Rescue of key features of the *p63*-null epithelial phenotype by inactivation of *Ink4a* and *Arf*

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Mice lacking *p63* cannot form skin, exhibit craniofacial and skeletal defects, and die soon after birth. The *p63* gene regulates a complex network of target genes, and disruption of *p63* has been shown to affect the maintenance of epithelial stem cells, the differentiation of keratinocytes, and the preservation of the adhesive properties of stratified epithelium. Here, we show that inactivation of *p63* in mice is accompanied by aberrantly increased expression of the *Ink4a* and *Arf* tumour suppressor genes. In turn, anomalies of the *p63*-null mouse affecting the skin and skeleton are partially ameliorated in mice lacking either *Ink4a* or *Arf*. Rescue of epithelialization is accompanied by restoration of keratinocyte proliferative capacity both *in vivo* and *in vitro* and by expression of markers of squamous differentiation. Thus, in the absence of *p63*, abnormal upregulation of *Ink4a* and *Arf* is incompatible with skin development.

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Introduction

p63, a *p53* family member, has multiple roles in the development of stratified epithelium (Mills *et al*, 1999; Yang *et al*, 1999; Koster and Roop, 2004; Ihrie *et al*, 2005; Carroll *et al*, 2006, 2007; Koster *et al*, 2007; Senoo *et al*, 2007). Deletion of *p63* in the mouse results in a generalized loss of stratified epithelium because of apparent defects in the maintenance of stem cell proliferative capacity, keratinocyte differentiation, and cell–cell adherence (Mills *et al*, 1999; Yang *et al*, 1999; Senoo *et al*, 2007). These functions are controlled by a vast

transcriptional network of genes regulated by *p63* (Vigano *et al*, 2006; Yang *et al*, 2006; Vigano and Mantovani, 2007), but the specific molecular mechanisms governing each of these processes remain largely ill defined.

The *Ink4a*-*Arf* locus encodes two tumour suppressor proteins, *p16^{Ink4a}* and *p19^{Arf}*, which induce apoptosis, cell-cycle arrest, or senescence by regulating the activities of the retinoblastoma protein and *p53*, respectively (Sherr and McCormick, 2002). Repression of *Ink4a*-*Arf* gene expression helps to maintain haematopoietic stem cell (HSC) and neural stem cell (NSC) function (Jacobs *et al*, 1999; Molofsky *et al*, 2003, 2005; Park *et al*, 2003; Bruggeman *et al*, 2005; Bracken *et al*, 2007). The *Ink4a*-*Arf* locus is epigenetically silenced by polycomb complexes in adult bone marrow-derived HSCs. Inactivation of the polycomb component, *Bmi1*, results in the apparent loss of HSC self-renewal capacity and leads to bone marrow aplasia in the first few weeks after birth, but this defect is largely alleviated when *Bmi1*-null mice are crossed onto an *Ink4a*-*Arf*-null background (Jacobs *et al*, 1999). Recent investigations indicate that inactivation of *Ink4a*-*Arf* increases the self-renewal capacity of multiple progenitor cells rather than affecting the frequency of HSCs from which they are derived (Akala *et al*, 2008). Self-renewal and differentiation of presumptive NSCs in *Bmi1*-null mice and in young mice lacking the *Hmga2* transcriptional regulator are also partially restored with loss of *Ink4a* or *Arf* (Molofsky *et al*, 2003, 2005; Nishino *et al*, 2008). Conversely, increased expression of *Ink4a*-*Arf* as mice age progressively limits the repopulating efficiencies of HSCs and NSCs and the proliferative capacities of B-lymphoid and pancreatic islet cells (Janzen *et al* 2006; Krishnamurthy *et al*, 2006; Molofsky *et al*, 2006; Signer *et al*, 2008). Although derepression of both products of the *Ink4a*-*Arf* locus each contribute to these phenotypes, *p16^{Ink4a}* and *p19^{Arf}* exert differential, cell type-specific effects during haematopoiesis, neurogenesis, and lymphopoiesis (Bruggeman *et al*, 2005; Molofsky *et al*, 2005, 2006; Miyazaki *et al*, 2008; Signer *et al*, 2008).

Negative regulation of *Ink4a*-*Arf* gene expression may be essential for tissue stem cell renewal and lineage commitment, but the putative repressors that might serve this function in many lineages have yet to be identified. We reasoned that *p63*, which is expressed at high levels in the basal layer of the epidermis (Senoo *et al*, 2007; Fuchs, 2008), might similarly have a function in suppressing *Ink4a*-*Arf* gene expression. Indeed, somatic deletion of *p63* in the mouse embryo from E8.5 onward or conditional inactivation of *p63* in cultured keratinocytes induces a senescence phenotype characterized in part by upregulation of *p16^{Ink4a}* (Keyes *et al*, 2005). However, shRNA-mediated knockdown of *p16^{Ink4a}* was insufficient to bypass senescence induced by *p63* ablation implying that other regulators must contribute to the observed phenotype. We, therefore, interbred *p63*^{+/-} mice with animals lacking either *Ink4a* or *Arf* to generate mice lacking both *Ink4a* and *p63* or *Arf* and *p63*. Mice with these compound gene deficiencies form stratified epithelium,

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and their keratinocytes exhibit a renewed capacity to proliferate and differentiate both *in vivo* and *in vitro*. These findings show that p63 negatively regulates the *Ink4a-Arf* locus and that abnormal upregulation of these genes in the absence of p63 inhibits skin development.

