p63 is upstream of IKKα in epidermal development

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Summary

The epidermis, the outer layer of the skin composed of keratinocytes, develops following the action of the transcription factor p63. The mouse *Trp63* gene contains two promoters, driving the production of distinct proteins, one with an N-terminal trans-activation domain (TAp63) and one without (ΔNp63), although their relative contribution to epidermal development is not clearly established. To identify the relative role of p63 isoforms in relation to IKKα, also known to be essential for epithelial development, we performed both molecular and in vivo analyses using genetic complementation in mice. We found that the action of TAp63 is mediated at the molecular level by direct and indirect transactivation of IKKα and Ets-1, respectively. We also found that ΔNp63 upregulates IKKα indirectly, through GATA-3. Our data are consistent with a role for p63 directly upstream of IKKα in epithelial development.

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Introduction

The epidermis is a multi-layered, stratified epithelium continuously regenerated by terminally differentiating keratinocytes (Candi et al., 2005; Fuchs and Watt, 2003; Owens and Watt, 2003), in which a major role is played by p63 (Yang et al., 1998), a member of the p53 family (Melino et al., 2002; Melino et al., 2003; Yang et al., 2002; Yang and McKeon, 2000). Indeed, mutations in the human *TP63* gene cause skin and limb defects (Celli et al., 1999), and $p63^{-/-}$ mice have no epidermis, no limbs and die at birth as a result of dehydration (Mills et al., 1999; Yang et al., 1999).

The expression of p63 proteins originates from two promoters, giving rise to two distinct isoforms, TAp63 and ΔNp63. In addition, both isoforms undergo alternative splicing at the C-terminus producing different TAp63 and ΔNp63 isoforms, respectively named α, β and γ, with α being the longest. Although p63 is involved in epithelial development (Mills et al., 1999; Yang et al., 1999), the relative contribution of these different isoforms to epidermal formation has been established at the molecular level only partially (Candi et al., 2006; Koster et al., 2004).

The formation of the epidermis requires the action of the IκB kinase-α (IKKα). IKKα shows both protein-kinase-dependent and -independent effects; the kinase-independent function is required for epidermal keratinocyte differentiation, skeletal and craniofacial morphogenesis, as shown by the $IKK\alpha^{-/-}$ mice (Hu et al., 1999; Li et al., 1999; Sil et al., 2004). Thus, loss of IKKα prevents terminal differentiation of keratinocytes by blocking the expression of late differentiation markers, such as loricrin and filaggrin (Hu et al., 1999; Hu et al., 2001; Li et al., 1999), and expression of IKKα, under the basal layer promoter cytokeratin 14 (K14) in $IKK\alpha^{-/-}$ mice, produced efficient rescue of the major morphological abnormalities (Sil et al., 2004). IKKα is also required for normal whisker and tooth development, where, in the developing oral cavity, the epithelium invaginates inward into the underlying mesenchyme (Ohazama et al., 2004). This appears to be independent of NF-κB. IKKα knockout mice also show an abnormal tooth cusp morphology very similar to *EdaA1*, *Edar* and *Edaradd* mutants. Changes in *Notch1*, *Notch2*, *Wnt7b* and *Shh* gene expression in incisor epithelium of IKKα-deficient mice suggest that this IKKα function is downstream of *EdaA1*/*Edar*/*Edaradd* and is mediated by *Notch*/*Wnt*/*Shh* signaling pathways. Interestingly, p63 is also required for whisker and tooth development (Laurikkala et al., 2006; Rufini et al., 2006). Here, *Bmp7*, *Fgfr2b*, *Jag1*, *Notch1* and *Edar* transcripts are co-expressed with ΔNp63, and are absent in $p63^{-/-}$ mice (Laurikkala et al., 2006). The involvement of p63 and IKKα in the development of the epidermis, whiskers and teeth, raises the question of the reciprocal molecular relationship between p63 and IKKα.

