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Δ Np63 is an ectodermal gatekeeper of epidermal morphogenesis

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Abstract

p63, a member of p53 family, plays 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. Here 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 E12.5) was hampered three weeks prior to corneal stem cell renewal (that begins at P14). Taken together, these data illustrate the significant role of *p63* in epithelial embryogenesis, prior to 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 Δ Np63 in the induction epidermal morphogenesis at E11.5. In the absence of p63, a mesodermal program is activated while epidermal morphogenesis does not initiate.

Introduction

At embryonic day 12.5 of mouse embryogenesis (E12.5), 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) (Fig. 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 senescence¹⁻⁴. 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}. While both mice strains were similarly born lacking limbs, epidermis and skin appendages, Yang et al. have reported that *p63*^{-/-} embryonic surface displayed rare clumps formed by superposed (packed) cells, positive for epidermal markers⁶. This observation led the authors to conclude that p63 is necessary for epidermal stem cell renewal. Consistently, the expression levels of Δ Np63 in epidermal stem/progenitor cells correlate with their proliferating capacity⁷.

In contrast, Mills et al showed no expression of epidermal differentiation markers during embryonic skin development⁵, suggesting that p63 is essential for epidermal

differentiation/stratification. Accordingly, ectopic expression of TAp63 induced *in vivo* stratification of single-layered lung epithelia⁸ and Δ Np63 induced epidermal commitment of embryonic stem (ES) cells^{9,10}. This major discrepancy resulted in a strong controversy concerning the function of p63 during skin development¹¹. 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.

Here we demonstrated that embryonic epithelia of the two p63-knockout mice strains are not exhausted due to 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 Δ Np63 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, three weeks prior to corneal renewal

Like the epidermis, the corneal epithelium originates from the 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 two weeks after birth (\sim P14)¹². During this static period (E12.5-P14), this epithelium remains mono/bi-layer without significant proliferation since corneal regeneration is not required yet (Fig. 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 (Fig. 1B, C), but failed to express corneal differentiation marker, K12 (Fig.

1D), corneal progenitor markers, K5 (Fig. 1E) and K14 (not shown), and remained positive for ectodermal markers, K8/K18 (Fig. 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 (Fig. S1).

Initiation of eyelids formation appeared normal in *p63*-null mice as the structures of protruding ridges and eye lids grooves were similar to wild type (WT) at E14. However, *p63*-deficient eyelids did not fuse and remained open after birth (Figure 1B, C, E). Interestingly, mice lacking *FGFR2* which is a *p63* target gene (Candi et al¹³ and Fig. 7C) also display similar ‘open eyelids at birth’ phenotype¹⁴. This phenotype was found in 129sv/C strain of *p63*-deficient mice as well (Fig. 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 (Fig. 2). Other epidermal regions that were examined displayed similar results (not shown).

At E18.5, WT epidermis appeared pluristratified (Fig. 3A) and showed strong expression of K5 (Fig. 2A) and K14 (not shown). In line with previous reports^{6,7}, a low and punctuated 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 (Fig. 2A). Surprisingly, however, we found similar staining in 129sv/C strain (Mills et al) of *p63*-deficient mice (Fig. 2A), in contrast to the original report⁵. Since the levels of K5 signal were relatively low in *p63*^{-/-} epidermal cells as compared to wild type epidermal cells (Fig. 2A), it is possible that they were not detected by Mills et al. due to 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 Fig. 7A) but not K5/K14 (not shown) while, at E14.5, WT epidermis was composed of 2-4 layers of cells (Fig. 3A) positive for K5 (Fig. 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 (Fig. 3A), rarely punctuated by K5 staining (Fig. 2B).

Next, we stained several epithelia at E14.5-18.5 for ectodermal markers (Fig. 2C, D). In contrast to WT epidermis that lost K8/K18 expression already at E14.5, *p63*-null epidermis abnormally maintained K8/K18 expression (Fig. 2C-D). Similar results were obtained using additional K18 specific antibodies (not shown). The apparent tissue detachment (Fig. 2C, lower panel) was probably due to the reduced expression of p63-dependent adhesion molecules in *p63*-deficient epithelia¹⁵.

Embryonic stratification begins at ~E12.5 and requires significant proliferation. This stage requires p63-dependent proliferation as only pluristratified WT epidermal cells expressed ki67, while *p63*^{-/-} epidermis remained non proliferative (Fig. 3B). **The loss of *p63*-null epidermis that initially developed but failed to be maintained, as suggested previously⁷, would have required massive apoptosis during first stratification cycle. As illustrated in Fig. 3C, there was no significant apoptosis in *p63*-deficient 'epidermis' at E14.5-E16.5 while 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 epidermis¹⁶ 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).

Likewise, oral *p63*-null epithelial tissues of both strains of *p63*-deficient mice remained a continuous monolayer of cells, incapable of stratification (Fig. 4). Wild type epithelia of the tongue and palate and vomeronasal organ expressed epithelial progenitors markers (K5 (Fig. 4A-C), and K14 (not shown)) as well as differentiation markers (K10 (Fig 4D)). In contrast, although at a lower level, K5 (and K14, not shown) was uniformly co-expressed with K8/K18 in large regions of *p63*-deficient tongue (Fig. 4A, B), but was absent in large regions of the palate (Fig. 4A, B), and of the vomeronasal organ (Fig. 4A, C). Nevertheless, in contrast to wild type, *p63*^{-/-} tongue and palate epithelia were a continuous monolayer of cells uniformly expressing K8/K18 (Fig. 4A, B) and completely missing K10 (Fig. 4D), Integrin β 4 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 co-expression of K8/K18, the absence of K10, Integrin β 4, 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 Fig 1-2 and Fig 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 (Fig. 5A). Patches of *p63*^{-/-} cells that expressed K5 (shown in Fig. 2A, B) and K14 (not shown) were substantially rare at E14.5 or E18.5 (quantified in Fig. 5B). Furthermore, early differentiation marker K10 or corneal differentiation marker K12 were infrequent or completely absent, respectively (Fig. 5C). We thus concluded that *p63*-deficient epithelia fail to develop beyond the ectodermal stage.

Rare 'differentiated' p63-deficient cells abnormally co-expressed K8/K18 but not integrin β 4 or laminin-5

We next questioned the epidermal nature of the rare K5⁺ cells found infrequently in *p63*-

null embryos (Fig. 2). E18.5 embryos (of C/BALB/c strain) were co-stained with K5 and K8/K18 (Fig. 6A), K10 and K8/K18 (Figure 6B) or K5 and integrin β 4 antibodies (Fig. 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, co-expressing K8/K18. Moreover, we never detected two canonical keratinocyte markers, namely integrin β 4 (Fig. 6C and Fig S2A) and laminin-5 (Fig S2B), in such K5-positive $p63$ -deficient cells. Similar results were found in 129sv/C strain of $p63$ -deficient mice (Fig. 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 $\Delta Np63$ in adult keratinocytes activated *de novo* K8/K18 expression and abolished stratification¹⁷. 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 epidermis¹⁸. 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 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 (prior to epidermal commitment of the ectoderm (Fig. 7A)), and at E14.5 (at early time of stratification (Fig. 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, fibronectin) in our samples (not shown). The top rank 51 genes that were up-regulated in WT epidermis at E14.5 as compared to WT tissue at E11.5 is shown in Fig. 7C. Interestingly, gene annotation (GO) analysis of these 51 up-regulated genes using DAVID algorithm¹⁹ 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 up-regulated genes, we found an array of 24 known epidermal genes (Fig. 7C, annotated in red) involved in epidermal gap junctions, tight junctions or adhesion (GJB6, GJA1, COL18A1, COL17A1, LGALS7, LGALS3, EMP1, IQGAP, PKP4), known epidermal stem/progenitor markers (e.g. K14, K17, LMO4, K15, FGF18), genes that are linked with epidermal stratification or differentiation (K10, KRTDAP, DMKN, S100A11, LOR, 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 shown in Figure 8A. In agreement with the array data (Fig. 7), the expression levels of K14, K10 and Loricrin increased at E14.5 only in wild type tissues while remained low in *p63*-null samples (Fig. 7A). In addition, over expression of $\Delta Np63$ in ES-EC cells induced K14 expression but did not affect K10 and Loricrin expression (Fig. 8A). Furthermore, these data were confirmed at the protein level by immunofluorescent staining (Fig. 8B). Again, the epidermis of wild type mice was

immunoreactive for K14, Loricrin and Galectin7 while the p63-null epidermis was not (Fig. 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 (Fig. 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 (Fig. 7E), clearly confirming that p63-deficient tissues could not develop beyond the ectodermal stage.

Embryonic signature of early Δ Np63-induced epidermal commitment

Δ Np63 expression induces epidermal commitment of ES-derived ectodermal progenitors (ES-EC)^{9,10}. We thus analyzed the global transcriptome profiling following ectopic expression of Δ Np63 in ES-EC cells (Fig. 7C, right panel). Since culture conditions did not allow stratification of ES-EC transfectants, the list of differentially regulated genes was considered as a defined subset of Δ Np63-responsive genes that are changed prior to and not as a consequence of stratification. Interestingly, 18 genes that were activated between E11.5 and E14.5 in wild type embryos *in vivo* were also activated during ES-derived epidermal commitment *in vitro* (see Fig. 7C and 7F (in blue and green)). Δ Np63 transfection induced the expression of genes that are known to be expressed in basal layer of the epidermis (e.g. KRT14, KRT17, LMO4, KRT15), and also some genes that are linked with keratinocyte differentiation (e.g; KRTDAP, S100A11, FGFR2), indicating that p63 also induces early keratinocyte differentiation. These genes most probably represent a specific embryonic signature of early Δ Np63-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 Fig. 7C and 7F). Among them, were factors that control adhesion and/or present in epithelial cell membranes (LYPD3, LY6G6C, ITM2B, NME2, RAB25), linked with different carcinomas (TRIM29, TACSTD2, TPD52), mitochondrial factors (GLDC, 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 (ITM2B, GLTP, TPD52, UQCRC1, LYPD, NPM1) that were enhanced at E14.5 and also induced by Δ Np63 (compare left and right panels of Fig. 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, approximately 55% (28/51) of the genes that were up-regulated at E14.5 contained p63-binding sites (Fig. 7C, annotated in blue). Among those p63-bound genes, we found 9 genes that were also induced by Δ Np63 transfection (see numbers in brackets in Fig. 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, PKP4) while the integral membrane 2B (ITM2B) and the glycolipid transfer protein (GLTP) 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 (Fig. 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 pathway²⁰ which is required to induce muscle fate²¹. In agreement, Δ Np63 has been shown to repress *Xenopus* mesoderm fate²². Interestingly, Barbieri et al. have shown that the loss of p63 leads to activation of several mesenchymal genes²³. These experiments are in line with our data, even though the effect of p63-siRNA was examined in human adult cancer cells while we studied 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 evidences clearly demonstrated that Δ Np63 is mandatory for the initiation of epidermal morphogenesis. In its absence, the ectoderm fail 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 Δ Np63 is a ‘gatekeeper’ of epidermal fate during embryogenesis.

Experimental procedures

Mice and animal care

p63 heterozygous ($p63^{+/-}$) mice were generated as described^{5,6}. $p63^{+/-}$ 129sv/C57BL/6 (129sv/C)⁵ were purchased from Jackson Laboratory (Bar Harbor, 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 Prof. 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 12h 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), and sections of 8-10µm were fixed in acetone and immunostained. Antibodies were Rabbit anti K5 (Covance), Rabbit anti K14 (Covance), Pig anti K8/K18 (Progen), Mouse anti K18 (Chemicon), Rabbit anti K10 (Covance), Mouse anti integrin β4 (R&D systems), Goat anti K12 (Santa Cruz Biotechnologies), Goat anti ki67 (Santa Cruz Biotechnologies). Rabbit anti Galectin7 and Loricrin were a kind gift from Dr. Thierry Magnaldo. Secondary antibodies were AlexaFluor 488nm, 593nm (Invitrogen) and Fluorescein anti Guinea Pig (Vector Laboratories).

Microscopy and quantification of staining

Fluorescent microscopy (Nikon Eclips Ti-RCP) was using imaging software ‘NIS-Elements AR 3.1’ at fixed exposure times, and for automated quantification of the surface intensity staining, 5 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), 100% xylene (1 min), and subjected to microdissector (Ziess P.A.L.M LCM). RNA was isolated (PicoPure RNA isolation kit (Arcturus)) followed by cRNA amplification (Total prep RNA amplification kit (Illumina)). Duplicate samples were concentrated and 3µl containing 750ng of RNA was hybridized onto Illumina chip (Mouse WG-6 v2 expression BeadChip kit, Illumina). Agilent microarray of ES-derived ectodermal (ES-EC) transfectants was as described previously²⁴.

