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p63 control of desmosome gene expression and adhesion is compromised in AEC syndrome

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Ankyloblepharon, ectodermal defects, cleft lip/palate (AEC) syndrome is a rare autosomal dominant disorder caused by mutations in the *p63* gene, essential for embryonic development of stratified epithelia. The most severe cutaneous manifestation of this disorder is the long-lasting skin fragility associated with severe skin erosions after birth. Using a knock-in mouse model for AEC syndrome, we found that skin fragility was associated with microscopic blistering between the basal and suprabasal compartments of the epidermis and reduced desmosomal contacts. Expression of desmosomal cadherins and desmoplakin was strongly reduced in AEC mutant keratinocytes and in newborn epidermis. A similar impairment in desmosome gene expression was observed in human keratinocytes isolated from AEC patients, in p63-depleted keratinocytes and in *p63* null embryonic skin, indicating that p63 mutations causative of AEC syndrome have a dominant-negative effect on the wild-type p63 protein. Among the desmosomal components, desmocollin 3, desmoplakin and desmoglein 1 were the most significantly reduced by mutant p63 both at the RNA and protein levels. Chromatin immunoprecipitation experiments and transactivation assays revealed that p63 controls these genes at the transcriptional level. Consistent with reduced desmosome function, AEC mutant and p63-deficient keratinocytes had an impaired ability to withstand mechanical stress, which was alleviated by epidermal growth factor receptor inhibitors known to stabilize desmosomes. Our study reveals that p63 is a crucial regulator of a subset of desmosomal genes and that this function is impaired in AEC syndrome. Reduced mechanical strength resulting from p63 mutations can be alleviated pharmacologically by increasing desmosome adhesion with possible therapeutic implications.

INTRODUCTION

The skin epidermis is a thin layer of stratified squamous epithelium that provides a structural and functional defence against dehydration, microorganisms, chemical substances and mechanical trauma (1). To serve these functions, epidermal cells undergo constant renewal in a highly regulated program of terminal differentiation. A master regulator of

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epidermal gene transcription is p63 that plays an essential function in controlling epidermal development (2–5), cell proliferation and stemness (5–7), stratification (2,7–9) and cell–matrix adhesion (2,10). The ΔNp63 alpha isoform is the most abundantly expressed p63 isoform in the basal proliferative layer of the epidermis (11), and acts as either an activator or a repressor depending on the target gene (12–14). *p63* and Δ*Np63* null embryos are born with similar phenotypes, namely abnormal craniofacial development, limb truncation and severe defects of stratified epithelia and their annexes (4,5,15). The surface epithelium of *p63* null embryos is thin and highly disorganized: epidermal cells do not properly stratify and are poorly adhesive.

Consistent with defects in *p63*-deficient mice, heterozygous mutations in the human *p63* gene cause a number of closely related autosomal dominant conditions mainly characterized by ectodermal dysplasia, ectrodactyly and/or syndactyly and cleft lip/palate syndromes (16). One of these, AEC syndrome (or Hay-Wells syndrome; OMIM 106260), is caused by missense or frame-shift mutations mostly affecting the carboxy-terminal portion of the p63 alpha protein, and differs from the other conditions in the occurrence of ankyloblepharon, the absence of ectrodactyly and in the severity of the skin phenotype (17,18). Skin involvement includes congenital erythroderma, skin fragility and severe skin erosions most prominently on the scalp that appear at or soon after birth and can last several years (17,19). Erosions typically involve the scalp, head and neck, skin folds, palms and/or soles and are often accompanied by crusting, granulation tissue and secondary infection. Healing is slow and recurrent breakdown is typical. Adult patients can be affected by palmoplantar hyperkeratosis and erosive palmoplantar keratoderma with bleeding after extensive walking (17,20). The biological mechanisms underlying the skin erosions remain unveiled, and treatment is limited to gentle wound care and antibiotic treatment to prevent or cure infections.

To maintain the structure and function of the epidermis, a number of intercellular junctions exist, including tight junctions, gap junctions, adherens junctions and desmosomes. Desmosomes are essential anchoring junctions that enforce adhesion through contacts to the intermediate filament cytoskeleton, forming a robust network among adjacent cells that confers strength and resiliency to the epidermis (reviewed in 21,22). These junctions are abundant in tissues that have to withstand continuous mechanical stress, such as the skin and the heart. The desmosomal cadherins, desmocollins (DSCs) and desmogleins (DSGs) are transmembrane proteins that form stable associations with similar cadherins in adjacent cells. Inside the cell, desmosomal cadherins associate with armadillo proteins, such as plakoglobin (JUP) and plakophilins (PKP1-PKP3), that in-turn bind desmoplakin (DSP). In the epidermis, DSP links the desmosomal plaque to the keratin cytoskeleton. Among the desmosomal cadherins DSC3 and DSG3 are typical of the basal layer, whereas DSC1 and DSG1 are expressed in the suprabasal layers of stratified epithelia (23–27).

Perturbations in desmosomal proteins are associated with epidermal blistering as observed in patients with pemphigus who have circulating autoantibodies directed against DSG3 (pemphigus vulgaris) or DSG1 (pemphigus foliaceus) (28). In addition a number of genodermatoses are caused by mutations in desmosomal genes (reviewed in 29), including the autosomal recessive disorders: skin fragility/ectodermal dysplasia syndrome (PKP1) (30), lethal congenital epidermolysis bullosa (JUP) (31), skin fragility-woolly hair with or without cardiomyopathy (DSP) (32) and lethal acantholytic epidermolysis bullosa (DSP) (33). In addition, heterozygous mutations in DSG1 and DSP cause striate palmoplantar keratoderma characterized by focal hyperkeratosis of the palms and soles that is exacerbated by mechanical trauma (34,35).

Knockout mouse models revealed similar essential functions for desmosomes in skin cell–cell adhesion. As predicted from their predominant localization in the basal layer, loss of *Dsc3* and *Dsg3* gene expression in the mouse epidermis causes impaired cell–cell adhesion, leading to intra-epidermal blistering between the basal and suprabasal compartment (36,37). Similarly, loss of *Dsp* in mouse skin results in epithelium peeling that is more pronounced within the basal and spinous layers, leaving large areas of denuded skin after mild mechanical stress (38). In contrast, loss of the suprabasal *Dsc1* causes disruption of cell adhesion in the upper layers of the epidermis associated with a hyperplastic phenotype (39,40).

Reduced desmosomal contacts are often observed in human epithelial carcinoma cell lines. Inhibition of epidermal growth factor receptor (EGFR) causes increased cell–cell adhesion and recruitment of desmosomal components to the cell borders (41–43). Induced desmosome assembly mediated by EGFR inhibition is thought to stabilize desmosomal components at the intercellular junctions by interfering with their accumulation in an internalized cytoplasmic pool. Similarly, EGFR activation causes loss of Dsp from desmosome and decreased adhesive strength in mouse keratinocytes (44).

