The carboxy-terminus of p63 links cell cycle control and the proliferative potential of epidermal progenitor cells

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ABSTRACT

The transcription factor p63 (Trp63) plays a key role in homeostasis and regeneration of the skin. The p63 gene is transcribed from dual promoters, generating TAp63 isoforms with growth suppressive functions and dominant-negative ΔNp63 isoforms with opposing properties. p63 also encodes multiple carboxy (C)-terminal variants. Although mutations of C-terminal variants have been linked to the pathogenesis of p63-associated ectodermal disorders, the physiological role of the p63 C-terminus is poorly understood. We report here that deletion of the p63 C-terminus in mice leads to reduced proliferative capacity of epidermal progenitor cells. Notably, unlike the ectodermal malformation and hypoplasia, accompanied by a reduced physiological role of the p63 C-terminus is poorly understood. We previously identified specific mechanisms that control their fate are not well understood. By alternative splicing, the p63 gene generates at least three C-terminus variants, termed Cα, Cβ and Cγ, for both the TAp63 and ΔNp63 isoforms (Senoo et al., 2007). Indeed, p63-null mice display severe defects in epithelial development (Mills et al., 1999; Yang et al., 1999). Genetic studies suggest that mutations in p63 contribute to the pathogenesis of a wide range of ectodermal dysplasias (EDs), characterized by ectodermal malformations and hypoplasias (Celli et al., 1999; Koster, 2010; Rinne et al., 2007; van Bokhoven and McKeon, 2002), and we have recently noted decreased p63 expression in chronic equine laminitis in which the proliferative epidermal layers appear dysplastic (Carter et al., 2011). Thus, it is clear that p63 plays a key role in both the normal physiology and pathophysiology of the epidermis.

INTRODUCTION

Adult stem cells are capable of long-term self-renewal and differentiation, enabling their use as an important therapeutic approach in regenerative medicine (Green, 2008; Pellegrini et al., 2009; Rama et al., 2010). Epidermal stem cells are essential for maintaining homeostasis and regeneration of the skin, but specific mechanisms that control their fate are not well understood. (Bickenbach et al., 2006; Blampain and Fuchs, 2009; Ghadially, 2012; Kaur, 2006; Watt and Jensen, 2009). We previously identified p63 (Trp63), a homolog of the tumor suppressor p53 (Trp53) (Osada et al., 1998; Schmale and Bamberger, 1997; Senoo et al., 1999; Trink et al., 1998; Yang et al., 1998), and showed that p63 expression in chronic equine laminitis in which the proliferative epidermal layers appear dysplastic (Carter et al., 2011). Thus, it is clear that p63 plays a key role in both the normal physiology and pathophysiology of the epidermis.

The p63 gene is transcribed from dual promoters, generating TAp63 isoforms that contain a transactivation domain with growth suppressive functions (Guo et al., 2009) and dominant-negative ΔNp63 isoforms that lack this domain and exhibit opposing oncogenic properties (Keyes et al., 2011). Studies on isoform-specific knockout (KO) mice revealed that loss of ΔNp63 leads to the identical epidermal hypoplasia observed in p63-null mice (Romano et al., 2012). As ΔNp63 is the predominant isoform expressed in epidermal progenitor cells, including stem cells and transit-amplifying cells, ΔNp63 appears to play the major role in maintaining the proliferative potential of epidermal progenitors (Senoo, 2013). However, ΔNp63-specific KO mice also show an early onset of terminal differentiation (Romano et al., 2012) that is not observed in p63-null mice, suggesting that ΔNp63 normally counterbalances TAp63-driven differentiation and the consequent reduction in proliferative potential of epidermal stem cells. Notably, although TAp63-specific KO mice show grossly normal epithelial development, their isolated epidermal progenitor cells show reduced proliferative capacity in vitro (Su et al., 2009). These results suggest that TAp63 opposes ΔNp63 function, thereby preventing a premature reduction in proliferative potential. Thus, it is likely that p63 function reflects a cooperative effect between TAp63 and ΔNp63 isoforms (Candi et al., 2006; Truong et al., 2006; Zhang et al., 2014).

Whereas the amino (N)-terminal functions of p63 are relatively well studied, carboxy (C)-terminal functions in vivo are poorly understood. By alternative splicing, the p63 gene generates at least three C-terminus variants, termed Cα, Cβ and Cγ, for both the TAp63 and ΔNp63 isoforms (Yang et al., 1998). Notably, Cα uniquely harnesses the sterile α-motif (SAM) domain (p63SAM), which is a protein-protein interaction domain (Qiao and Bowie, 2005; Thanos and Bowie, 1999), and the transcription inhibitory (TI) domain (p63TI) (Serber et al., 2002). The significance of Cα is evident from genetic studies of p63-associated EDs, showing that mutations in either the p63SAM or p63TI domain or a complete absence of Cα/β but not Cγ cause ankyloblepharon-ectodermal defects-cleft lip/palate (AEC) syndrome, ectodactyly-ectodermal dysplasia-clefting (EEDC) syndrome and limb-mammary syndrome (LMS) (Barrow et al., 2002; Celli et al., 1999; Rinne et al., 2009; van Bokhoven et al., 2001). A recent study has shown that a point...
mutation in the p63\textsuperscript{SAM} domain reduces the number of epidermal progenitor cells (Ferone et al., 2012), highlighting the significance of Cα in regulating stem cell properties. However, how Cα exerts its function and the relative contribution of Cα in TAp63 and ΔNp63 isoform functions remain largely unknown.

