The epidermis develops from a single layer of ectodermal progenitor cells through a tightly regulated series of events occurring during embryonic development. In mice, this process is initiated at about embryonic day (E) 8.5 to E9.5, when the cells of the surface ectoderm, which express keratin 8 (K8) and K18, are specified to an epidermal cell fate and switch to express a different set of keratin proteins, K5 and K14 (Fuchs, 2007; Koster and Roop, 2007; Nagarajan et al., 2008). After this initial commitment step, the newly formed basal layer receives instructive developmental cues to form the spinous layer. This stage is marked by the expression of differentiation markers K1 and K10 and occurs at ~E15.5. Subsequently, at E16.5, this program continues and markers of late differentiation, such as loricrin, are expressed in the outermost cells representing the granular layer. Epidermal differentiation nears completion with the expression of filaggrin at ~E17.5 and the generation of corneocytes. Finally, by E18.5, the epidermis is a fully mature, stratified squamous epithelium with complete barrier function (Candi et al., 2005). This entire process requires a precise balance between proliferation and differentiation to ensure that the terminally differentiated cells that are sloughed off are replaced by progeny of stem cells located in the inner basal layer, which divide, differentiate and migrate outward towards the surface of the skin (Blanpain and Fuchs, 2009). The genetic mechanisms controlling this intricate process have been linked to the transcription factor p63 (Trp63).

p63 exhibits high sequence and structural homology to the tumor suppressor protein p53 (Trp53), and to another member of this family p73 (Trp73) (Yang et al., 1998). Through the use of alternative promoters, the p63 gene generates transcripts encoding two major classes of protein isoform: TAp63 and ∆Np63. TAp63 contains an N-terminal transactivation (TA) domain, whereas ∆Np63 lacks this domain but is still transcriptionally active (Crum and McKeon, 2010; King and Weinberg, 2007). In addition, the TAp63 and ∆Np63 transcripts are alternatively spliced at the 3′ end to generate proteins with unique C-termini, designated α, β and γ (Yang et al., 1998).

The crucial contribution of p63 to embryonic morphogenesis is underscored by the striking abnormalities observed in animals deficient in p63, including limb truncations, craniofacial malformations and the lack of an intact epidermis. Moreover, these animals are born without teeth, mammary glands, prostate or skin appendages – structures that are highly dependent on proper epithelial-mesenchymal interactions (Mills et al., 1999; Yang et al., 1999). These phenotypes have been observed in two independently derived p63−/− mouse models, each generated by the disruption of exons common to both the TAp63 and ∆Np63 isoforms, thereby ablating all isoforms of p63. However, the analysis and
interpretation of the skin phenotype were reported differently by each group. In the p63+/− animals developed by the McKeon laboratory, scattered clumps of skin cells covering the dermis expressed markers of differentiation including loricin, filaggrin and involucrin. These observations led to the conclusion that the loss of p63 does not affect the commitment or differentiation potential of keratinocytes, but rather results in a defect in epithelial stem/progenitor cell self-renewal (Yang et al., 1999). Conversely, in the p63−/− mice generated by the Bradley laboratory, uncommitted ectodermal cells expressing K8 and K18 were found to cover the underlying dermis in place of the committed epidermal cells expressing K5 and K14. Given this altered keratin profile and the failure to detect any markers of keratinocyte differentiation, the p63-null phenotype was attributed to a block in lineage commitment of the ectodermal cells to an epithelial fate (Koster et al., 2004; Mills et al., 1999). Recent reports that the mouse model developed by the Bradley laboratory might represent a hypomorphic allele of p63 have sparked additional debate about the shortcomings of the existing genetic models (Mikkola et al., 2010; Talos et al., 2010; Wolff et al., 2009).

The conflicting mechanisms suggested for the role of p63 in epithelial development have been further clouded by the existence of multiple isoforms of p63. This has raised valid questions regarding the expression levels and roles of the two principal isoforms of p63: TAp63 and ΔNp63 (Crum and McKeon, 2010). Most researchers contend that, in mouse, ΔNp63 is highly expressed during the early stages of epidermal morphogenesis and continues to be expressed in the basal layer of the skin as well as in many other types of epithelial tissue (Laurikkala et al., 2006; Romano et al., 2007; Romano et al., 2009). By contrast, TAp63 isoforms are believed to be only weakly expressed in skin. Functional analyses using transgenic mouse models demonstrate that forced expression of both ΔNp63 and TAp63 can induce transformation of single-layered lung epithelium into a stratified epithelium accompanied by the expression of markers normally associated with the skin epidermis (Koster et al., 2004; Romano et al., 2009). However, when transgenic mice expressing either TAp63 or ΔNp63 were bred to the p63−/− background, those expressing ΔNp63, but not TAp63, were able to partially rescue basal epidermal gene expression (Candi et al., 2006; Romano et al., 2009). These data, coupled with the fact that isoform-specific deletion of TAp63 in mice does not cause any overt epidermal developmental defects, are consistent with the notion that it is the ΔNp63 isoforms that are crucial in epidermal development (Guo et al., 2009; Su et al., 2009; Suh et al., 2006).