In the present study, we investigated a possible involvement of desmosomes in the skin fragility phenotype observed in AEC syndrome, given the similarity between the macroscopic lesions observed in patients affected by desmosome-related disorders and those affected by AEC syndrome, including peeling of the skin with a predominant localization on the scalp and palmoplantar keratoderma. We used a knock-in mouse model for AEC syndrome ($p63^{+/L514F}$) that closely resembles the human disorders at both the genetic and phenotypic levels (45). AEC mice are born with ectodermal dysplasia, hypoplastic and fragile skin and cleft palate, resulting in perinatal lethality. During embryonic development, progenitor cell expansion is affected due to impairment of fibroblast growth factor signalling leading to a defective epidermal stem cell compartment (45). Despite epidermal hypoplasia, epidermal differentiation occurs normally in $p63^{+/L514F}$ newborn mice, and the impermeable epidermal barrier is established at the appropriate developmental time-point consistent with little or no alteration of terminal differentiation observed in the skin of AEC patients (18,46). Here, we demonstrate that in AEC syndrome skin fragility is associated with acantholysis and reduced desmosomes between the basal and suprabasal layers of the epidermis. Basal and, to a lesser extent, suprabasal desmosomal cadherins and DSP are reduced in AEC mouse skin and in human keratinocytes derived from AEC patients. Similarly, loss of p63 expression leads to down-regulation of desmosomal cadherin and Dsp expression, with *Dsc3* and *Dsp* being transcriptionally regulated by p63. Reduced mechanical strength of mutant keratinocytes

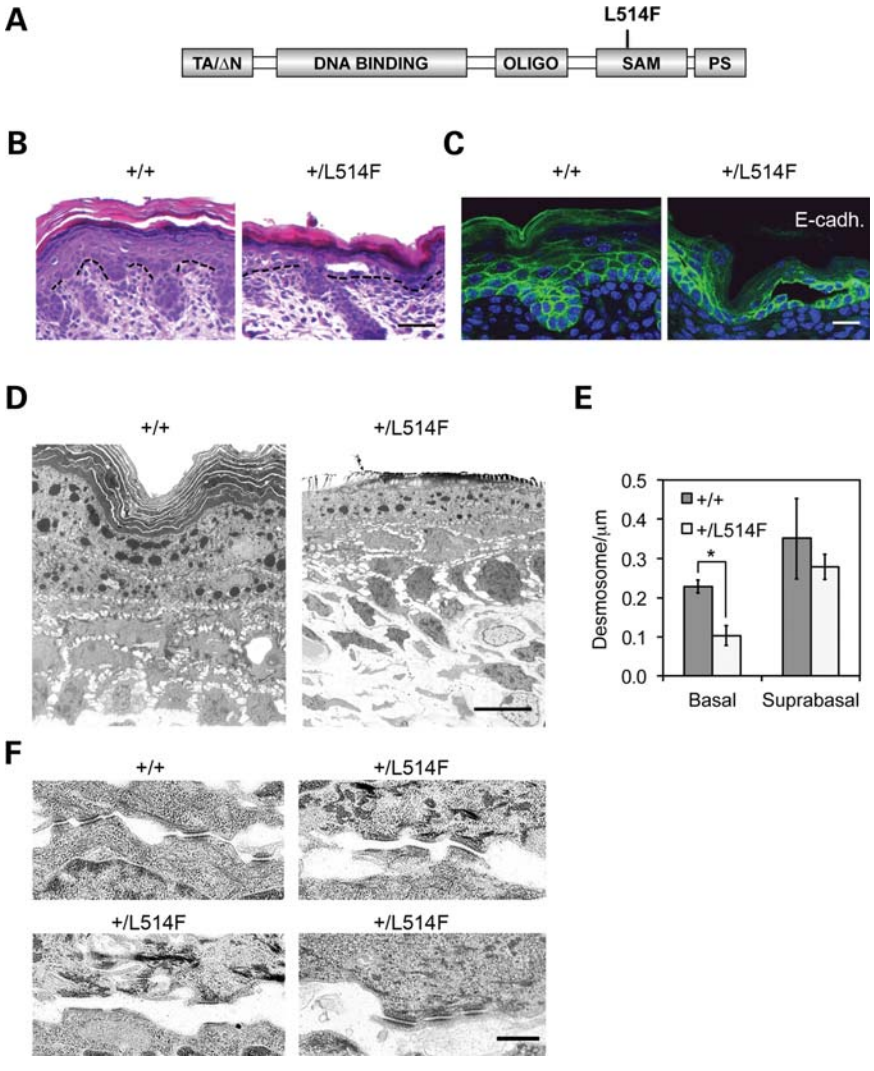

Figure 1. Intra-epidermal blistering and desmosomal defects in AEC mutant mice. (**A**) Schematic representation of AEC mutant protein generated by knock-in strategy. The L514F mutation falls in the Sterile Alpha Motif (SAM) at the carboxyl-terminus of the p63 alpha isoform. The other p63 domains are also indicated: the TA or the ΔN are two mutually exclusive transactivation domains at the N-terminus of the p63 protein. Oligo: oligomerization domain. PS, post-SAM domain. (**B**) H&E staining of the skin reveals blistering between the basal and suprabasal compartment in mutant (+/L514F) but not in wild-type (+/+) epidermis. Dashed lines indicate the border between epidermis (top) and dermis (bottom). Scale bar: 50 μm. (**C**) Immunofluorescence staining for E-cadherin reveals normal localization of this adherens junction component, and confirms basal to suprabasal blistering. Scale bar: 20 μm. (**D**) Transmission electron microscopy of skin samples reveals reduced basal–basal and basal–suprabasal cell contacts in mutant epidermis. Scale bar: 8 um. (**E**) Desmosomes between basal–basal and basal–suprabasal cells (basal) are significantly less abundant in mutant (white) versus wild-type epidermis (grey). $^{*}P$-value = 0.015; $n = 6$. Desmosomes between suprabasal cells are not significantly affected. (**F**) Loss of desmosomes in AEC mutant epidermis occurs by disruption of extracellular contacts between adjacent cells, or less frequently by disruption between the desmosome plaque and the intermediate filaments (lower right panel). Scale bar: 500 nm.

in culture was alleviated by inhibition of EGFR signalling, which was previously reported to enhance desmosomal assembly.

RESULTS

Intra-epidermal blistering and reduced desmosomes in AEC mutant mice

We previously generated a knock-in mouse model for AEC syndrome ($p63^{+/L514F}$) carrying a phenylalanine substitution in position 514 (L514F), a missense mutation in the p63 alpha terminal portion causative of AEC syndrome (Fig. 1A) (18,45). A significant fraction of newborn $p63^{+/L514F}$ mutant pups (21%; $n = 28$) displayed histologically detectable intra-epidermal blisters between the basal and suprabasal cell layers (Fig. 1B and C), whereas macroscopic skin blistering was rarely observed (45). E-cadherin, the core components of the adherens junction, was appropriately localized at the cell membrane even in blistered areas (Fig. 1C). However, ultrastructural evaluation of cell–cell contacts obtained by Electron Microscopy (EM) indicated that basal and suprabasal cells were more widely spaced in mutant when compared with wild-type epidermis with loss of desmosomal contacts (Fig. 1D). In mutant epidermis, electron-dense desmosomal plaques were significantly reduced in number particularly between basal cells and between basal and suprabasal cells (Fig. 1E). At high magnification the space between