A precise coupling between cell proliferation and differentiation is required during embryonic development and self-renewal of adult tissues. The cyclin-dependent kinase (CDK) inhibitor p21\textsuperscript{Waf1/Cip1} is required during embryonic development and self-renewal of adult isoform functions remain largely unknown.

Generation of mice lacking the C-terminus of p63

To further investigate the global function of the p63\textsuperscript{SAM} and p63\textsuperscript{TI} domains, we have generated mutant mice lacking Cα/β by gene targeting and found that homozgyous mutant (referred to here as p63\textsuperscript{C−/−}) mice show multiple phenotypes including ectodermal hypoplasia, limb malformation and orofacial clefting. We further demonstrate that mice with p63 C-terminus deficiency show reduced cell cycle progression and enhanced p21\textsuperscript{Waf1/Cip1} expression in epidermal progenitor cells, leading to their decreased proliferative capacity. Although the function of p63 is complex owing to the existence of multiple isoforms as well as inter- and intramolecular interactions, our present study shows that loss of Cα both promotes transcriptional activity of TAp63 and reduces the dominant-negative activity of ΔNp63 in the control of p21\textsuperscript{Waf1/Cip1} expression. Based on these data, we propose that p63 links cell cycle control and proliferative potential of epidermal progenitor cells through C-terminus-dependent mechanisms that balance TAp63 and ΔNp63 isoform functions.

RESULTS

Generation of mice lacking the C-terminus of p63

The SAM and TI domains of p63 are encoded by exons 12-14 of the p63 gene (Fig. 1A). To generate mice lacking these two domains, we deleted exon 12 of p63 by gene targeting (supplementary material Fig. S1). This strategy allowed us to delete both p63\textsuperscript{SAM} and p63\textsuperscript{TI} from Cα while leaving the Cγ isoform intact, as it is encoded by alternative exon 10 (Fig. 1A). As Cα and Cβ share exon 12, these mice also lack full-length p63β isoforms. We confirmed that expression of both full-length Cα and Cβ was absent in homozgyous mutant (p63\textsuperscript{C−/−}) mice, whereas expression of Cγ was similar between p63\textsuperscript{C−/−} and the wild-type (WT) control (Fig. 1B).

To analyze alternative splicing at the C-terminus resulting from the deletion of exon 12, we sequenced the fragments amplified from p63\textsuperscript{C−/−} epidermal cell cDNA (Fig. 1B,C). Our data show that the major transcript was encoded by exon 11 spliced to exon 13 (termed Cα'), while a minor transcript resulted from splicing of exon 11 to exon 14 (termed Cβ'). In both transcripts, stop codons appear shortly after the splicing sites by frameshift, resulting in the addition of only one and eight amino acids after exon 11, respectively (Fig. 1C; supplementary material Fig. S2). These Cα' and Cβ' isoforms in p63\textsuperscript{C−/−} mice were collectively referred to as the ΔC isoform (Fig. 1D).

To determine whether targeting of exon 12 influences p63 gene promoter activities, we performed qPCR using TAP63- and ΔNp63-specific primers (Fig. 1E). Our data show that relative expression of TAP63 and ΔNp63 was unchanged in p63\textsuperscript{C−/−} mice compared with WT littermates, indicating that the manipulation of the p63 locus at the distal region does not affect p63 gene promoter activities.

We next investigated p63 protein expression in p63\textsuperscript{C−/−} mice by western blot (Fig. 1F). As ΔNp63α is the predominant p63 isoform expressed in WT epidermal cells, the major p63 isoform detected in p63\textsuperscript{C−/−} keratinocytes likely represents the ΔNp63 isoform lacking Cα (hereafter referred to as ΔNp63ΔC). Together, these data demonstrate that p63\textsuperscript{C−/−} mice lack both p63\textsuperscript{SAM} and p63\textsuperscript{TI} domains from Cα without significant changes to either p63 gene isoforms.
promoter activity or p63γ isofrom expression. Thus, p63C−/− mice are useful for elucidating the function of Cα/β in the context of both the TAp63 and ΔNp63 isoforms.