In an attempt to resolve the controversies surrounding the role of p63, specifically that of ΔNp63, we have generated a mouse strain (ΔNp63300/400) in which the coding region of the ΔNp63 exon has been replaced with GFP. Using GFP as a surrogate for ΔNp63 expression, we have confirmed high expression levels of this isoform in the basal layer of several epithelial tissues including the skin. ΔNp63-null (ΔNp63300/400) animals, which die within hours after birth, are born with severe abnormalities including limb truncations, craniofacial malformations and the lack of a mature stratified epidermis. Despite a failure to develop a mature epidermis, ΔNp63-null mice display isolated patches of epithelial cells covering the exposed dermis. Interestingly, these clumps of keratinocytes are able to progress through the differentiation program, albeit in an accelerated fashion. However, there are clusters of skin keratinocytes that fail to undergo the proper cellular transition to an epithelial cell lineage and retain expression of the simple epithelial cell markers K8 and K18.

Together, our data are consistent with the notion that in the absence of ΔNp63 some ectodermal cells are capable of committing to an epidermal cell fate and subsequently undergo accelerated differentiation, which is likely to be due to hyperactive Notch signaling, whereas other cells fail to commit and retain their simple epithelial characteristics. The ΔNp63300/400 animals provide crucial insights into the biological activities of the ΔNp63 isoform and offer a new tool in the genetic arsenal to better understand fate and lineage choices, particularly of epithelial cell types.

**MATERIALS AND METHODS**

**Generation of ΔNp63300/400 mice and animal procedures**

Mice were generated using standard ES cell technology as previously described (Choi et al., 2009; Packard et al., 2011). To facilitate efficient homologous recombination, the targeting vector was designed to include two homologous arms, which were generated by PCR using BAC DNA. The 5′ homologous arm included a 5.1 kb fragment that ended just before the start codon of ΔNp63 in exon 3′. The open reading frame of exon 3′ of ΔNp63 was replaced by the coding sequences of GFP. Two correctly targeted ES cell clones were used to generate chimeras. ES cell work and the generation of chimeras were performed by the Gene Targeting and Transgenic Core Facility at the Roswell Park Cancer Institute. After generating germline chimeras, correct homologous recombination was further confirmed by Southern blot and PCR analysis. Genotyping of progeny was performed by PCR analysis of tail DNA using three primers. For wild-type animals, an 842 bp product was obtained using primer A (5′-CCATGGAGTGGAGCCAGTTG-3′) and primer B (5′-CAGTCGGAGTACTAAGGATGC-3′). Primers A and B generated a 1.7 kb PCR product in ΔNp63300/400 animals. Primer C (5′-CTCCAGGACCAGTATGAGG-3′) is located within the GFP cassette and generated a 952 bp fragment in heterozygous animals when used with primer A. The Brdm2 p63−/− mice have been characterized previously (Mills et al., 1999). Protocols for mouse experimentation were performed according to Roswell Park Cancer Institute IACUC protocols. Mice of appropriate genotype were mated and noon of the day the vaginal plug was observed was considered E0.5.

**Chromatin immunoprecipitation (ChIP)**

Mouse keratinocytes were obtained from CELLineTEC and were grown to 80% confluence in CnT-07CF media (Bern, Switzerland). Cells were cross-linked with 1% formaldehyde and processed according to the Magna ChIP G Chromatin Immunoprecipitation Kit (Millipore). Antibodies used were RR14 (Romano et al., 2006), H129 (Santa Cruz) and normal rabbit IgG. Purified immunoprecipitated DNA was used for real-time qPCR. Primer sequences are provided in supplementary material Table S1.

**Semi-quantitative and quantitative RT-PCR**

Total RNA from whole embryos taken at various time points from ΔNp63+/+ , ΔNp63300/400 and ΔNp63300/400 animals was isolated as previously described (Romano et al., 2009). RNA was reverse transcribed into cDNA using the Thermoscript RT-PCR system (Invitrogen) according to the manufacturer’s instructions. Real-time RT-PCR was performed on an iCycler iQ PCR machine using iQ SYBR Green Supermix (Bio-Rad). All real-time RT-PCR assays were performed in triplicate in at least two independent experiments using two different animal samples. Relative expression values of each target gene were normalized to beta-2 microglobulin (β2m) expression. Primer sequences are provided in supplementary material Table S1.

