IKKα Is a p63 Transcriptional Target Involved in the Pathogenesis of Ectodermal Dysplasias

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The transcription factor p63 plays a pivotal role in the development and differentiation of the epidermis and epithelial appendages. Indeed, mutations in p63 are associated with a group of ectodermal dysplasias characterized by skin, limb, and craniofacial defects. It was hypothesized that p63 exerts its functions by activating specific genes during epidermal development, which in turn regulate epidermal stratification and differentiation. We have identified I-kappaB kinase alpha (IKKα) as a direct transcriptional target of p63 that is induced at early phases of terminal differentiation of primary keratinocytes. We show that the ΔNp63 isoform is required for IKKα expression in differentiating keratinocytes and that mutant p63 proteins expressed in ectodermal dysplasia patients exhibit defects in inducing IKKα. Furthermore, we observed reduced IKKα expression in the epidermis of an ankyloblepharon ectodermal dysplasia clefting patient. Our data demonstrate that a failure to properly express IKKα may play a role in the development of ectodermal dysplasias.

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INTRODUCTION

The transcriptional activator p63 is a p53 paralog that is expressed in basal cells and somatic stem cells of stratified epithelia, in myoepithelial cells of the breast, salivary glands, and in the proliferative compartment of gastric mucosa (Yang *et al.*, 1998, 1999). Owing to the use of two promoters and complex alternative splicing, p63 encodes six isoforms (Yang *et al.*, 1998). Of these, TA isoforms structurally resemble p53 and contain an N-terminal transactivation domain, whereas Delta-N (ΔN) isoforms lack this transactivation domain (Yang *et al.*, 1998). Dominantly inherited mutations in the p63 gene are found in a number of human ectodermal dysplasias, including ectrodactyly ectodermal dysplasia-cleft lip/palate (EEC) syndrome, limb-mammary syndrome (LMS), ankyloblepharon ectodermal dysplasia clefting (AEC) and non-syndromic split-hand/split-foot malformation (SHFM) (Rinne *et al.*, 2006). Genotype–phenotype correlations exist in that mutations causing EEC syndrome, for example, are not found in AEC, LMS, or SHFM (van Bokhoven and Brunner, 2002). Moreover, there is a clear correlation between the position where the mutation occurs and the observed syndrome. The majority of mutations found in the EEC syndrome are missense mutations generating amino acid substitutions in residues predicted to contact DNA (Rinne *et al.*, 2006). As the DNA binding domain is present in all p63 isoforms, all isoforms of p63 are affected by these mutations. Mutations in exon 13 and exon 14, affecting only the α-isoforms of p63, are almost exclusively associated with AEC (McGrath *et al.*, 2001; Rinne *et al.*, 2006). DNA-binding mutants often act as dominant-negative molecules, although whether this is also the case for mutant p63 proteins remains to be determined.

Mice lacking p63 die soon after birth with several developmental defects, particularly in limb and skin development (Mills *et al.*, 1999; Yang *et al.*, 1999). Defects in limb morphogenesis in p63 null mice were evident as early as embryonic day 9.5 (E9.5). In wild-type (wt) mice, during this interval, p63 is expressed in the surface ectoderm as well as in the ectoderm covering the limb buds and branchial arches. Although still a matter of debate (Laurikkala *et al.*, 2006; Suh *et al.*, 2006), it has been found that TAp63 isoforms, the first p63 isoforms expressed during epidermal development, are required for the commitment to stratification while they inhibit terminal differentiation (Koster and Roop, 2004; Koster *et al.*, 2004). After commitment to stratification has occurred, ΔNp63 isoforms induce the expression of genes that are required for later stages of epidermal morphogenesis (Koster *et al.*, 2007).

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Abbreviations: Ab, antibody; AEC, ankyloblepharon ectodermal dysplasia clefting; ChIP, chromatin immunoprecipitation; EEC, ectrodactyly ectodermal dysplasia-cleft lip/palate; IKKα, I-kappaB kinase alpha; LMS, limb-mammary syndrome; NHEK, normal human epidermal keratinocytes; SHFM, split-hand/split-foot malformation; siRNA, small interfering RNA

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Interestingly, I-kappaB kinase alpha (IKKα)-deficient mice display developmental defects, including skin, craniofacial, and limb defects, showing some similarities with p63 null mice (Hu *et al.*, 1999; Li *et al.*, 1999; Mills *et al.*, 1999; Takeda *et al.*, 1999; Yang *et al.*, 1999). Although IKKα is a protein kinase that is part of the IKK complex, its role in epidermal differentiation is independent of its kinase activity or NF-κB activation (Hu *et al.*, 1999). Interestingly, expression of IKKα in the epidermis is required not only for epidermal development but also for the development of tissues derived from the mesoderm and neural crest (limbs and craniofacial structures) (Sil *et al.*, 2004) and to switch on the differentiative program by favoring keratinocyte cell cycle arrest (Descargues *et al.*, 2008). A recent study (Candi *et al.*, 2006) has identified IKKα as a transcriptional target for p63 with TAp63 being a better activator than ΔNp63 in transcriptional assays. In our previous work (Koster *et al.*, 2007), we showed that epidermal-specific ΔNp63 downregulation in mice leads to increased keratinocyte proliferation, which correlates with reduced expression of IKKα. Furthermore, these mice display a skin phenotype characterized by skin fragility that may resemble that of AEC patients (Koster *et al.*, 2007). We thus hypothesized that IKKα and p63 may genetically also interact in human cells, with IKKα functioning downstream of p63. Furthermore, we hypothesized that mutant p63 proteins expressed in ectodermal dysplasia patients may interfere with the induction of IKKα expression and that the alteration of this pathway may contribute to the phenotype observed in ectodermal dysplasia patients. In this study, we show that p63 directly induces IKKα transcription and that mutant p63 proteins expressed in ectodermal dysplasia patients interfere with IKKα induction.

RESULTS

IKKα is a direct transcriptional target of p63 in keratinocytes

IKKα promoter contains at least three potential p63 responsive elements (Osada *et al.*, 2005a, b; Candi *et al.*, 2006). To determine whether p63 could induce IKKα transcription, we generated a luciferase reporter construct (IKKα-luc) containing a 1.2 kb fragment of the IKKα promoter and performed transient transactivation assays. Exogenously expressed ΔNp63α, ΔNp63β, ΔNp63γ, and TAp63γ strongly induced the IKKα reporter construct, whereas TAp63α and TAp63β weakly induced this reporter (Figure 1a). We also observed activation of the IKKα promoter by exogenously expressed ΔNp73α and TAp73α (Figure 1a). In contrast, p53 expression did not induce transcription from the IKKα promoter either in U2OS cells (Figure 1a) or in the immortalized keratinocyte cell line HaCaT (data not shown). Although this is in disagreement with the previously described role of p53 as a transcriptional repressor of IKKα in an acute lymphoblastic leukemia cell line (Gu *et al.*, 2004), this discrepancy could potentially be explained by cell-type specificity.