Gene ontology annotation and correlation to potential direct target genes of *p63*

Gene Ontology analysis was performed using DAVID Bioinformatics Resources 6.7 (<http://david.abcc.ncifcrf.gov/>)¹⁹. Differentially regulated genes identified in mice were mapped for their human homologues using bioDBnet database (National Cancer Institute at Frederick) (<http://biodbnet.abcc.ncifcrf.gov/dbInfo/>). The human homologues 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}.

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Figure legends

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 E12.5 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 three weeks until corneal stratification begins two weeks after birth (P14) upon 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 hematoxylin and eosin (H&E) staining (C) or immunofluorescent staining for the indicated markers (D-E) at E18.5. Higher magnification of H&E staining is shown in the lower panels (C). Each panel in E contains 3 channels of the same field and a merge of the green and red co-staining. Similar results were found in 129sv/C strain (Fig.S1). The dashed line indicates the dermal-epidermal junction. Scale bar for C and E is 500 μ m, and for D is 250 μ m. Abbreviations: EL, eyelids; S, stroma; E, corneal epithelium; L, lens.

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, C) or E14.5 (B, D) using anti K5 antibodies (A-B) or K8K18 antibodies (C-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. Abbreviations: epidermis (ep), tongue (t), palate (p), eyelids (el) and cornea (c) olfactory (olf) and vomeronasal (vn). Scale bars for A and B are 500 μ m. Comparable observations were found in both $p63^{-/-}$ strains (129sv and C/BALB/c strains).

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 Hematoxylin 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. Abbreviation: Der, dermis; Ep, epidermis. Scale bars for B and C are 100 μ m.

Figure 4.

Immunostaining of oral epithelia.

Oral tissues (E18.5, C/BALB/c mouse strain) were subjected to co-staining 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 μ m and 100 μ m for B-D. Abbreviations: tongue (t), palate (p).

Figure 5.

Quantification of the immunofluorescent staining

Computerized analysis of the immunostaining shown in Figs 1-3. The surface intensity values of immunofluorescence staining were determined using computerized analysis as described in Methods. The relative expression was normalized as a percentage of values recorded for WT (B, C) or $p63^{-/-}$ (A).

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 co-staining of the indicated proteins (A-C). Each panel contains 3 channels of the same field and a merge of the green and red co-staining is shown (A-B). The dashed line indicates the dermal-epidermal junction. Scale bars for A and B are 100 μ m and 50 μ m for C. Comparable observations were found in 129sv strain.