**Statistical analysis**

Results are reported as mean ± s.d. Statistical comparisons were performed using unpaired two-sided Student’s t-test with unequal variance assumption.

**Immunofluorescence**

Whole embryo antibody stainings were performed on paraffin and frozen embedded sections as previously described (Romano et al., 2009; Romano et al., 2010). Primary antibodies used were: K5 (1:200, American Research
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RESULTS

Generation and validation of ΔNp63 knockout mice

The p63 gene is transcribed using two alternative promoters, generating TAp63 transcripts from an upstream (P1) promoter and ΔNp63 variants that are transcribed using the downstream promoter (P2) embedded within intron 3 (supplementary material Fig. S1A). Translation of the ΔNp63 protein isoforms is initiated at an AUG codon located in exon 3', an exon that is unique to the ΔNp63 isoform (supplementary material Fig. S1A). To target ΔNp63 while ensuring the TAp63 isoforms remain unaffected, the coding sequence of the ΔNp63-specific exon was replaced by GFP (supplementary material Fig. S1B). This knock-in strategy also allowed for expression of GFP in place of ΔNp63 while under the influence of the endogenous ΔNp63 regulatory control elements. ΔNp63Δp63;/ES cells were generated by homologous recombination and subsequently used to derive mice that were either heterozygous or homozygous for this mutation (supplementary material Fig. S1C). Importantly, using RT-PCR we showed that the ΔNp63 mRNA was absent in the homozygous mutant mice, whereas TAp63 mRNA transcripts remain unaffected, demonstrating selective inactivation of the ΔNp63 isoforms (supplementary material Fig. S1D). Loss of ΔNp63 was further verified by performing immunofluorescence on whole embryo tissue sections from E14.5 ΔNp63Δp63; mice using a ΔNp63-specific antibody (supplementary material Fig. S1E, top). Moreover, immunostaining on sections of ΔNp63Δp63; ovaries with anti-TAp63 antibodies showed robust TAp63 expression in the follicles, consistent with previous reports demonstrating high levels of this isoform in ovaries and confirming that the expression of TAp63 proteins is not disrupted in these animals (supplementary material Fig. S1E, bottom) (Suh et al., 2006).

In order to confirm that the GFP expression mirrored that of endogenous ΔNp63, we performed immunofluorescence staining on various epithelial tissues from heterozygous ΔNp63Δp63; animals. Examination of dorsal skin samples from postnatal day 7 ΔNp63Δp63; mice clearly revealed that GFP expression colocalized with ΔNp63. More specifically, GFP was expressed in the basal layer of the epidermis and outer root sheath of the hair follicles, which have been shown to express high levels of ΔNp63 (supplementary material Fig. S2). Similar colocalization of GFP and ΔNp63 was also detected in oral tissues, such as the buccal mucosa and the dorsal surface of the tongue, in ΔNp63Δp63; mice (supplementary material Fig. S2).

ΔNp63 mutant animals phenocopy mice lacking all p63 isoforms and fail to develop a normal stratified epidermis

Upon intercrossing of ΔNp63Δp63; animals, all resulting ΔNp63Δp63; null mice died shortly after birth, presumably owing to severe developmental defects and dehydration. In order to investigate the
loss of ΔNp63 in epithelial morphogenesis, embryos were harvested at various stages of development beginning at E14.5. Heterozygous ΔNp63<sup>+/−</sup> mice taken at all time points examined appeared indistinguishable from wild-type littermates. By contrast, ΔNp63<sup>+/−</sup> mice were smaller than their wild-type counterparts and exhibited obvious defects in limb and craniofacial development and a lack of epidermis that resulted in an overall shiny, translucent appearance (Fig. 1, left). Of the 40 homozygous mutant embryos examined so far, all animals exhibited arrested hind limb and forelimb development, albeit with some degree of variability in the extent of defects (supplementary material Fig. S3). Closer examination of paraffin-embedded sections of whole embryos revealed a failure of keratinocytes to develop into a mature stratified epidermis in the ΔNp63<sup>+/−</sup> animals as compared with wild-type mice (Fig. 1, middle). Interestingly, at E14.5, ΔNp63-null embryonic skin consisted of a thin layer of intact epithelial cells, which appeared hypoplastic (~2 cell layers in thickness) and disorganized. By contrast, control E14.5 wild-type skin was typically 3-4 cell layers thick. This disorganized pattern of epithelial keratinocytes persisted at E16.5, along with obvious defects in epidermal organization. By E18.5, ΔNp63<sup>+/−</sup> surface keratinocytes were present in sparse clumps, which covered portions of the exposed lower dermis and failed to show overt signs of normal epidermal stratification and maturation, as surface keratinocytes remained 1-2 cell layers in thickness in most of these areas (Fig. 1, middle). Moreover, there were no signs of hair follicle and sebaceous gland morphogenesis in the ΔNp63 knockout animals at any of the embryonic stages examined.