The ability of p63 to induce endogenous IKKα expression was tested in transient expression experiments in U2OS cells. As shown in Figure 1b, exogenously expressed ΔNp63 and ΔNp73 isoforms induced an increase in the levels of endogenous IKKα mRNA (left panel) and protein (right panel) better than the TA isoforms did. Expression levels of different p63 and p73 isoforms are shown in Figure S1A.

To determine if p63 isoforms directly interact with the IKKα promoter, we carried out chromatin immunoprecipitation (ChIP) experiments after transient transfection of U2OS cells with myc-tagged p63 expression plasmids. Cross-linked chromatin was immunoprecipitated with specific antibodies (Abs), and the presence of recovered IKKα promoter fragments was analyzed by PCR using primers specific for the proximal region of the putative IKKα promoter. Quantification of p63-bound IKKα promoter fragments after normalization to the amount of transfected p63 protein (Figure S1B) indicated that ΔNp63α, TAp63γ, and ΔNp63γ had a higher affinity for the IKKα promoter than TAp63α did (Figure 1c, left panel). The ChIP assay was repeated in primary human keratinocytes placed under proliferating or differentiating conditions (Figure 1c, right panel). We observed a transient increase in p63 binding to IKKα promoter peaking 8 hours after differentiation stimulus (Figure 1c, right panel).

These data indicate that p63 isoforms can induce IKKα transcription, acting most likely through direct binding to its promoter.

ΔNp63 induces IKKα during keratinocyte differentiation

To study the regulation of IKKα transcription during keratinocyte differentiation, we examined IKKα mRNA levels by real-time quantitative PCR (RT-qPCR) in normal human epidermal keratinocytes (NHEK) subjected to a differentiation stimulus (2 mM $CaCl_2$). IKKα mRNA levels increased at early times (8 and 24 hours) after the induction of NHEK differentiation (Figure 2a, left panel). As expected, ΔNp63 mRNA levels decreased upon differentiation (Figure 2a, right panel), suggesting that p63 may be required to induce IKKα transcription but not to maintain it. The early induction of IKKα transcription upon keratinocyte differentiation correlates with high levels of IKKα protein expression in the nuclei of basal and suprabasal keratinocytes in normal human skin (Figure 2b, upper panels) and colocalization of IKKα with p63 in most cells of the basal and suprabasal layers (Figure 2b, lower panels). To determine whether the subcellular localization of IKKα was also affected by differentiation of human keratinocytes, we performed immunoblotting using an anti-IKKα Ab on cytoplasmic and nuclear extracts of normal human keratinocytes. We observed an increase in the nuclear fraction of IKKα protein upon keratinocyte differentiation (Figure 2c), confirming previous observations obtained in mouse keratinocytes (Sil *et al.*, 2004; Descargues *et al.*, 2008).

To determine whether ΔNp63 proteins are involved in IKKα induction, we used small interfering RNAs (siRNAs) to selectively downregulate ΔNp63 transcripts in primary keratinocytes. The specificity of siRNAs was determined by transient transfection experiments performed in U2OS cells in which we co-transfected expression plasmids encoding for ΔNp63 together with control siRNAs (GFPsiRNA), ΔNp63-specific, or TAp63-specific siRNAs. We observed a high efficiency of ΔNp63-specific siRNA in downregulating ΔNp63 protein expression (Figure 3a, left panel). The same