Figure 7.***Transcriptome analyses embryonic epidermal tissues and of ES-derived ectodermal-like cells transfected with $\Delta Np63$***

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-D, left panel). In addition, murine ES-derived ectodermal cells (ES-EC) transfected with empty vector (Ctl) or $\Delta Np63\alpha$ ($\Delta Np63$), were subjected to transcriptome analysis (C-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-D). The average expression of the 24 epidermal genes is presented in E. (F) Venn diagram categorizing the 51 genes that were up-regulated in WT at E14.5 (gray), among them known epidermal genes (red), $\Delta Np63$ -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 μ m.

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 Fig. 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 μ m.

Table 1.

Top GO terms of differentially up-regulated genes

* *P* value < 0.01

Figure S1.

The heads of *p63*-deficient mice (of 129sv/C strain, at E18.5) were used for immunostaining using the indicated antibodies. White arrows indicate for the corneal epithelium. Arrow indicates the corneal epithelium. Abbreviation: el, eyelid.

Figure S2.

Epidermal tissues of the indicated genotypes (of C/BALB/c strain at E15.5) were used and used for immunostaining of integrin β 4 (A) or laminin 5 (B). The dashed line indicates for dermal-epidermal junction. Abbreviation: Der, dermis; Ep, epidermis.

Figure S3.

Epidermal tissues of *p63*-deficient mice (of 129sv/C strain) were obtained at E16.5 and used for co-immunostaining using the indicated antibodies. The dashed line indicates for dermal-epidermal junction. Abbreviation: Der, dermis; Ep, epidermis.

Figure S4.

A. RNA extractions of wild type and p63-deficient (129sv/C (Brdm2) strain) mice skin was used as a template for reverse transcription polymerase chain reaction (RT-PCR) using the appropriate primers¹⁸ as illustrated in B, or primers of house keeping gene (36B4) as control .

B. Schematic representation (adapted from Wolff et al¹⁸) of p63 gene in wild type (WT) and mutated (Brdm2 (129sv/C)) mice. The corresponding fragments that were amplified in A are illustrated below.

References

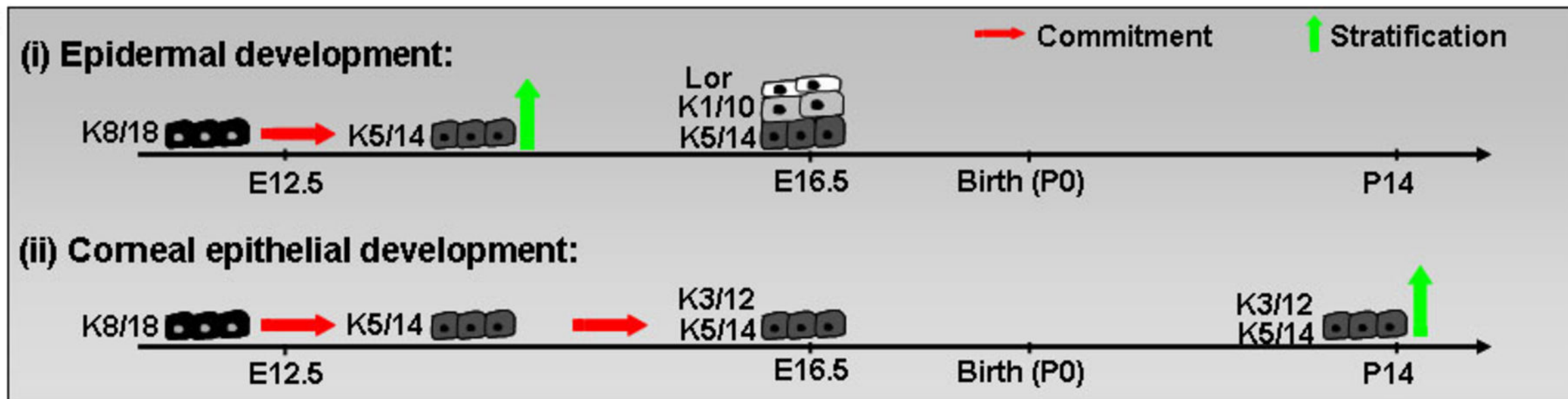
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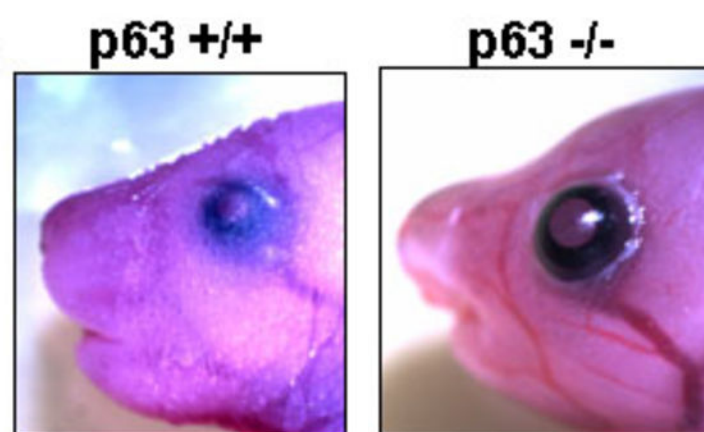
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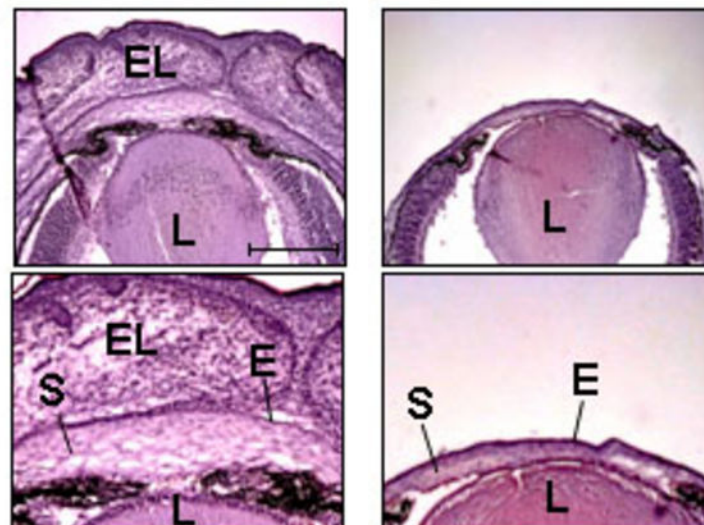
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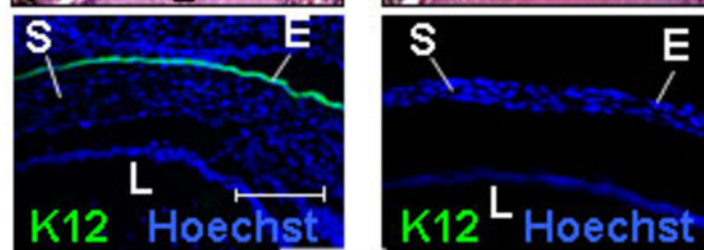
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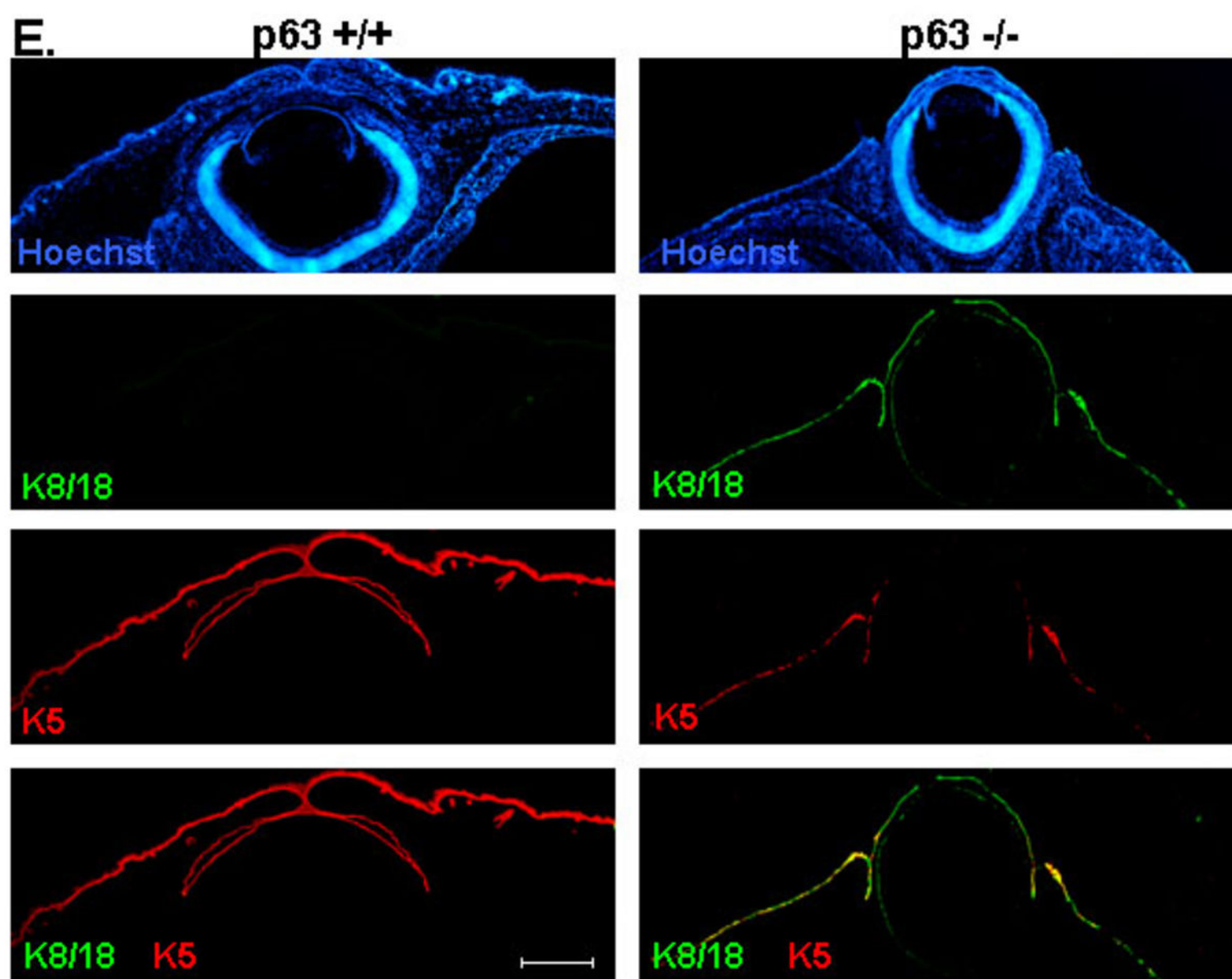
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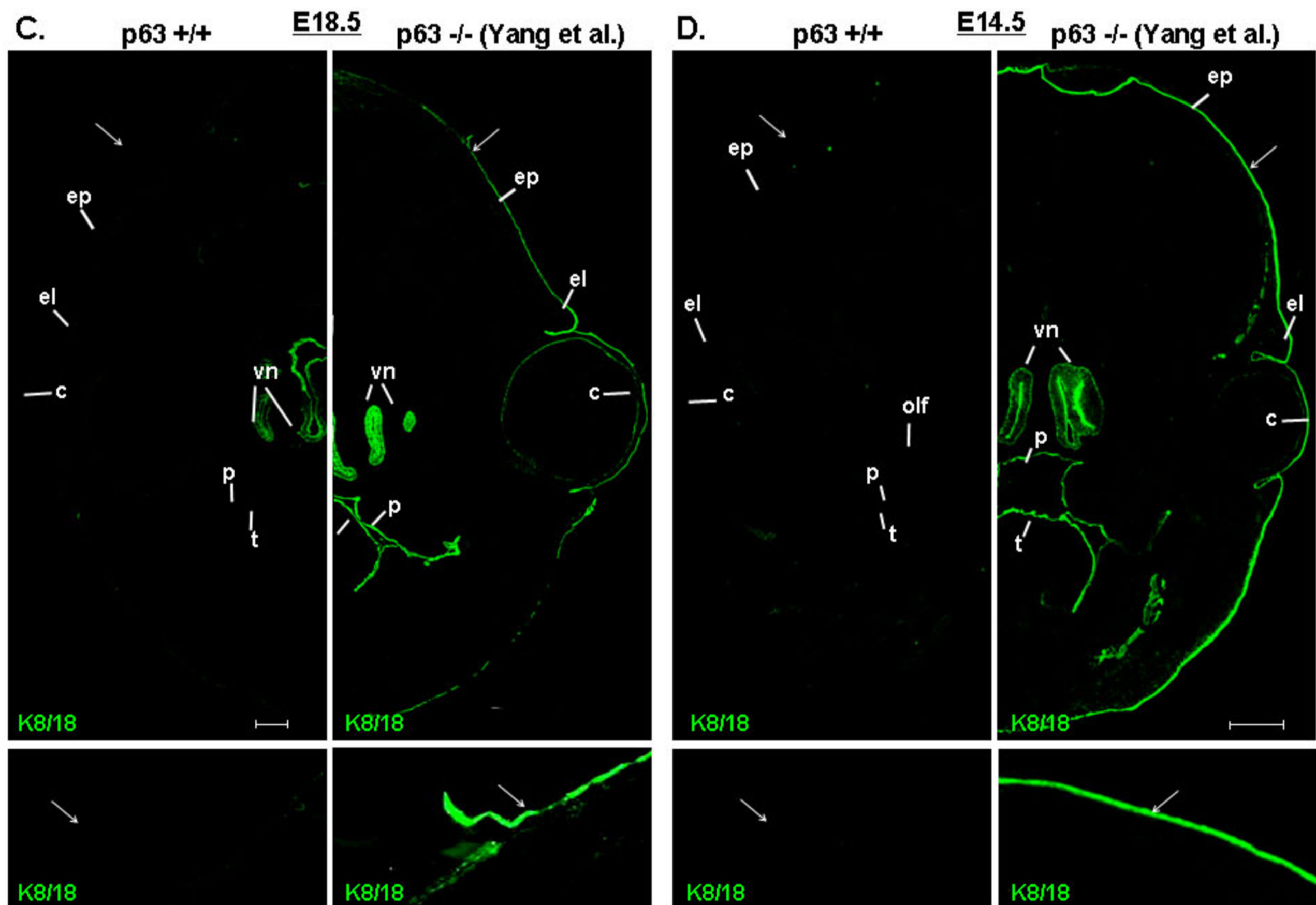
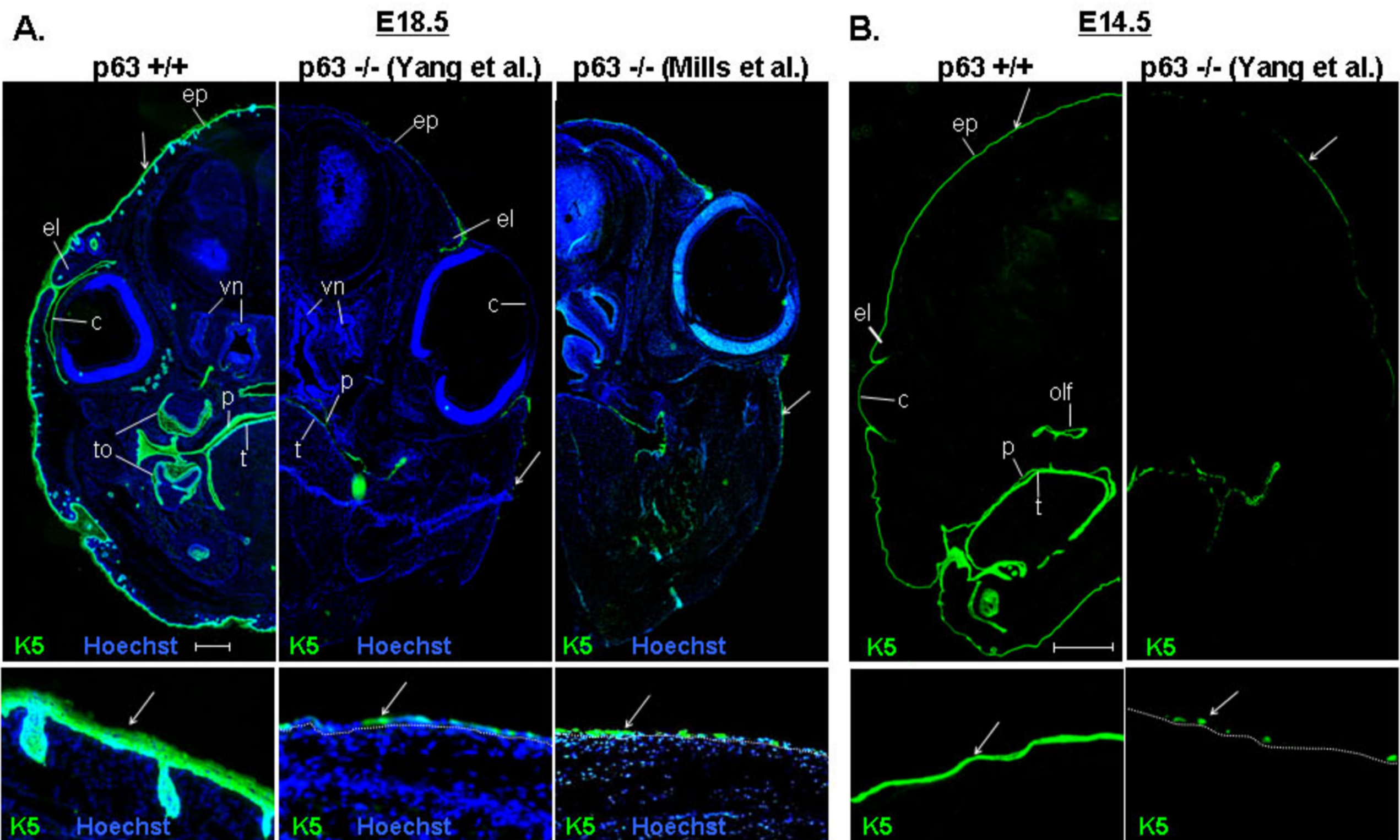