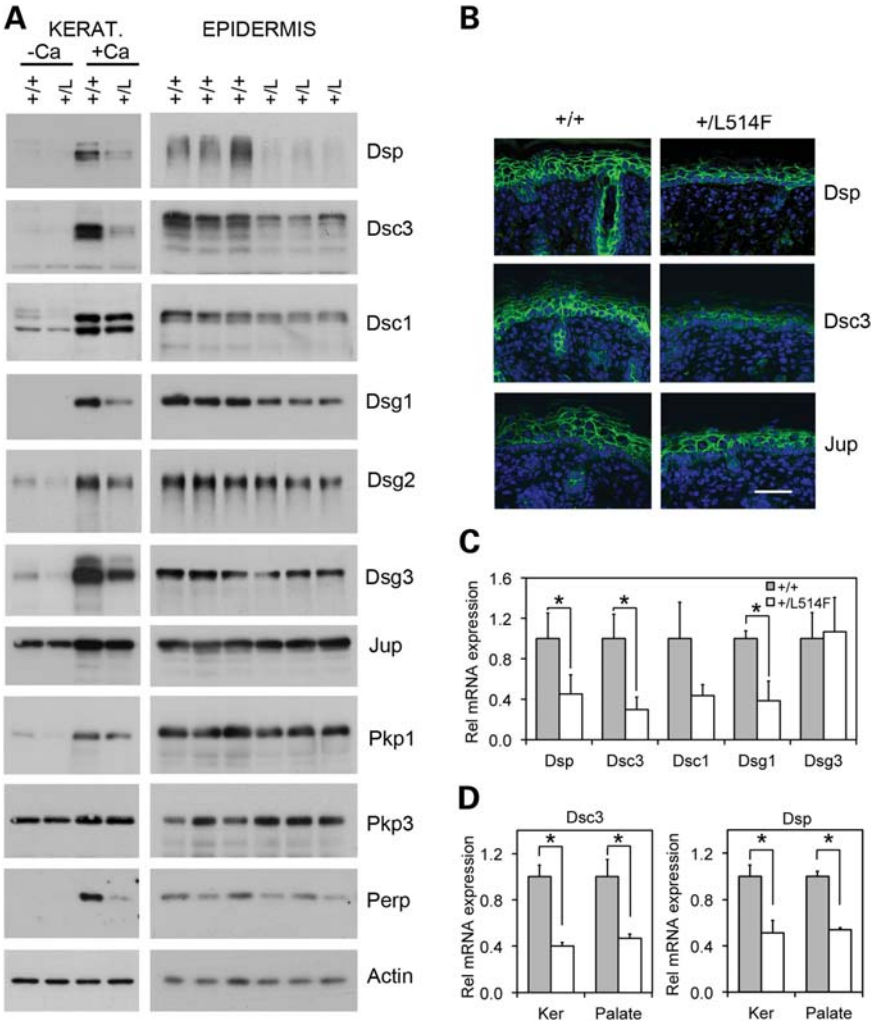

Figure 2. Expression of several desmosomal components is impaired in AEC mutant mice. (**A**) Immunoblotting of desmosomal proteins in total cell extracts of wild-type (+/+) and AEC mutant (+/L) primary keratinocytes (left panel) or newborn epidermis (right panel). Keratinocytes were cultured in low-calcium conditions (−Ca) or switched to 0.6 mM calcium (+Ca) for 24 h. (**B**) Immunofluorescence of the indicated desmosomal proteins was performed on frozen sections of neonatal epidermis. Scale bar 50 μM. (**C**) Real-time RT–PCR on RNA isolated from embryonic skin at E.14.5 from mutant (white bars) and control embryos (grey bars). Data are normalized for β-actin mRNA levels and are represented as mean +/− SD normalized mRNA levels. $^{*}P$-value < 0.05; $n = 6$. (**D**) Real-time RT–PCR on RNA isolated from primary neonatal keratinocytes and embryonic palate at E13.5. Data are normalized and represented as in **C**) $^{*}P$-value < 0.05; $n = 4$.

desmosomal plaques of two adjacent cells was wider, indicating reduced adhesion between the desmosomal cadherins, although occasionally desmosomal plaques were associated entirely with only one of the two cells (Fig. 1F), suggesting that the attachment of the plaque to the intermediate filament was also affected. No difference in the plaque length was observed.

Taken together these data indicate that desmosomes are compromised in their strength and organization in $p63^{+/L514F}$ epidermis.

Impaired desmosomal gene expression in AEC mutant epidermal cells

Skin lesions are a distinctive sign of AEC syndrome and the observed desmosome alterations may explain at least in part the skin fragility and erosions. To determine the molecular defect underlying desmosome alterations, we measured desmosomal cadherins, plakins and armadillo proteins by immunoblotting analysis using total protein extracts from primary keratinocytes under growing conditions (low calcium) and under conditions that allow for the formation of desmosomes (high calcium), as well as from newborn epidermis. Several desmosomal components were significantly reduced in AEC mutant keratinocytes, including Dsp, Dsc3, Dsg1, Dsc1 and Perp, encoding for a previously recognized *bona fide* p63 target gene (47). In contrast, the armadillo proteins Jup, Pkp1 and Pkp3 were unaffected (Fig. 2A). In mutant, newborn epidermis Dsc3 and Dsp were the most reduced desmosomal proteins as shown by immunoblotting and immunofluorescence (Fig. 2A and B).

To determine whether desmosomal genes were affected at the RNA level, AEC mutant cells and tissues were compared with wild-type controls. In AEC embryonic skin Dsp, Dsc3

and Dsc1 were significantly reduced at the RNA level compared with wild-type controls (Fig. 2C). In contrast, Dsg3 mRNA levels were unaffected, suggesting that reduced Dsg3 protein was a consequence of destabilized desmosome assembly. A similar significant decrease in Dsc3 and Dsp mRNA was also observed in mutant primary keratinocytes and in embryonic palate (Fig. 2D), in which p63 and these desmosomal genes are co-expressed.

To rule out the possibility that adherens junction may also be affected in AEC mutant mice and thereby contribute to weakening of cell adhesions, two crucial components of the adherens junction, P-cadherin (Cdh3), a well-established p63 target gene (48) and β-catenin (Ctnnb1) were measured. Similarly to E-cadherin, these adherens junction components were not affected in AEC mutant epidermis either at the RNA or protein levels (Supplementary Material, Fig. S1A and B). Furthermore immunofluorescence staining in differentiated AEC and wild-type keratinocytes revealed a similar expression and localization of both adherens junction components Cdh3 and Ctnnb1, as well of the desmosomal component Jup, whereas Dsp expression and localization was profoundly affected (Supplementary Material, Fig. S1C).

To test whether changes in desmosomal components observed in mouse were relevant to the human condition, their expression was measured in primary keratinocytes derived from two AEC patients and healthy controls. Similar to mouse keratinocytes, human keratinocytes derived from AEC patients cultured under conditions that favour desmosome assembly had reduced expression of DSP and desmosomal cadherins when compared with control samples, whereas PKP1 and E-cadherin were unaffected (Fig. 3A). At the RNA level a similar reduction in DSP, DSC3, DSC1 and DSG1 expression was observed, whereas similarly to what occurred in mutant mouse keratinocytes DSG3 was not affected (Fig. 3B).

Taken together these data demonstrate that in contrast to adherens junction components, the expression of DSP and several desmosomal cadherins is profoundly reduced both at the RNA and protein levels in the AEC mouse model and in human keratinocytes derived from individuals affected by AEC syndrome, with *DSC3* and *DSP* being the most significantly affected genes.