**Craniofacial and skeletal phenotypes in p63C−/− mice**

Heterozygous mutant (p63C−/+ ) mice were grossly normal and fertile. Although homozygotes were born at near Mendelian ratios (p63C−/+ :p63C−/−:p63C+/− =27.9:43.7:28.4, n=197) they died shortly after birth due to a significant loss of the skin, resulting in dehydration and neglect by mothers. Although this neonatal lethality is similar to that observed in p63-null mice (Mills et al., 1999; Yang et al., 1999), we noted that craniofacial defects in p63C−/− embryos were less significant than those in p63-null mice (Fig. 2A). Whereas all p63-null mice show both cleft lip and cleft palate, some p63C−/− mice (35%, n=23) lack cleft lip although cleft palate is still evident (Fig. 2B). As described above, p63C−/− mice show more advanced limb development than p63-null mice, suggesting that epidermal cells in p63C−/− embryos are still capable of supporting epithelial-mesenchymal interactions. Indeed, macroscopic examination revealed that, whereas p63-null embryos are translucent and devoid of epidermis by embryonic day (E) 17.5 (supplementary material Fig. S3), these data indicate that, in contrast to the p63-null condition, p63Cα proteins support the initial development of limbs but that alterations in p63 C-terminus function restrict further progression of limb development.

**Epidermal phenotypes in p63C−/− mice**

Normal development of limbs requires a close interplay between the ectoderm and underlying mesenchyme (Johnson and Tabin, 1997). As described above, p63C−/− embryos show more advanced limb development than p63-null mice, suggesting that epidermal cells in p63C−/− embryos are still capable of supporting epithelial-mesenchymal interactions. Indeed, macroscopic examination revealed that, whereas p63-null embryos are translucent and devoid of epidermis by embryonic day (E) 17.5 (supplementary material Fig. S3), skin covered 60-80% of the body of p63C−/− embryos at the same developmental stage, although it was severely eroded (Fig. 3A). Microscopic analysis using Hematoxylin and Eosin (H&E) staining showed that p63C−/− mouse epidermis is hypoplastic and significantly thinner than that of WT littermates and lacks hair follicle development (Fig. 3B,C).

To characterize epidermal cell differentiation in p63C−/− embryos, we stained sections of the epidermis with markers of basal cells [cytokeratin 14 (K14; also known as keratin 14, Krt14) and K5], a marker of the spinous layer (K10) and markers of terminally differentiated epithelium (loricrin and involucrin) (Eckert and Green, 1986; Freedberg et al., 2001; Hohl et al., 1991; Ivanyi et al., 1989). Our data show that marker expression was comparable in p63C−/− and WT epidermis (Fig. 3D), suggesting that the hypoplastic epidermis in p63C−/− mice is not due to a block in differentiation. Notably, however, staining of E15.5 epidermis with anti-p63 antibody showed that, whereas a large number of p63-positive cells were found in the suprabasal layer of WT epidermis, the majority of p63-positive cells in p63C−/− mice remained in the basal layer (Fig. 3D, top). This hypoplastic characteristic of progenitor cells was also seen in other epithelial tissues in p63C−/− mice (supplementary material Fig. S4). For instance, developing eyelids remained thinner and unfused in p63C−/− mice, whereas the eyelids of WT littermates are densely populated by p63-positive epithelial progenitor cells and are completely fused at the midline. Likewise, dental epithelial progenitors expand and develop into a large number of outer and inner enamel epithelia of the molar in WT.
embryos, but they remain in only a few cell layers in p63C−/− mice. Together, these data indicate that epithelia remain hypoplastic in many tissues of p63C−/− mice due to reduced expansion of epithelial progenitor cells.

We have shown previously that phosphorylation of serine residues at position 66/68 of ΔNp63 is associated with expansion of epithelial progenitor cells (Suzuki and Senoo, 2012, 2013). To determine whether S66/68 phosphorylation of ΔNp63 is reduced in p63C−/− epidermal cells, we co-stained E15.5 epidermis with anti-pan-p63 and anti-S66/68 antibodies (Fig. 3E). Our data show that, whereas virtually all basal cells in WT epidermis exhibit relatively high levels of S66/68 phosphorylation, the majority of p63-positive cells in p63C−/− mice were low to negative for this marker (Fig. 3E,F), suggesting that epithelial progenitor cells in p63C−/− mice are less proliferative than those in the WT control. We extended our analysis of S66/68 phosphorylation to tongue epithelium, another epithelial tissue showing hypoplasia in p63C−/− mice, and obtained similar results (supplementary material Fig. S5A).

To determine whether increased apoptosis promotes the hypoplastic epidermis observed in p63C−/− mice, we stained sections prepared from p63C−/− and WT embryos with anti-cleaved caspase 3 antibody. However, no evidence of enhanced apoptosis in p63C−/− mouse epidermis was detected at E15.5 (supplementary material Fig. S6), indicating that increased apoptosis does not account for the hypoplastic epidermis of p63C−/− mice. Collectively, these data demonstrate that the C-terminus of p63 plays a crucial role in regulating the expansion of epithelial progenitor cells but is dispensable for their differentiation and survival.