Given the strong expression pattern of p63 in the basal cells of many stratified epithelial tissues, we next examined the effects of the loss of ΔNp63 in the oral cavity, a tissue with high levels of ΔNp63 expression. Similar to what we observed in the skin, the tongue, palate and the buccal mucosa of E18.5 ΔNp63-null animals lacked a mature stratified epithelium, confirming an essential role for this p63 isoform in the formation of these stratified epithelial tissues (Fig. 1, right). In general, judging by morphological and histological analysis, the ΔNp63<sup>+/−</sup> animals appear to largely phenocopy the epithelial aplastic phenotype of the previously reported pan-p63-null animals (Mills et al., 1999; Yang et al., 1999).

**ΔNp63-null keratinocytes undergo accelerated differentiation, as evident by the premature developmental expression of terminal differentiation markers**

To better define the specific role of ΔNp63 during embryonic skin development and to help resolve the lingering questions regarding the overall cellular nature of the phenotypic changes that result from the loss of p63, we next examined the ΔNp63<sup>+/−</sup> skin in greater detail using a battery of established markers. To investigate whether cells of the surface ectoderm of ΔNp63<sup>+/−</sup> animals were able to commit to an epidermal cell lineage, we analyzed skin sections at E13.5, a stage at which this process is already initiated. As expected, we identified robust expression of K5 in the epidermis of control animals, with expression of K8, a marker of the simple epithelium, being completely absent (Fig. 2, left). This was true for all the subsequent developmental time points that we examined in the control animals. In the ΔNp63<sup>+/−</sup> mice, keratinocytes that covered the surface of the embryo also expressed K5, albeit to a weaker extent and in a patchy fashion compared with control animals. These data confirm the ability of at least some cells lacking ΔNp63 to commit to an epidermal cell fate. Interestingly, we also identified clusters of keratinocytes that, although they expressed K5, also retained expression of K8 (Fig. 2, middle). This was particularly prominent in anatomical areas that are normally protected from abrasion and friction, such as the ventral neck and chest. In some areas that were more exposed, such as the dorsal back and head regions, we found a single layer of cells expressing K8 (Fig. 2, right). These K8-retaining epithelial cells were present at multiple stages of embryonic development. Together, these data suggest that, in the absence of ΔNp63, keratinocytes in at least some areas of the embryonic skin are able to commit to an epidermal lineage. However, there also appears to be an intermediate state, suggesting a developmental conundrum in which the epithelial cells simply fail to commit or co-express simple epithelial (K8) and basal keratinocyte (K5) specific markers.

Given the ability of the keratinocytes of ΔNp63<sup>+/−</sup> mice to commit to an epidermal cell fate, as demonstrated by the expression of K5 and K14 (Fig. 2; data not shown), we sought to further confirm this observation by investigating the status of the transcription factor AP-2α (T Parses – Mouse Genome Informatics), which is highly expressed in basal keratinocytes and is important for basal gene expression (Maytin et al., 1999; Sinha et al., 2000; Wang et al., 2006). Robust expression of AP-2α was evident in both the wild-type and ΔNp63<sup>+/−</sup> skin samples (supplementary material Fig. S4), verifying that, in the absence of this specific p63 isoform, keratinocytes can form a basal layer and express some of its distinctive markers.

After confirming formation of the basal layer in the absence of ΔNp63, we next sought to investigate whether the cells were able to progress through to subsequent stages of keratinocyte differentiation. We co-stained ΔNp63<sup>+/−</sup> skin sections for K5 and K1 or K10, which allowed us to simultaneously mark the basal (K5) and spinous (K1, K10) layers. As expected, we observed a
clear separation between these layers at E15.5 in control animals (supplementary material Fig. S5). By contrast, keratinocytes in several areas of ΔNp63<sup>gfp/gfp</sup> skin exhibited co-expression of K5 with K1 and K10, suggesting an altered differentiation state. Although the boundary between the basal and spinous layer was blurred in ΔNp63-null skin, the keratinocytes were able to initiate the formation of a multilayered stratified epithelium, as evident by small pockets of cells that were stacked on top of each other (Fig. 3; supplementary material Fig. S5). The formation of the spinous layer in ΔNp63<sup>gfp/gfp</sup> keratinocytes was further confirmed by the expression of the transcription factor Gata3, which normally marks this layer (de Guzman Strong et al., 2006; Kaufman et al., 2003; Kurek et al., 2007) (supplementary material Fig. S4, bottom). Interestingly, we also observed the expression of markers associated with terminal differentiation, including loricin, filaggrin and involucrin in the ΔNp63-null E15.5 embryos. Such expression at this early developmental window was notably premature given that these markers were conspicuously absent in the counterpart wild-type control littermate embryos (Fig. 3). We confirmed these results by quantifying the expression levels of several of these markers of differentiation by qRT-PCR at E14.5 and E15.5 (supplementary material Fig. S6A).