Results

Loss of *Arf* or *Ink4a* rescues features of the phenotype of *p63*^{-/-} mice

Mice lacking functional p63 are born with defects in the epidermis, have cleft lip and palate, and craniofacial abnormalities (Mills et al, 1999; Yang et al, 1999). These mice die within hours after birth because of desiccation resulting from the lack of a stratified epidermis covering the mouse. We hypothesized that loss of *Arf* or *Ink4a* could lead to the formation of a complete epithelium in the *p63*^{-/-} mice; therefore, *Arf*^{GFP/GFP} (Zindy et al, 2003) and *Ink4a*^{-/-} (Sharpless et al, 2001) mice were intercrossed with the *p63*^{+/-} mice (Yang et al, 1999) to first obtain double heterozygous mice (*Arf*^{GFP/+}; *p63*^{+/-} and *Ink4a*^{+/-}; *p63*^{+/-}). Subsequent matings by intercrossing compound heterozygous mice gave rise to *Arf*^{GFP/GFP}; *p63*^{-/-} and *Ink4a*^{-/-}; *p63*^{-/-} mice, respectively. The *Arf*^{GFP/GFP} knock-in allele is functionally null but yields green fluorescent cells when the *Arf* promoter is activated (Zindy et al, 2003). Macroscopic examination of 10 neonates of each genotype showed the presence of skin covering 80–95% of the body, but these animals did not survive after birth because of maternal neglect. Day 18.5 embryos (E18.5) of *Arf*^{GFP/GFP}; *p63*^{-/-} and *Ink4a*^{-/-}; *p63*^{-/-} mice had also developed a thin layer of skin covering 85–90% of the body (Table I). Twenty *Arf*^{GFP/GFP}; *p63*^{-/-} and 20 *Ink4a*^{-/-}; *p63*^{-/-} E18.5 embryos had dull and opaque skin with partially sloughed stratified epithelium (Figure 1C and D), compared with the shiny and translucent appearance of the *p63*^{-/-} embryos devoid of such epithelium (Figure 1B). In addition, both double mutant embryos exhibited rudimentary limbs and a long tail (Figure 1C and D). Strikingly, 16 of 20 *Arf*^{GFP/GFP}; *p63*^{-/-} and 18 of 20 *Ink4a*^{-/-}; *p63*^{-/-} mice had normal facial features including a complete palate, lip, and fully developed ears (Figure 1C and D) (Table I).

A more rigorous analysis of the skeletal structures of 10 embryos of each genotype was therefore undertaken (Figure 1E–P) (Table II). In the *Arf*^{GFP/GFP}; *p63*^{-/-} and *Ink4a*^{-/-}; *p63*^{-/-} embryos, the scapula, humerus, and ulna were well developed and in most cases comparable in size to wild-type embryos (Figure 1I–L) (Table II). In addition, rudimentary digits were apparent in both *Arf*^{GFP/GFP};

p63^{-/-} and *Ink4a*^{-/-}; *p63*^{-/-} embryos (Figure 1K and L), whereas the forelimbs were significantly smaller in the *p63*^{-/-} embryos (Figure 1J) (Table II). Similarly to the *p63*^{-/-} embryos, the *Arf*^{GFP/GFP}; *p63*^{-/-} and *Ink4a*^{-/-}; *p63*^{-/-} embryos did not develop hindlimbs; however, their pubic bones were comparable in size to those of wild-type mice and were fused (compare Figure 1O and P with Figure 1M) (Table II), a process that did not occur in the *p63*^{-/-} mice (Figure 1N). Thus, inactivation of *Ink4a* and *Arf* contributes independently to partially reverse cardinal features of the *p63*-null phenotype.

Loss of *Arf* or *Ink4a* leads to formation of a uniform layer of skin in the absence of p63

From macroscopic examination of double mutant embryos, we reasoned that loss of *Arf* or *Ink4a* alone had rescued epidermal defects detected in the *p63*^{-/-} embryos. The epidermis of the embryos was characterized by microscopic analysis using haematoxylin and eosin (H&E) staining and immunofluorescence (IF) for the epidermal markers, keratin 5 (K5), keratin 14 (K14), keratin 10 (K10), keratin 15 (K15), and filaggrin (Figure 2; Table I). The H&E-stained sections of E18.5 *Arf*^{GFP/GFP}; *p63*^{-/-} embryos showed an intact basal layer of epithelium surrounding the mice (Figure 2C). Twenty *Arf*^{GFP/GFP}; *p63*^{-/-} embryos had an average coverage of 85% as determined by H&E staining. The extent of rescue in the 20 *Ink4a*^{-/-}; *p63*^{-/-} embryos examined was even more striking with the appearance of structures that resembled hair follicles (Figure 2D; Table I); 10 ± 2 rudimentary hair follicles per centimetre were apparent in all 20 of the *Ink4a*^{-/-}; *p63*^{-/-} embryos as compared with 64 ± 4 cm in the wild type and none in the *Arf*^{GFP/GFP}; *p63*^{-/-} and *p63*^{-/-} mice (Table I). These data highlight the fact that inactivation of *Ink4a* and *Arf* exerts differential phenotypic effects in the *p63*-null background.

The structure of the epithelium was further analysed by IF using antibodies to K5 and K14, markers of the basal layer, K10, a marker of the spinous layer, and filaggrin, a marker of the granular layer of the epithelium. Immunofluorescence was also performed using markers for the basement membrane, collagen IV (Figure 2I–L), and for cellular adhesion, E-cadherin (Figure 2M–P). All of these markers are completely absent or are only weakly detected in the *p63*^{-/-} mice (Mills et al, 1999; Yang et al, 1999). Consistent with earlier published data (Yang et al, 1999), 3 of the 20 *p63*^{-/-} embryos had an average of 2% K5-positive or 5% K14-positive cells in patches above the dermis (Figure 2F, J, and N) (Table I). K5-positive and K14-positive cells in the *Arf*^{GFP/GFP}; *p63*^{-/-} and *Ink4a*^{-/-}; *p63*^{-/-} mice encircled the embryos (Figure 2G, H,

Table I Quantification of epithelial structures in day 18.5 embryos

Genotype	Embryos analysed	K5	K14	K15	K10	Filaggrin	Embryos w/hair follicle	Embryos w/cleft palate	Embryos w/forelimbs
Wild type	20	100%	100%	100%	100%	100%	20	0	20
<i>p63</i> ^{-/-}	20	2%	5%	0%	0%	0%	0	20	3
<i>Arf</i> ^{GFP/GFP} ; <i>p63</i> ^{-/-}	20	85%	85%	90%	75%	50%	0	4	18
<i>Ink4a</i> ^{-/-} ; <i>p63</i> ^{-/-}	20	85%	85%	100%	85%	75%	20	2	20
<i>p53</i> ^{-/-} ; <i>p63</i> ^{-/-}	6*	0%	0%	0%	0%	0%	0	6	0

Shown are the numbers of embryos analysed per genotype. Numbers in columns with % represent the mean from 20 embryos analysed per group. The asterisk (*) indicates that only 6 of 20 *p53*^{-/-}; *p63*^{-/-} embryos could be analysed at E18.5; the remaining 14 embryos died *in utero* at day 15.5.