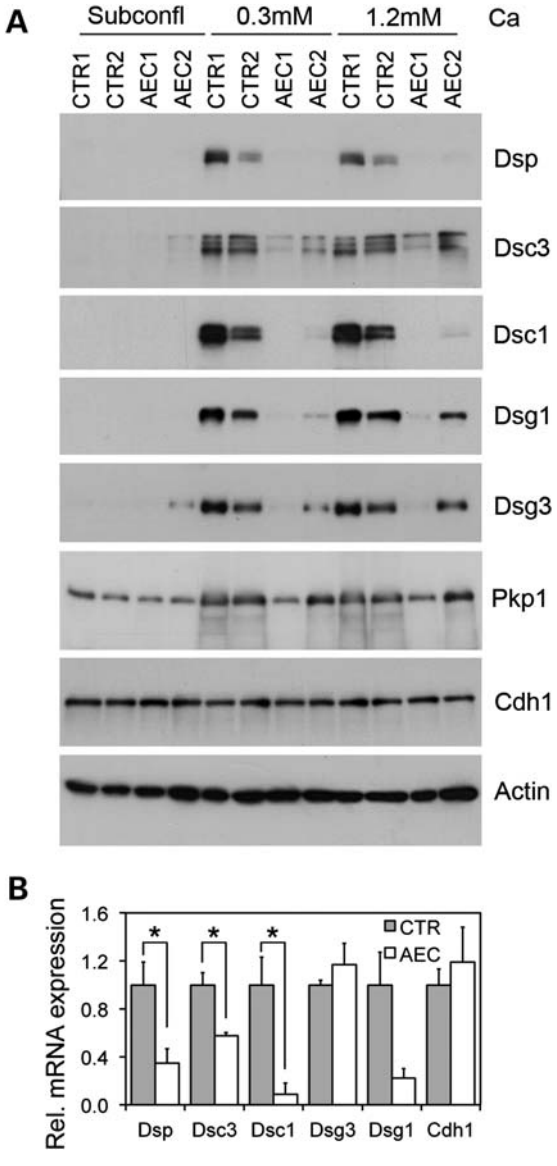

Figure 3. Reduced expression of desmosomal genes in human keratinocytes isolated from AEC syndrome patients. (**A**) Immunoblotting of total cell extracts from human keratinocytes isolated from humans affected by AEC syndrome (AEC1 and AEC2) or unaffected controls (CTR1 and CTR2) using antibodies against the indicated desmosomal proteins. Keratinocytes were cultured in low-calcium conditions (−Ca) and collected before reaching confluency or cultured at confluency for 24 h in the presence of 0.3 mM and 1.2 mM calcium (+Ca). (**B**) Real-time RT–PCR on RNA isolated from human keratinocytes from AEC patients and from controls. Data are normalized for RPLP0 RNA levels and are represented as mean +/− SD normalized mRNA levels (*P-value ≤ 0.05; $n = 4$; for Dsg1 P-value = 0.06).

Dsc3, *Dsp* and *Dsg1* are *bona fide* p63 target genes

In spite of the crucial role of desmosomes in maintaining cell–cell adhesion in the epidermis, little is known about regulation of their gene expression. Since p63 is a crucial transcriptional regulator of several epidermal genes and it has been demonstrated that AEC mutants can affect this function (45,49–51), we hypothesized that p63 may directly control expression of a subset of desmosome genes in addition to PERP, a previously identified p63 target gene (47). First, we evaluated whether p63 or ΔNp63 depletion may affect the expression of desmosomal proteins by transfecting mouse keratinocytes with specific siRNA. Expression of Dsp and desmosomal cadherins was strongly reduced by p63 and ΔNp63 knockdown, whereas E-cadherin was unaffected (Fig. 4A).

The previously reported role of Irf6 in keratinocyte differentiation (52,53) and its down-regulation in AEC skin samples both in human and mice (45,54,55) prompted us to investigate the contribution of Irf6 in desmosomal gene regulation. Irf6 protein was down-regulated by p63 and ΔNp63 knockdown, whereas p63 was strongly up-regulated by Irf6 knockdown (Fig. 4A), confirming reciprocal regulation between p63 and Irf6 (54–56). Irf6 knockdown did not cause a reduction in any of the desmosomal protein tested, but rather an increase likely due to p63 up-regulation (Fig. 4A). Concomitant p63 and Irf6 knockdown had no additional effects when compared

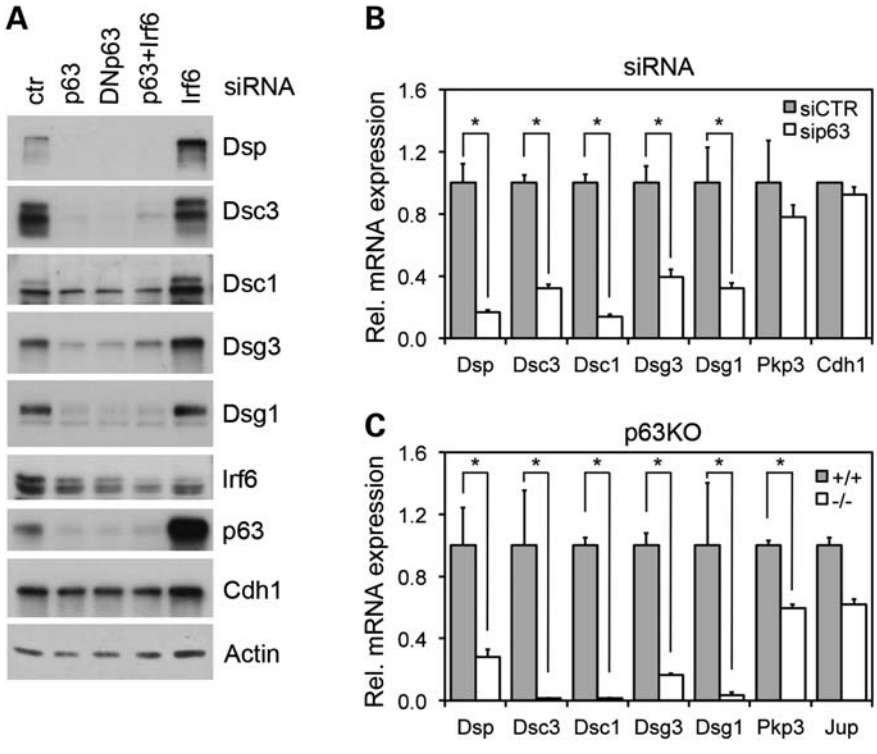

Figure 4. Desmosome cadherins and desmoplakin are regulated by p63 but not by Irf6. (**A**) Immunoblotting for the indicated proteins in total cell extracts of mouse primary keratinocytes transfected with p63, ΔNp63 and Irf6 specific siRNA or controls. After transfection keratinocytes were kept in culture for 24 h and then treated with 0.6 mM calcium for 24 h before collection. (**B**) Real-time RT–PCR on RNA isolated from mouse keratinocytes treated with siRNA for p63 (sip63) or control siRNA. Data are normalized for actin mRNA levels and are represented as mean +/− SD normalized mRNA levels (*P-value < 0.05; $n = 4$). (**C**) Real-time RT–PCR on RNA isolated from *p63* null (−/−) or wild-type (+/+) mouse skin at E14.5. Data are normalized as in (B) (*P-value < 0.05; $n = 6$).

with p63 knockdown alone, indicating that Irf6 is unlikely to play a role in desmosome regulation downstream of p63.

Consistent with the observed changes at the protein level, mRNA of Dsp and the desmosomal cadherins was strongly down-regulated in p63 knockdown keratinocytes compared with controls, whereas Pkp3 and E-cadherin remained unaffected (Fig. 4B). Desmosomal gene expression was also evaluated in *p63* null embryonic skin at E14.5. Consistent with the observed changes in primary keratinocytes Dsp and desmosomal cadherins were all severely reduced in *p63* null skin, whereas Pkp3 and Jup were modestly affected (Fig. 4C). These data indicate that p63 is a crucial regulator of Dsp and several desmosomal cadherins.

To explore the possibility that desmosomal genes may be regulated by p63 at the transcriptional level, we analysed previously generated ChIP-seq data obtained in human keratinocytes (57). In the DSP genomic locus, a p63-binding region was present in the first intron and corresponded to a genomic region enriched in positive histone marks associated with active transcription, and to a cluster of DNase hypersensitive sites (Supplementary Material, Fig. S2A) (58,59). Desmosomal cadherin genes are located in two adjacent clusters with opposite directions on human chromosome 18 spanning altogether ∼580 kb. This genomic cluster contained 12 p63-binding regions enriched in human keratinocytes for positive histone marks and for DNase hypersensitive sites (Supplementary Material, Fig. S2B), whereas only two p63-binding regions with similar characteristics were present in a 2.4 Mb region aside of the DSC–DSG cluster in spite of the presence of several genes.