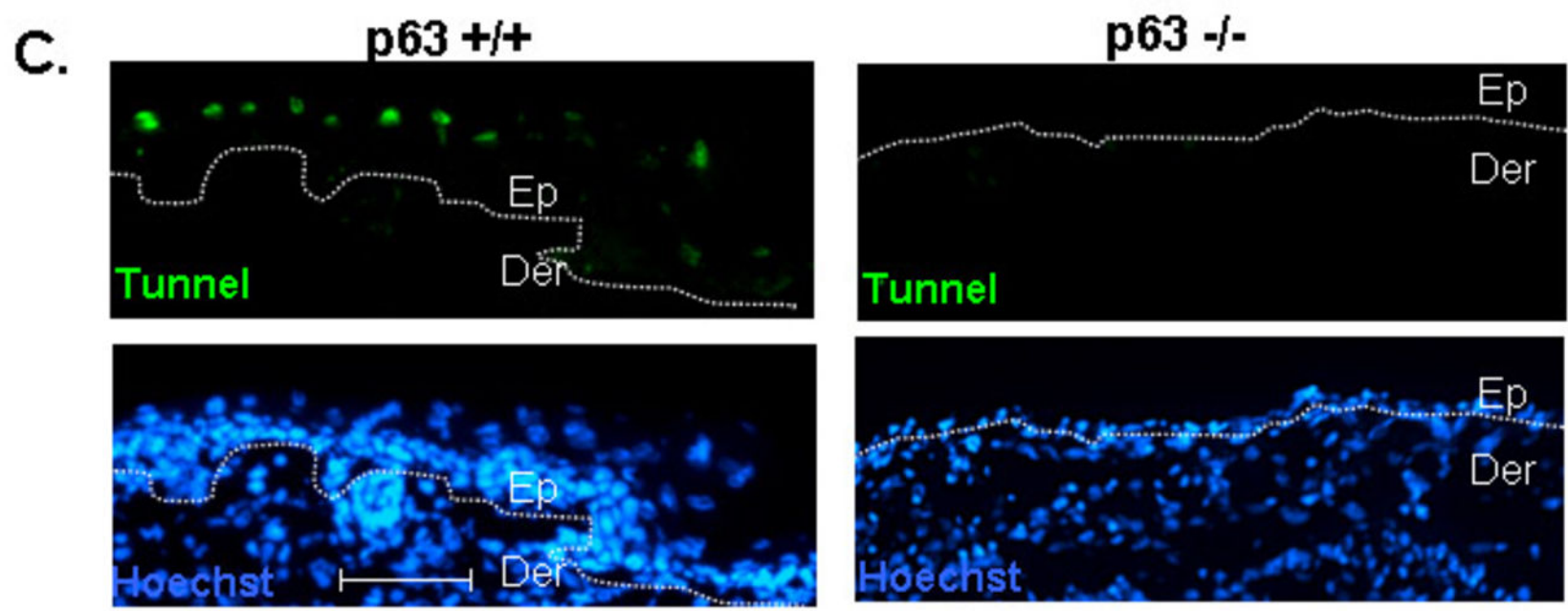
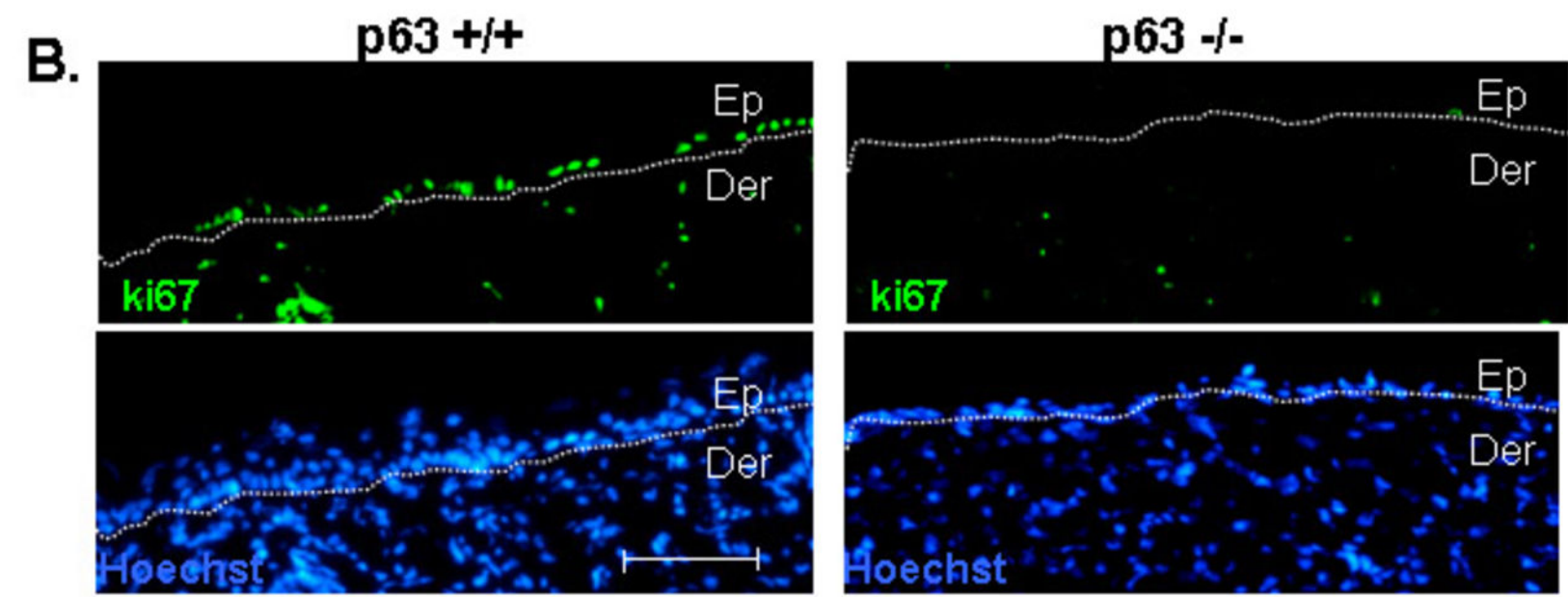
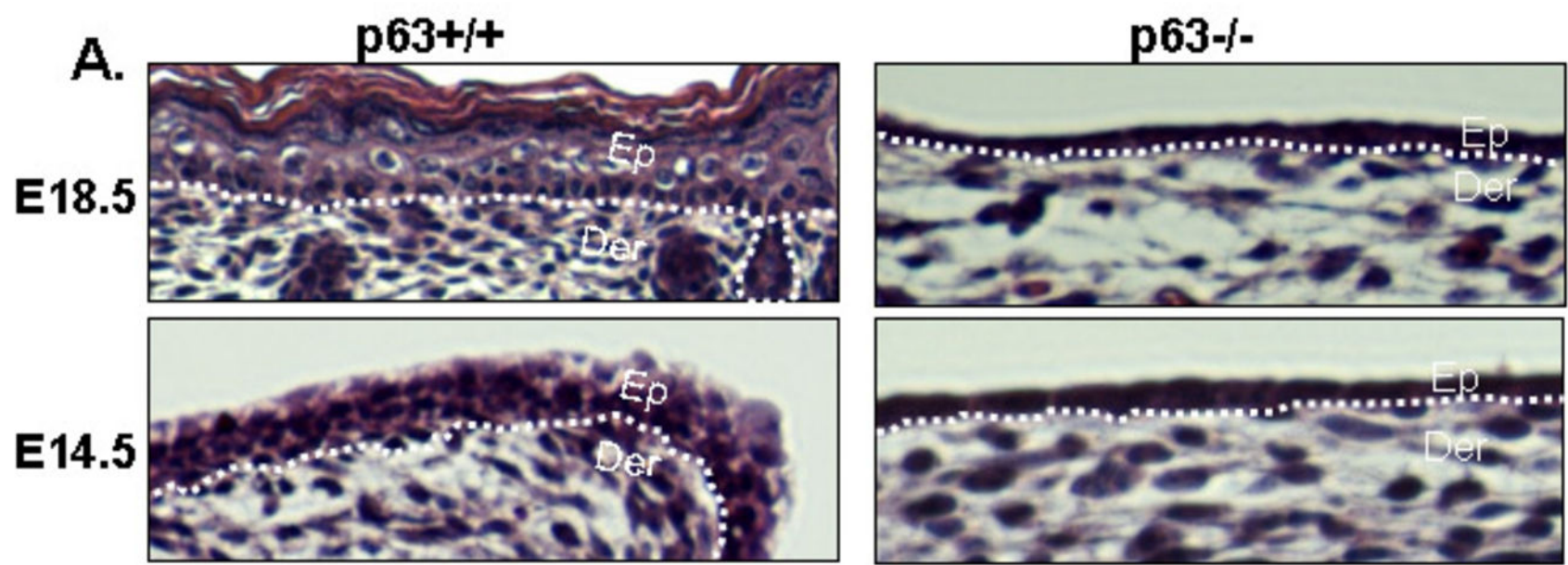
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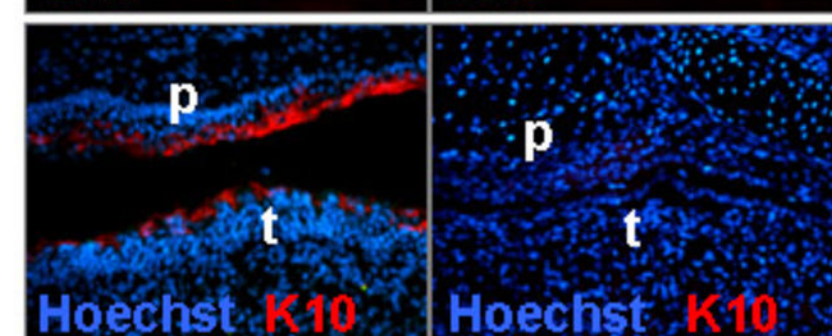
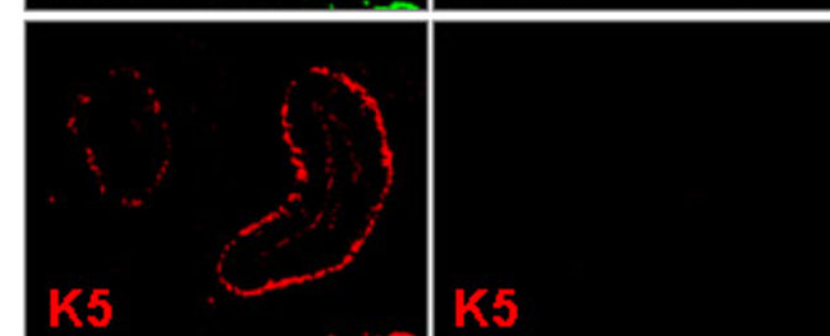
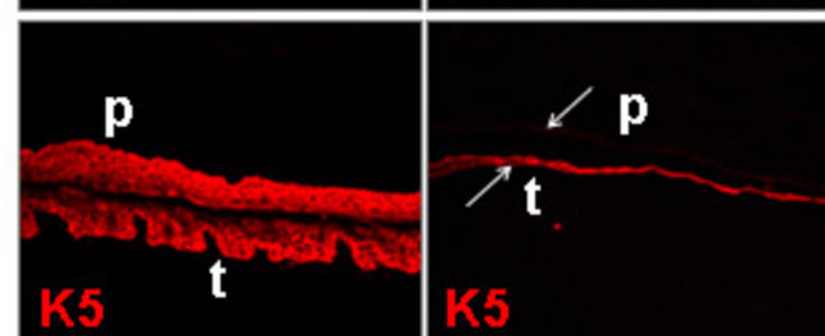