Figure 1 Macroscopic analysis of embryos at day E18.5. (A–D) Illustrate images of embryos with their respective genotypes indicated above each panel; WT indicates wild type. The white arrow in (B) points to cleft lip and palate in *p63*^{−/−} embryo. White arrows in (C, D) designate strings of desquamated epithelium and forelimbs. (E–P) Show skeletal preparations from E18.5 embryos stained with Alcian Blue for cartilage and alizarin red for bone. Whole embryos (E–H) were of the following genotypes: (E), wild type; (F), *p63*^{−/−}; (G), *Arf*^{GFP/GFP}; *p63*^{−/−}; and (H), *Ink4a*^{−/−}; *p63*^{−/−}. Analysis of forelimbs (I–L) from (I), wild type; (J), *p63*^{−/−}; (K), *Arf*^{GFP/GFP}; *p63*^{−/−}; and (L), *Ink4a*^{−/−}; *p63*^{−/−} embryos. The arrowheads in (K) and (L) point to rudimentary digits. Designated bones include the scapula (s), humerus (h), radius (r), and ulna (u). Analysis of pubic bones and hindlimbs (M–P) from (M), wild type; (N), *p63*^{−/−}; (O), *Arf*^{GFP/GFP}; *p63*^{−/−}; and (P), *Ink4a*^{−/−}; *p63*^{−/−} embryos. Designated bones include the ilium (il), ischium (is), and pubic bone (p). A 2 mm scale bar is included in the corner of (P).

Table II Quantification of length of limbs in day 18.5 embryos

Genotype	Scapula	Humerus	Ulna	Pubic bone
Wild type	4.8 ± 0.2	4.4 ± 0.1	4.8 ± 0.2	3.9 ± 0.3
<i>p63</i> ^{−/−}	3.8 ± 0.1	0.5 ± 0.1	1.5 ± 0.2	2.6 ± 0.1
<i>Arf</i> ^{G/G} ; <i>p63</i> ^{−/−}	5.0 ± 0.1	1.5 ± 0.2	2.8 ± 0.3	3.9 ± 0.1
<i>Ink4a</i> ^{−/−} ; <i>p63</i> ^{−/−}	5.0 ± 0.1	2.8 ± 0.3	4.5 ± 0.3	3.9 ± 0.2

Shown are the mean ± s.d. measured in mm of the indicated skeletal structures. Measurements were taken from 10 embryos per genotype.

K, L, O and P) (Table I) and formed a multi-layer stratum basale. Double IF for K14 and collagen IV further showed a multi-layer stratum basale above the basement membrane marked by collagen IV in the *Arf*^{GFP/GFP}; *p63*^{−/−} and

Ink4a^{−/−}; *p63*^{−/−} embryos (Figure 2K and L). Immunostaining for K14 and E-cadherin showed cellular disorganization in the stratum basale and somewhat disrupted cell–cell adhesion in the double mutant embryos (Figure 2O and P). In addition, K10 and filaggrin staining were apparent, consistent with further differentiation of suprabasal cells (Figure 2S, T, W and X) (Table I). In the *Ink4a*^{−/−}; *p63*^{−/−} mice, the appearance of a multi-layer stratified epithelium, which stained positively for K5, K14, K10, and filaggrin (Figure 2H, L, P, T and X), was even more apparent, and hair follicle-like structures that stained positively for K15, a marker of epithelial stem cells residing in the hair follicle bulge (Liu *et al*, 2003), were also visible (Figure 2Y, AA, and BB). K15 staining was apparent in the stratum basale of the epidermis of *Arf*^{GFP/GFP}; *p63*^{−/−} and *Ink4a*^{−/−}; *p63*^{−/−} embryos (Figure 2AA and BB) indicating the presence of epidermal precursor cells,

Figure 2 Microscopic analysis of the epithelium of embryos at E18.5. Cross sections of skin stained with haematoxylin and eosin (A–D) and immunofluorescence signals using antibodies to keratin 5 (red) (E–H), keratin 14 (green) and collagen IV (red) (I–L), keratin 14 (green) and E-cadherin (red) (M–P), keratin 10 (red) (Q–T), filaggrin (red) (U–X), and keratin 15 (green) (Y–BB) and counterstained with DAPI (blue) are illustrated. The genotypes for each vertical set of panels are indicated at the top of the figure. Broken lines define the border between the dermis and epidermis.

as was evident in the developing epidermis of wild-type embryos (Figure 2Y).

To determine whether loss of *p53* could likewise rescue the *p63*^{−/−} phenotype, *p53*^{−/−};*p63*^{−/−} embryos were also analysed at E18.5. Fourteen of 20 *p53*^{−/−};*p63*^{−/−} embryos died *in utero* at day E15.5. Some had neural tube closure defects as has been reported earlier for *p53*^{−/−} mice (Sah *et al*, 1995). However, 6 of 20 embryos were viable at day E18.5 and had a similar phenotype to *p63*^{−/−} embryos (Table I), raising the possibility that rescue of the *p63*-null phenotype by *Arf* inactivation might be mediated, at least in part, through a p53-independent pathway.

Proliferation of *p63*^{−/−} keratinocytes is restored in the absence of *Arf* or *Ink4a*

Owing to grossly abnormal epithelialization in *p63*^{−/−} mice, it is difficult to explant and culture keratinocytes from these embryos. Indeed, when we attempted to culture keratinocytes on J2-3T3 feeder cells under conditions that normally allow expansion of clones from epidermal precursors (Barrandon and Green, 1987; Flores *et al*, 2000), cells could be extracted from the surface of *p63*^{−/−} embryos, but they did not proliferate (Koster *et al*, 2004). In contrast, when epidermis was isolated from *Arf*^{GFP/GFP};*p63*^{−/−} and *Ink4a*^{−/−};*p63*^{−/−} embryos at E18.5, recovered keratinocytes formed colonies much similar to those formed by wild-type keratinocytes

Figure 3 Keratinocyte cultures derived from embryos at E18.5. (A–E) Show light micrographs of keratinocytes cultured on J2-3T3 feeder cells with their respective genotypes indicated to the left of the panels. Black arrows indicate the positions of keratinocyte colonies. (F–J) Show immunofluorescence obtained with an antibody to keratin 5 (red), a marker of basal epithelial cells; cells were counterstained with DAPI (blue). (K) Indicates the proliferation rates of cultured keratinocytes of the following genotypes: wild type (WT, blue), *Arf*^{GFP/GFP} (*Arf*, red), *Ink4a*^{-/-} (*Ink4a*, yellow), *p63*^{-/-} (*p63*, green), *p53*^{-/-}; *p63*^{-/-} (brown), *Arf*^{GFP/GFP}; *p63*^{-/-} (*Arf*; *p63*, purple), and *Ink4a*^{-/-}; *p63*^{-/-} (*Ink4a*; *p63*, orange). (L) Computes the cumulative population doublings of keratinocyte cultures passaged every 5 days for seven passages. The average of results obtained with three independent keratinocyte lines each assayed in triplicate is shown. The genotypes are indicated as in (K).