Six p63-binding regions that were conserved between human and mouse were tested by ChIP-qPCR in primary mouse keratinocytes (Supplementary Material, Table S1). Binding regions located in the first intron of *Dsp*, upstream of *Dsc3*, upstream of the *Dsg1* gene were strongly enriched in ChIP-qPCR with anti-p63 antibodies compared with control antibodies, whereas the other tested binding regions were not enriched (Fig. 5A). Binding of p63 to *Dsp*, *Dsc3* and *Dsg1* genomic regions was confirmed in embryonic skin at E14.5 (Fig. 5B), indicating that p63 binds these genomic regions both *in vitro* and *in vivo*. Sequence analysis of the p63-binding regions revealed the presence of 'canonical' p63-binding sites that were conserved between human and mouse (Fig. 5C). We focused on *Dsp* and *Dsc3* p63-binding regions that were most significantly reduced in AEC mouse epidermis. To determine whether these p63-binding regions were functional, their potential enhancer activity was tested by the luciferase assay. Both *Dsc3* and *Dsp* p63-binding regions displayed a significant enhancer activity in mouse primary keratinocytes, which was reduced in p63 knockdown keratinocytes (Fig. 5D). In addition, response to p63 knockdown was abolished by a point mutation in the p63-binding site in both Dsc3 and Dsp enhancer regions, indicating that p63 directly controls the enhancer

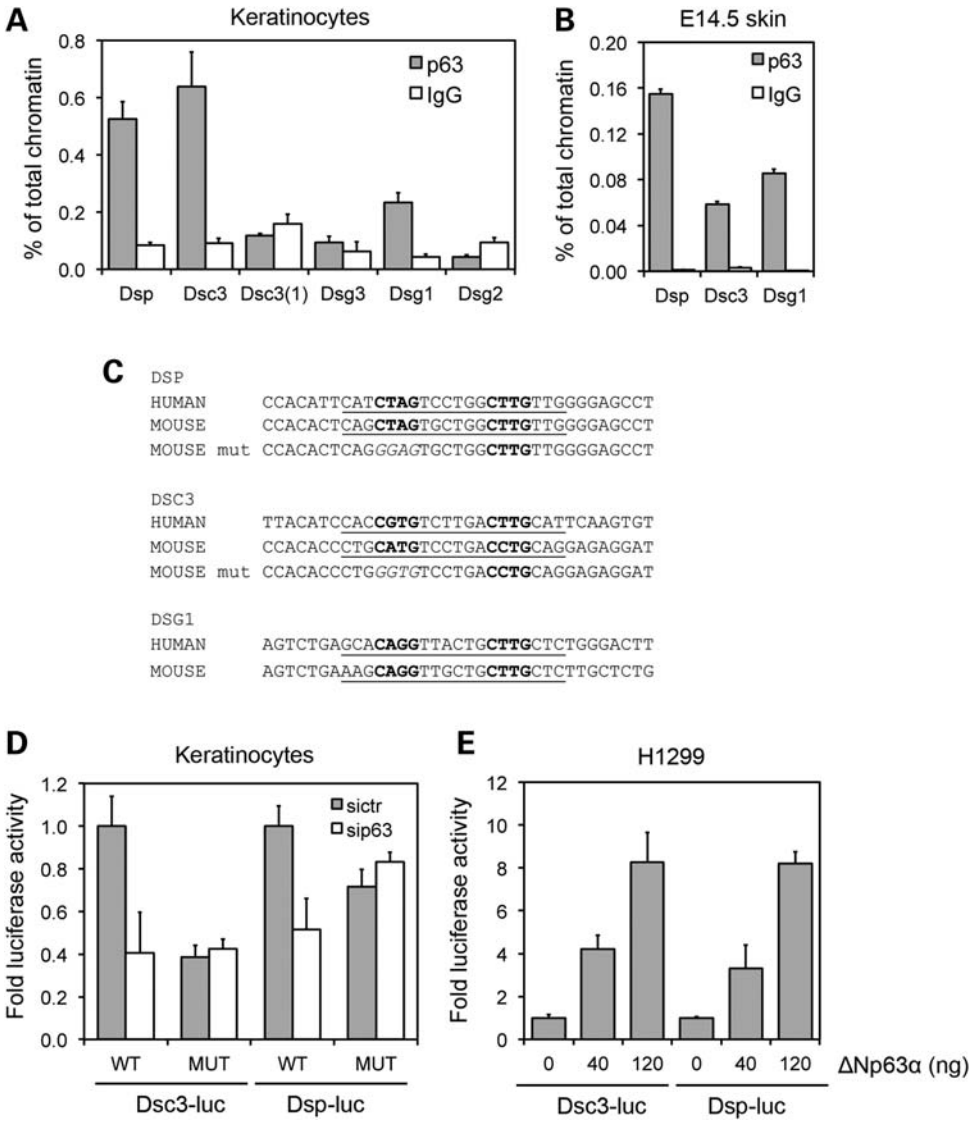

Figure 5. Identification of desmosomal genes directly controlled by p63. (**A**) ChIP-qPCR of mouse keratinocyte chromatin was performed using p63 polyclonal antibodies (H-137) (grey bars) and rabbit IgG (white bars) as negative control. Strong p63 binding was observed in mouse genomic regions corresponding to the human p63-binding regions in *Dsp* and *Dsc3*. Binding was observed also in a genomic region upstream of *Dsg1*. Data are representative of three independent experiments. Error bars denote SEM. (**B**) ChIP-qPCR of E14.5 skin chromatin showing that p63 efficiently bound *Dsp*, *Dsc3* and *Dsg1* regulatory regions *in vivo*. Data are represented as in (A). (**C**) Sequence of the conserved p63-binding motif in human and mouse in the *Dsp*, *Dsc3* and *Dsg1* regulatory regions is underlined. The core consensus is in bold. p63-binding site in the regulatory regions was identified by MatInspector (http://www.genomatix.de/). Two nucleotides in the core sequence were mutated in the *Dsp* and *Dsc3* core sequence as indicated (p63 mut) for the luciferase assay. (**D**) Luciferase activity of the *Dsp* and *Dsc3* regulatory regions cloned in the pGL3-Luc vector (WT) is strongly inhibited in p63 knockdown (white bars) when compared with control mouse keratinocytes (grey bars). In contrast, *Dsp* and *Dsc3* regulatory regions with a mutant p63-binding site as in (C) (MUT) were no longer responsive to p63 knockdown. Data are expressed relative to the WT p63-binding site treated with the siRNA control (siCTR). (**E**) The luciferase assay in H1299 cells transfected with the indicated concentrations of ΔNp63alpha expressing vector and *Dsp* and *Dsc3* luciferase reporters. Data are representative of three independent experiments. Error bars denote SEM.

activities. Consistently, in human H1299 cells that lack p63 and the other members of the p53 family, *Dsc3* and *Dsp* enhancer regions were responsive to exogenous p63 in a dose–response manner (Fig. 5E).

To test the possibility that L514F mutation may affect p63 function, we performed ChIP-qPCR assays in AEC mutant versus wild-type embryonic skin. p63 binding to the enhancer regions of both Dsp and Dsc3 was reduced in mutant embryonic skins when compared with the wild-type ones (Supplementary Material, Fig. S3A). Consistent with reduced binding, mutant p63 displayed a reduced ability to transactivate the Dsp enhancer region in H1299 cells when compared with wild-type p63. Transactivation was also affected, although to a lesser extent, when equimolar amounts of wild-type and mutant p63 were co-transfected (Supplementary Material, Fig. S3B).