**Decreased proliferative capacity of epithelial progenitor cells in p63C−/− mice**

Having excluded obvious alterations in differentiation and apoptosis, we next investigated whether hypoplastic epidermis in p63C−/− embryos is accompanied by decreased proliferation of epithelial progenitor cells. We first investigated the proliferation of p63C−/− epidermal progenitor cells in vivo by injecting BrdU into pregnant mice of p63C+/− intercrosses 2 h prior to sacrifice, followed by sectioning and staining of WT and p63C−/− mouse epidermis with anti-p63 and anti-BrdU antibodies (Fig. 4A). Notably, our data show that the relative frequency of p63+ BrdU+ epithelial progenitor cells was significantly lower in p63C−/− mice than in WT littermates at E15.5 (Fig. 4B), indicating that the C-terminus of p63 is crucial for the proliferation of epithelial progenitors.

Next, to determine the long-term consequence of loss of the p63 C-terminus for the proliferative capacity of epithelial progenitor cells, we isolated epithelial cells from E14.0 WT and p63C−/− embryos and cultured them at clonal density in a defined keratinocyte medium. Our data show that, in contrast to the densely packed and highly proliferative WT keratinocytes, p63C−/− keratinocytes became large and flattened within the first few days, with reduced growth as determined by Ki67 staining and high expression of involucrin (Fig. 4C,D). These data are consistent with our in vivo observation that p63C−/− epidermal cells undergo premature differentiation with a reduced ability to propagate into suprabasal cell populations (Fig. 3D,E). Accordingly, extended cultivation of these keratinocytes showed that, whereas WT cells grew into large clones, p63C−/− cells did not yield any macroscopically visible clones at 3 weeks of culture as determined by Rhodamine B staining (Fig. 4E). Together, these data indicate that p63C−/− epithelial progenitor cells have a reduced proliferative capacity compared with their WT counterparts.

**Increased expression of p21Waf1/Cip1 in epithelial progenitor cells of p63C−/− mice**

As epithelial progenitor cells in p63C−/− mice showed reduced cell cycle progression (Fig. 4), we next investigated expression of the CDK inhibitor p21Waf1/Cip1, a well characterized downstream target of p63 (Su et al., 2013) with growth suppressive function in keratinocytes (Di Cunto et al., 1998; Missero et al., 1996). We first stained sections of E15.5 WT and p63C−/− epidermis with anti-p21Waf1/Cip1 antibody and found that, unlike the WT control, p63C−/− epidermis had a high frequency of epithelial cells with high p21Waf1/Cip1 expression (Fig. 5A). We confirmed that
expression of p21Waf1/Cip1 was significantly higher in p63C−/− mice than in WT littermates by western blot using whole epidermal cell extracts (Fig. 5B). Expression of p53, a strong activator of p21Waf1/Cip1 expression, was undetectable in epidermal cells from either p63C−/− or WT mice (supplementary material Fig. S7), indicating that increased expression of p21Waf1/Cip1 in p63C−/− mice is not due to deregulated p53 expression. We also showed that expression of two other closely related CDK inhibitors, p27Kip1 (Cdkn1b) and p57Kip2 (Cdkn1c), was similar in p63C−/− and WT mice (Fig. 5B). As p57Kip2 is a known p63 target gene (Beretta et al., 2005; Su et al., 2009), these data indicate that alterations to p63 C-terminus function selectively influence a subset of p63 target genes.

To determine whether the increase in p21Waf1/Cip1 expression in p63C−/− mice is specific to the skin epithelium or is common to other epithelia, we performed additional staining of sections of WT and p63C−/− mouse esophagus, a tissue that expresses high levels of p63, like the epidermis, the esophageal epithelium in p63C−/− mice exhibited high p21Waf1/Cip1 expression, whereas cells with detectable p21Waf1/Cip1 levels in WT mice were rare (Fig. 5C). These data suggest that an increase in p21Waf1/Cip1 expression in p63C−/− mice is common among different epithelial cell types that normally express p63.

Finally, our data further show that esophageal epithelial cells in p63-null mice had no detectable p21Waf1/Cip1 expression (Fig. 5C), indicating that increased expression of p21Waf1/Cip1 in p63C−/− mice does not result from the loss of function of p63 but is instead likely to reflect altered functions of p63AC isoforms. Collectively, these data suggest that p63AC proteins, in the context of either the TAp63 or ΔNp63 isoform or a combination of both, promote p21Waf1/Cip1 expression in p63C−/− epithelia.