Here, we provide evidence that p63 is directly upstream of IKKα in epidermal development. Using HA-tagged p63-inducible Tet-on Saos-2 cell lines (see Gressner et al., 2005), we demonstrate that TAp63 induces the expression of IKKα, through the p53-like responsive element on its promoter. In addition, TAp63 also drives the expression of Ets-1, another factor which transactivates the IKKα promoter. Finally, we identified four GATA-3 binding sites located in the human IKKα proximal promoter, and because both TAp63 and ΔNp63 are able to transactivate the GATA-3 promoter, this provides a further indirect mechanism allowing TAp63 and ΔNp63 to modulate IKKα expression. Consequently, we observed that the double complemented mice [transgenic TAp63α and

ΔNp63α mice under the keratin 5 promoter crossed into $p63^{-/-}$ mice, thus named $p63^{-/-}$;ΔN;TA, generated in our laboratory (Candi et al., 2006)] show higher levels of epidermal IKKα expression compared with $p63^{-/-}$ mice. This provides evidence for p63 being directly upstream of IKKα in epidermal development.

Results and Discussion

IKKα is a transcriptional target of p63

In order to elucidate the molecular mechanism through which p63 regulates the formation of the epidermis, we investigated its ability to induce IKKα. The human IKKα promoter contains three specific p53-like responsive elements that could be recognised by p63 proteins (Fig. 1A). This promoter region was cloned in a plasmid upstream of a luciferase reporter gene. We observed that, in Tet-inducible Saos-2 cells (Candi et al., 2006; Gressner et al., 2005), TAp63α significantly increased luciferase activity in a dose-dependent manner (Fig. 1B, lanes 2-3), whereas ΔNp63α did not (Fig. 1B, lanes 4-5). In addition, TAp63β and TAp63γ also significantly increased luciferase activity (Fig. 1C, lanes 3-4) compared with ΔNp63β and ΔNp63γ isoforms (Fig. 1C, lanes 6-7). Transactivation of the IKKα promoter by TAp63 was also demonstrated in HEK293 cells (data not shown). Microarray studies, performed on TAp63- and ΔNp63-inducible Saos-2 cells (Candi et al., 2006; Gressner et al., 2005), have shown that TAp63α (3.2 times the control level by microarray activation; 6.3 times the control level by real-time PCR at 24 hours), but not ΔNp63α (0.3-fold over control level by microarray activation; 1.1 times the control level by real-time PCR at 24 hours) also drives expression of Ets-1 (Candi et al., 2006) (data not shown). Expression of Ets-1 alone in both types of Saos-2 cells activates the IKKα promoter (Fig. 1B, lanes 1, 6) and the IKKα promoter contains an Ets-1 consensus motif (Fig. 1A). Co-expression of TAp63α with Ets-1 results in a greater enhancement of IKKα promoter activity compared with that produced by TAp63α alone (Fig. 1B, lanes 7, 3). However, co-expression of ΔNp63α with Ets-1 did not increase the activity of the IKKα promoter over that produced by Ets-1 alone (Fig. 1B, compare lanes 8 and 6).

Using the inducible HA-tagged TAp63α and ΔNp63α Saos-2 cell lines (Gressner et al., 2005), we demonstrated that TAp63α induces IKKα through direct binding to the previously described p53-like responsive elements in its promoter (Gu et al., 2004) as demonstrated by chromatin immunoprecipitation