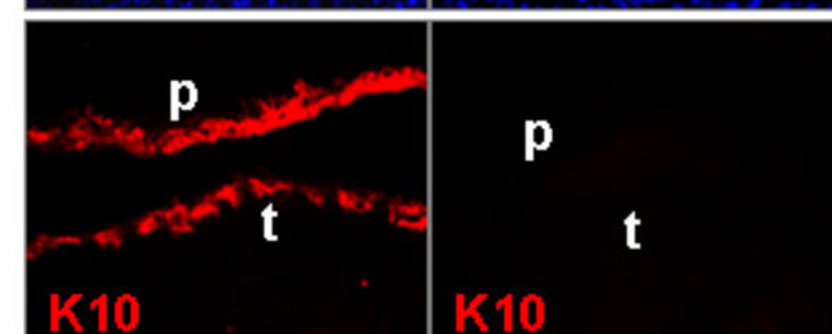
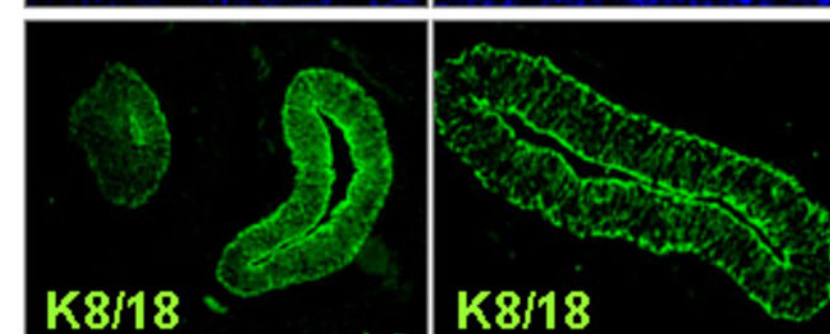
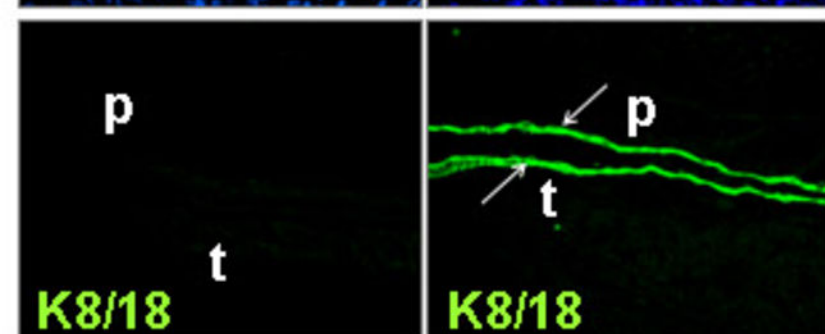
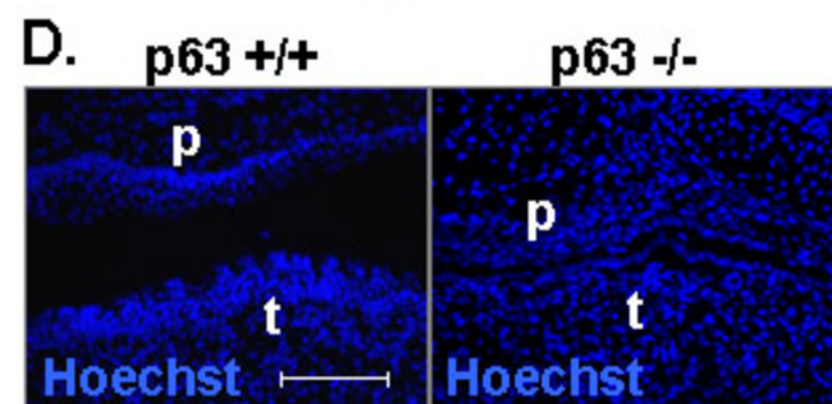
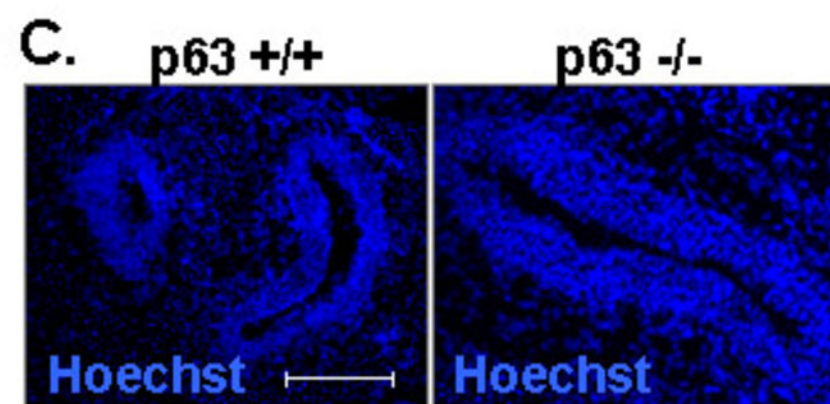
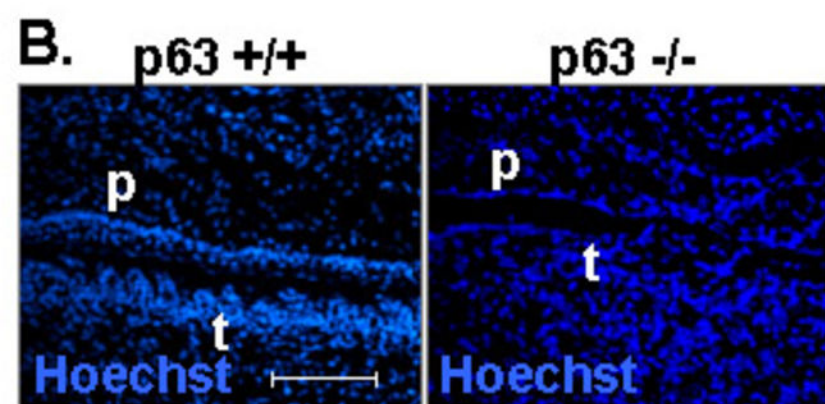
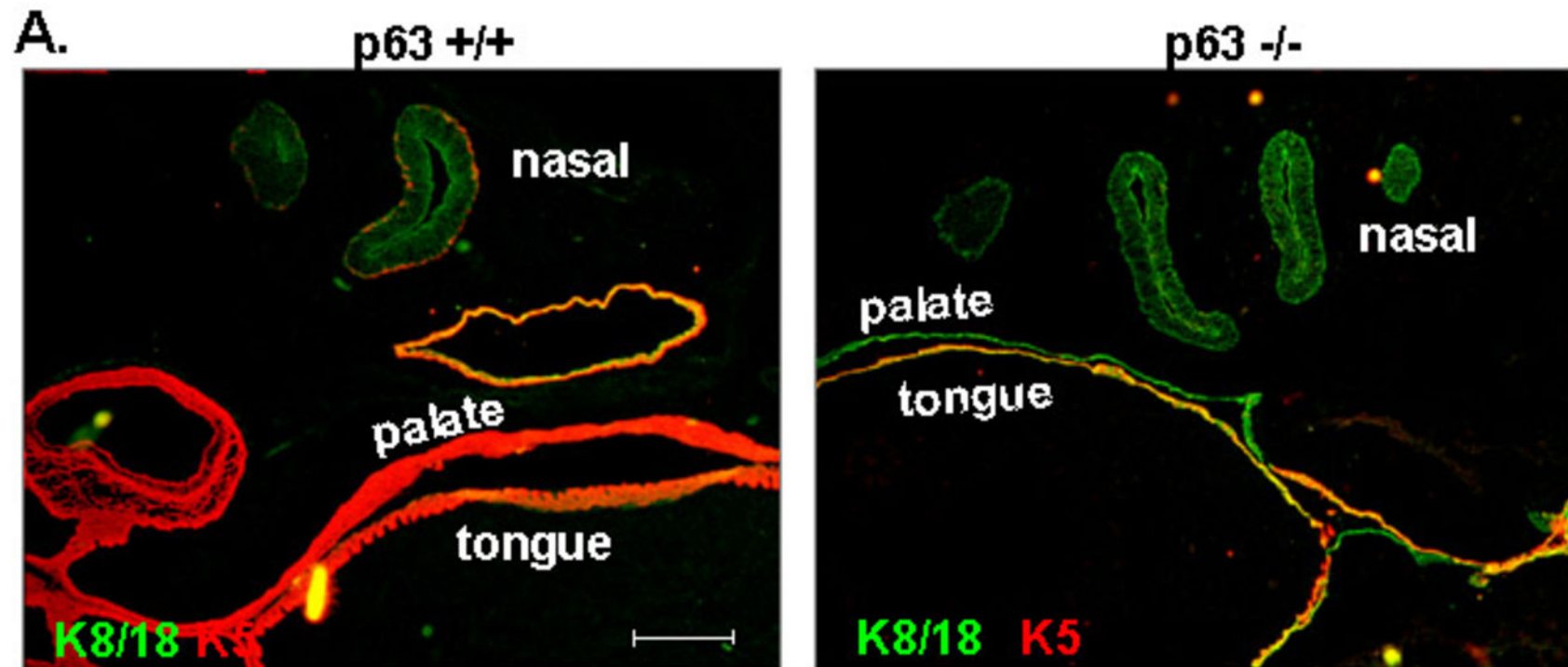