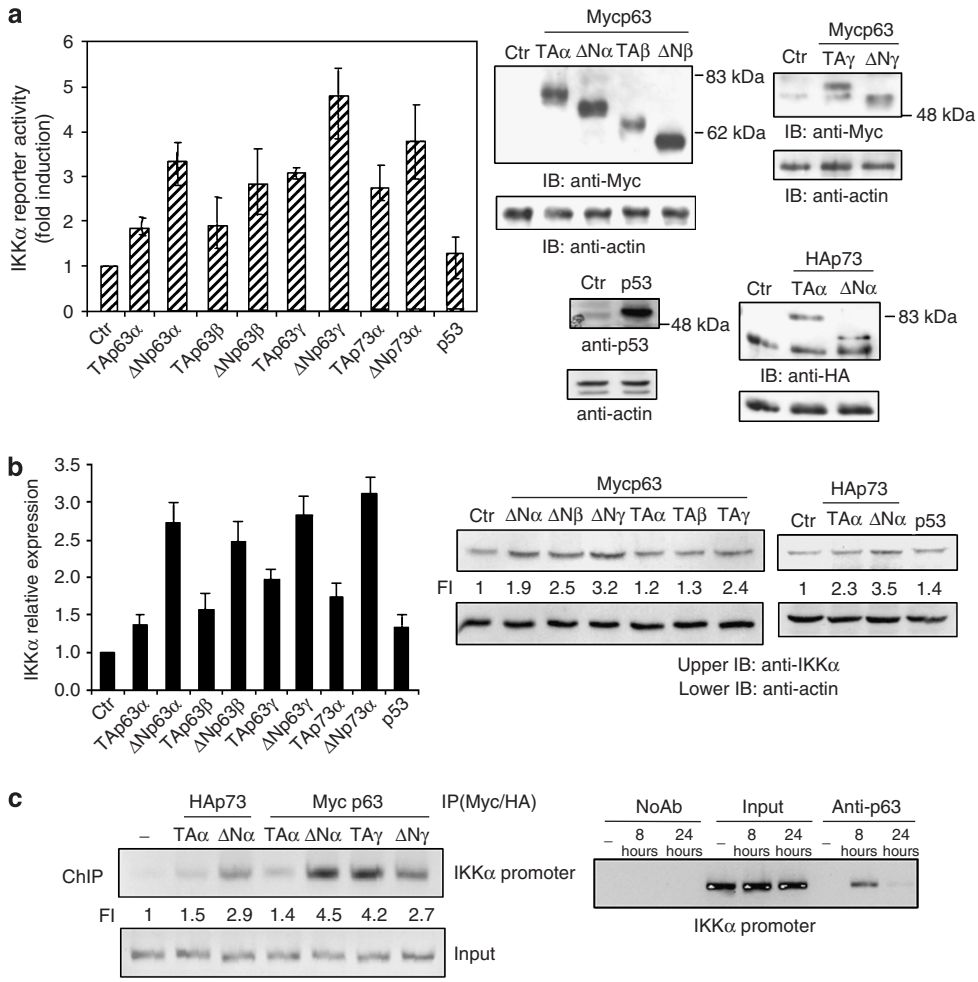

Figure 1. p63 activates IKKα transcription. (**a**) p63 activates IKKα reporter gene. U2OS cells were transfected with IKKα reporter plasmid (0.1 μg), pRL-null plasmid (0.02 μg), and expression vectors for different p63 and p73 isoforms or p53 (1.5 μg). After 24 hours, cells were harvested and luciferase activity was determined (left panel). Protein expression was evaluated by western blot analysis with anti-Myc, anti-HA, and anti-p53 antibody (Ab) (right panel). (**b**) Exogenously expressed p63 and p73 isoforms induce IKKα mRNA and protein expressions. U2OS cells were transfected with 2 μg of the indicated plasmid in 35 mm dishes. Cells were harvested 24 hours after transfection and total mRNA was extracted and used for RT-qPCR. Results are expressed as IKKα expression relative to ARP (human acidic ribosomal protein P0) expression (left panel). In a parallel experiment, U2OS cells were transfected with 2 μg of the indicated plasmid in 35 mm dishes. Cells were harvested 24 hours after transfection, and total cell lysates (25 μg) were subjected to immunoblot analysis with an anti-IKKα Ab to determine IKKα expression. Actin expression was determined as a loading control. Levels of expression relative to pCDNA (Ctr)-transfected cells are indicated (right panel). (**c**) p63 and p73 directly interact with the IKKα promoter. U2OS cells were transfected with the indicated plasmids. Cells were subjected to ChIP, and the recovered chromatin was amplified with IKKα promoter-specific primers and normalized to protein expression (Figure S1). The ChIP assay was repeated on primary human keratinocytes under proliferating or differentiating conditions using anti-p63-specific polyclonal Abs (right panel).

siRNAs were used to downregulate endogenous ΔNp63 in primary human keratinocytes (Figure 3a and b) or in primary mouse keratinocytes (Figure S2). The transfection of ΔNp63 siRNAs resulted in a marked downregulation of ΔNp63 expression in both proliferating and differentiating keratinocytes. Downregulation of ΔNp63 had no effect on the basal levels of IKKα protein, whereas calcium-induced levels of IKKα were significantly decreased in ΔNp63-silenced cells (Figure 3a, right panel). ΔNp63 downregulation also decreased the expression of K1 early differentiation marker (Figure 3a, right panel). These results were confirmed by evaluating the levels of IKKα mRNA in keratinocytes in which ΔNp63 was downregulated (Figure 3b), and suggest that ΔNp63 is involved in the induction of IKKα at early phases of keratinocyte differentiation. Indeed, the downregulation of ΔNp63 also resulted in a failure to induce early differentiation markers of keratinocytes such as K1 (Figure 3b, right panel), indicating that ΔNp63 controls the formation of suprabasal layers. We also confirmed these results in primary mouse keratinocytes (Figure S2). In addition, as ΔNp63 expression levels are reduced in the suprabasal layers of the epidermis (Yang *et al.*, 1998), these data are consistent with the existence of ΔNp63-independent mechanisms for maintaining IKKα expression in terminally differentiated cells.

In addition to reduced levels of K1 differentiation markers, ΔNp63-downregulated cells also display increased proliferation as determined by BrdU incorporation assay (Figure 3c). This is consistent with the observation made on the ΔNp63-