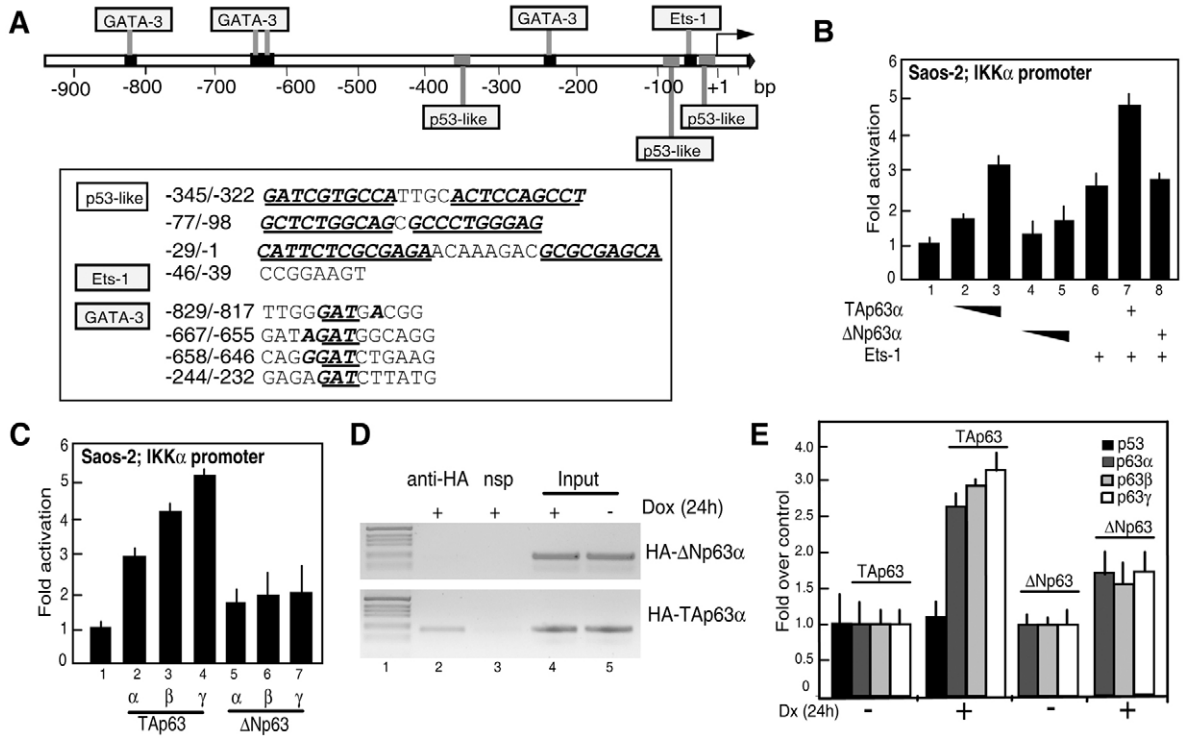

Fig. 1. TAp63 isoforms bind and transactivate the IKKα promoter. (A) Schematic structure of the IKKα promoter showing the different putative p53-like, Ets-1 and GATA-3 responsive elements. (B) Luciferase assay analysis showing the ability of TAp63α to induce IKKα expression. The ratios of IKKα promoter to transcription factor are: IKKα:TAp63α/ΔNp63α, 1:0.5; IKKα:Ets-1, 1:2; IKKα:TAp63α/ΔNp63α:Ets-1, 1:2:2. The luciferase assay shown was performed in Saos-2 cells, and similar results were also obtained in HEK293 cells (data not shown). Results are shown as mean ± s.d. of three independent experiments. (C) Luciferase assay analysis showing the ability of TAp63 and ΔNp63 isoforms to transactivate IKKα in Saos-2 cells. Results are similar to those obtained in Saos-2 Tet-on inducible cells in B. The ratio of IKKα promoter to TAp63/ΔNp63 isoforms was 1:2. Results are shown as mean ± s.d. of three independent experiments. (D) ChIP showing the binding of p63 protein to the IKKα promoter. Lane 1, marker; lane 2, specific antibody (anti-HA); lane 3, non-specific antibody (nsp); lane 4, input+Dox; lane 5, input–Dox, (representative result of two independent experiments is shown). (E) Real-time PCR performed on Dox (Tet-on) inducible Saos-2 clones. TAp63 isoforms already induce a significant increase of IKKα RNA upon 24 hours of Dox treatment, whereas ΔNp63α and p53 do not significantly change IKKα RNA levels. Results are mean ± s.d. of three independent experiments.