Ca balances TAp63 and ΔNp63 functions in the control of p21Waf1/Cip1 expression

To gain insight into how deletion of the p63 C-terminus promotes p21Waf1/Cip1 expression, we first compared the transcriptional activity of TAp63AC with that of other TAp63 isoforms in the non-small cell lung carcinoma cell line H1299 using a minimal p21Waf1/Cip1−luciferase reporter construct that harbors the p63/p53 binding site (Suzuki et al., 2012). H1299 cells are ideal for our study as they have a homozygous deletion of the p53 gene with no detectable endogenous p63 expression and thus induce p21Waf1/Cip1 gene expression in response to ectopic p63 (Zeng et al., 2002).

Unlike TAp63AC, our data show that TAp63AC upregulates p21Waf1/Cip1 reporter activity to a similar extent as transcriptionally active TAp63β and TAp63γ isoforms (Fig. 6A). We also show that the stimulation of the p21Waf1/Cip1 reporter reflects endogenous p21Waf1/Cip1 expression at both mRNA and protein levels (Fig. 6B,C). This raises the possibility that the loss of the p63 C-terminus enhances the total activity of TAp63 in stimulating p21Waf1/Cip1 gene expression in p63C−/− mice as compared with the WT control. However, as
ΔNp63α normally counterbalances TAp63-dependent transcriptional activity in a WT setting (Yang et al., 1998), we next determined whether ΔNp63αC has an impaired dominant-negative activity against TAp63γ and TAp63αC, two transcriptionally active TAp63 isoforms expressed in p63C−/− mice. Indeed, our data show that, whereas ΔNp63αC inhibited both TAp63β- and TAp63γ-dependent transcriptional activation of the p21Waf1/Cip1 reporter in a dose-dependent manner, ΔNp63αC lacked such ability against both the TAp63γ and TAp63αC isoforms (Fig. 6D). We confirmed that changes in p21Waf1/Cip1 reporter activity correlated with those of endogenous p21Waf1/Cip1 expression at both the mRNA and protein levels (Fig. 6E,F). We conclude that α balances TAp63 and ΔNp63 isoforms in the control of p21Waf1/Cip1 expression.

DISCUSSION

Studies of mice deficient for p63 have shown that it is a key regulator of ectodermal development (Mills et al., 1999; Yang et al., 1999). In humans, mutations in the p63 gene (TP63) cause at least seven syndromic and non-syndromic disorders, each with different combinations of ectodermal dysplasia, split hand/foot malformation and orofacial clefting (Rinne et al., 2007). Our data show that p63C−/− mice exhibit all of these characteristics (Figs 2 and 3), indicating that the C-terminus is central to p63 function during embryonic development. α is frequently mutated or deleted in AEC patients and indeed p63C−/− mice show AEC-like severe skin erosion and fragility (Fig. 3A). However, the presence of abnormalities in orofacial and limb development in p63C−/− mice indicates that they will also be useful for assessment of additional p63-associated disorders. Specifically, relatively rare mutations that lead to the generation of p63C-like proteins are found in patients with EEC syndrome and LMS, both of which exhibit the three p63-associated characteristics mentioned above (Celli et al., 1999; van Bokhoven et al., 2001). Our studies reveal that loss of α leads to the dysregulation of both TAp63 and ΔNp63 isoform functions (Fig. 6). Although these changes can be explained by the loss of function of the T1 domain, the SAM domain also plays a key role in regulating p63 function in vivo (Ferone et al., 2012). Thus, it will be important to determine the specific roles of the p63SAM and p63T1 domains and whether a mutation in one domain affects the function(s) of the other during development. Answering these questions will contribute to our understanding of the molecular mechanisms underlying p63-associated EDs.

As a member of the p53 family of transcription factors, p63 is likely to carry out its function through controlling the expression of genes that are crucial for stem cell properties (Yang et al., 2006). p21Waf1/Cip1 is one of the best-characterized downstream targets of p63, and the growth-suppressing function of this CDK inhibitor is well established. However, how p21Waf1/Cip1 expression is regulated in epidermal progenitor cells in vivo remains an open question (Ohtani et al., 2007). In the present study, we found that p63 can regulate p21Waf1/Cip1 gene expression through a mechanism that is dependent on the function of the p63 C-terminus. Our data suggest that the p63 C-terminus regulates the functional equilibrium between TAp63 and ΔNp63 isoforms in controlling target gene expression (Fig. 6). However, as p63C−/− epidermal cells show significantly elevated levels of p21Waf1/Cip1 but not of the closely related p63 target p57Kip1 (Beretta et al., 2005; Su et al., 2009), our studies indicate that the p63 C-terminus influences only a subset of downstream target genes. Further investigation of the mechanistic basis for this effect will provide significant insight into stem cell regulatory pathways governed by p63.

