627
GO:0051094	Positive regulation of developmental processes	5	9.8	0.003267	0.88311957
GO:0008284	Positive regulation of cell proliferation	5	9.8	0.008830	0.99702754

*P-value <0.01.

p63 leads to activation of several mesenchymal genes. These experiments are in line with our data, even though the effect of p63 siRNA was examined in human adult cancer cells while our studies included mouse embryonic untransformed tissues. The two lists of p63-repressed mesenchymal genes were distinct, which strongly suggests that the genes we have identified could represent an embryonic signature of p63-deficient ectodermal cells.

Taken together, our accumulative evidence clearly demonstrated that ΔNp63 is mandatory for the initiation of epidermal morphogenesis. In its absence, the ectoderm fails to engage a large battery of genes that are required for epidermal lineage commitment, proliferation and differentiation and commits into mesodermal fate. These observations indicate that ΔNp63 is a 'gatekeeper' of epidermal fate during embryogenesis.

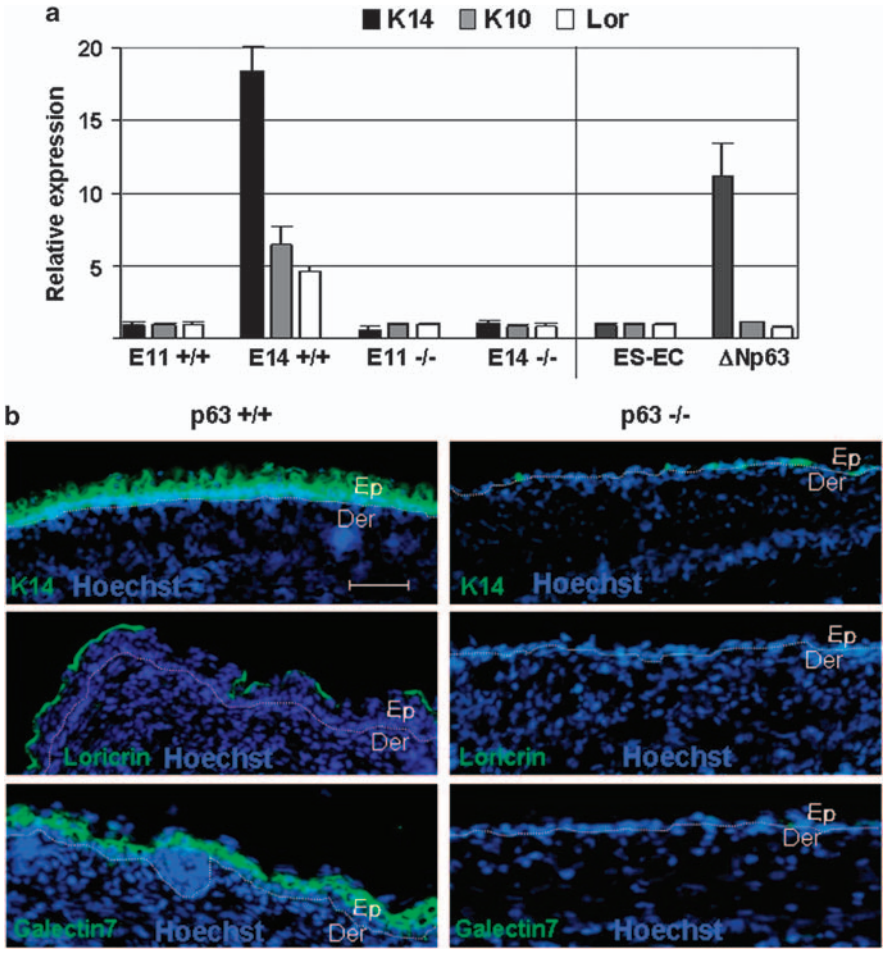

Figure 8 Validation of transcriptome analyses by qPCR and immunofluorescent staining. (**a**) Real-time PCR analysis was performed to amplify the indicated transcripts using the same RNA samples that were used for the micro array assay that is described in Figure 6. (**b**) Epidermal tissues of the indicated genotypes (of C/BALB/c strain) were immunostained for K14, Loricrin and Galectin7. The dashed line indicates the dermal-epidermal junction. Scale bar for **b** is 100 μm