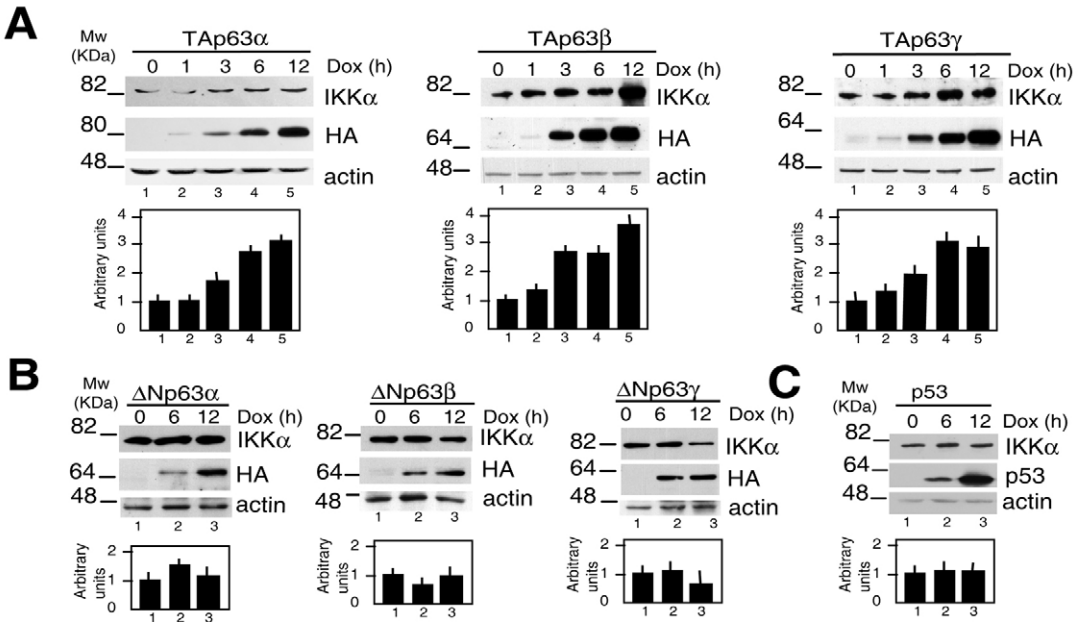

Fig. 2. TAp63 isoforms induce IKKα protein expression. (A) Western blot in Saos-2 Tet-on inducible cell lines (Dox 2 μg/ml) overexpressing TAp63 or ΔNp63 isoforms. All TAp63 isoforms show an induction of IKKα at the protein level (from 1.3- to 3.7-fold). The actin western blot is shown in the lower panel as a loading control. IKKα expression was normalised to actin by densitometry and results are reported as fold change over zero time (see graphics below actin bands). (B) ΔNp63 isoforms expression do not change (ΔNp63α) or produce a minor downregulation (ΔNp63β and γ) of IKKα protein after 12 hours of Dox induction. IKKα expression was normalised to actin by densitometry and results are reported as fold change over zero time. (C) Western blot in Saos-2 Tet-on inducible cell lines (Dox 2 μg/ml) overexpressing p53. p53 does not induce IKKα at either protein or RNA level (see also Fig. 1) after 6 hours and 12 hours of induction. IKKα expression was normalised to actin by densitometry and results are reported as fold change over the level at zero time. All results obtained from the densitometry are shown as mean ± s.d. from three independent experiments.

(ChIP) experiments (Fig. 1D). Immunoprecipitation, using anti-HA antibodies, of protein-DNA complexes containing HA-tagged TAp63 but not ΔNp63, yielded DNA fragments containing IKKα sequences (Fig. 1D, compare lanes 2 in lower and upper panels). No IKKα sequence was amplified from immunoprecipitates prepared with the control anti-K5 antibody (Fig. 1D, compare lanes 2 and 3). RT-PCR analysis of IKKα expression levels in the inducible TAp63 and ΔNp63 Saos-2 cell lines confirms that expression of all TAp63 isoforms upregulates the transcription of IKKα mRNA after 24 hours of induction with Dox, whereas ΔNp63 isoforms and p53 do not (Fig. 1E).

The induction of IKKα by TAp63 isoforms was also confirmed by western blot in different Saos-2-inducible clones (Fig. 2A,B). Induction of HA-tagged TAp63α, β and γ in inducible Saos-2 cells is associated with a parallel induction of IKKα at the protein level, which is detectable after 3 hours of Dox treatment. No change in IKKα expression was elicited by p53 (Fig. 2C). IKKα promoter activation by TAp63 but not ΔNp63 isoforms was also confirmed by transient transfection experiments in human keratinocytes, HaCaT