Taken together, these data indicate that p63 binds and functionally controls putative enhancer regions in Dsp and Dsc3 genes, and that the presence of mutant p63 partially impairs both DNA binding and transactivation activities.

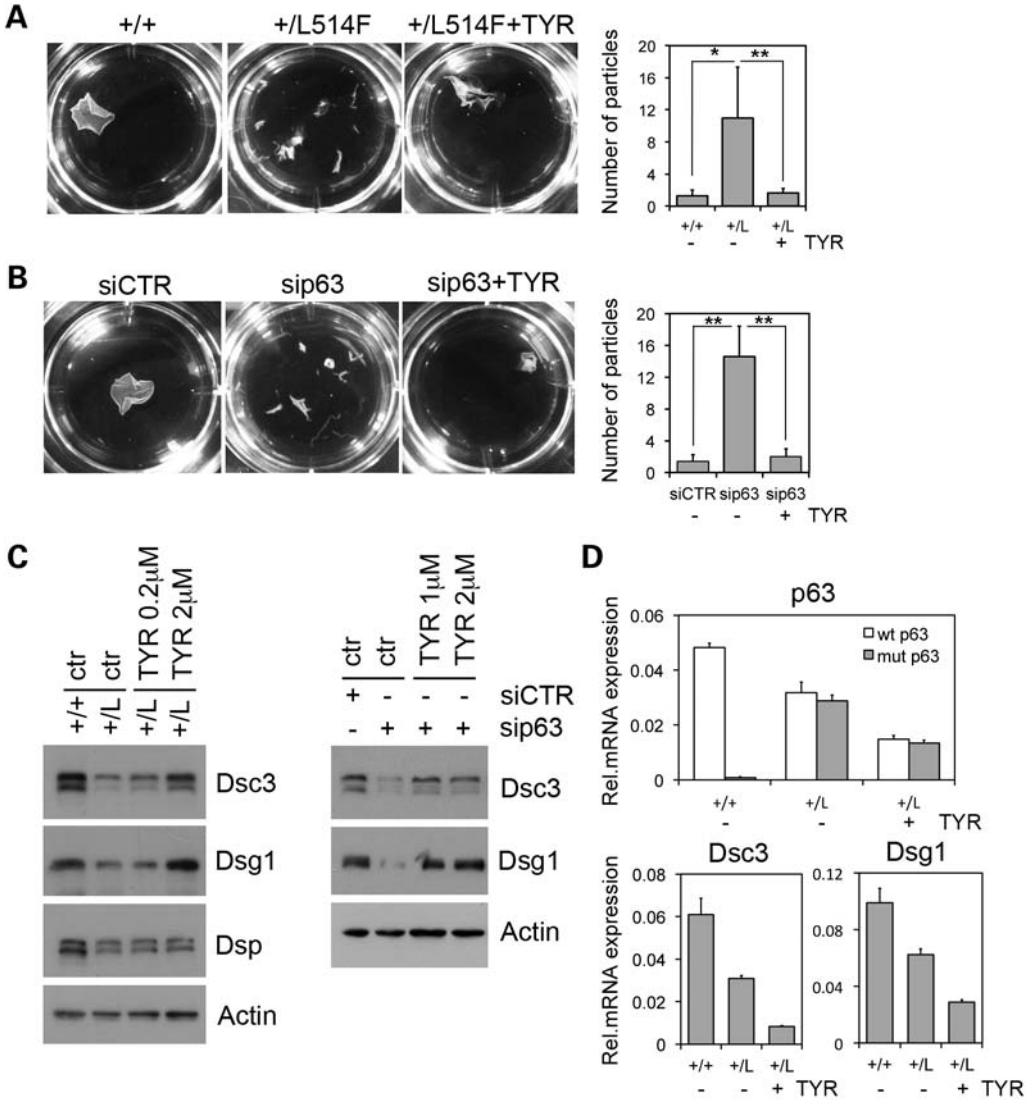

Figure 6. p63 is required for mechanical stress resistance in mouse keratinocytes. (**A**) AEC mutant and wild-type mouse keratinocytes were cultured in 0.6 mM calcium for 24 h, either in the presence or in the absence of 2 mM Tyrphostin (TYR), and then treated with dispase. Detached monolayers were subjected to mechanical stress, and resulting fragments of the cell sheet were imaged and counted. Quantitative evaluation of particles generated in the experiment is shown on the right (*P-value <0.05, **P-value <0.01; $n = 10$). Error bars denote SD. (**B**) p63-depleted (sip63) or control (siCTR) keratinocytes were treated as in (A). (**P-value <0.01; $n = 10$). Error bars denote SD. (**C**) Immunoblotting of desmosomal proteins in total cell extracts of wild-type (+/+) and AEC mutant (+/L) primary keratinocytes (left panels) or control (siCTR) and p63 knockdown keratinocytes (sip63) (right panels) were cultured in 0.6 mM calcium for 24 h and either untreated (ctr) or treated with AG1478 (TYR) at the indicated concentrations (left panel) 2 h before calcium addition. (**D**) Real-time RT–PCR on RNA isolated from wild-type (+/+) and mutant keratinocytes (+/L). Mutant (grey bars) and wild-type p63 (white bars), Dsc3 and Dsg1 mRNA were measured in keratinocytes treated as in (C). TYR was used at 2 μM. Data are normalized for actin mRNA levels and are represented as mean +/− SD normalized mRNA levels.

AEC keratinocytes exhibit cell–cell adhesion defects that can be alleviated by EGFR inhibition

At the functional level, desmosome defects are associated with weaken cell–cell adhesion and reduced mechanical stress resistance of keratinocyte monolayers, a phenotype observed for instance in *Dsc3* null keratinocytes (36). To determine whether defects in desmosome gene expression resulted in weakened cell–cell adhesion, the mechanical stress resistance of mutant or *p63*-deficient keratinocyte monolayers in culture was compared with controls. Consistent with reduced expression of several desmosomal proteins and reduced desmosome contacts *in vivo*, cell sheets of newborn AEC mutant keratinocytes generated under high-calcium conditions were significantly less resistant to mechanical stress than control wild-type keratinocytes in a shearing assay (Fig. 6A). A similar impairment in mechanical resistance was observed for p63 knockdown keratinocytes (Fig. 6B).

To determine that the defective epidermal adhesion seen in AEC syndrome is primarily due to reduced expression of desmosomal components, we attempted to rescue monolayer fragmentation by enhancing desmosome assembly. EGFR inhibition induces desmosome stabilization and assembly in human keratinocytes and in epithelial cancer cells

(41–43,60). Accordingly, treatment with the selective EGFR inhibitor Tyrphostin AG1478 restored mechanical resistance in both AEC mutant and p63 knockdown keratinocytes (Fig. 6A and B). To further demonstrate that EGFR inhibition is at the basis of increased mechanical resistance, two independent EGFR siRNA were co-transfected with p63 siRNA. Concomitant knockdown of p63 and EGFR lead to a complete rescue of mechanical resistance when compared with p63 knockdown alone (Supplementary Material, Fig. S4).

Dsc3 and Dsg1 protein expression was restored by AG1478 treatment in AEC mutant keratinocytes, whereas EGFR inhibition had no effect on DSP protein levels (Fig. 6C), as previously reported (43). A similar rescue of Dsc3 and Dsg1 expression was observed in p63 knockdown keratinocytes treated with AG1478.

In spite of restored protein levels, EGFR inhibition was unable to re-establish normal levels of Dsg1 and Dsc3 mRNA in AEC mutant keratinocytes (Fig. 6D), but instead further inhibited their mRNA levels possibly as a consequence of reduced p63 expression, a previously reported consequence of EGFR inhibition (61), consistent with the ability of EGFR inhibitors to stabilize desmosome formation rather than acting at the transcriptional level.