Given that such a precocious differentiation program of embryonic p63-null keratinocytes had not been reported previously, we examined the status of the markers of late differentiation in the Brdm2 mouse model, which lacks all isoforms of p63 (Mills et al., 1999). We were unable to detect the expression of any of the late markers of differentiation at E15.5 in the Brdm2 mouse (supplementary material Fig. S7), a feature that was consistently evident in our ΔNp63-null animals. This result suggests that there are functionally distinct outcomes in these two mouse models, which could reflect the effects of remnant p63 levels, isoform-specific activities or differences in strain background. Importantly, we demonstrate that, although keratinocytes are able to undergo terminal differentiation in the absence of ΔNp63, this process appears unbalanced and accelerated.

**ΔNp63 maintains the proliferative potential of basal keratinocytes in embryonic epidermis**

During the early stages of skin morphogenesis, epidermal keratinocytes undergo massive proliferation in order to maintain adequate cell coverage of the quickly growing embryo. Since only small patches of the epidermis were present in the skin surface of the ΔNp63<sup>gfp/gfp</sup> embryos, we wondered whether the proliferative potential of the epidermis was compromised. We examined ΔNp63<sup>gfp/gfp</sup> skin samples for expression of the proliferation marker Ki67 from two distinct stages when the cells are normally undergoing rapid proliferation (E13.5 and E16.5). Although both the ΔNp63<sup>gfp/gfp</sup> and wild-type epidermis showed Ki67-positive nuclear staining, a dramatic reduction in Ki67 expression was evident in the ΔNp63<sup>gfp/gfp</sup> skin (quantified in supplementary material Fig. S8A,B, top and middle). These data thus demonstrate that the proliferative potential of keratinocytes is reduced in the absence of ΔNp63, which is consistent with previous knockdown studies in cell culture (Truong et al., 2006).

Given the hypoplastic nature of the ΔNp63<sup>gfp/gfp</sup> skin, we wondered whether this phenotype was also affected by alterations in the rate of cellular apoptosis. Immunostaining with an antibody recognizing cleaved caspase 3 revealed no evidence of enhanced apoptosis in the skin of ΔNp63 knockout animals at E13.5 or E16.5 (supplementary material Fig. S8A,B, bottom).

**Loss of components of the extracellular matrix and cellular junctions in the absence of ΔNp63**

One of the key components necessary for proper epithelial formation and integrity is the proper formation of the extracellular matrix (ECM)-rich basement membrane (Fuchs and Raghavan, 2002). The basement membrane is generated from the cells of the basal layer, which are responsible for the production, secretion and assembly of this important structure. Given the lack of a mature stratified epidermis in our mutants, we wondered whether there was a defect in the formation of the basement membrane in these animals. We examined the expression of a panel of protein markers
important, we found several prominent basal ECM proteins, including laminin α5 and collagen IV, to be severely reduced in the ΔNp63<sup>−/−</sup> mice as compared with control animals (Fig. 4). We also found reduced levels of nidogen, heparan sulfate proteoglycan core protein (HSPG; Hspg2 – Mouse Genome Informatics), β1-integrin and E-cadherin in the mutant animals as compared with control littermates (Fig. 4). Importantly, similar results were obtained with sections from both the dorsal and ventral surfaces of the skin (data not shown) in the ΔNp63<sup>−/−</sup> animals, as compared with the controls, suggesting no differences between these anatomical regions. A quantification of the mRNA expression levels for a subset of these ECM genes (supplementary material Fig. S6B) matched the corresponding protein expression profiles.