Notably, p21Waf1/Cip1 KO mice have an increased number of epidermal progenitor cells with high proliferative potential compared with WT mice (Topley et al., 1999), indicating that p21Waf1/Cip1 plays a key role in restricting the proliferative capacity of epidermal progenitors. The function of p21Waf1/Cip1 in stem cell maintenance is operative in other cell types, including hematopoietic and neuronal progenitor cells (Cheng et al., 2000; Kippin et al., 2005). However, as these cells do not express p63, it would be interesting to investigate whether cell type-specific regulators of stem cell maintenance (Lessard and Sauvageau, 2003; Molofsky et al., 2003; Park et al., 2003) are involved in the regulation of p21Waf1/Cip1 expression. We show in the present study that epidermal progenitor cells derived from p63C−/− mice have reduced proliferative capacity with enhanced p21Waf1/Cip1 expression (Figs 4 and 5). Thus, a connection has been established between p63 C-terminus functions and cell cycle progression in control of the proliferative potential of epidermal progenitor cells. However, as we have reported previously, a complete absence of all p63 isoforms also reduces the proliferative capacity of epidermal progenitor cells (Senoo et al., 2007). Thus, it is likely that alternative pathways exist by which p63 regulates the proliferative potential of epidermal progenitor cells independently of p21Waf1/Cip1.
function. Deciphering gene programs altered by p63 C-terminus deficiency versus total loss of p63 function will provide further insight into the molecular mechanisms that regulate proliferative potential.

Finally, cell proliferation and differentiation are coupled during embryonic development and in self-renewing tissues of the adult, including the skin. Skewing this balance toward proliferation can cause tumorigenesis (Owens and Watt, 2003; Perez-Losada and Balmain, 2003). In this regard, p63 and p21Waf1/Cip1 might share common tumor-suppressive machineries. For instance, loss of p21Waf1/Cip1 cooperates with Ras oncogene (Hras) in the induction of aggressive and relatively undifferentiated tumors in vivo (Missiro et al., 1996). Conversely, Ras-driven transformation of p53-deficient cells is counteracted by ectopic expression of TAp63 in a p21Waf1/Cip1-dependent manner (Guo et al., 2009), whereas the ΔNp63 isoform cooperates with Ras to promote tumor-initiating stem-like proliferation (Keyes et al., 2011). It will be important to determine the molecular mechanisms by which p63 and loss of p21Waf1/Cip1 cooperate in the regulation of genes required for the proliferative potential of epidermal progenitor cells versus those favoring malignant transformation. Thus, an exciting area of future study is the close interplay between the classical cell cycle and stem cell pathways in the control of self-renewal and tumorigenesis of epithelia.

MATERIALS AND METHODS

Animals
All procedures were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania. For staged embryos, the day of the vaginal plug was designated E0.5.

Generation of p63C−/− mice
Genomic p63 DNA was isolated from BAC clones (Children’s Hospital Oakland Research Institute, Oakland, CA, USA). The targeting vector consists of a 3.2 kb HindIII/HincII homologous fragment containing exon 11 followed by a PGK-Neo5 cassette flanked by loxP sites and a 2.3 kb homologous fragment containing exon 13, and a PGK-TK cassette at the 3′-end. Gene targeting was performed in V6.5 (129B6 F1 hybrid) embryonic stem cells (ESCa) (Eggan et al., 2001). Two correctly targeted ESC clones were microinjected into C57BL/6 blastocysts to obtain chimeric mice. Male chimeras were mated to female C57BL/6 mice to achieve embryonic stem cells (ESCs) (Eggan et al., 2001). Two correctly targeted mice were crossed with Actβ-Cre transgenic mice in a FVB/N background (Jackson Labs) to remove the Neo cassette (ΔNeo allele). Genotyping of progeny was performed by PCR using tail DNA and the Neo and ΔNeo allele and Cre transgene primers listed in supplementary material Table S1. Mutant mouse colony was maintained by intercrossing heterozygous mutant mice in the absence of the Actβ-Cre transgene

Generation of p63-null mice
Mice deficient for all p63 isoforms were generated and maintained in the same genetic background as described above. The 5′ homologous arm included a 5.0 kb fragment extending to the end of the coding sequences of p63 exon 5, ligated in frame to a start codon (ATG)-deleted EGFP expression cassette derived from pEGFP-N1 (BD Biosciences). A 4.2 kb SphI fragment harboring p63 exons 8 and 9 was used as the 3′ homologous arm. Mutant p63 alleles were identified by PCR using tail DNA and the primer pairs listed in supplementary material Table S1.

Southern blot
Genomic DNA was digested with restriction enzymes, separated in 0.8% agarose gels and transferred to nylon membranes (Sigma-Aldrich). To confirm homologous recombination at the C-terminus of p63, 1.4 kb and a 560 bp genomic fragments outside the targeting vector were radiolabeled with [32P]dCTP (GE Healthcare) using the Rediprime II DNA Labeling System (GE Healthcare) and used as probes. To confirm homologous recombination at the p63 DNA-binding domain, 600 bp and 350 bp genomic DNA fragments outside the targeting vector were radiolabeled and used as probes.