(Figure 3A–E), and these stained positively with antibodies to the stratum basale marker, K5 (Figure 3F–J). However, the double null colonies were less compact than wild-type colonies (Figure 3C, E, H, and J), and the cells seemed to be less adherent to one another, based on their propensity to be more easily detached from the plate and dispersed by trypsinization. This feature is not surprising, given the discontinuous staining of E-cadherin in *Arf*^{GFP/GFP}; *p63*^{-/-} and *Ink4a*^{-/-}; *p63*^{-/-} embryos and that *p63* is known to regulate genes involved in cell adhesion (Ihrie *et al*, 2005; Carroll *et al*, 2006).

Although *p63*^{-/-} keratinocytes at passage-2 did not proliferate further, cells of all the other genotypes continued to accumulate over an 8-day time course (Figure 3K). The most rapidly expanding keratinocyte populations were those derived from the *Ink4a*^{-/-} and *Arf*^{GFP/GFP} embryos, but the *Arf*^{GFP/GFP}; *p63*^{-/-}, and *Ink4a*^{-/-}; *p63*^{-/-} keratinocytes divided as rapidly as wild-type keratinocytes. In addition to measuring their proliferative rates, we passaged the cultured keratinocytes every 5 days, and assessed their cumulative population doublings for seven passages (Figure 3L). The *p63*^{-/-} keratinocytes senesced at passage-1, whereas cells of

all other genotypes continued to proliferate on further passage. The wild-type keratinocytes senesced at passage-3, but the *Ink4a*^{-/-}, *Arf*^{GFP/GFP}, *Ink4a*^{-/-}; *p63*^{-/-}, and *Arf*^{GFP/GFP}; *p63*^{-/-} keratinocytes reached 12–14 accumulated population doublings by passage-7 (Figure 3L). Notably, in both assays, the *p53*^{-/-}; *p63*^{-/-} keratinocytes behaved similarly to *p63*^{-/-} keratinocytes (Figure 3K and L) providing further evidence that loss of *p53* does not rescue the ability of *p63*^{-/-} keratinocytes to proliferate. Taken together, these results show that loss of either *Arf* or *Ink4a* can rescue the proliferative defect of *p63*-deficient keratinocytes and that this rescue is not phenocopied by *p53* inactivation.

The ability of rescued epidermal cells to proliferate *in vivo* was determined by the incorporation of the thymidine analogue, bromodeoxyuridine (BrdU), into the DNA of the developing epidermis of wild type, *p63*^{-/-}, *Arf*^{GFP/GFP}; *p63*^{-/-}, and *Ink4a*^{-/-}; *p63*^{-/-} embryos at day E18.5 (Figure 4A–H). BrdU-positive cells could be detected in multiple layers of the epidermis in *Arf*^{GFP/GFP}; *p63*^{-/-} and *Ink4a*^{-/-}; *p63*^{-/-} embryos (Figure 4E–H). Consistent with the findings made with cultured keratinocytes, inactivation of *Ink4a* or *Arf* significantly rescued the proliferation of *p63*-null cells, with elimination of *Ink4a* having the greater effect and restoring the proliferative capacity of the cells to wild-type levels (Figure 4I).

p63 downregulates p19^{Arf} and p16^{Ink4a} to rescue the proliferation and differentiation defects of cells lacking p63

The failure of *p63*^{-/-} mice to generate proliferative keratinocytes together with the rescue of this process in *Arf*-null and *Ink4a*-null backgrounds suggests that p63 negatively regulates p19^{Arf} and p16^{Ink4a} expression. Presumably, in the absence of p63, the untimely expression of p16^{Ink4a} and p19^{Arf} inhibits the outgrowth of keratinocytes, leading to the profound defects in epidermal development. Indeed, upregulation of p16^{Ink4a} in cultured primary keratinocytes was observed earlier after conditional p63 ablation, but in this setting, shRNA-mediated knockdown of *Ink4a* expression did not bypass cellular senescence; p19^{Arf} expression was not studied (Keyes *et al*, 2005). We made use of the GFP reporter built into the *Arf*^{GFP/GFP} knock-in mouse. *Arf* expression is not detectable during normal mouse epithelial development but is induced when cells are explanted into culture (Zindy *et al*, 2003). Direct measurements of GFP fluorescence indicated that *Arf*^{GFP/GFP}; *p63*^{-/-} keratinocytes expressed significantly higher GFP levels than those detected in age-matched *Arf*^{GFP/GFP} keratinocyte cultures (Figure 5A–C). Most pertinent, GFP levels were significantly elevated in the epithelium of *Arf*^{GFP/GFP}; *p63*^{-/-} embryos when compared with those in *Arf*^{GFP/GFP} embryos at E18.5 (Figure 5D–F). Immunoblotting of lysates from cultured keratinocytes documented a two-fold increase in GFP protein expression in *Arf*^{GFP/GFP}; *p63*^{-/-} cells versus that in their *Arf*^{GFP/GFP} counterparts (Figure 5G, lanes 3 and 4). Strikingly, the difference in the level of p19^{Arf} expressed in wild type versus *p63*^{-/-} cells was much greater (Figure 5G, lanes 1 and 2) indicating that p63 suppressed p19^{Arf} expression. The p19^{Arf} protein levels were also elevated in MEFs lacking p53 (Figure 5G, lane 5) as reported earlier (Kamijo *et al*, 1998; Stott *et al*, 1998). Likewise, similar increases in the levels of p16^{Ink4a} were detected in the

Figure 4 *In vivo* proliferation of epidermal cells in embryos at E18.5. (A, C, E, G) Show cross sections of skin labeled with BrdU (brown) and counterstained with haematoxylin (purple). Black arrows indicate positive cells and blue arrows indicate cells that stain weakly for BrdU. (B, D, F, H) Show immunofluorescence staining of skin sections with BrdU (red) and counterstained with DAPI (blue). Genotypes of the embryos are as follows: wild type (A, B), *p63*^{-/-} (C, D), *Arf*^{GFP/GFP}; *p63*^{-/-} (E, F), and *Ink4a*^{-/-}; *p63*^{-/-} (G, H). White arrows indicate positive cells. Both were used to calculate the mitotic index shown in the bar graph in (I). The mitotic index is shown on the x-axis as the percentage of BrdU-positive cells on the surface of the epidermis.

absence of p63 also indicating that p63 downregulates p16^{Ink4a} expression (Figure 5H).