In addition to the ECM, cell-cell adhesion junctions play an important role in maintaining epidermal integrity and homeostasis (Green et al., 2010). Given the disorganized appearance of the epidermis of the ΔNp63<sup>−/−</sup> animals, we investigated the status of hemidesmosome and desmosome junctions, which are crucial components of the cell-substrate and cell-cell adhesion machinery, respectively, in the skin. We found reduced staining for integrins α6 and β4 in both the dorsal and ventral surfaces of the ΔNp63<sup>−/−</sup> skin, suggesting that hemidesmosomes were compromised (Fig. 5). We next examined the expression profiles of several desmosomal components, including plakophilin 2, which is normally expressed in the basal layer, and desmoglein 1 and desmoglein 2, which are expressed in the suprabasal layers (Kottke et al., 2006). As shown in Fig. 5, at E15.5 we discovered a dramatic reduction in desmoglein 1 expression and moderate reductions in desmoglein 2 in both the dorsal and ventral surfaces of the skin of ΔNp63<sup>−/−</sup> animals as compared with control mice. However, we did not observe appreciable differences in plakophilin 2 staining between ΔNp63<sup>−/−</sup> and wild-type skin. These results were further substantiated by examining the corresponding mRNA levels by qRT-PCR (supplementary material Fig. S6C). Taken together, our data indicate that in the absence of ΔNp63 there is a generalized failure of keratinocytes to properly form the ECM, desmosome and hemidesmosome junctions. It is likely that these broad defects, in combination with the reduction in cellular proliferation, contribute to the loss of epithelial cell integrity and impaired skin development observed in our ΔNp63 mutant animals.

**Loss of ΔNp63 is associated with alterations in the Notch signaling pathway**

We next asked whether the epidermal differentiation phenotype observed in the ΔNp63<sup>−/−</sup> animals is associated with changes in Notch signaling given its importance in mammalian epidermal differentiation and the well-established molecular link between p63 and Notch (Blanpain et al., 2006; Laurikkala et al., 2006; Nguyen et al., 2006; Watt et al., 2008; Williams et al., 2011). Furthermore, an accelerated epidermal differentiation phenotype similar to that observed in the E15.5 ΔNp63<sup>−/−</sup> skin has been reported in animals with targeted deletion of Hes1, a crucial Notch effector (Moriyama et al., 2008). We began our initial investigation by examining the gene expression levels of various components of the Notch signaling pathway by qRT-PCR at E14.5 and E15.5 in ΔNp63<sup>−/−</sup> and control embryos (supplementary material Fig. S6D). In the absence of ΔNp63, the expression of several components of the Notch signaling pathway was reduced, including Hes1, Jag1 and Notch1, a trend that was particularly evident in E15.5 embryos. Interestingly, several of these genes have previously been identified as direct transcriptional targets of p63 (Dotto, 2009; Nguyen et al., 2006; Sasaki et al., 2002; Yang et al., 2006). However, to our surprise, we also observed elevated levels of Notch2, Notch3 and Rbpj mRNAs in ΔNp63<sup>−/−</sup> embryos, suggesting that the loss of ΔNp63 has opposing effects on the expression of individual Notch receptors and related genes within this pathway. To examine whether these changes occurred specifically in the skin epidermis, we performed immunofluorescence staining on dorsal skin sections of E15.5 control and ΔNp63<sup>−/−</sup> mice. Interestingly, we found reduced levels...
of Hes1 and Notch1 in the ΔNp63<sup>gfp/gfp</sup> skin sections, whereas suprabasal Notch2 and Notch3 staining was stronger than in control animals (Fig. 6). These data confirm our qRT-PCR observations and reveal that the loss of ΔNp63 results in altered expression levels of various components of the Notch signaling pathway.

Whereas the relationship between p63 and Notch1 has been well established by prior studies, the link between p63 and Notch2 and Notch3 has not received much attention, particularly in the context of keratinocyte development and differentiation (Nguyen et al., 2006). The elevated levels of Notch2 and Notch3 in ΔNp63<sup>gfp/gfp</sup> animals prompted us to carefully investigate genomic data sets generated by the ENCODE project (Myers et al., 2011) and p63 ChIP-seq experiments performed with human keratinocytes (Kouwenhoven et al., 2010). We specifically focused on regulatory regions that were in an open chromatin confirmation and contained histone modification signatures associated with regulatory elements. More importantly, because no such data are currently available from mouse, we also focused on regulatory regions that were evolutionarily conserved between human and mouse.

Whereas none of the potential regulatory elements surrounding the Notch2 gene met such strict criteria (data not shown), one region within intron 2 of Notch3, as identified by ChIP-seq experiments, was worthy of closer examination (Fig. 7A). This region in normal human epidermal keratinocytes (NHEKs) is likely to be in an open chromatin conformation as suggested by DNase-seq data. Concomitantly, there is also enrichment for chromatin modification marks that are typically associated with enhancers [H3K4me1 and H3K4me2 (Barski et al., 2007; Bernstein et al., 2005; Birney et al., 2007; Heintzman et al., 2007)] and active regulatory regions [H3K9ac and H3K27ac (Bernstein et al., 2005; Heintzman et al., 2009)] (Fig. 7A). Closer examination of this DNA region revealed high sequence conservation between human and mouse, as shown by the Genome Vista alignment (Fig. 7B) and the presence of two closely spaced potential p63 binding motifs (data not shown). To test whether p63 can bind to the corresponding site of the mouse Notch3 gene, we performed ChIP followed by qPCR experiments with cross-linked DNA isolated from mouse keratinocytes grown in culture. p63-bound chromatin was immunoprecipitated with an anti-ΔNp63 antibody [RR14 (Romano et al., 2006)] and an anti-p63 antibody that recognizes the alpha isoforms of p63 (H129). Quantitative PCR was then performed with primers spanning the p63 binding sites within this region. In both of the p63 immunoprecipitations, we found strong enrichment of the putative p63 response segment in the Notch3 gene relative to the IgG control after normalization to a random genomic locus (Fig. 7C). These data strongly suggest direct binding of ΔNp63 to a critical regulatory region within intron 2 of Notch3.