Materials and Methods

Mice and animal care. p63-heterozygous ($p63^{+/-}$) mice were generated as described.[5,6] $p63^{+/-}$ 129sv/C57BL/6 (129sv/C)[5] was purchased from Jackson Laboratory (Bar Harbor, ME, USA) in C57BL/6 genetic background. Mice colony of p63-heterozygous mice C57BL/6-BALB/c (C/BALB/c)[6] that were kindly provided by Professor F McKeon was established by mating with pure C57Bl/6 mice that were purchased from Charles River Italy (Lecco, Italy). Each generation of mice was genotyped. Animals were maintained under standard conditions with a 12 h light/dark cycle, provided food and water *ad libitum*.

Tissue processing and tissue staining. To examine embryos at different developmental stages, embryos were collected at E11–16, E18.5 and at birth. Three embryos of each developmental stage of each *p63*-knockout strains (129sv/C and C/BALB/c) were used. Tissues were embedded in frozen specimen embedding media (Thermo Scientific, Rockford, IL, USA), and sections of 8–10 μm were fixed in acetone and immunostained. Antibodies were Rabbit anti-K5 (Covance, Paris, France), Rabbit anti-K14 (Covance), Pig anti-K8/K18 (Progen, Heidelberg, Germany), Mouse anti-K18 (Chemicon, Billerica, MA, USA), Rabbit anti K10 (Covance), Mouse anti-integrin-β4 (R&D systems, Minneapolis, MN, USA), Goat anti-K12 (Santa Cruz Biotechnologies, Paris, France) and Goat anti-ki67 (Santa Cruz Biotechnologies). Rabbit anti-Galectin7 and Loricrin were a kind gift from Dr. Thierry Magnaldo. Secondary antibodies were AlexaFluor 488, 593 nm (Invitrogen, Paris, France) and Fluorescein anti-Guinea Pig (Vector Laboratories, Burlingame, CA, USA).

Microscopy and quantification of staining. Fluorescent microscopy (Nikon Eclips Ti-RCP, Nikon Inc., Melville, NY, USA) was performed using imaging software 'NIS-Elements AR 3.1' at fixed exposure times, and for automated quantification of the surface intensity staining, five independent sections were used for each condition.

Laser capture microdissection and transcriptome analysis. Sections were sequentially incubated in acetone (1 min), primary (1/20) and secondary (1/40) antibodies, 70% ethanol (30 seconds), 95, 100% ethanol (repeated twice, 2 min) and 100% xylene (1 min), and subjected to

microdissector (Ziess P.A.L.M LCM, Paris, France). RNA was isolated (PicoPure RNA isolation kit (Arcturus, Paris, France)) followed by cRNA amplification (Total prep RNA amplification kit (Illumina; Applied Biosystems, Courtaboeuf, France)). Duplicate samples were concentrated and 3 μl containing 750 ng of RNA was hybridized onto Illumina chip (Mouse WG-6 v2 expression BeadChip kit, Illumina, San Diego, CA, USA). Agilent microarray of ES-EC transfectants was as described previously.[24]

Gene ontology annotation and correlation to potential direct target genes of p63. Gene ontology analysis was performed using DAVID Bioinformatics Resources 6.7 (http://www.david.abcc.ncifcrf.gov/).[19] Differentially regulated genes identified in mice were mapped for their human homologs using bioDBnet database (National Cancer Institute at Frederick) (http://biodbnet.abcc.ncifcrf.gov/dbInfo/). The human homologs of the differentially regulated genes were correlated to genes with p63-binding sites detected in human primary keratinocytes (Kouwenhoven *et al*., 2010) using Galaxy (http://main.g2.bx.psu.edu/).[25,26]

Conflict of interest

The authors declare no conflict of interest.

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