Quantitative real-time (qRT) PCR was performed on RNA extracted from embryonic skin containing the dermis and attached epidermal cells of embryos of the indicated genotypes (Figure 5I–K). Notably, the analysis of RNA expressed

Figure 5 Expression of p19^{Arf}, p16^{Ink4a}, and GFP in keratinocytes and stratified epithelium. (A–C) Illustrate GFP expression in keratinocytes of the indicated genotypes (top). Cells were counterstained with DAPI (blue). Representative colonies of cultured *Arf*^{GFP/GFP}; *p63*^{-/-} keratinocytes (B, C) were vividly fluorescent when compared (matched exposures) with cultures at the same passage derived from *Arf*^{GFP/GFP}; *p63*^{+/+} mice (A). (D–F) Show cross sections of stratified epithelium from E18.5 embryos stained with antibodies to GFP (red). DAPI (blue) was used as a counterstain. The respective genotypes correspond to those indicated above (A–C). Matched exposures are shown. (G, H) Illustrate immunoblotting analysis of selected proteins (indicated at the left) identified in detergent lysates of age-matched passage-2 keratinocytes (B) of the indicated genotypes (top). Passage-2 *p53*^{-/-} MEFs were used as a positive control (G). Actin was used as a loading control. (I–K) Show results from qRT-PCR of the indicated mRNAs (top) extracted from E18.5 dermis/epidermis of the indicated genotypes (bottom). GAPDH was used as an internal control.

in dermal/epidermal tissues taken directly from embryos bypasses any inductive effects of ‘culture shock’ seen with explanted cells propagated *in vitro*. Indeed, we found that levels of GFP RNA were 18-fold higher in *Arf*^{GFP/GFP}; *p63*^{-/-} versus *Arf*^{GFP/GFP} embryos (Figure 5I). Similarly, *Arf* levels were found to be 15-fold higher in *p63*^{-/-} compared with wild-type embryos (Figure 5J). Likewise, *Ink4a* RNA levels were increased in the absence of *p63*, although to a lesser extent (Figure 5K).

p63 directly represses *Ink4a* and *Arf* expression

Both the *Ink4a* and *Arf* promoters were found to have elements that match the p53 consensus binding site (Figure 6A and C). Chromatin immunoprecipitation (ChIP) analysis

using an antibody for p63 and primers to amplify the discovered sites by qRT-PCR identified p63-binding sites upstream of the *Ink4a* and *Arf* promoters (Figure 6). Two sites were identified in both promoters (Figure 6B and D). The sites bound by p63 on the *Ink4a* promoter were located between nucleotides -4835 and -4621 (Figure 6A and B[I]) and -1850 and -1539 (Figure 6A and B[III]). Other sites analysed within the *Ink4a* promoter from nucleotides -3996 and -3845 (Figure 6A and B [II-UP]), between -3632 and -3362 (Supplementary Figure 1A[II-DOWN]), and -1519 and -1157 (Supplementary Figure 1B[IV]) did not show p63 binding. The p63-binding sites identified on the *Arf* promoter reside between -1533 and -1331 (Figure 6C and D[I]) and between -1276 and -943 (Figure 6C and D[IIA and IIB]). Other

Figure 6 p63 binds to response elements located on the *Ink4a* and *Arf* promoters. (A) Illustrates two p53/p63 consensus binding sites in the *Ink4a* promoter upstream of exon 1 α . (B) Shows the percentage of total input DNA bound (DNA percentage bound) to p63. A graph for each region amplified (I-nucleotides -4835 to -4621, II-UP-nucleotides -3996 to -3845, and III-nucleotides -1850 to -1539) is shown. (C) Shows three p53/p63 consensus binding sites in the *Arf* promoter upstream of exon 1 β . (D) Shows the percentage of total input DNA bound (DNA percentage bound) to p63. A graph for each region amplified (I-nucleotides -1533 to -1331, IIA and IIB-nucleotides -1276 to -943, and UP denotes a site upstream of site (I) from nucleotides -1811 to -1622). Genotypes of keratinocytes analysed are indicated below each bar on the graph. Nucleotides shown in red are those that do not match the consensus. Nucleotides in green correspond to those found in the spacer region. If the spacer was greater than 5, N(X) is shown where X = number of nucleotides in the spacer region.

amplicons analysed upstream, -1811 and -1622 (Figure 6C and D[UP]), or in intervening sequences, -1417 and -1303 (Supplementary Figure 1C[INT]), were negative for p63 binding. As a positive control, ChIP analysis was performed using an antibody for p63 and primers to amplify known transcriptional targets of p63, *p21*, and *Perp*, by qRT-PCR (Supplementary Figure 2).

Transfection of the p63 isoforms in *Arf*^{GFP/GFP}; *p63*^{-/-} MEFs showed a marked repression of GFP protein and RNA levels (Figure 7A, C, and E) with Δ Np63 α and - γ having the greatest effect. Similarly, introduction of the p63 isoforms into wild type and *p63*^{-/-} MEFs repressed expression of *Arf* protein and mRNA (Figure 7B, D, and F). p63 can also repress *Ink4a*. *p63*^{-/-} MEFs express *Ink4a* mRNA and protein at high levels. Transfection of the p63 isoforms resulted in a reduction in *Ink4a* mRNA (Figure 7G). Although all isoforms of p63 repressed *Ink4a*, the Δ Np63 isoforms repressed to the greatest extent (Figure 7G).

These data, taken together, indicate that p63 directly represses *Ink4a* and *Arf* gene expression and that the ectopic expression of these genes when p63 is absent results in a failure to form stratified epithelium.

Discussion

The p63 protein regulates an extensive transcriptional network of genes required for the formation and integrity of epithelium in the skin and other organs, such as the breast, prostate, thymus, and urothelium (Mills *et al*, 1999; Yang *et al*, 1999). Complete inactivation of p63 globally compromises epithelial development and leads as well to craniofacial and limb deformities. However, it is particularly difficult to discern whether ablation of p63 affects epithelial stem cell self-renewal, lineage commitment, proliferation, keratinocyte differentiation, cell adhesion, or as seems likely, several of these functions.