Taken together these data indicate that impaired p63 function leads to reduced resistance to mechanical stress of keratinocyte monolayers. Desmosome assembly and function can be restored in AEC keratinocytes by EGFR inhibition without restoring desmosomal gene expression at the RNA levels.

DISCUSSION

Here, we demonstrate that p63 positively regulates desmosome adhesion by directly controlling the expression of several desmosome genes, including *Dsp, Dsc3* and *Dsg1*, and that such regulation is altered in both human and mouse AEC keratinocytes. At the functional level, our data indicate that AEC keratinocytes are less adhesive than the wild-type counterparts *in vitro* as well as in the context of newborn epidermis. Impairment in desmosomal function is likely to significantly contribute to reduced mechanical resistance in AEC syndrome.

Several clinical and experimental observations are consistent with a defect in desmosome function in AEC syndrome. Intra-epidermal blistering with areas of both acantholysis and cytolysis was reported in the perilesional skin subjected to mild mechanical stress obtained from a newborn AEC patient (62), a phenotype consistent with the reduced number of desmosomes, split desmosomes and occasional disruption of the plasma membrane at the desmosome level observed in the AEC mouse model. While the splitting into two desmosomes has been observed in *Dsc3* null mice (36), breaks at the plasma membrane in correspondence to desmosome has been observed in *Dsp* null mice (38), consistent with the molecular defect that we observed in AEC mice. In addition, skin erosions in AEC syndrome tend to be more severe on the scalp, a feature shared with desmosomal diseases such as pemphigus, possibly due to the regional expression of desmosomal proteins (63). Furthermore, skin fragility observed in AEC syndrome is reminiscent of the phenotype observed in the ectodermal dysplasia and skin fragility syndrome due to mutations in desmosome component plakophilin 1 (30). The palmoplantar keratoderma observed in AEC syndrome is typical of disorders linked to mutations in either desmosomal genes or components of the associated keratin cytoskeleton (29,34,35,64). An involvement of intermediate filaments or adherens junctions in the phenotype observed in the AEC mouse is unlikely as the main keratins of the basal (keratin K5, K14) and spinous layers (K1, K10) and the adherens junction components E-cadherin, P-cadherin and β-catenin are expressed at a similar level in the AEC mutant skin compared with wild-type (45 and present work).

In AEC mutant mice, blistering is observed primarily between basal and suprabasal layers, in spite of reduced expression of both basal and suprabasal cadherins. Compensatory mechanisms may be in place in the suprabasal compartment that compensate for the reduced desmosomal function. In agreement with this possibility, Dsp null mice display severe blistering more severe in the inner layers in spite of a broad expression of DSP in all layers of the skin (38). An alternative possibility is that as-yet-unidentified defects may contribute to the basal layer fragility to exacerbate skin blistering. Since p63 controls several biological processes, other defects beside reduced desmosomes may contribute to the complex phenotype of the skin erosions in AEC syndrome, including cell–matrix adhesion defects. Clinical evaluation of erosions in AEC patients suggest that most of the erosions tend to be relatively superficial, consistent with a desmosome defect more than a defect in attachment to the basement membrane (19 and references therein). Defects in the dermal–epidermal junction in the AEC mouse skin were not observed at the histological level or in EM studies, in spite of the known role of p63 in regulating expression of a number of integrins, and hemidesmosome and extracellular matrix components (2,10,65,66).

The first cell–cell adhesion gene identified as a p63 direct target was *Perp*, which encodes a tetraspan membrane protein that localizes to the desmosomes (47). *Perp* null mice exhibited frequent basal-suprabasal blistering in the skin and oral cavity (47), and developed ectodermal dysplasia and palmoplantar keratoderma (67). A normal pattern of PERP membrane staining was observed in most AEC patients, although in two unrelated patients PERP expression was reduced in the basal and suprabasal layers of the skin (68), consistent with either reduced PERP expression or with protein delocalization due to alterations of other desmosomal components. Similarly, in mouse mutant epidermis Perp was not affected, even though AEC mouse keratinocytes had significantly reduced Perp protein levels. More in general, changes in desmosomal gene expression were less strong *in vivo*, suggesting that compensatory mechanisms may be in place especially in the postnatal skin to ensure appropriate desmosomal gene expression. This possibility would be consistent with the amelioration and resolution of skin erosions observed in AEC children. The relatively low penetrance of intra-epidermal blistering observed in AEC mice is likely due to the mixed genetic background in which the AEC mice were generated. Owing to perinathal lethality in heterozygosity the influence of genetic background was not studied further; however, a

similar highly variable skin erosive phenotype has been reported in AEC patients carrying the same mutation and within the same family (17).

The desmosomal cadherins and DSP show complex patterns of expression, and thus are likely to be highly regulated at the transcription level, although, little is known about this regulation (47,69,70). We show here that p63 binds to *Dsp*, *Dsc3*, *Dsg1* regulatory regions. Consistent with our data, Dsg1 expression was strongly reduced in Δ*Np63* null embryonic skin (15). Several p63-binding regions in the Dsg/Dsc locus in human chromosome 18 have been identified by ChIP-seq experiments (57). While we were able to demonstrate direct p63 binding only for some of the tested corresponding mouse genomic regions possibly because of little conservation between human and mouse, the highly significant number of binding sites in the desmosomal cadherin locus indicate that this cluster might be coordinately regulated by p63.

Mutations in the SAM domain are thought to act in a dominant-negative fashion (45 and reference therein) with a mechanism that has not yet been elucidated. Here, we report that not only mutant p63 has an impaired transactivation ability, but also that in mutant skin p63 binds less efficiently Dsp and Dsc3 enhancers. These data are consistent with previous observations showing that in contrast to wild-type p63, mutations in the SAM domain impaired p63 ability to interfere with p53 binding to the DNA in a transactivation assay (18). Understanding how mutations in the SAM domain affect DNA binding, and whether partial impairment of DNA binding is the cause of reduced transactivation ability will require further studies. Another aspect that needs further clarification regards the mutant selectively, as some well-established p63 target genes, such as Perp (47) and P-cadherin (48), are unaffected in AEC mutant epidermis.

Beside p63 another putative regulator of desmosome gene expression is Irf6, whose genomic binding sites are enriched in cell adhesion genes in human keratinocytes (71). However, in spite of the fact that *Irf6* is a p63 target gene and its expression is reduced in AEC syndrome (45,54,55), our data indicate that Irf6 is not directly involved in desmosome gene expression at least in mouse keratinocytes.

Defective resistance to mechanical stress observed *in vitro* in AEC mutant keratinocytes can be overcome by inhibiting EGFR expression or activation. While EGFR activation is required for keratinocyte proliferation (72 and ref. therein), its inhibition favours cell adhesion by promoting the recruitment of DSP and desmosomal cadherins to the cell borders, and stabilizing desmosomal cadherins (42–44,60). We show here that a similar induction of Dsc3 and Dsg1 proteins occurs upon EGFR inhibition in AEC mouse keratinocytes, thus ameliorating the mechanical strength defect. EGFR signalling is also required to prevent terminal differentiation by suppressing Notch1 transcription and function (72). Thus, EGFR inhibition may exert its effect in part by enhancing differentiation in addition to stabilizing desmosome. Independently of its mechanisms of action, EGFR inhibition may elicit a beneficial effect *in vivo* when desmosome function is partially impaired as in the case of AEC syndrome. Whether treatment with EGFR inhibitors may be a therapeutic option for AEC patients will require more studies.