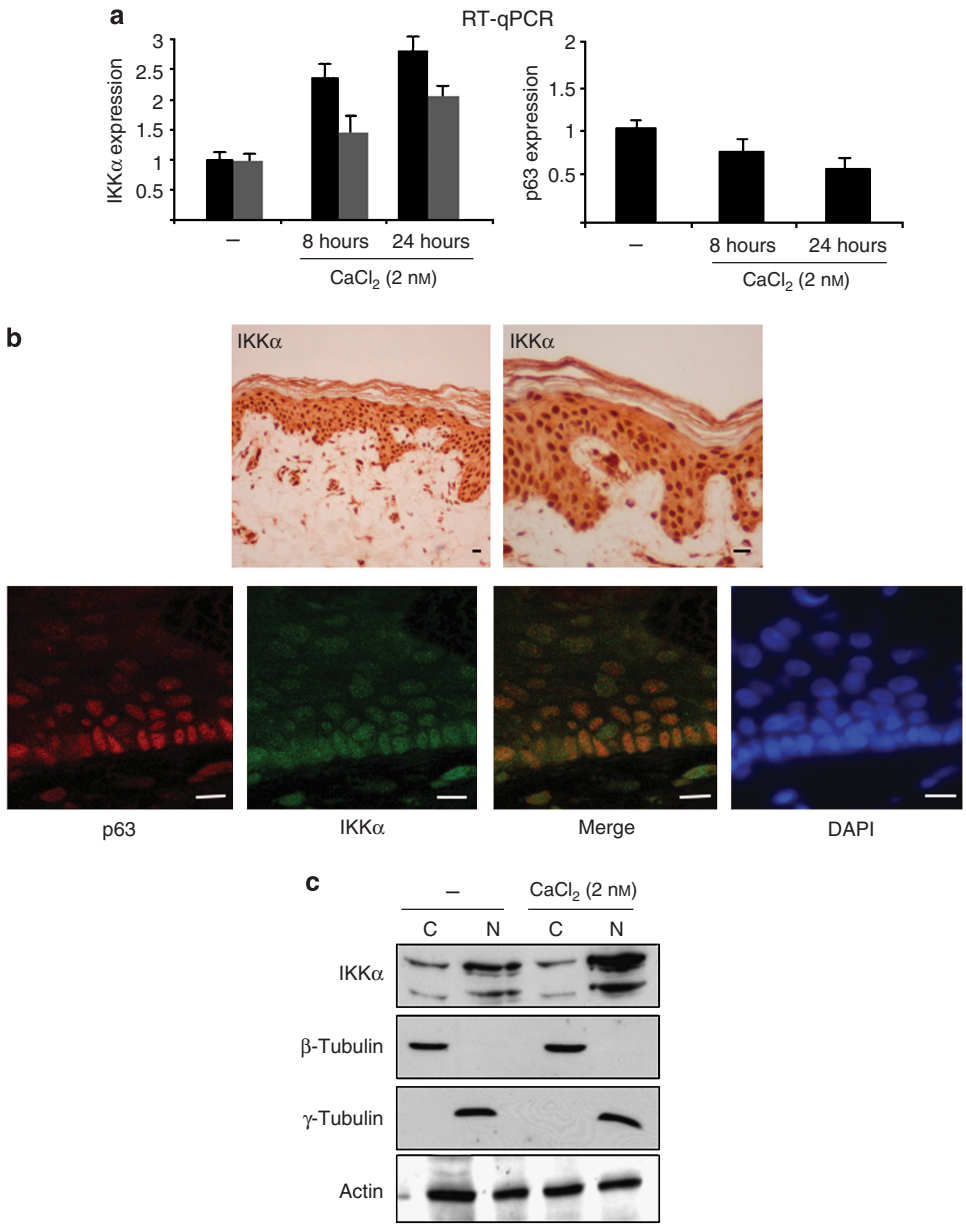

Figure 2. Nuclear IKKα expression increases at the onset of keratinocyte terminal differentiation. (**a**) IKKα expression increases at the onset of keratinocyte differentiation. Human primary keratinocytes were treated with 2 mM $CaCl_2$ for 8 or 24 hours. Relative mRNA abundance was determined by RT-qPCR for IKKα, K1 (left panel), or ΔNp63 (right panel). (**b**) p63 and IKKα colocalize in keratinocytes in human epidermis. Immunohistochemical staining of normal skin sections was performed with a monoclonal anti-IKKα antibody (Ab) (see the Materials and Methods section), and upper panels represent original magnifications of ×10 and ×40 acquisition of the same section. Analysis of IKKα and p63 expressions in the human epidermis was performed by confocal microscopy after immunofluorescence staining (scale bar = 20 μm). (**c**) IKKα nuclear translocation is induced by $CaCl_2$ treatment. Human primary keratinocytes were treated with 2 mM $CaCl_2$. Cells were harvested 24 hours after treatment, and nuclear/cytoplasmic extracts were analyzed by western blot analysis using an anti-IKKα Ab. Actin, β-tubulin, and γ-tubulin expressions were used as a loading control to determine the purity of cytoplasmic extracts.

knockdown mice skin (Koster *et al.*, 2007) and with the phenotype of AEC skin (Figure 5c). Given the role of IKKα as a critical regulator of cell cycle exit in keratinocytes (Koster *et al.*, 2007; Descargues *et al.*, 2008), we attempted to rescue the correct proliferation rate by reintroducing IKKα in ΔNp63-deficient keratinocytes. To this aim, human keratinocytes were transfected with control or ΔNp63-specific siRNAs and then infected with mock or IKKα-expressing adenoviruses and placed under differentiation conditions. Twenty-four hours after infection, BrdU assay was performed to determine the proliferation rate. As shown in Figure 3c, the reintroduction of IKKα in keratinocytes restored low proliferation rate. These data suggest that IKKα may act as the ΔNp63 target gene required for correct exit from the cell cycle upon differentiation stimulus, a step necessary to achieve terminal differentiation.

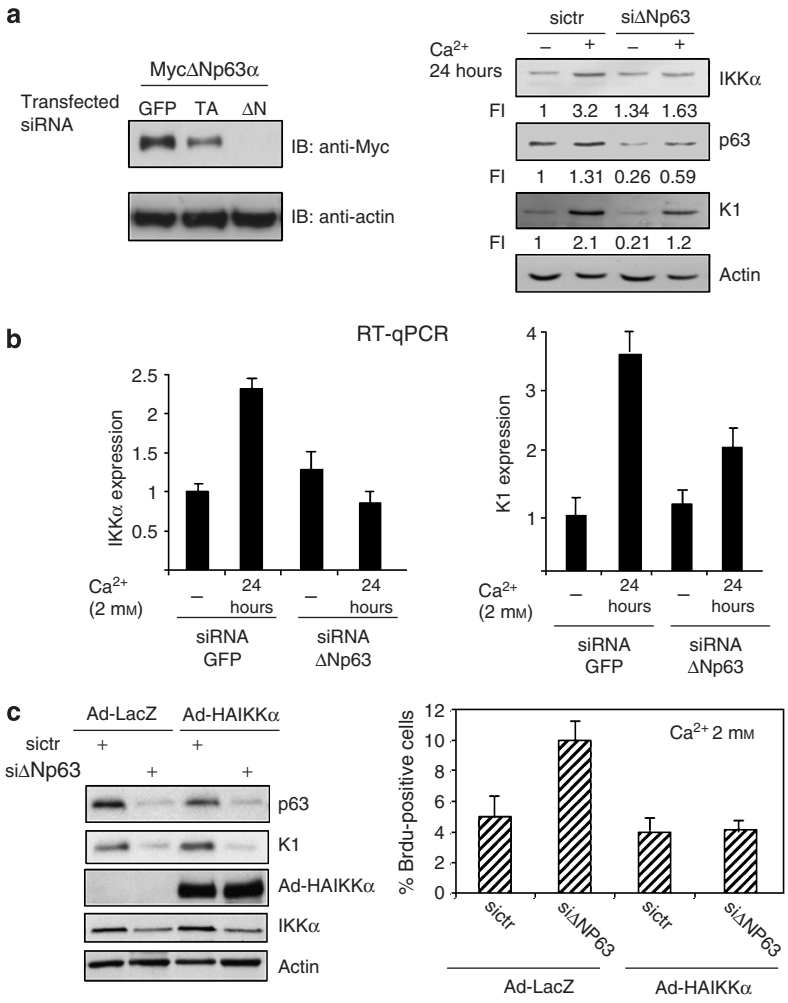

Figure 3. ΔNp63 is required for induction of IKKα expression by differentiation. (**a**) Specificity of ΔNp63 siRNAs. U2OS cells were co-transfected with 1 μg of Myc-tagged ΔNp63α expression plasmids, and the indicated siRNAs and cell lysates were subjected to immunoblotting with anti-myc or anti-actin antibodies (left panel); primary human keratinocytes were transfected with specific siRNAs and then kept under proliferating or differentiating (2 mM $CaCl_2$) conditions. After 24 hours, cells were harvested and 25 μg of total lysates were subjected to immunoblot analysis to verify p63, IKKα, K1, or actin expression levels (right panel). Expression levels relative to control (first lane) were quantified. (**b**) The downregulation of ΔNp63 isoforms abrogates $CaCl_2$-induced activation of IKKα transcription. Primary human keratinocytes maintained under proliferating or differentiating (2 mM $CaCl_2$) conditions were transiently transfected with specific siRNAs. Relative mRNA abundance was determined by RT-qPCR for IKKα (left panel) and K1 (right panel). (**c**) Reintroduction of IKKα in ΔNp63-deficient keratinocytes restores calcium-induced exit from the cell cycle. Primary keratinocytes were transfected with indicated siRNAs. Sixteen hours after transfection, cells were infected with Ad-IKKα. The next day, the cells were induced to differentiate by the addition of calcium (2 mM). BrdU was then added for 6 hours and its incorporation measured by a specific kit. Results are expressed as percentage of positive cells and error bars represent SD. Results are representative of three independent experiments.

p63 mutant proteins expressed in ectodermal dysplasia patients influence IKKα transcription