The p63 gene encodes various isoforms that contain (TA) or lack (Δ N) a transactivation domain. The TAp63 α , TAp63 β , and TAp63 γ isoforms are the first to be expressed during embryogenesis and are required for the initialization of epithelial stratification (Koster *et al* 2004). In contrast, the Δ Np63 α isoform is the predominant species expressed in the epidermis, and it is detected in the bulge region of hair follicles and intermittently along the basal layer of the inter-follicular epidermis, two regions in which epidermal stem

Figure 7 p63 represses *Arf* and *Ink4a*. (A) Shows GFP expression in passage-2 *Arf^{GFP/GFP};p63^{-/-}* MEFs transfected with the indicated expression vectors (top). (B) Illustrates p19^{Arf} expression in passage-2 wild-type MEFs transfected with the indicated expression vectors (top). Both western blots in (A, B) were probed with a p63 antibody (4A4) specific for all isoforms of p63. The asterisk denotes a non-specific band. Actin was used as a loading control. (C, D) Show quantification of western blots, including the ones shown in (A, B), performed in triplicate. Actin was used as a loading control. (E–G) Show results from qRT-PCR of the indicated mRNAs (top) extracted from the specified MEFs transfected with the indicated expression vectors (bottom). GAPDH was used as an internal control. The asterisk denotes statistical significance ($P < 0.01$).

cells are known to reside (Clayton *et al*, 2007; Senoo *et al*, 2007; Fuchs 2008). Once developing epidermal cells become suprabasal, Δ Np63 α expression is extinguished, pointing to its possible involvement in gating stem cell proliferation (Fuchs, 2008). When Δ Np63 α synthesis is knocked down with shRNAs, cultured epidermal progenitors form smaller colonies with reduced rates of proliferation (Senoo *et al*, 2007). However, opinions differ as to the relative contributions of the various p63 isoforms in skin development (Candi *et al*, 2007) and as to whether the failure of the primitive ectoderm to differentiate in *p63*-null mice reflects a defect in stem cell self-renewal (Yang *et al*, 1999; Senoo *et al*, 2007) or an early block in epidermal differentiation (Mills *et al*, 1999; Keyes *et al*, 2005; Koster *et al*, 2007).

Here, we now provide evidence that germ line disruption of either *Ink4a* or *Arf* significantly alleviates the phenotypic consequences of *p63* ablation during embryonic development. Double mutant mice lacking *p63* and either *Ink4a* or

Arf produced K5-, K14-, K10-, K15-, and filaggrin-expressing epidermal cell sheets that encircled the embryos. Rescue was more impressive in the *Ink4a^{-/-};p63^{-/-}* mice, which also exhibited rudimentary hair follicle formation not detected in their *Arf^{-/-};p63^{-/-}* counterparts. However, the skin of these mice was fragile and was easily detached from the embryo as shown by discontinuous expression of E-cadherin. Given that p63 also regulates genes involved in cell adhesion (Ihrie *et al*, 2005; Candi *et al*, 2007), our findings suggest that loss of *Ink4a* or *Arf* only counters the initial proliferative defect of keratinocytes deficient in *p63* but not the additional consequences of *p63* inactivation that are manifested as the double null cells differentiate.

Primary keratinocytes explanted from *p63*-null embryos cannot be propagated in culture, but those derived from *Ink4a^{-/-};p63^{-/-}* or *Arf^{GFP/GFP};p63^{-/-}* embryos exhibited a proliferative capacity approaching those of their wild-type counterparts. Conversely, and consistent with the notion that

p63 negatively regulates *Ink4a* and *Arf* gene expression to facilitate skin development, expression of *Ink4a* and *Arf* mRNA, their encoded proteins, and of the product of the *Arf*^{GFP} allele were upregulated in cultured keratinocytes lacking p63. Strikingly, GFP was expressed *in situ* in the epidermis of genotypically *Arf*-null *Arf*^{GFP/GFP};p63^{-/-} embryos, arguing that induction of the *Arf* promoter was physiologically relevant and not simply because of explantation of primary keratinocytes into culture. Negative regulation of the *Ink4a-Arf* locus by p63 is reminiscent of the effects of Bmi-1, which has an essential role in the maintenance of stem cell function in HSCs and NSCs (Jacobs et al, 1999; Molofsky et al, 2003, 2005; Park et al, 2003; Bruggeman et al, 2005; Bracken et al, 2007) but has no such function during skin development.

p53 is also a potent negative feedback regulator of *Arf* gene expression (Kamijo et al, 1998; Stott et al, 1998), and yet despite a decade of investigation, there is still no evidence that p53 binds directly to *Arf* promoter sequences. We found that p63 can bind to potential response elements upstream of the transcriptional start sites of both *Arf* and *Ink4a*. These sequences conform to p53/p63 consensus binding sites that have been predicted to negatively regulate transcription (Johnson et al, 2001; Yang et al, 2006), raising the possibility that p63 acts as a direct repressor of the *Ink4a-Arf* locus.

p63 has been shown to have p53-dependent and -independent activities that promote cell survival and stem cell maintenance (Truong et al, 2006). Intriguingly, although *Arf* disruption in the mouse germ line partially rescues the p63-null epidermal phenotype, germ line inactivation of p53 exerts no such effects (Keyes et al, 2005; this paper). This implies that *Arf* ablation during early embryonic development affects a crucial p63-dependent but p53-independent function. Many p53-independent functions have been attributed to *Arf*, although much of the extant data remain controversial (Sherr, 2006). However, there is some evidence that *Arf* and p53 loss-of-function can contribute independently to the formation of carcinogen-induced squamous cell carcinomas (Kelly-Spratt et al, 2004). In contrast, cellular senescence induced by the acute inactivation of p63 in cultured primary keratinocytes was rescued when p53 expression was ablated by use of shRNA (Keyes et al, 2005). Thus, in the absence of p63, *Arf* inactivation may well exert both p53-independent and p53-dependent effects that are, respectively, manifested during early stages of skin development *in utero* or, differentially, in mature keratinocytes in which an *Ink4a-Arf*-dependent senescence programme is abruptly induced by somatic p63 disruption.

p63^{+/-} mice have a shortened lifespan and display many features of accelerated aging and declining fitness (Flores et al, 2005; Keyes et al, 2005). Whether the aging phenotype depends on accelerated expression of the *Ink4-Arf* gene cluster is unknown, although it is now well appreciated that p16^{Ink4a} and p19^{Arf} upregulation can induce age-dependent declines in the regenerative potential of various tissues (Janzen et al, 2006; Krishnamurthy et al, 2006; Molofsky et al, 2006; Signer et al, 2008). Unfortunately, though inhibition of *Ink4b-Arf-Ink4a* functions may counter the impact of advancing age on tissue stem cell function, cancer development, including the formation of malignant skin tumours (Krimpenfort et al 2007), is a consequence.