Skeletal staining
Skeletal staining was performed as previously described (Wallin et al., 1994) with some modifications. Briefly, embryos were eviscerated and the skin removed, then fixed in 95% ethanol for 5 days and then in 100% acetone for an additional 2 days at room temperature. Specimens were incubated in staining solution (0.015% Alcian Blue and 0.005% Alizarin Red S in 90% ethanol and 5% acetic acid) for 3 days at 37°C. Subsequently, specimens were rinsed in water and kept in 1% KOH for 2 days at room temperature, followed by clearing in a series of 0.8% KOH/20% glycerol, 0.5% KOH/50% glycerol and 0.2% KOH/80% glycerol for 3-5 days each. After complete clearing, specimens were stored in 100% glycerol.

Antibodies
The primary antibodies used in this study were mouse anti-p63 (4A4, Santa Cruz Biotechnology; 1:250), rabbit anti-phosphorylated p63 (64901, corresponding to S66/68 of ΔNp63α, Cell Signaling Technology; 1:100), mouse anti-p21Waf1/Cip1 (F-5, Santa Cruz Biotechnology; 1:50 for immunohistochemistry and immunofluorescence, and 1:300 for western blotting), mouse anti-p27Kip1 (p27/Kip1, BD Biosciences; 1:2000), rabbit anti-p57Kip2 (BNP1-61640, Novus Biologicals; 1:2000), mouse anti-p53 (PAH421, EMD Millipore; 1:2000), rabbit anti-mouse Ki67 (NB600-1209, Novus Biologicals; 1:50), rat anti-BrdU (ICR1, Abcam; 1:100), rabbit anti-cleaved caspase 3 (5A1E, Cell Signaling Technology; 1:200), rabbit anti-cytokeratin 5 (EP1601Y, Abcam; 1:4000), mouse anti-cytokeratin 14 (LL001, Santa Cruz Biotechnology; 1:300), mouse anti-cytokeratin 10 (RSKE60, Abcam; 1:400), rabbit anti-loricrin (PRB-145, Covance; 1:1000), mouse anti-involucrin (SY5, NeoMarkers; 1:1000), mouse anti-pan-cytokeratin (AE1/ AE3, Thermo Fisher Scientific; 1:200), rabbit anti-pan-cytokeratin (PAS21985, Thermo Fisher Scientific; 1:100) and mouse anti-tubulin α (12G10, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA; 1:500). Secondary antibodies used for immunofluorescence were Alexa 488-goat anti-mouse IgG, Alexa 488-goat anti-rabbit IgG, Alexa 594-goat anti-mouse IgG and Alexa 594-goat anti-rabbit IgG (Molecular Probes). When a combination of mouse and rat primary antibodies was used, pre-adsorbed DyLight 594-goat anti-mouse IgG and pre-adsorbed DyLight 488-goat anti-rat IgG antibodies (Abcam) were used to avoid cross-reactivity. For DAB staining and western blot, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (KPL) and HRP-conjugated goat anti-rabbit IgG (Cell Signaling Technology) were used.

Immunohistochemistry
Tissues were fixed in 10% buffered formalin overnight at 4°C. Paraffin-embedded sections were cut into 6 μm sections and antigen retrieval was performed by incubating the slides in 0.01 M citric acid buffer (pH 6.0) at 95°C for 20 min. Sections were then blocked with 10% fetal bovine serum (FBS) (Atlanta Biologicals) at room temperature for 30 min. Cultured epidermal cells were fixed in 10% buffered formalin for 10 min and permeabilized in 0.1% Triton X-100 in phosphate buffered saline (PBS) for 5 min, followed by blocking at room temperature for 30 min. Subsequently, samples were incubated with primary antibodies overnight at 4°C. After three washes in PBS containing 0.1% Tween 20, samples were incubated with either fluorescently labeled secondary antibodies for 1 h at room temperature followed by counterstaining with Hoechst 33342 (Molecular Probes), or HRP-conjugated secondary antibodies followed by detection of the antigens using a DAB reagent kit (KPL).

BrdU labeling and detection
Pregnant mice received an intraperitoneal injection of 100 mg/kg body weight 5-bromo-2′-deoxyuridine (BrdU) (BD Biosciences). Two hours after injection, embryos were collected, fixed, dehydrated and embedded in paraffin blocks. Sections were treated with 2 N HCl for 10 min at 37°C and
washed three times in PBS, followed by treatment with 0.025% trypsin for 15 min at 37°C. Staining was then performed as described (Suzuki and Senoo, 2013).