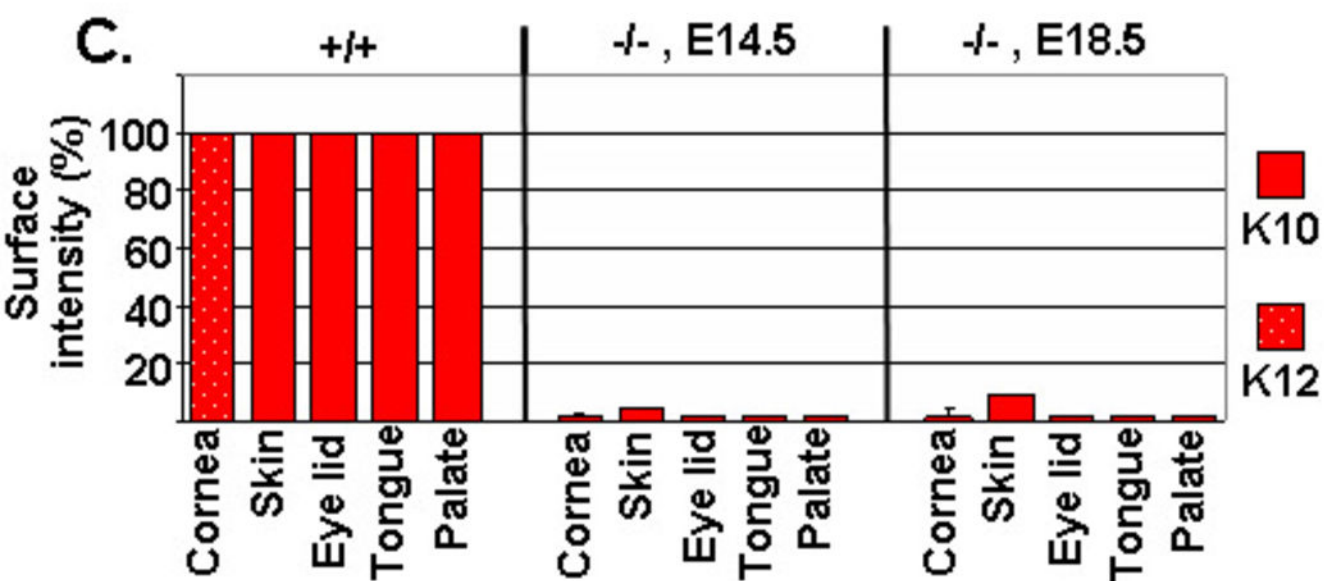
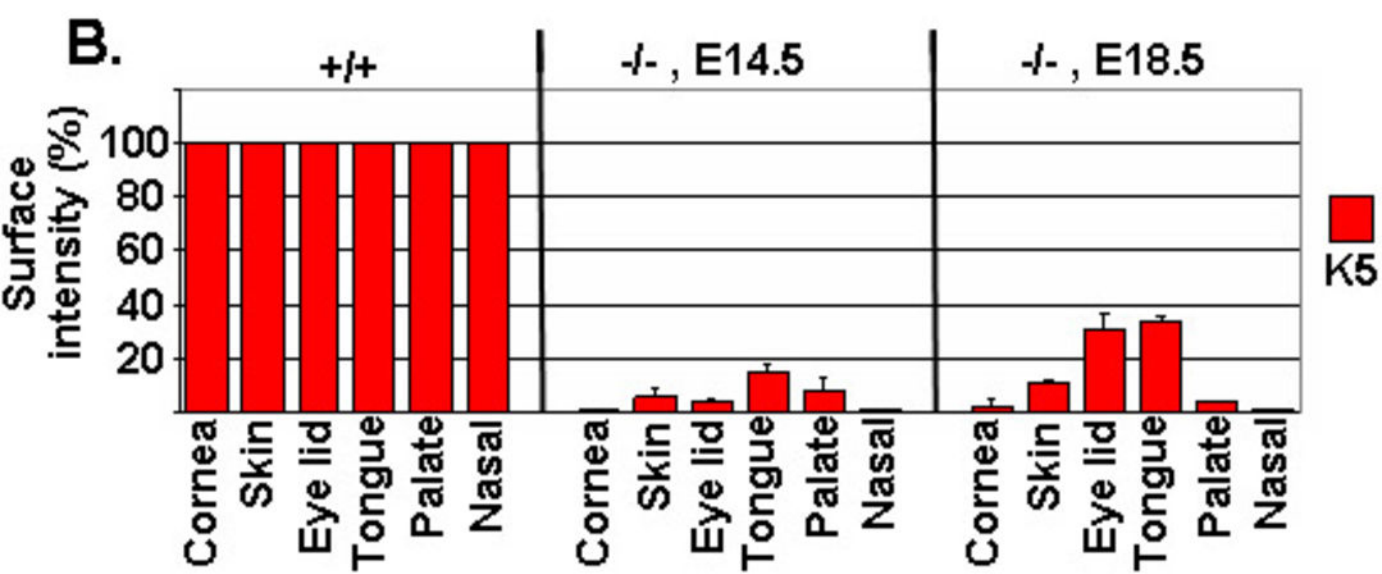
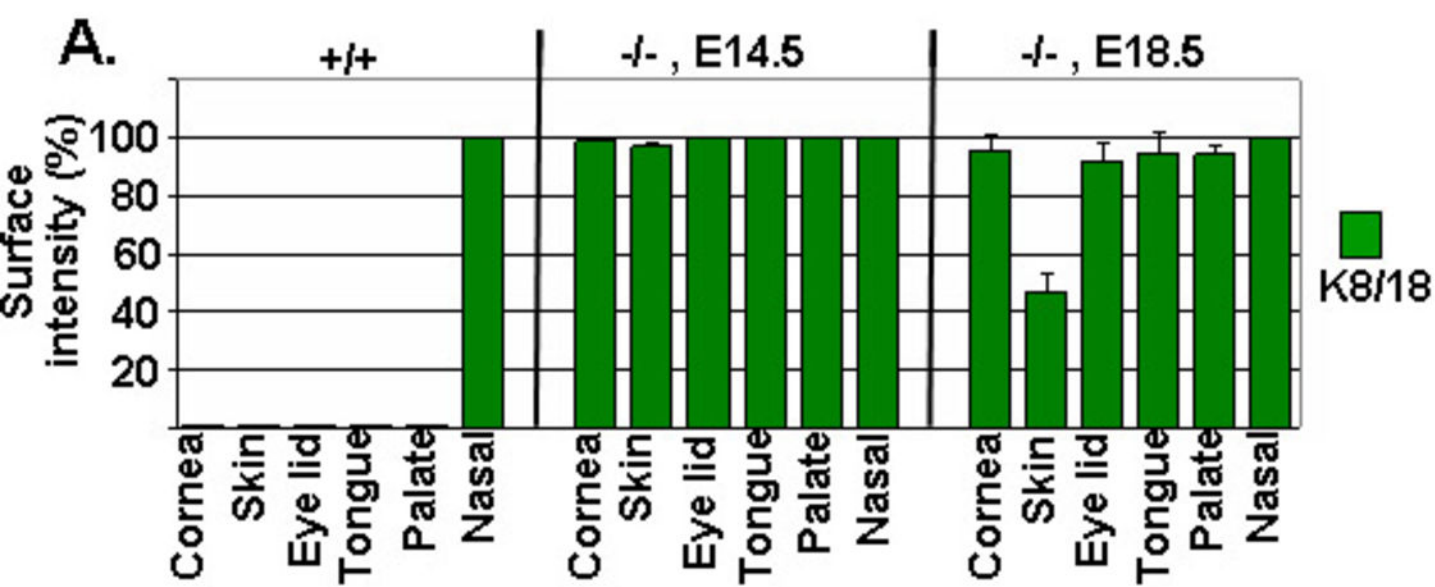
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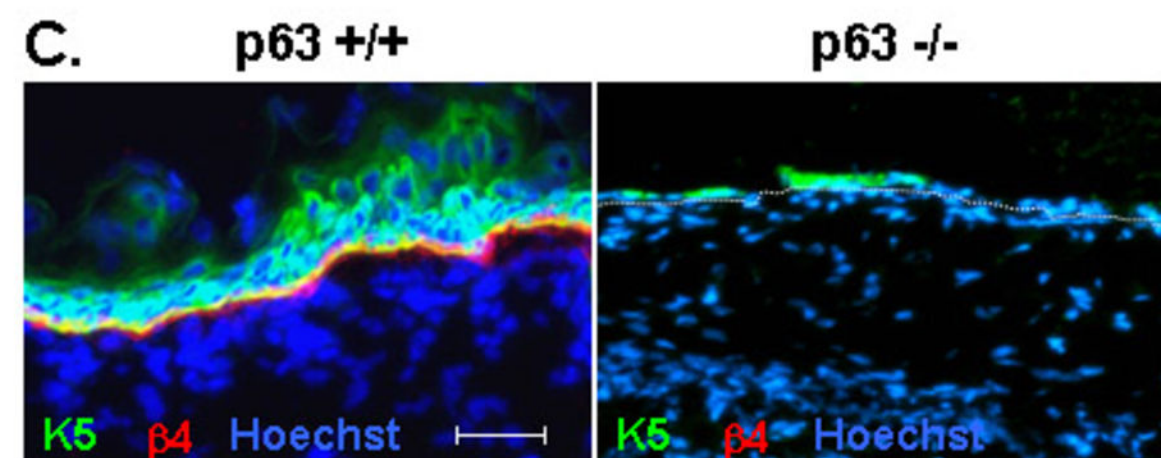
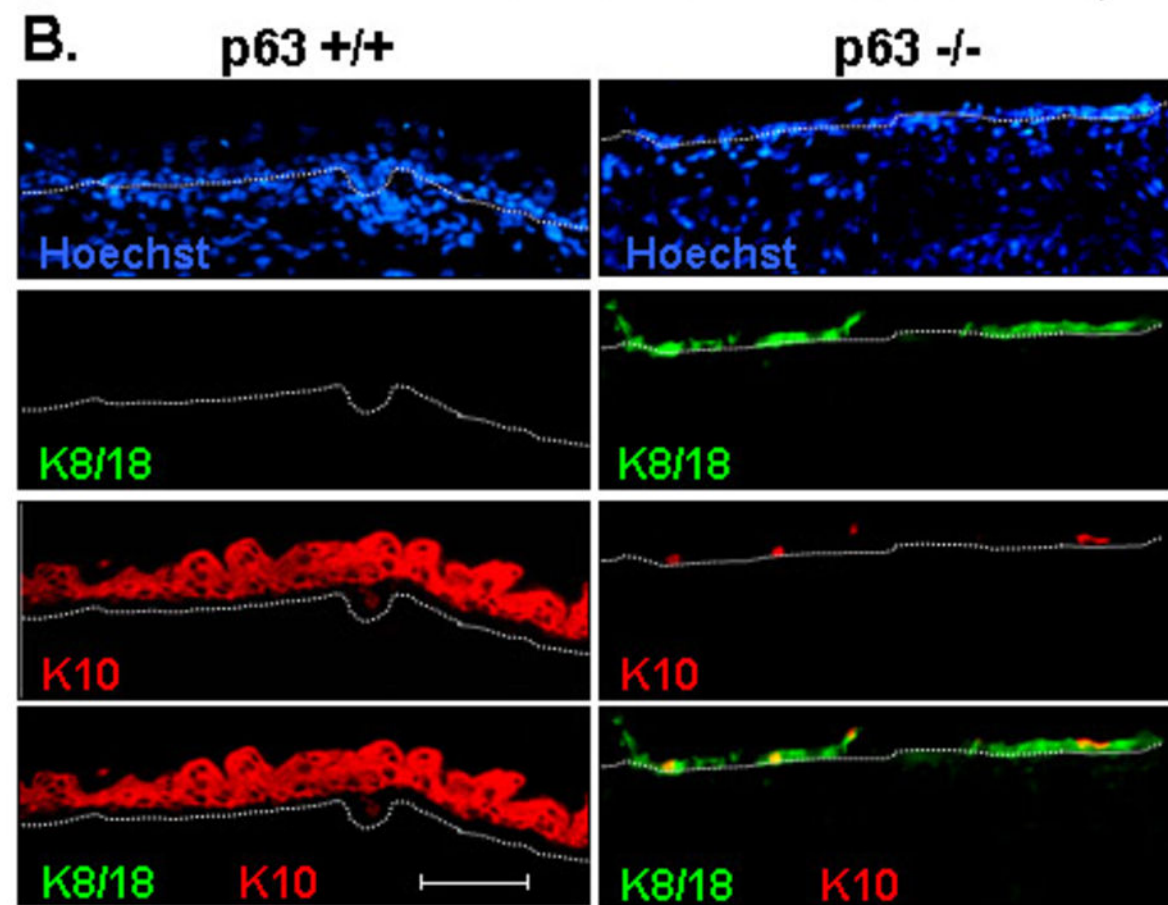
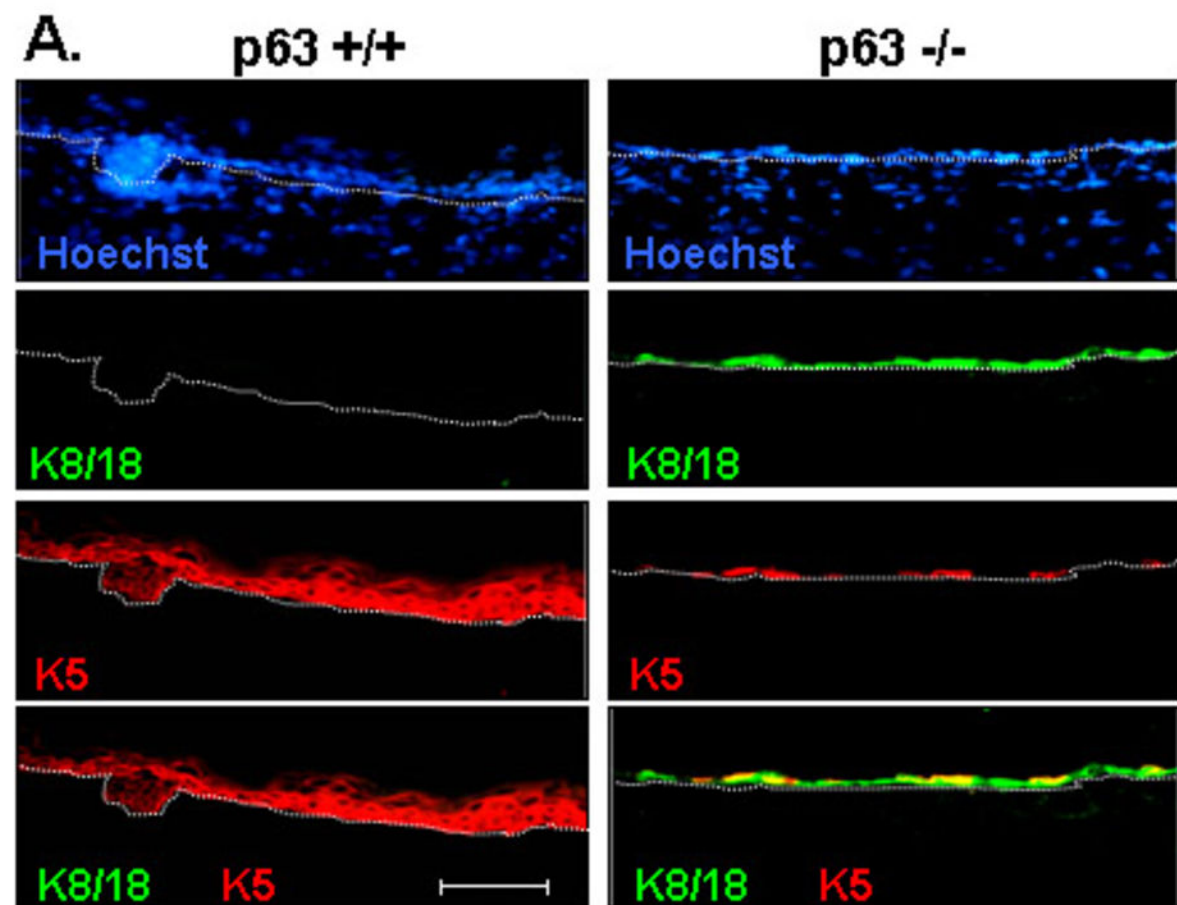