Materials and methods

Mouse husbandry

p63^{+/-} mice on (C57BL/6 × 129SvJae) background enriched for C57BL/6 (95%) (Yang et al, 1999; Flores et al, 2005) were intercrossed with *Ink4a*^{-/-} (Sharpless et al, 2001), *Arf*^{GFP/GFP} (Zindy et al, 2003) or p53^{-/-} (Jacks et al, 1994) mice to obtain compound mutant mice and embryos of the following genotypes: *Ink4a*^{-/-}; p63^{-/-}, *Arf*^{GFP/GFP};p63^{-/-}, and p53^{-/-};p63^{-/-}. Wild type and p63^{-/-} mice were also generated from each cross and analysed. All animal studies were approved by the Institutional Animal Care and Use Committee.

Histology and immunostaining of E18.5 embryos

Pregnant females at day 18.5 of gestation were injected three times at 1-h intervals with 100 µg of BrdU per gram of total body weight. Embryos were extracted 1 h later and fixed in 10% formalin at room temperature overnight, sectioned, and stained with H&E for microscopic analysis. For IF, paraffin-embedded sections were rehydrated in xylene and decreasing concentrations of ethanol. Sections were subjected to antigen unmasking in citrate buffer unmasking solution (Vector Laboratory) followed by a 1-h incubation with blocking solution, and overnight incubation at 4°C with primary antibodies to K5 (1:1000) (Abcam), K14 (1:500) (LifeSpan BioSciences), collagen IV (1:80) (Abcam), E-cadherin (1:200) (Abcam), K10 (1:1000) (Covance), K15 (1:500) (Covance), and filaggrin (1:1000) (Abcam). For staining with collagen IV antibody, unmasking was performed with Protease XXV (Fisher, AP-9006-002) at 37°C for 10 min. Visualization was performed using an anti-rabbit secondary antibody conjugated to Texas-red (1:5000) (Jackson ImmunoResearch Laboratories), an anti-guinea pig secondary antibody conjugated to FITC (1:1000, Jackson ImmunoResearch), or an anti-chicken secondary antibody conjugated to Alexa 488 (1:1000) (Molecular Probes) followed by counterstaining with DAPI (Vector Laboratory). Incorporation of BrdU was quantified using the BrdU detection kit II (Roche). For BrdU analysis by IF, an anti-rabbit secondary antibody conjugated to Texas Red was used with the BrdU detection kit II (Roche). GFP immunofluorescence was performed on frozen sections prepared from E18.5 embryos. Briefly, the epidermis was peeled from day 18.5 embryos and frozen in OCT compound (Tissue Tek). Frozen sections were fixed in 2% formaldehyde/0.2% glutaraldehyde solution for 2 min before incubation with anti-GFP (1:1000) (Invitrogen) at 4°C overnight followed by incubation with anti-rabbit secondary antibody conjugated to Texas-red (1:2000) (Jackson ImmunoResearch Laboratories) and counterstaining with DAPI (Vector Laboratory).

Skeleton preparation and staining

Embryos were collected at E18.5. Skin and internal organs were discarded, and the remaining embryo was fixed overnight in 95% ethanol at room temperature. The following day, the cartilage was stained in 0.05% alcian blue (Sigma) in 95% ethanol in acetic acid for 6 h and then destained in 1% KOH overnight. Bone staining was performed in 0.015% alizarin red (Sigma) in 1% KOH. The embryonic skeleton was washed in 20% glycerol/1% KOH for 5 days and changed twice per day until the solution was clear.

MEF and keratinocyte isolation and culture

Wild type, p53^{-/-}, p63^{-/-}, *Arf*^{GFP/GFP}, and *Arf*^{GFP/GFP};p63^{-/-} MEFs were isolated from embryos at day 13.5 and cultured in DMEM with 10% FBS as described earlier (Flores et al, 2002). Wild type, p63^{-/-}, p53^{-/-};p63^{-/-}, *Arf*^{GFP/GFP};p63^{-/-}, *Arf*^{GFP/GFP};p63^{-/-}; *Ink4a*^{-/-}, and *Ink4a*^{-/-};p63^{-/-} keratinocytes were isolated from E18.5 embryos. Briefly, the skin was peeled off and incubated in dispase II (Roche) for 18 h at 4°C to separate the epidermis from the dermis. Epidermal sheets were cut into small pieces and incubated in 0.25% trypsin-EDTA (Gibco-BRL) for 20 min at 37°C. Cells were plated on J2-3T3 feeder cells and cultured in F media (Sigma) supplemented with 0.4 µg/ml hydrocortisone, 24 ng/ml adenine, 8.4 ng/ml cholera toxin, 5 µg/ml insulin, 13 ng/ml 3,3',5-triiodo-L-thyronine, and 10 ng/ml EGF as described earlier (Barrandon and Green, 1987; Flores et al, 2000).

Immunostaining of keratinocytes

Keratinocytes seeded at a density of 4 × 10⁴ cells/6 cm diameter dish were fixed after 8 days in culture in 10% formalin for 30 min.

Immunostaining was performed by blocking and permeabilization of cells using 8% bovine serum albumin in 0.3% Triton X-100 for 30 min at room temperature. Keratinocytes were incubated with anti-K5 (1:1000) (Abcam) or anti-GFP (1:200) (Invitrogen) overnight at 4°C in a humidified chamber. Visualization of K5 was performed using an anti-rabbit secondary antibody conjugated to Texas-red (1:500) (Jackson ImmunoResearch laboratories) and GFP using an anti-rabbit secondary antibody conjugated to FITC (1:500) followed by counterstaining with DAPI (Vector Laboratory).