Western blot
Cells were lysed in a protein lysis buffer containing 50 mM Tris-HCl (pH 6.8), 5% 2-mercaptoethanol, 2% sodium dodecyl sulfate, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride and 1 mM sodium orthovanadate. After boiling for 5 min, whole cell extracts were subjected to 8-12% SDS-PAGE followed by western blot.

Cell culture
Human embryonic kidney 293T (HEK293T) cells and human lung carcinoma H1299 cells were grown in DMEM containing 10% FBS, 10 µg/ml penicillin and 100 µg/ml streptomycin in a humidified chamber at 37°C with 5% CO₂. Primary epidermal cells were isolated from E14.0 mouse embryos. Briefly, embryos were collected into 1.5 ml microcentrifuge tubes and incubated in 0.25% trypsin at 37°C for 20 min with gentle agitation every 5 min without disrupting embryo structures. Cell suspensions were transferred into new tubes, neutralized and centrifuged at 1000 rpm (180 g) for 5 min. Cells were then suspended in Cm-57 basal keratinocyte medium supplemented with growth supplements (Celltice) and 1×10⁵ cells were seeded onto 35 mm dishes coated with Matrigel (BD Biosciences) and incubated at 37°C with 5% CO₂. After 3 weeks of culture, epidermal cell clones were fixed in 10% buffered formalin and visualized by staining with 1% Rhodamine B (Sigma-Aldrich).

qPCR and RT-PCR
Total RNA was purified using Trizol reagent (Life Technologies) and 1 µg was reverse transcribed using the ProtoScript M-MuLV First Strand cDNA Synthesis Kit (New England BioLabs). Real-time PCR was performed using SYBR Green Master Mix (Life Technologies) in an ABI 7900 HT machine. Relative expression of each gene to the housekeeping gene Rps18 or GAPDH was determined by the ΔΔCt method. Primer sequences used for qPCR are listed in supplementary material Table S1.

To analyze alternative splicing at the p63 C-terminus in p63C-Δmice, epidermal cell cDNAs were amplified with a primer pair Exon 11-forward and Exon 14-reverse. PCR products were separated in 1.2% agarose gels and DNA fragments corresponding to Cα (494 bp) and Cβ (400 bp) were purified and directly sequenced with primer 5′-GTGAGCCAGCTTATCAACC-3′. Note that although the exon 11-forward/Exon 14-reverse primer pair is also able to detect Cβ transcripts, they were below detection level in WT mice, probably owing to low expression and competition with Cα transcripts. Therefore, a second primer pair was used to detect Cβ transcripts: Exon 6-forward and Exon 12/14-reverse (852 bp). Cγ transcripts were amplified using Exon 6-forward and Exon 10-reverse (648 bp). Primers are listed in supplementary material Table S1.

Construction of p63 plasmids
All p63 cDNAs used in this study were of human origin. ΔNp63 isoforms (α, β and γ) were myc-tagged at the N-terminus and subcloned into the pcDNA3 vector (Life Technologies), while TAp63 isoforms (αm, βm and γm) were 2× HA-tagged at the N-terminus and subcloned into the pcDNA3.1 vector (Life Technologies). To generate p63ΔC isoforms, site-directed mutagenesis was performed using ΔNp63α in pUK21 as template with primer pair 5′-TTTCTTAAGCAGGTGGGCTGTC-3′ and 5′-TGTTGGGCCTCCTACATGCAAGA-3′, followed by self-ligation and direct sequencing to verify correct amplification. Mutant C-terminus was excised as a BsrGI/Xhol fragment and replaced with that in TAp63α and ΔNp63α.

Transient transfection and reporter assay
H1299 and HEK293T cells were seeded into 12-well plates at 1×10⁵ cells/well the day before transfection. Transfections were performed using SuperFect transfection reagent (Qiagen). Twenty-four to 36 h later, cells were harvested and whole cell extracts and total RNA were prepared for western blot and qPCR, respectively. For reporter assays, H1299 cells were transiently transfected with p63-expressing plasmids along with 0.5 µg minimal p21(Waf1/Cip1) reporter and 0.3 µg Renilla luciferase vector pRL-TK (Promega). The minimal p21(Waf1/Cip1) reporter was constructed by inserting the 72 bp HindIII-SacI fragment harboring the p63/p53 binding site (Suzuki et al., 2012) of WWP-luc (El-Deiry et al., 1993) into the pGL3-Basic vector (Promega). Firefly luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activities were normalized to Renilla luciferase control activities. In co-transfection experiments, pcDNA3 was used as carrier so that all wells received the same total amount of DNA.

Statistical analysis
Student’s t-tests were performed and P<0.05 was considered statistically significant.

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Competing interests
The authors declare no competing financial interests.

Author contributions
D.S. designed and performed the majority of the experiments and analyzed data. N.A.L., R.S., D.S. and M.S. generated mutant mice. M.S. conceived the project, performed experiments, analyzed data and wrote the manuscript with input from coauthors.

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Supplementary material
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References