We extended our studies to the murine Rbpj gene because it was consistently upregulated in our qRT-PCR analysis of ΔNp63<sup>gfp/gfp</sup> animals. One evolutionarily conserved element was identified in a region 3' of Rbpj, which was marked with distinctive chromatin states typical of regulatory elements (supplementary material Fig. S9A). ChIP experiments performed in mouse keratinocytes using the RR14 and H129 antibodies revealed specific enrichment of this region, providing evidence of ΔNp63 directly regulating this gene (supplementary material Fig. S9B).
Collectively, our studies, coupled with those from prior investigations, support the notion that p63 directly regulates various components of the Notch signaling pathway and suggest that these cumulative effects of altered Notch signaling in part contribute to the accelerated keratinocyte differentiation phenotype observed in ΔNp63<sup>frfr</sup> animals.

**DISCUSSION**

In the p63 knockout animals generated thus far, the expression of either TA or both the TA and AN isoforms has been ablated, leaving the specific role of ΔNp63 unexplored. Interestingly, recent conditional knockout studies have revealed that TAp63 is dispensable for embryonic skin development and differentiation, in agreement with its weak expression in skin under physiological conditions (Guo et al., 2009; Su et al., 2009; Suh et al., 2006). By contrast, AN isoforms are by far the most abundant p63 proteins in keratinocytes and accumulating data suggest that the AN isoforms might mediate a significant portion of the biological function of p63. To date, however, the specific role of ΔNp63 has never been directly addressed. Here, using a genetic knockout mouse model that specifically targets ΔNp63, we provide irrefutable evidence that, during embryonic development, the loss of ΔNp63 isoforms results in a profound block in the development of stratified epithelia coupled with severe aplasia of multiple ectodermal appendages and limb defects. These broad developmental defects very much resemble the previously described phenotypes of pan-p63 knockouts, thus putting to rest the debate surrounding the importance of the ΔNp63 isoform.

Our thorough analysis of the ΔNp63-null phenotype allowed us to revisit important questions concerning the nature, extent and the likely cause of the p63-null skin phenotype – controversial issues that are still being debated (Crum and McKeon, 2010; Senoo et al., 2007; Shalom-Feuerstein et al., 2011; Wolff et al., 2009). For example, a recent re-examination of the two pan-p63 knockout mice strains concluded that the embryonic epithelia do not in fact suffer from a non-regenerative epidermal phenotype, but rather fail to develop beyond the ectodermal stage (Shalom-Feuerstein et al., 2011). Our analysis, as supported by the examination of specific markers, reveals that in the absence of ΔNp63 the epithelium encasing the embryo is in an altered developmental state. In certain pockets there are keratinocytes that retain expression of the simple epithelial marker K8 as late as E18.5, a clear sign of a failure in the K8/K18-to-K5/K14 switch that normally occurs early in embryogenesis during the process of commitment to an epidermal fate. Interestingly, a subset of ΔNp63-null keratinocytes co-expresses K8 and K5, confirming that these cells are indeed stuck in an intermediate state of identity crisis. We also find distinct areas of the ΔNp63-null skin where not only basal markers, including K5 and AP-2α, are expressed, but also the keratinocytes can differentiate as evident by the appearance of spinous markers such as K10 and Gata3. We surmise that the ΔNp63-null keratinocytes can progress through the terminal differentiation program, as judged by the expression of loricrin, filaggrin and involucrin. To reconcile such a wide range of distinct cellular fates observed in both the ΔNp63-null and pan-p63-null skin, it is tempting to suggest a unifying model whereby the absence of p63 results in both progenitor cell exhaustion and epidermal commitment failure of the skin keratinocytes.