Given that some defects observed in ectodermal dysplasias caused by p63 mutations are also present in IKKα knockout mice, we hypothesized that mutant p63 proteins expressed in ectodermal dysplasia patients may have a reduced ability to induce IKKα. To test this hypothesis, we determined the ability of mutant ΔNp63 proteins to activate the IKKα reporter gene and to induce endogenous IKKα expression. To this aim, we used expression plasmids expressing different p63 mutants found in ectodermal dysplasias including DNA binding domain mutants (ΔNp63α-306) and truncation mutants (ΔNp63α-fs) of EEC syndrome, ΔNp63α-518 and ΔNp63α-536 SAM domain mutants of AEC syndrome, ΔNp63α-DAA of LMS, and ΔNp63α-639 of SHFM (Figure 4a). We initially performed transcriptional assays in the epithelial cell line TE1 (North *et al.*, 2002) and observed that mutant ΔNp63α proteins expressed in EEC and AEC patients (ΔNp63α-EEC and ΔNp63α-AEC, respectively) displayed a reduced ability to activate the IKKα reporter gene (Figure 4b). In particular, ΔNp63α-AEC proteins had a reduced ability to activate the IKKα reporter construct. Similarly, mutant ΔNp63α-EEC proteins showed a reduced ability to activate the IKKα reporter construct. Mutant ΔNp63α proteins expressed in LMS and SHFM patients (ΔNp63α-LMS and ΔNp63α-SHFM, respectively), on the contrary, did not show significant differences in their ability to activate the IKKα reporter (Figure 4b).

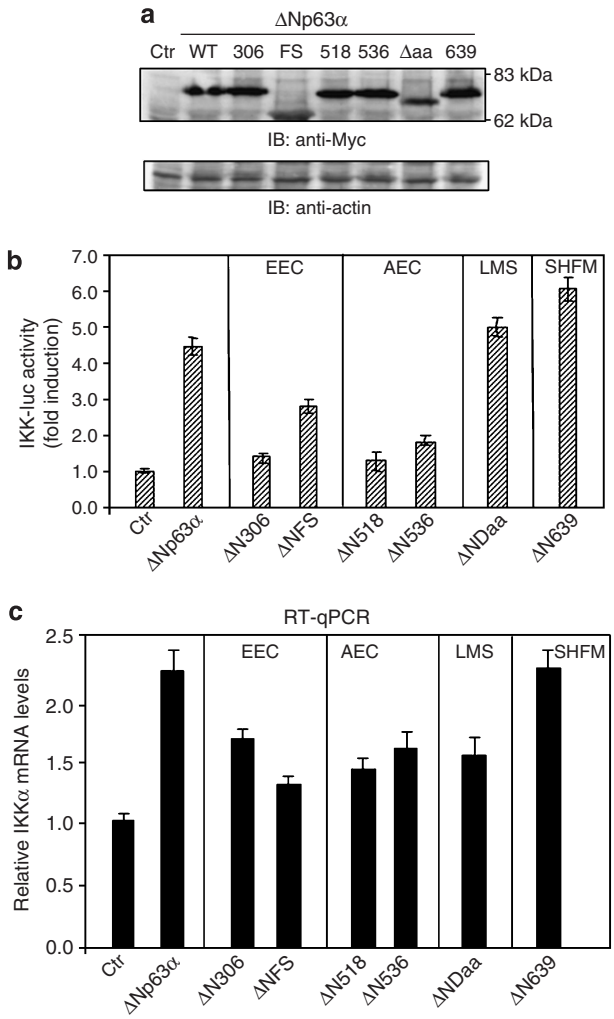

Figure 4. Transcriptional regulation of the IKKα promoter by ΔNp63α mutants expressed in ectodermal dysplasias. Transcriptional regulation of the IKKα promoter by ΔNp63α mutants expressed in ectodermal dysplasias. (**a**) Expression levels of myc-tagged wt ΔNp63α and ΔNp63α mutants L518V, Q536L, FS, C306R, 1743 DelAA, and E639X were analyzed in TE1 cells by immunoblot with an anti-myc antibody. (**b**) TE1 cells were co-transfected with the IKKα reporter plasmid (0.1 μg), pRL-null plasmid (0.02 μg), and expression vectors for ΔNp63α wt and mutants L518V, Q536L, FS, C306R, 1743 DelAA, and E639X (1.5 μg). After 24 hours, cells were harvested and luciferase activity was determined. (**c**) TE1 cells were transfected with the indicated plasmids and subjected to total mRNA extraction. IKKα mRNA expression was determined by RT-qPCR after normalization to ARP expression.

The analysis of IKKα mRNA transcript levels in TE1 cells transfected with ΔNp63α-AEC or ΔNp63α-EEC constructs confirmed the defective ability of ΔNp63α-AEC and ΔNp63α-EEC to induce IKKα transcription and showed reduced ability of the LMS mutant to induce accumulation of IKKα mRNA (Figure 4c). Similar results were obtained in the U2OS cell line (data not shown).

p63 mutations that cause ectodermal dysplasias are heterozygous, and mutant p63 proteins expressed in ectodermal dysplasia patients may have a dominant-negative effect toward wt p63 on the activation of p63 target genes. To test this hypothesis, we determined the ability of mutant p63 proteins to interfere with IKKα induced by exogenously expressed wt ΔNp63α in TE1 cells. As shown in Figure 5a, ΔNp63α-AEC, ΔNp63α-EEC, and ΔNp63α-LMS proteins impaired the ability of wt ΔNp63α to induce IKKα mRNA, indicating their capacity to act in a dominant-negative manner. To further confirm this observation, we analyzed the expression levels of IKKα protein in TE1 cells transfected with ΔNp63α in the presence of mutant ΔNp63α proteins (Figure 5b). Densitometric quantification of IKKα expression levels confirmed that ΔNp63-FS (EEC) and ΔNp63-536 (AEC) exert a dominant-negative activity, that ΔNp63-306 (EEC) has low interference potency, that and ΔNp63-639 (SHFM) does not interfere at all with ΔNp63α-induced IKKα expression. The dominant-negative activity of p63 mutants is also demonstrated by their ability to block differentiation-induced activation of IKKα reporter transcription (Figure S3).

To confirm that ΔNp63α-AEC proteins function as dominant-negative molecules, we analyzed the epidermis of an AEC patient carrying the I537T mutation. We determined the expression of IKKα, K1, and Ki-67 by immunohistochemistry on the lesional skin. As shown in Figure 5c, IKKα expression is drastically reduced in AEC skin as compared with normal skin of a control subject (Figure 5c, left panels). Furthermore, we observed delayed K1 expression, which is present only in the upper layers of AEC skin, and an increase in the expression of the proliferation marker Ki-67 (Figure 5c, middle and right panels). IKKα down-regulation in AEC skin is most likely due to reduced transcription, as determined by the analysis of IKKα mRNA levels extracted from AEC skin tissue (Figure 5c right histogram).