MATERIALS AND METHODS

p63$^{+/L514F}$ mice, histology, immunofluorescence and immunoblotting

The *L514F* mutation was inserted into the *p63* locus in E14TG2a ES cells (129/Ola strain) by recombineering, and injected into C57BL/6 blastocysts as previously described (45). Chimeric mice were generated by mating with C57BL/6 female mice. *p63*$^{+/L514F}$ mice were born in the normal Mendelian ratio, but died few hours after birth because of cleft palate. Offspring was genotyped for germline transmission of the *p63L514F* wild-type and mutant allele by PCR on tail genomic DNA with specific primers (Supplementary Material, Table S2). *p63*+/− mice (4) were obtained from Jackson Laboratory. All mouse experiments were approved by the Italian Ministry of Health.

For immunoblotting, newborn epidermis was obtained by floating skin biopsies in 0.5 M ammonium thiocyanate (NH_4SCN) in phosphate buffer, pH 6.8 for 20 min on ice. Tissue was pulverized in liquid nitrogen, lysed in sample buffer and immunoblotted as described (73). For histology mouse, tissue was fixed in 4% paraformaldehyde (PFA) and embedded in paraffin wax; 6 μm paraffin sections were cut and stained with Haematoxylin and Eosin (H&E) and monitored under a Zeiss Axioskop2 plus image microscope. For immunofluorescence tissue was embedded in O.C.T. compound (Tissue-TekII; Lab-Tek Products) and frozen at −70°C. Cryosections (6 μm) were fixed in 4% PFA and processed as previously described (45). Sections were examined using a Zeiss confocal microscope LSM510meta. The following primary antibodies were used for rabbit polyclonal antibodies for DSC1 and DSC3 (kindly provided by D. R. Garrod), mouse antibodies to p63 (4A4, Santa Cruz Biotechnology), DSG1 4B2 (kindly provided by Kathleen Green), DSG2 33–3D and DSP 115-F (kindly provided by D. R. Garrod), DSG3 AK18 (MBL International), PKP 1, PKP 3 and DSP (Progen Biotechnik GmbH), rabbit anti-Irf6 antibodies (kindly provided by B. C. Schutte), β-actin (Santa Cruz Biotechnology); rat antibodies to E-cadherin (Invitrogen); chicken antibodies to Jup 1408 (gift of K. Green). For immunoblotting, secondary antibodies were donkey anti-rabbit and sheep anti-mouse IgG conjugated to horseradish peroxidase (HRP) (GE Healthcare); goat anti-rat HRP (Santa Cruz Biotechnology); anti-chicken HRP (Sigma). Immunoblotting was performed using ECL (GE Healthcare). Secondary antibodies used for immunofluorescence staining were Alexa Fluor 488 goat anti-mouse, rabbit or chicken (Invitrogen).

EM

Fresh skin was dissected into small pieces (1 × 1 mm) and fixed by immersion in 2% PFA) plus 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) containing 0.15 M sucrose and 2 mM calcium chloride. Tissues were fixed for 24 h at 4°C and then washed four times with cacodylate buffer. They were then post-fixed with 1% osmium tetroxide for 2 h, dehydrated through an ethanol series, cleared in propylene oxide and embedded in 100 resin (Agar Scientific Ltd.). Ultrathin sections were contrasted with uranyl acetate

and lead citrate and examined on a Philips 400 *FEI Tecnai 12 Biotwin* electron microscope (40).

Cell culture

Wild-type and AEC mutant primary mouse keratinocytes were isolated from newborn mice by dispase/trypsin treatment and cultured under low Ca^{2+} conditions (0.03 mM) as previously described (45). p63 and Irf6 knockdown were obtained by transient transfection of 100nM small interfering RNA (siRNA) for pan-p63, ΔNp63 (73), IRF6 (Mm_Irf6_2; Qiagen) or negative control (Invitrogen). Six days after plating (24 h after transfection), growing keratinocytes were switched to high-calcium medium (0.6 mM calcium) for 24 h to induce desmosome assembly. Rescue experiments were performed by treating cells with 200 nM, 1 μM, 2 μM of the EGFR inhibitor, Tyrphostin AG1478 (Cell Signaling), 2 h before the calcium switch. Human keratinocytes obtained from AEC patients Q11X (74) and T533P (18), as well as control keratinocytes obtained from unaffected individuals were plated at a density of 10^4cells/cm^2 and cultured in KBM Gold medium (Lonza) until they reach confluence. Cells were then treated with 0.3 mM or 1.2 mM calcium for 24 h to induce desmosome assembly for subsequent RNA or protein analysis.

Real-time RT–PCR

Total RNA was extracted from mouse epidermis and mouse and human keratinocytes with TRIzol reagent (Invitrogen) and was employed as a template for cDNA synthesis performed with SuperScript Vilo (Invitrogen) according to manufacturer's instructions.

Two-step real-time reverse transcription RT–PCR was performed using the SYBR Green PCR master mix in an ABI PRISM 7500 (Applied Biosystems). Levels of the target genes were quantified with specific oligonucleotide primers and normalized for β-actin expression (for mouse and human primer sequences, respectively, see Supplementary Material, Tables S3 and S4).

Chromatin immunoprecipitation

Approximately 3×10^6 primary keratinocytes were fixed with 1% formaldehyde to perform ChIP-qPCR analysis using rabbit antibody to p63 (H-137 or H-129 Santa Cruz Biotechnology) and rabbit IgG as previously described (75). For primer sequences, see Supplementary Material, Table S5.

Cloning, mutagenesis and luciferase reporter assay

Mouse p63-binding regions located in intron 1 of *Dsp* (478 bp) and at −24 kb (479 bp) from *Dsc3* TSS were amplified by PCR, inserted in NheI-KpnI in the pGL3 basic vector (Promega) and sequence verified. Dsp and Dsc3 reporter constructs were mutagenized in the putative p63-binding site using the QuikChange mutagenesis kit (Promega). For oligonucleotides used for cloning and mutagenesis, respectively, see Supplementary Material, Tables S6 and S7. Primary keratinocytes seeded on 12-well plates were transiently cotransfected with reporter vectors listed above (250 ng), stealth siRNA (Invitrogen) for p63 or Negative control (100 nM) and pTK-Renilla (20 ng), using Lipofectamine 2000, following the manufacturer's protocol. Similarly, H1299 cells seeded in 24-well plate, were transiently cotrasfected with reporter constructs listed above and CMV-FLAG-ΔNp63α expression vector or empty CMV-FLAG vector as a control, and pTK-Renilla (5 ng). Luciferase activity was determined 48 h after transfection with the dual-luciferase reporter assay kit (Promega). *Renilla* luciferase activity was used to normalize transfection efficiency.

Dispase-based dissociation assay

Mechanical integrity of cell monolayers was assessed as described previously (76,77). Keratinocytes were grown to confluence in low-calcium medium containing 0.05 mM $CaCl_2$ and switched to medium containing 0.6 mM $CaCl_2$ for 1–3 days. At that time, keratinocyte sheets were lifted from the culture dishes by treating with 2.4 U/ml Dispase II (Roche) for 30 min at 37°C. Intact sheets were transferred to 15 ml conical tubes containing 5 ml of PBS. Tubes were placed in a rack and inverted were inverted on a rotor at 0.28 *g* for 30–50 inversion cycles. Cellular fragments were transferred to 35 mm tissue culture plates and counted using a dissecting microscope.

Statistics

Statistical significance of the gene expression studies was assessed by an unpaired two-tailed Student's *t* test. *P*-values are indicated in figure legends. All quantitative results are presented as mean ± standard deviation (SD) or standard error (SEM) as indicated in the figure legends, and were calculated by the Excel software.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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