We are particularly intrigued by the observation that in the ΔNp63-null animals the isolated pockets of keratinocytes show an accelerated and precocious differentiation program. Indeed, at E15.5, ΔNp63-null keratinocytes already express terminal differentiation markers, which do not appear before E16.5 in wildtype counterparts. Surprisingly, this phenotype has not been described thus far for any of the previously generated p63 knockout animals, as confirmed by our own analysis of one of the pan-p63-null mutants that lack both TAp63 and ΔNp63. This suggests that there is a complex functional interplay between the ΔNp63 and TAp63 isoforms and that an imbalance might influence developmental processes. This functional interaction and transcriptional cross-talk is also likely to extend to other p53 family members, which share a large number of target genes and are intimately interlinked through direct physical heteromerization and cross-regulation (Dotsch et al., 2010). The premature differentiation of the ΔNp63-null skin is reminiscent of the epidermal phenotype of Hes1-null animals and, at first glance, is expected given the differentiation-promoting effects of Notch (Moriyama et al., 2008; Nguyen et al., 2006). However, when we probed further into the molecular mechanisms underlying this phenotype, we found reduced expression levels of several genes involved in Notch signaling, including Notch1, Hes1 and Jag1. By contrast, ΔNp63 loss leads to elevated levels of Notch2, Notch3 and Rbpj transcripts in the embryo and increased suprabasal immunostaining of Notch2 and Notch3 in E15.5 skin. Our ChIP experiments using mouse keratinocytes confirmed binding of ΔNp63 to regulatory genomic regions of Notch3 and Rbpj and suggest that these genes are under repressive control of ΔNp63. We posit that p63 modulates Notch activity in a complex fashion, with both activating and repressive

**Fig. 6. Effects of ΔNp63 loss on components of the Notch signaling pathway.** Dorsal skin sections at E15.5 reveal increased expression levels of Notch2 and Notch3, whereas Hes1 and Notch1 appear modestly reduced, in ΔNp63<sup>frfr</sup> compared with wild-type mice. Insets show higher magnification views. Sections are co-stained for K5 and counterstained with DAPI. Scale bars: 37.5 μm.
attributes that are dependent on cell type context and the differentiation state. Furthermore, we postulate that the hyperactive suprabasal Notch signaling mediated by increased Notch2 and Notch3 and the canonical Notch mediator Rbpj might be the underlying trigger for the accelerated keratinocyte differentiation program in ΔNp63gfp/gfp animals.

Given the wide range of signaling pathways that intersect with p63 function, and the fact that ΔNp63 can act both as a transcriptional repressor and activator for a large number of genes, it is difficult to pinpoint the primary molecular cause of the underlying skin phenotype. One specific aspect that we have examined here is the effect of ΔNp63 loss on cell-cell and cell-substratum adhesion. Our investigations revealed a severe reduction in laminin α5 and collagen IV expression, two crucial basal extracellular membrane proteins. We also observed reduced levels of other important components of the basement membrane, including nidogen and HSPG. In addition to a failure of the ΔNp63-null keratinocytes to form a proper ECM, there is an apparent deficiency of these cells to maintain proper desmosome and hemidesmosome junctions. Interestingly, we found the hemidesmosome junctions, which serve as the major cell surface attachment sites for cell-substratum contacts, to be severely compromised. Our findings are in good agreement with prior studies that found integrins α6 and β4, two major components of the hemidesmosome junctions in the skin, to be direct transcriptional targets of ΔNp63 (Carroll et al., 2006). Indeed, we suspect a wide cohort of the genes that encode basement membrane and cell-cell adhesion molecules to be direct targets of p63, a notion that is well supported upon thorough examination of the growing p63 target database. The loss of keratinocyte cell organization and integrity and their apparent inability to form functional cell-cell and cell-substratum contacts offer a likely explanation for the fragile nature of the clusters of ΔNp63-null epidermal cells that are particularly prominent at later stages of embryonic development.

Although there is no denying the crucial role that p63 plays in epithelial development, the relative contributions and roles of the ΔN and TA isoforms have not been conclusively established in a...
defined genetic system. The generation and characterization of the ΔNp63gfpΔp63 animals as reported in this study finally puts to rest a lingering debate that has clouded the field, establishing ΔNp63 as the key p63 isoform in epidermal development and differentiation. The ΔNp63gfpΔp63 knock-in animals should provide a valuable asset for future investigations of the transcriptional control mechanisms that dictate epithelial cell fate, lineage decisions and stem/progenitor functions.

Acknowledgements
We thank Drs Caterina Missero and Lee Ann Garrett-Sinha for their thoughtful comments on the manuscript. We are especially grateful to Irene Kulik for excellent technical assistance and to Kenji Unno for help with TAp63 staining.

Funding
This work was supported in part by grants from the National Institutes of Health [R01AR049238 to S.S., R01CA154358 to T.K.] Deposited in PMC for release after 12 months.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at http://dev.biologists.orglookup/suppl?doi=10.1242/dev.071191+/DC1

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