Taken together, our data demonstrate that ΔNp63α directly induces expression of IKKα and that reduced IKKα expression, caused by expression of mutant p63 proteins, may contribute to the development of the clinical phenotype observed in ectodermal dysplasias.

DISCUSSION

Epidermal morphogenesis is a tightly regulated process that involves the temporally coordinated activation of specific sets of genes required for keratinocytes to achieve the subsequent differentiation step (Koster and Roop, 2004). In addition, many genes required for the execution of the epidermal stratification program also mediate epithelial–mesenchymal interactions, thus contributing to the formation of epithelial appendages. Analysis of the phenotypes of p63 and IKKα knockout mice indicated that p63 and IKKα belong to this category of genes (Li *et al.*, 1999; Mills *et al.*, 1999; Takeda *et al.*, 1999; Yang *et al.*, 1999; Koster and Roop, 2004; Sil *et al.*, 2004).

Our results demonstrate that p63 and IKKα are part of the same genetic pathway, with p63 directly inducing transcription of IKKα. The transcriptional activation of IKKα occurs early upon differentiation of cultured keratinocytes. The subsequent translocation of IKKα to the nucleus is required for the role of IKKα in keratinocyte differentiation. All three ΔNp63 isoforms and, to a lesser extent, TAp63γ can transcriptionally activate IKKα when overexpressed.

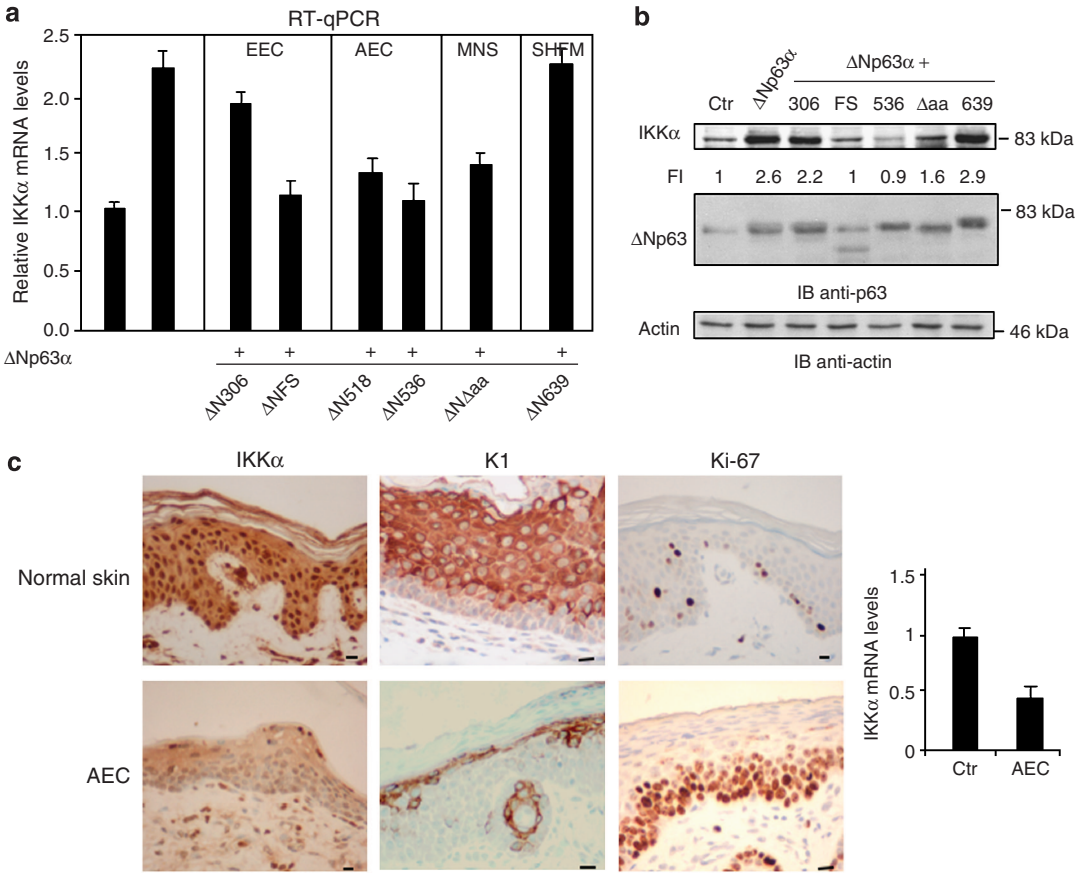

Figure 5. Dominant-negative activity of p63 mutants on ΔNp63-induced IKKα expression. (**a**) TE1 cells were transfected with wild-type ΔNp63α alone or in combination with the indicated expression plasmids. After 24 hours, cells were harvested and the IKKα mRNA expression normalized to ARP levels was determined by RT-qPCR. (**b**) TE1 cells were transfected with wild-type ΔNp63α alone or in combination with the indicated expression plasmids. Cells were harvested after 36 hours, and total cell lysate was immunoblotted with specific antibodies (Abs). The relative amount of endogenous IKKα protein was quantified by densitometric analysis. (**c**) Skin sections from healthy donors (upper panels) and from the lesional skin of a patient affected by AEC syndrome (I537T mutation in the SAM domain of p63) (lower panels) were subjected to immunohistochemistry with specific Abs to reveal the pattern of expression of IKKα (left panels), K1 (middle panels), and the proliferation marker Ki-67 (right panels). Scale bar = 20 μm. Total RNA extracted from normal skin sections and from AEC skin sections was subjected to RT-qPCR to determine IKKα mRNA expression levels (right histogram). Bars represent the mean expression (± SD) obtained from three different sections of normal subjects and of the AEC patient.

However, experiments performed in primary human keratinocytes, in which ΔNp63 was selectively downregulated, clearly indicate that ΔNp63α is required for the induction of IKKα during keratinocyte differentiation, confirming the essential role of ΔNp63 in the regulation of proliferation and differentiation of mature keratinocytes (Truong *et al.*, 2006; Koster *et al.*, 2007). The role of IKKα as a critical regulator of keratinocyte proliferation was further demonstrated by its ability to rescue normal cell cycle exit in ΔNp63-downregulated cells. The same pathway was independently identified by Candi *et al.* (2006); however, their data suggest that both classes of p63 isoforms, TA and ΔN, could induce IKKα expression when overexpressed in SAOS2 cells. This apparent discrepancy with our data can be explained by the use of different cell types.