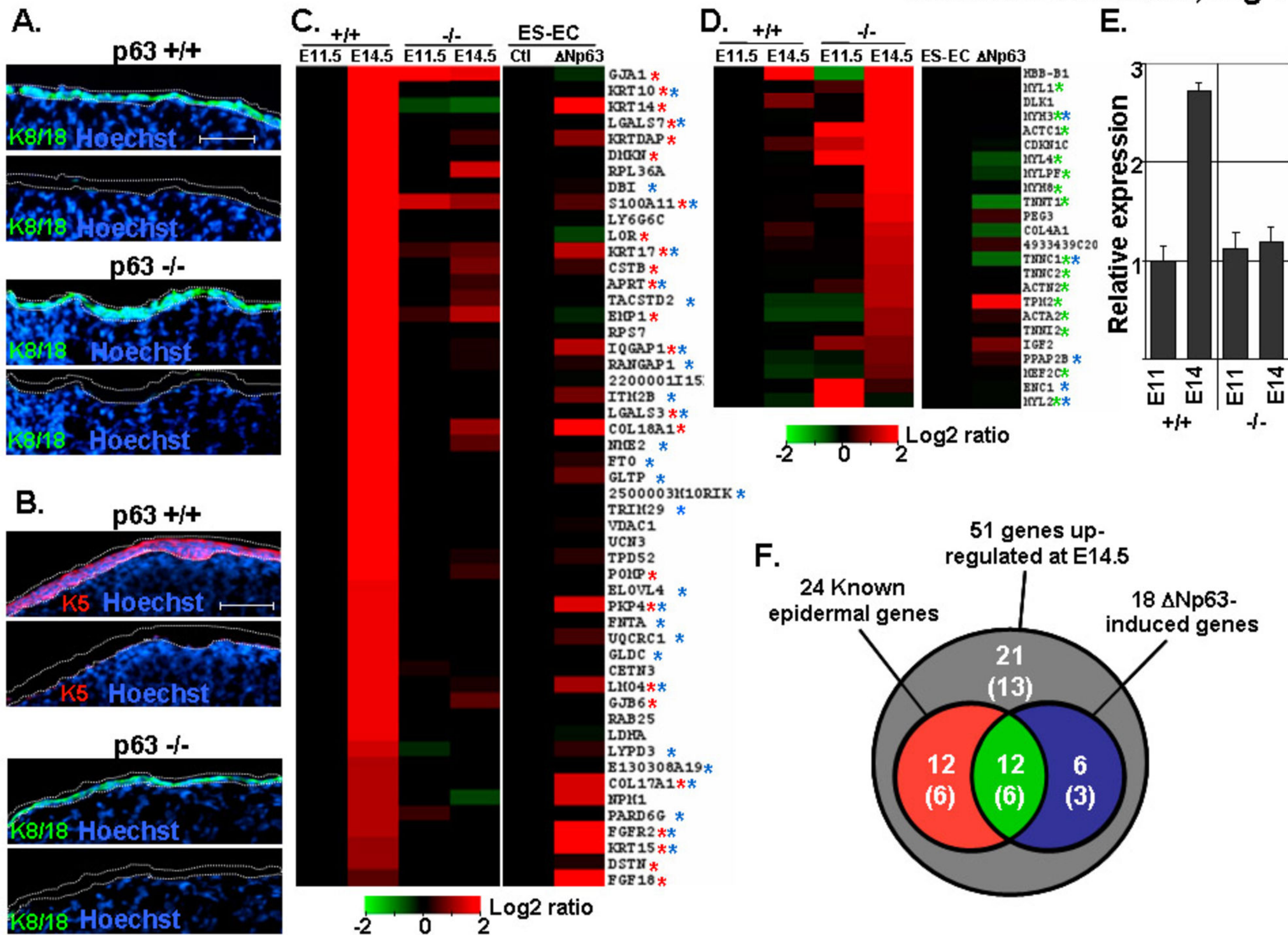


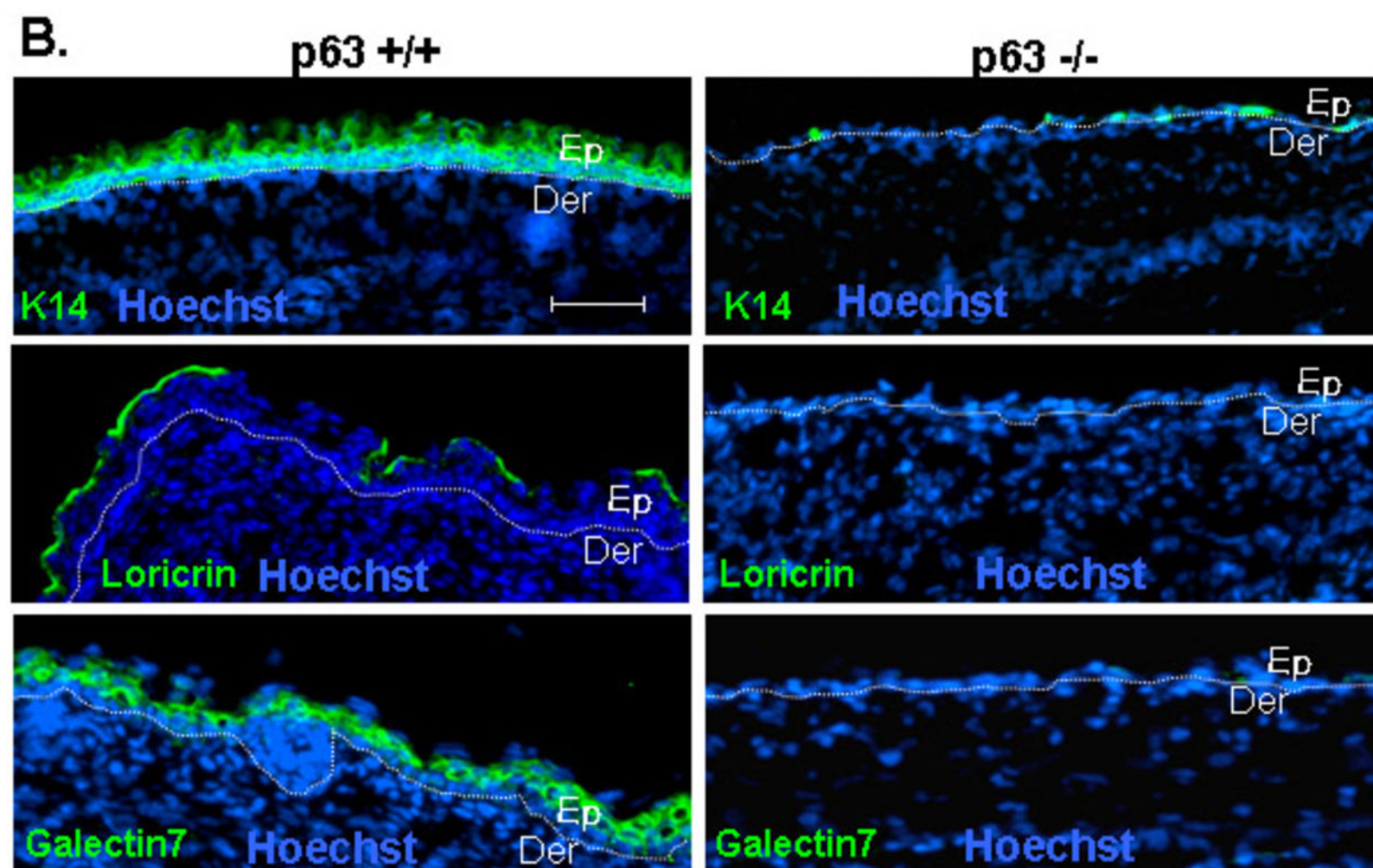
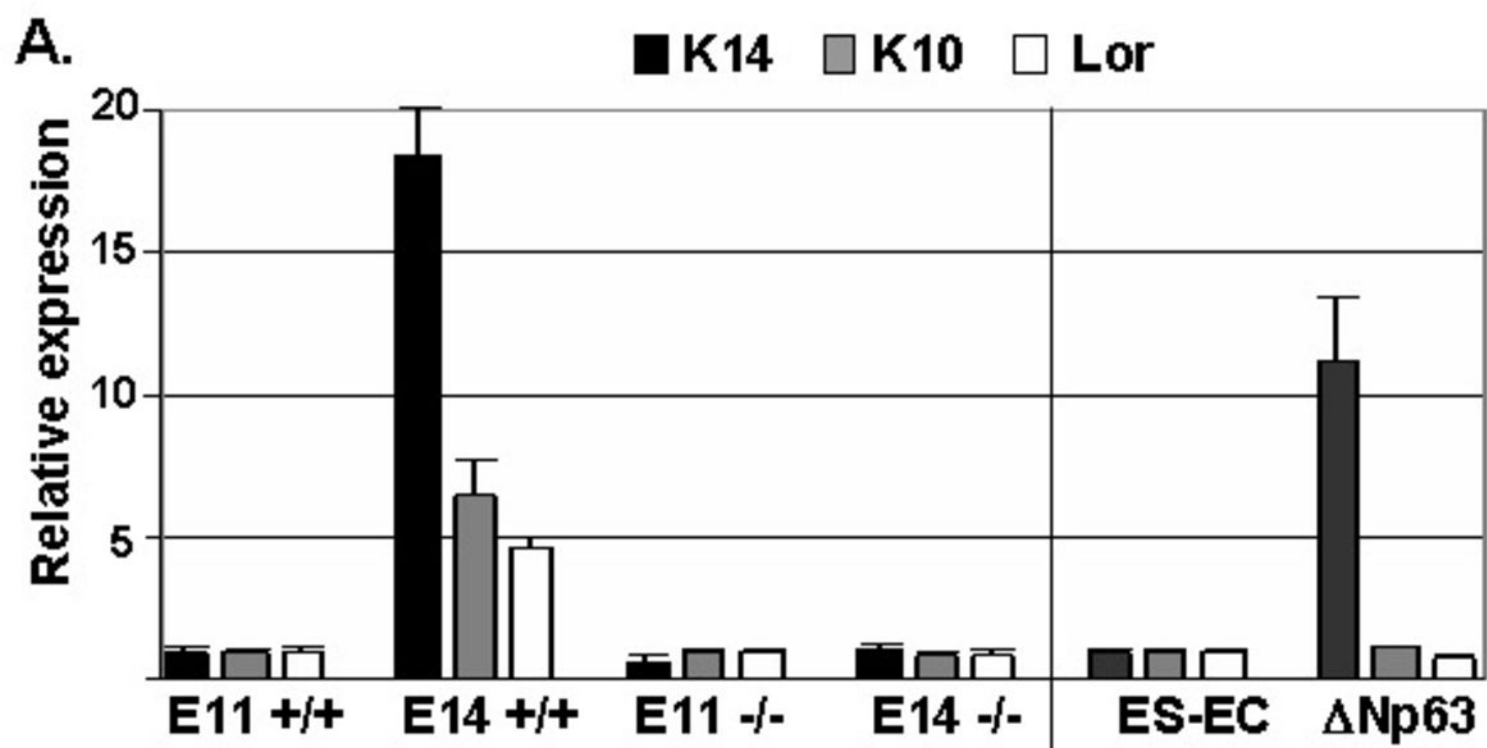


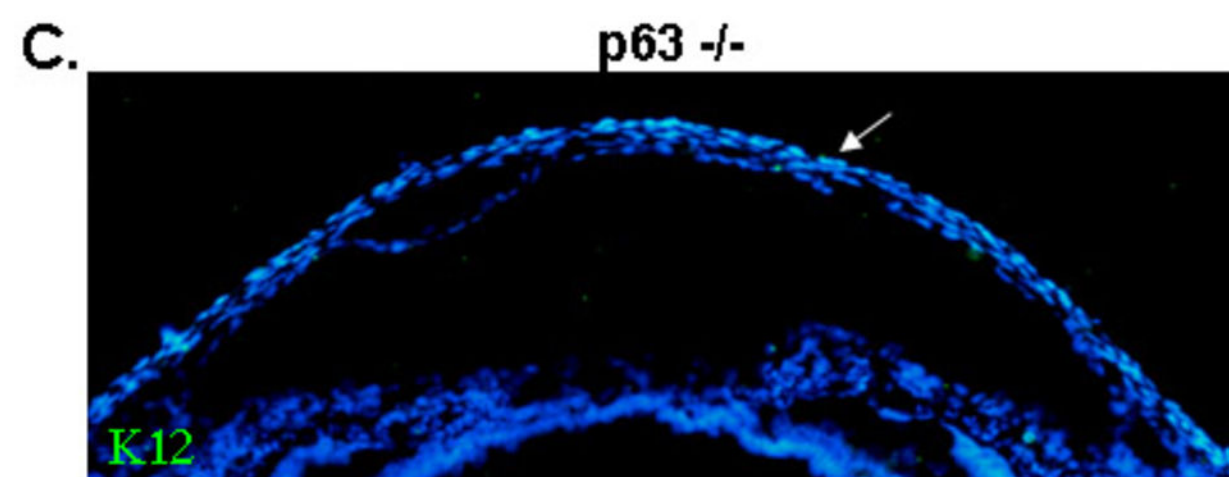
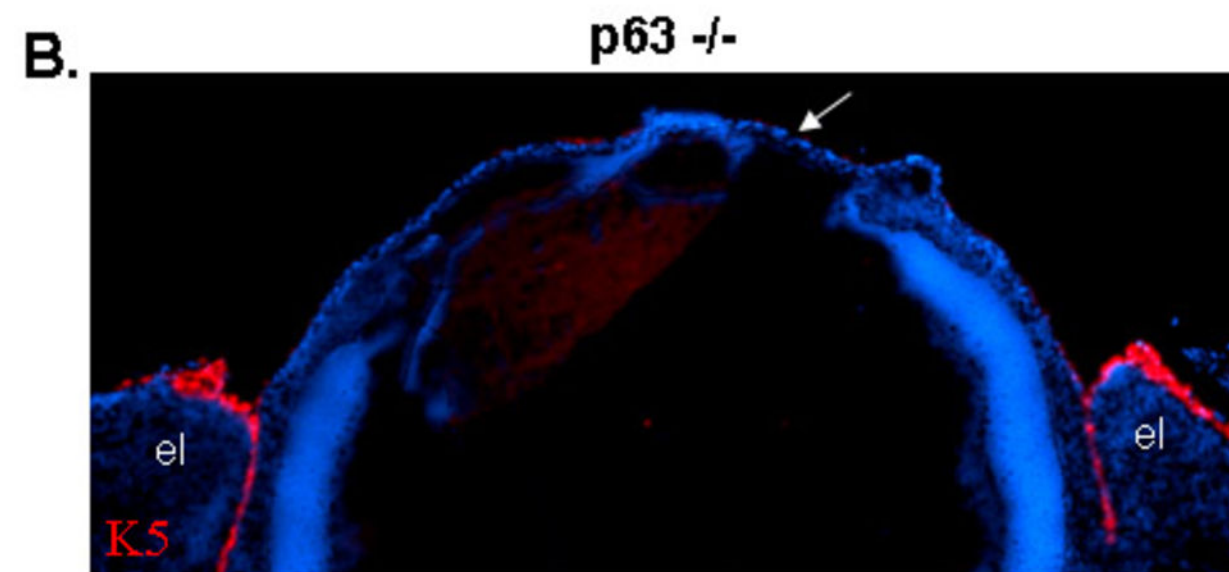
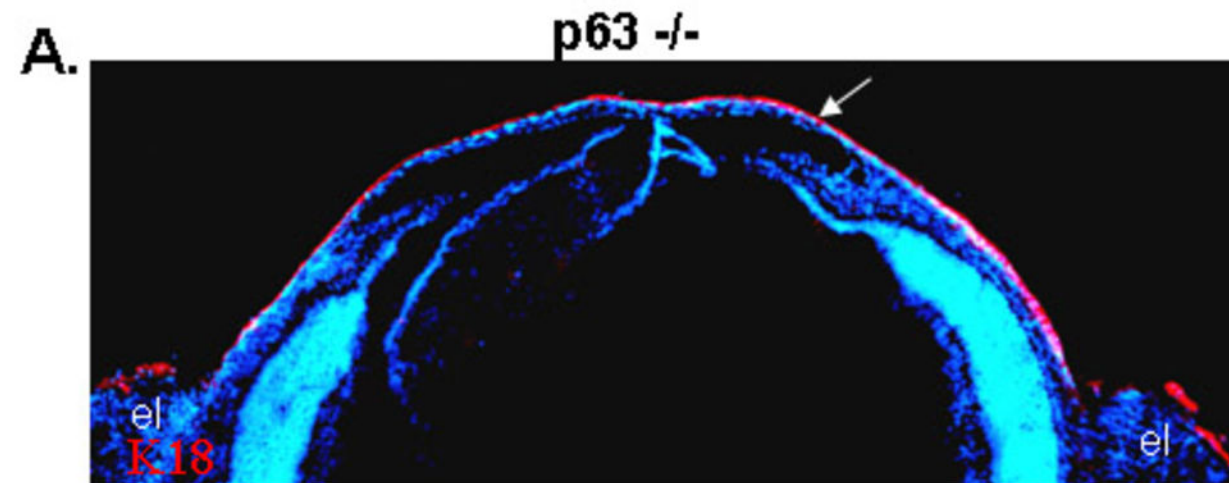




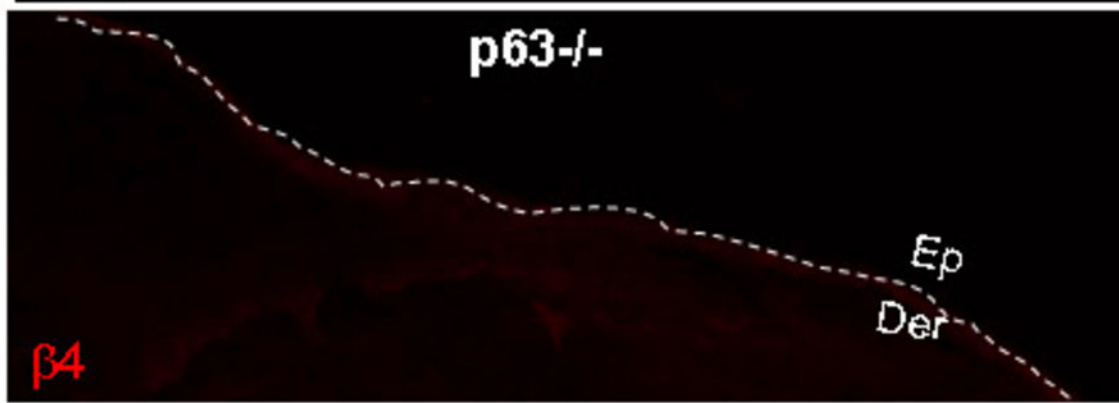
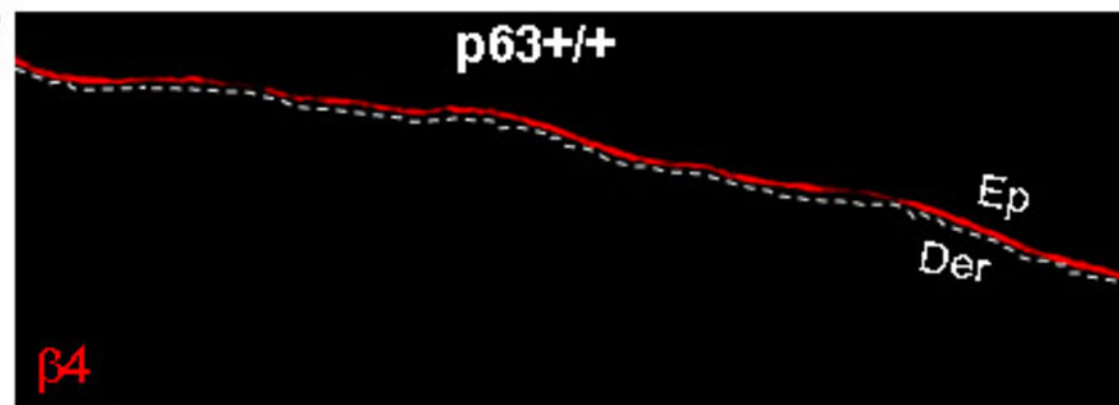