Keratinocyte proliferation assays

Passage-2 keratinocytes of 1×10^4 were plated in triplicate on J2-3T3 feeder cells. Keratinocytes were counted every day for 8 days. Keratinocytes were also subjected to seven serial passages at 5-day intervals. After isolation, 1×10^4 keratinocytes were plated in triplicate, passaged, and the cumulative number of population doublings was calculated.

Immunoblotting

MEFs and keratinocytes were lysed in buffer (100 mM Tris–HCl pH 8.0, 100 mM NaCl, 1% NP40, protease inhibitor cocktail tablet (Roche), and 0.2 M phenylmethylsulfonyl fluoride) and 100 µg of lysate were resolved by electrophoresis on a 12.5% SDS–PAGE gel, transferred to a PVDF membrane and probed with antibodies against p19^{Arf} (5C3-1) (Bertwistle *et al*, 2004), GFP (1:1000) (Invitrogen), p16^{Ink4a} (1:200) (Santa Cruz), p63-4A4 (1:500) (Santa Cruz), or Actin (1:5000) (Sigma) followed by goat anti-rat immunoglobulin G (IgG) or goat anti-rabbit IgG secondary antibodies conjugated to HRP (1:5000) (Jackson ImmunoResearch).

RNA extraction and real-time PCR

RNA was prepared from the dermis/epidermis peeled from wild type, p63^{−/−}, Arf^{GFP/GFP}, and Arf^{GFP/GFP}; p63^{−/−} 18.5 day embryos using an RNeasy plus kit (Qiagen Ltd.). cDNA was synthesized from 800 ng of total RNA using the SuperScript[®] III First-Strand Synthesis kit (Invitrogen) according to the manufacturer's protocol. The RNA levels of *Ink4a*, *Arf*, and GFP encoded by the cellular *Arf* promoter were determined by qRT–PCR and performed in triplicate using an ABI 7900 machine. The following primers were used for performing real-time PCR: *Ink4a* sense 5'-CGTACCCCGATTACAGGTGAT-3', *Ink4a* antisense 5'-TTGAGCAGAAGAGCTGCTACGT-3' (Bruggeman *et al*, 2005), GFP sense 5'-GTCCGCCCTGAGCAAAGA-3', GFP antisense 5'-TCCAGCAGGACCATGTGATC, and p19^{Arf} sense 5'-GCCGCACCGGATCCCT-3', p19^{Arf} antisense 5'-TGGAGCAGAAGAGCTGCTACGT-3' (Bruggeman *et al*, 2005). Primers for GAPDH were used as an internal control.

Chromatin immunoprecipitation

Wild type and Arf^{GFP/GFP}; p63^{−/−} keratinocytes were grown to near confluence on J2-3T3m feeder cells in F media as described above. Twenty-four hours before collecting chromatin, the feeder cells were removed with 0.02% EDTA. Cellular proteins were crosslinked to DNA using 1% formaldehyde and chromatin was prepared as described earlier (Flores *et al*, 2002). p63–DNA complexes were diluted 10-fold in ChIP dilution buffer and incubated overnight at 4°C with 2 µg of pan-p63 antibody (4A4, Abcam) or 2 µg IgG (no antibody). Resulting chromatin was resuspended in 300 µl of double-distilled H₂O. Each ChIP was performed in triplicate using keratinocytes from three embryos from each genotype. qRT–PCR was performed by using primers specific for the indicated regions of the *Ink4a* and *Arf* promoters (primer sequences below), p21 (Flores *et al*, 2002), and *Perp* promoters (Ihrie *et al*, 2005) using a 96 well plate ABI Step One Plus real-time PCR machine and 1 × SYBR green PCR master mix (Applied Biosystems). The percentage DNA bound was calculated using the following formula:

$$\text{Percentage total bound} = \frac{(2^{\Delta CT}) \times 2.5}{(2^{\Delta CT}) \times 2.5} \text{p63 antibody} - \frac{(2^{\Delta CT}) \times 2.5}{(2^{\Delta CT}) \times 2.5} \text{no antibody}$$

where $\Delta CT = CT_{\text{input chromatin}} - CT_{\text{sample}}$ and CT is cycle threshold.

Primers for qRT–PCR are as follows:

A. *Ink4a* promoter

- (1) Site I
-4835F-5'-ACCTCTCCAGGGTGGTCACAGGC-3'
-4621R-5'-AAAGGCCGAGTCAGATGATCTGGCC-3'
 - (2) Site II-upstream (II-UP)
-3996F-5'-ACACTGCAGTGAAGAGGACATCA-3'
-3845R-5'-GTTAAAAATGTGTAGACATGGGCC-3'
 - (3) Site II-downstream (II-DOWN)
-3632F-5'-CCACAGTTTGAACAGCGTGAAGCA-3'
-3362R-5'-AGGAAACGGATGTGATCCCAGGAA-3'
 - (4) Site III
-1850F-5'-AACTAGTAGAAGGGAGATTACTTTATG-3'
-1539R-5'-ATAAACTATGTAAATTTCTGAGGC-3'
 - (5) Site IV
-1519F-5'-TGCTAGCAGCTTCTAACCCAGCA-3'
-1157R-5'-TTTGTATGTGTGTGGTGAGAATGTC-3'
- #### B. *Arf* promoter
- (1) Upstream regions (UP)
-1811F-5'-AGACATCAGGCAACAGGGAATGGA-3'
-1622R-5'-TTCGGGCTACCTACCTGGAATTT-3'
 - (2) Site I
-1533F-5'-ATACTCAGGGAGCCCTCAAACTC-3'
-1331R-5'-TAGACATAGGAAGATGGCAAGGCC-3'
 - (3) Intervening sequences (INT)
-1417F-5'-ACCTGCCCTGCGGCTCTCAGTTGC-3'
-1303R-5'-TCTTATAGAGCGGATCCAAATGC-3'
 - (4) Sites IIA and IIB
-1276F-5'-ATTGCTACTTTACTGCAGCCAGAC-3'
-943R-5'-TTGATCGGAGCGCCGCCGGGGAC-3'

Transfection of MEFs

MEFs were plated on a six-well dish at a density of 4×10^5 cells/well. Twenty-four hours later, 2 µg of pcDNA3-p63 expression vectors (Tap63α, Tap63γ, ΔNp63α, or ΔNp63γ) were individually transfected using FuGENE HD Transfection Reagent (Roche) following the manufacturer's protocol. Cells were collected 24 h after transfection for analysis by western blotting and qRT–PCR.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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