The EEC, SHFM, AEC, and LMS syndromes are caused by mutations in the p63 gene. These syndromes show clinical variability, with sparse hair, dry skin, pilosebaceous gland dysplasia, and oligodontia as well as variable abnormalities of hands and feet, ranging from severe ectrodactyly in EEC patients to mild defects in AEC patients (Brunner *et al.*, 2002; Rinne *et al.*, 2006). We hypothesized that alterations in IKKα expression levels, caused by the inability of mutant p63 proteins to activate IKKα transcription at critical steps during epidermal development, may contribute to the development of some of the clinical phenotypes observed in ectodermal dysplasia patients. Indeed, our data demonstrate that most of the mutant p63 proteins used in this study displayed an altered ability to induce transcription from an IKKα reporter and to induce expression of IKKα mRNA. These defects are particularly evident in ΔNp63 proteins carrying mutations found in patients with AEC, which display skin abnormalities, and less in ΔNp63 proteins carrying mutations found in ectodermal dysplasias lacking a skin phenotype (SHFM). Moreover, a subset of mutant ΔNp63 proteins expressed in EEC (ΔNp63FS), AEC (ΔNp63-536), or LMS (ΔNp63DAA) patients exhibit a dominant-negative activity toward the induction of IKKα transcripts by wt ΔNp63α. This effect

correlates with the observation of the heterozygous nature of p63-dependent ectodermal dysplasias supporting the hypothesis that some clinical defects observed in ectodermal dysplasia patients may be due to the ability of mutant p63 proteins to prevent gene activation by wt p63. Recent observations confirm that the selective and conditional downregulation of ΔNp63 in the epidermis of newborn mice results in IKKα mRNA downregulation and in a clinical phenotype similar to that observed in AEC syndrome (Koster *et al.*, 2007). Furthermore, IKKα downregulation was also observed in the lesional skin of AEC patients at both protein and mRNA levels (Figure 5c), further reinforcing the idea that IKKα activation by p63 may play a role in the pathogenesis of skin defects in ectodermal dysplasias. Although additional *in vivo* models (for example, AEC knockin mice) are necessary to confirm our conclusions, the data reported in this study suggest that IKKα may act as a critical p63 downstream target gene in the regulation of epidermal morphogenesis and in the development of a subset of ectodermal dysplasias.

MATERIALS AND METHODS

Cell lines

U2OS (wt-p53 osteosarcoma cells) were grown in DMEM containing 10% regular fetal bovine serum and antibiotics (100 U ml^{-1} penicillin and 10 mg ml^{-1} streptomycin) at 37 °C. TE1 cells (esophageal SCC cell line) were cultured in RPMI medium containing 10% regular fetal bovine serum and antibiotics (100 U ml^{-1} penicillin and 10 mg ml^{-1} streptomycin) at 37 °C. Primary mouse keratinocytes were isolated from newborn mice and cultured at 37 °C in low calcium (0.05 mM $CaCl_2$) keratinocyte basal medium (Clonetics, San Diego, CA) and EGF (10 ng ml^{-1}). Cryopreserved NHEKs were obtained from Clonetics and grown on calf skin collagen (Sigma, Milan, Italy) coated dishes in serum-free keratinocyte basal medium. Third-passage NHEK cells were used for transfection experiments.

Reporter plasmids, transient transfections, viral infection, and luciferase assays

Expression plasmids encoding for TAp63α, TAp63β, TAp63γ, ΔNp63α, ΔNp63β, ΔNp63γ, and ΔNp63α mutants L518V and Q536L (AEC), FS (1689insA) and C306R (EEC), 1743 DelAA (LMS), and E639X (SHFM) were obtained from L. Guerrini (University of Milan, Italy). The p53-encoding plasmid was a kind gift of M. Oren (The Weizmann Institute). IKKα-expressing adenovirus was already described (Hu *et al.*, 1999). Viral infections were conducted by incubating primary keratinocytes in serum- and Ca^{2+}-free Eagle's minimum essential medium with the appropriate adenovirus at a multiplicity of infection of 50 for 3 to 4 hours, followed by culture in normal growth medium. Under these conditions, ⩾90% of primary mouse keratinocytes were infected, as confirmed by X-gal staining in cells infected with Ad-LacZ.

To generate the IKKα-luc reporter plasmid, normal human genomic DNA was amplified with primers (hIKKαprom sense 5′-CAGTGCTCAACATTCTGGTTGC-3′ and hIKKαprom antisense 5′-GCCTCAGGTTCCACAGTTGT-3′) spanning the −1,182 to +45 region relative to the IKKα transcription initiation site. The PCR product was cloned into the *Sma*I site of the pGL3 basic plasmid (Promega Inc., Madison, WI) and verified by sequencing. The analysis of putative p53 responsive elements was performed using PatSearch software (http://bighost.area.ba.cnr.it/BIG/PatSearch/) and the following strings:

p1 = rrrcwwgyyy[3,0,0] 0.13 p2 = rrrcwwgyyy[3,0,0];
p1/p2:(p3 = rrrcwwgyyyrrrcwwgyyy[3,0,0]

Transfection and luciferase activity assay

For reporter gene assays, cells were transiently co-transfected with the IKKα-luc reporter and mammalian expression plasmids encoding for TAp63α, TAp63β, TAp63γ, ΔNp63α, ΔNp63β, ΔNp63γ, p53, and ΔNp63α mutants L518V, Q536L, FS, C306R, 1743 DelAA, and E639X. Cells were seeded in 12-well plates and transfected with 0.1 μg IKKα-luc, 0.02 μg of pRL-null plasmid, and 1.5 μg of the indicated expression plasmids using Lipofectamine 2000 (Invitrogen Inc., Carlsbad, CA). At 24 hours post-transfection, cell extracts were prepared with 1 × lysis buffer, and the luciferase activity was measured using the Dual-Luciferase Kit (Promega Inc.) on a Triathler luminometer (Hidex). Results from experiments, performed three times in triplicate wells, are expressed as relative luciferase activity after normalization with the pRL-null plasmid as internal control. Basal activity of the reporter was set to 1. Each histogram bar represents the mean of three independent transfection experiments performed in triplicate. Standard deviations are indicated.

RNA interference

siRNA duplexes targeting ΔNp63, TAp63, and GFP were obtained from MWG-Biotech (Ebersberg, Germany). Sequences are available in the Supplementary Online Material

Primary keratinocytes plated on collagen-coated 35 mm dishes were transfected with 0.5 μg of siRNA per dish using Lipofectamine 2000 (Invitrogen).

Antibodies, immunoblotting, and cell fractionation

The Abs used were polyclonal anti-actin (C-11), monoclonal β-tubulin (3F3-G2), monoclonal γ-tubulin (14C11), monoclonal anti-p63 (4A4), polyclonal anti-p63 (H-137), monoclonal anti-p53 (DO-1), and monoclonal anti-HA (F-7), and they were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); monoclonal anti-IKKα and anti-Ki67 Ab was from BD Pharmingen (San Diego, CA); anti-K1 was purchased from Covance (Princeton, NJ).