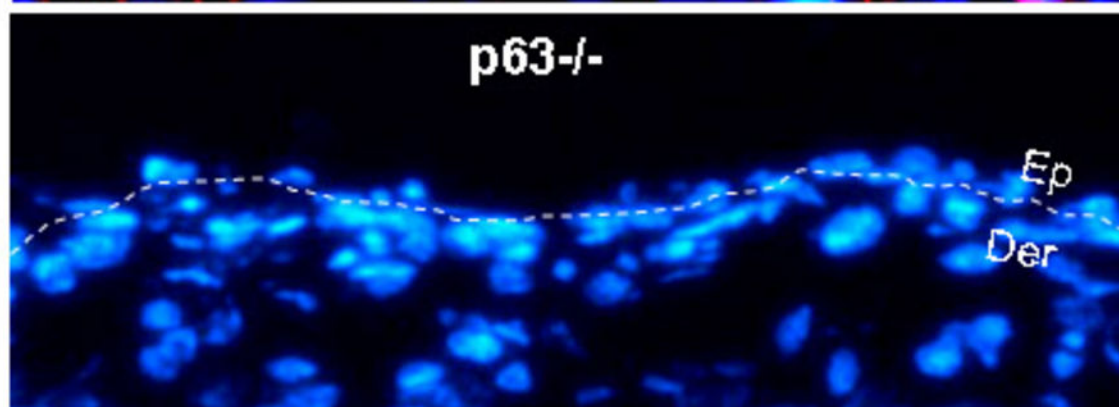
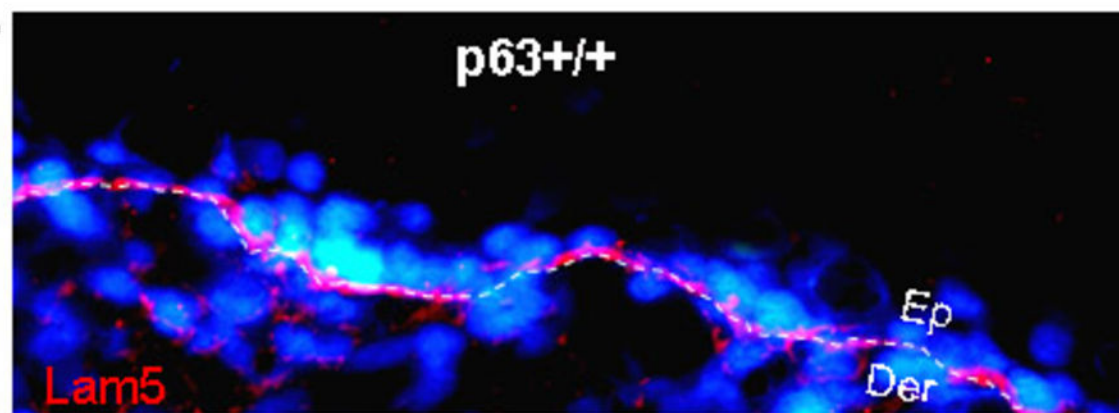


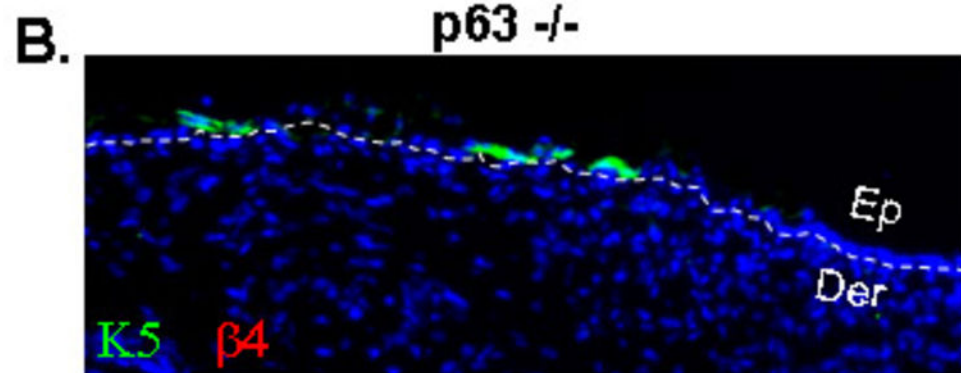
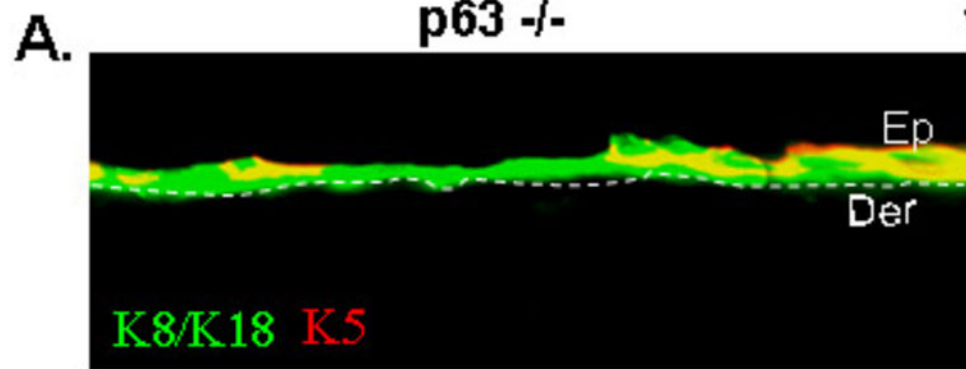


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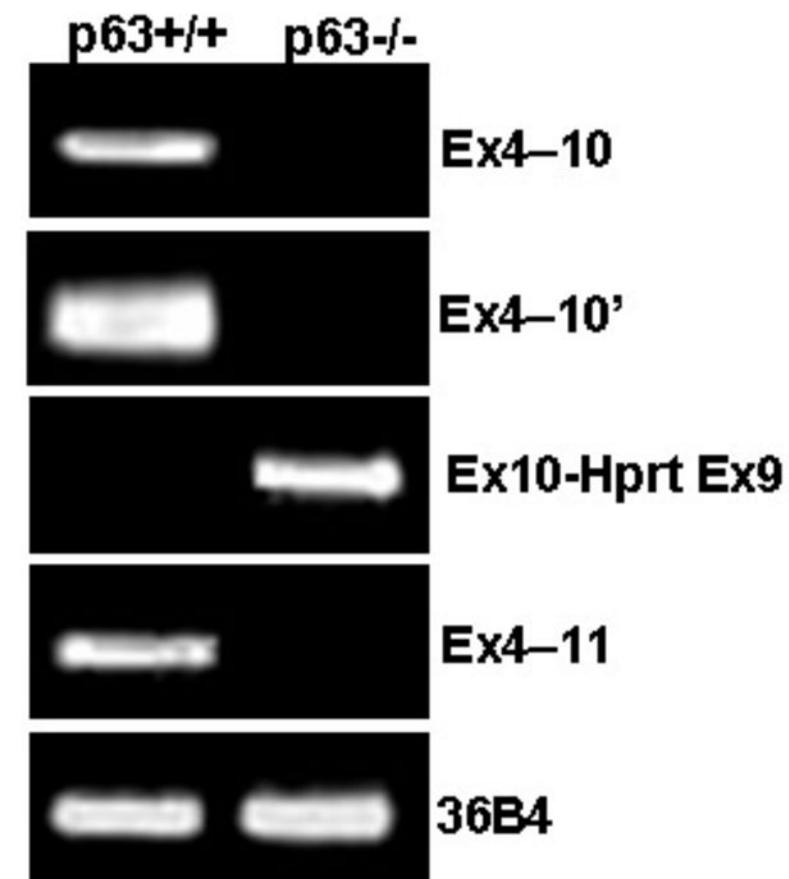


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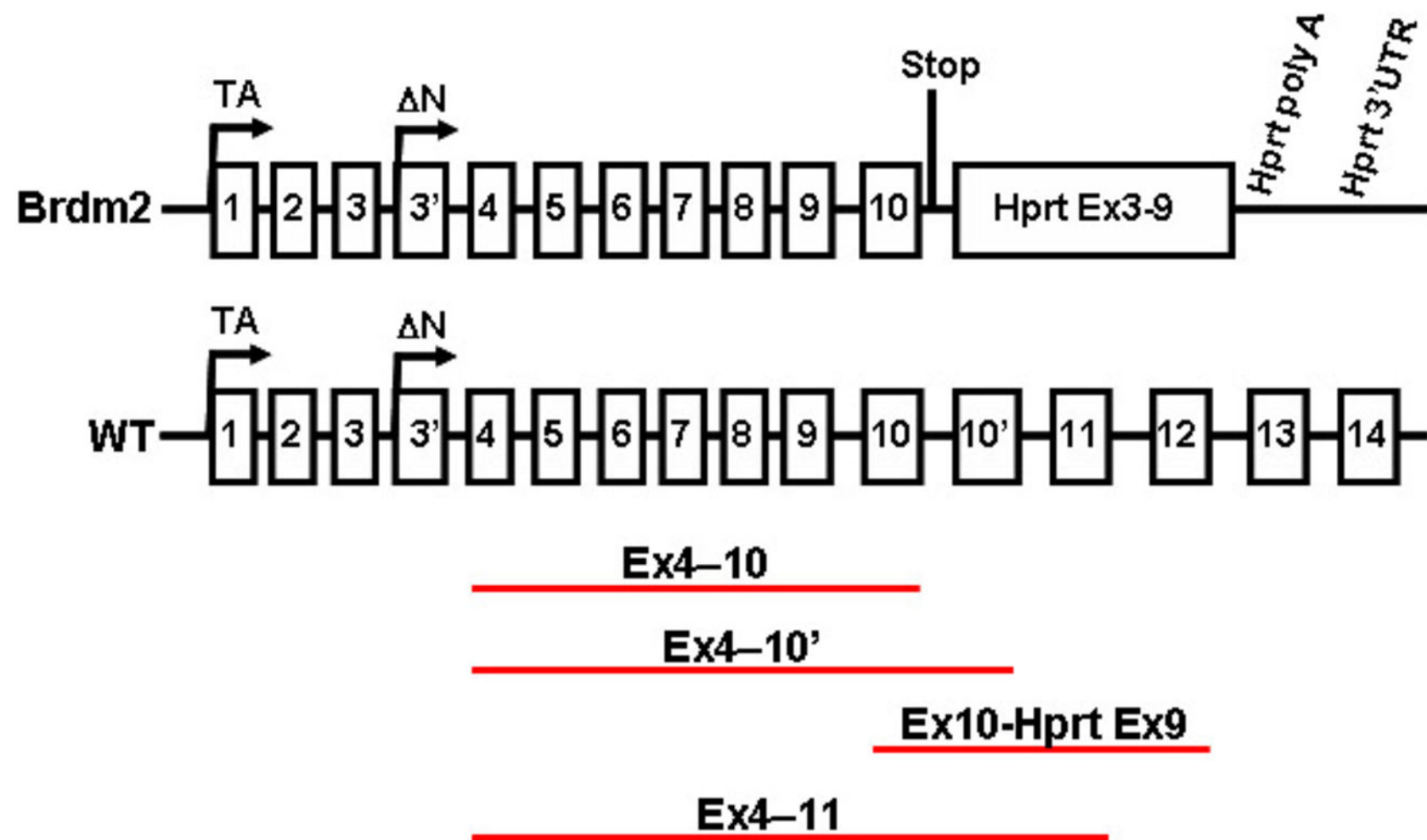




A.



B.



GO Term No.	GO Terms	No. of genes	% of analysed genes	P Value*	Bonferroni
GO:0030855	epithelial cell differentiation	6	11.76470588	2.61E-05	0.016958476
GO:0060429	epithelium development	7	13.7254902	1.16E-04	0.073536236
GO:0009888	tissue development	9	17.64705882	4.04E-04	0.232656265
GO:0051094	positive regulation of developmental proc	5	9.803921569	0.00326691	0.88311957
GO:0008284	positive regulation of cell proliferation	5	9.803921569	0.00883024	0.997027539

* *P* value < 0.01