For immunoblotting assays, cells were lysed in 50 mM Tris, pH 8, 120 mM NaCl, and 0.5%. NP-40, and protein concentration was determined by the Bio-Rad dye-binding assay (Irvine, CA). Total cell extract of 50 μg was loaded on denaturing SDS-PAGE gels and blotted onto a polyvinylidene difluoride membrane. Western blot analysis was performed using the enhanced chemiluminescence system (Amersham Pharmacia Biotech Inc., Uppsala, Sweden).

For cyto/nuclear extraction, cells were washed twice in ice-cold PBS. Cells were then pelleted by centrifugation at 1,400 r.p.m. for 5 minutes, washed once in (1 ml) buffer A (10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (pH 7.9), 10 mM KCl, 0,1 mM EDTA, 0,1 mM EGTA (ethylene glycol bis(β-aminoethyl-ether)-*N,N,N′,N′*,-tetraacetic acid), 1 mM PMSF (phenylmethyl-sulfonyl fluoride), and 1 mM dithiothreitol), and centrifuged at 10,000 r.p.m. for 10 minutes. Cell pellets were resuspended in buffer A containing 0.1% (v/v) NP-40 for 10 minutes on ice and lysed. Subsequently, cells were centrifuged at 10,000 r.p.m. for 10 minutes. The supernatant was harvested and the nuclear pellet was extracted

with buffer C (20 mM Hepes (pH 7.9), 400 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20% (w/v) glycerol, and 1 mM PMSF) for 15 minutes on ice. After incubation, the nuclei were centrifuged at 10,000 r.p.m. for 10 minutes and the supernatant was diluted with four volumes of buffer D (10 mM Hepes (pH 7.9), 50 mM KCl, 0.2 mM EDTA, 25% (w/v) glycerol, 0.5 mM PMSF, and 1 mM DTT).

mRNA expression analysis

For quantitative real-time PCR, total RNA was extracted from cells using the Trizol Reagent (Invitrogen). One microgram of total RNA was reverse transcribed with GeneAmp RNA PCR (Applied Biosystems Italy, Milan, Italy). For RT-qPCR in mouse keratinocytes, the TaqMan Universal PCR Master Mix (Applied Biosystems Italy) was used for PCR amplification of the cDNA using the Applied Biosystem 7300 real-time PCR system. Each mRNA was normalized to the levels of cyclophilin mRNA for each reaction, and the relative quantification of each gene was determined using the comparative C_T method. Primer sequences for IKKα and K10 are available in Supplementary Online Material.

For RT-qPCR in human cells, the reverse transcriptase reaction products were used for quantitative real-time PCR amplification, which was performed with the MyiQ Single-Color Real-Time Detection System for quantification with SYBR Green and melting curve analysis (Bio-Rad, Richmond, CA). The hARP gene (human acidic ribosomal protein P0) was used for normalization. Total RNA was extracted from formalin-fixed, paraffin-embedded sections by the Optimum FFPE RNA Isolation Kit (Ambion, Austin, TX) according to the manufacturer's protocol. Primer sequences for IKKα, K1, p63, and ARP are available in Supplementary Online Material.

ChIP assay

For ChIP assays, primary human keratinocytes (NHEK) and U2OS cells were used. ChIP assays were performed as described previously (Costanzo *et al.*, 2002). Briefly, after fixing in 1% formaldehyde, cells were lysed for 5 minutes in 50 mM Tris, pH 8.0, 2 mM EDTA, 0.1% NP-40, and 10% glycerol supplemented with protease inhibitors. Nuclei were re-suspended in 50 mM Tris, pH 8.0, 1% SDS, and 5 mM EDTA. Chromatin was sheared by sonication, centrifuged, and diluted 10-fold in 50 mM Tris, pH 8.0, 0.5% NP-40, 0.2 M NaCl, and 0.5 mM EDTA. After pre-clearing with a 50% suspension of salmon sperm-saturated protein A, lysates were incubated at 4 °C overnight with anti-p63, anti-HA, or anti-Myc Abs. Immune complexes were collected with sperm-saturated protein A, washed three times with high salt buffer (20 mM Tris, pH 8.0, 0.1% SDS, 1% NP-40, 2 mM EDTA, and 500 mM NaCl), and three times with 1 × Tris/EDTA (TE). Immune complexes were extracted in 1 × TE containing 1% SDS, and protein–DNA cross-links were reverted by heating at 65 °C overnight. DNA was extracted by phenol–chloroform, and 1/20th of the immunoprecipitated DNA was used in each PCR reaction.

PCR reactions were performed for 25–35 cycles of denaturation at 95 °C for 45 seconds, annealing at 55–57 °C for 45 seconds, and extension at 72 °C for 45 seconds.

Primer sequences are reported in online Supplementary Material.

Immunohistochemistry and immunofluorescence

Punch biopsy specimens (4 mm) were taken from normal skin of healthy volunteers ($n = 4$). The Declaration of Helsinki protocols were followed and patients gave written approved consent before biopsy. A patient affected by AEC (I537T mutation in p63) was subjected to skin biopsy on the lesional skin after obtaining informed consent from his parents. Paraffin-embedded 5 mm skin sections were kept at 60 °C for 1 hour and then incubated at 80 °C overnight in citrate buffer pH 6 (DAKO Italia, Milan, Italy). The sections were incubated for 1 hour at room temperature with the appropriate dilution of anti-human IKKα monoclonal Ab, anti-K1, anti-Ki67, or control mouse Igs. Sections were stained with an avidin–biotin–peroxidase technique (DAKO Italia) by using 3,3′-diaminobenzidine as a substrate (DAKO Italia). For BrdU incorporation assay, we made use of BrdU Immunohistochemistry Assay Kit (Invitrogen) according to the manufacturer's protocol. For indirect immunofluorescence labeling, double labeling was performed on skin biopsies after deparaffination and rehydration. The primary Abs used were monoclonal anti-p63 (4A4) (1:100) and rabbit polyclonal anti-IKKα (1:50). Secondary Abs were Alexa 488-conjugated goat anti-rabbit (Molecular Probes, Milan, Italy) and Texas Red-conjugated goat anti-mouse (Molecular Probes). To determine nonspecific binding, staining control experiments with secondary Ab without primary Ab were also performed. Samples were examined with the use of a Zeiss LSM 410 laser-scanning confocal microscope. The picture shown in Figure 2b is representative of four different biopsies taken from four healthy donors.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Figure S1. Total cell lysate (20 μg) from experiment described in Figure 1B right panel was immunoblotted with specific antibodies to detect expression levels of p63 isoforms, p73 isoforms and p53.

Figure S2. Primary mouse keratinocytes were transfected with indicated siRNA and then kept under proliferating or differentiating (2 mM $CaCl_2$) conditions.

Figure S3. Dominant-negative activity of p63 mutants on differentiation-induced IKKα transcription.

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