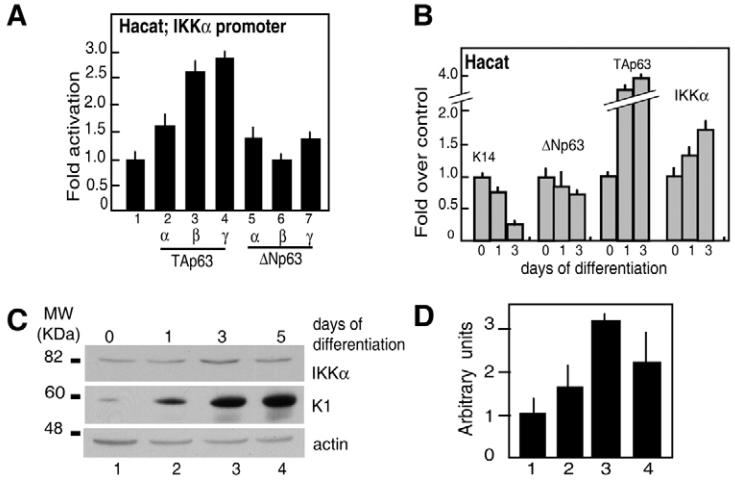

Fig. 3. Regulation of IKKα during keratinocyte differentiation. (A) Luciferase assay showing the ability of p63 isoforms to transactivate IKKα in HaCaT cells. The ratio IKKα promoter to TAp63/ΔNp63 isoforms was 1:2. Results are shown as mean ± s.d. of three independent experiments. (B) Quantitative real-time PCR performed on HaCaT cells induced to differentiate in high Ca^{2+} for 1 and 3 days. ΔNp63α and K14 mRNA decreases indicating that the cells underwent differentiation. Results are shown as mean ± s.d. from three independent experiments. (C) Western blot using anti-IKKα and anti-keratin 1-antibody (K1) on HaCaT cells induced to differentiate in high Ca^{2+} for 1 to 5 days. K1 is shown as control for keratinocyte differentiation; actin is shown as loading control. (D) Quantification of the western blot by densitometry.

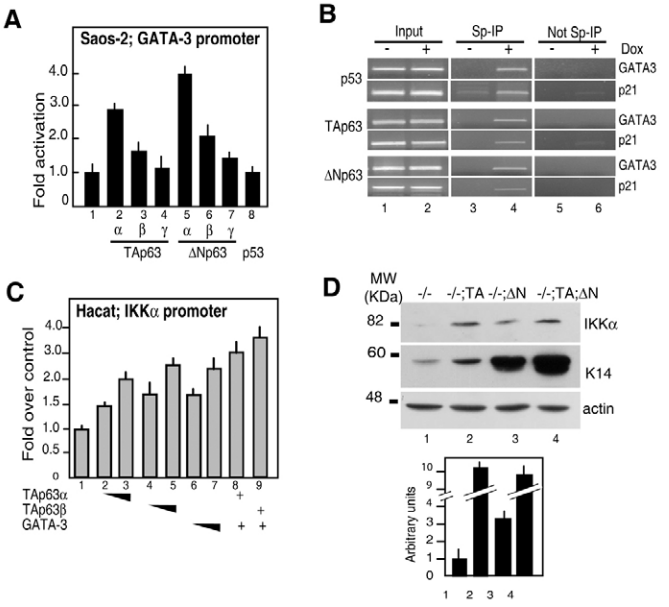

Fig. 4. IKKα regulation by GATA-3, and overexpression in ΔNp63/TAp63 genetically complemented mice. (A) Galactosidase assay showing the ability of TAp63α and ΔNp63α to activate the GATA-3 promoter. The ratio of GATA-3 promoter to transcription factor is 1:3. The in vitro transcription assay shown was performed in Saos-2 cells, and similar results were also obtained in HEK293 cells (data not shown). Results are shown as mean ± s.d. from three independent experiments. (B) ChIP showing the binding of p53 and p63 proteins to the GATA-3 promoter. Lanes 1 and 2, input; lanes 3 and 4, specific antibody (Sp-IP, anti-GATA-3 and anti p21); lanes 5 and 6, non-specific antibody (Not Sp-IP). A representative result of two independent experiments is shown. (C) Luciferase assay showing the ability of GATA-3 to directly induce the IKKα promoter. The ratio of IKKα promoter to transcription factor is 1:1 and 1:3. In lanes 8 and 9 the ratio was 1:1:1. The in vitro transcription assay shown was performed in HaCaT cells, and similar results were also obtained in HEK293 cells (data not shown). Results are shown as mean ± s.d. from three independent experiments. (D) Western blot of epidermal cell extracts from $p63^{-/-}$, $p63^{-/-}$;TA, $p63^{-/-}$;ΔN, $p63^{-/-}$;ΔN;TA mice (see 13). Double $p63^{-/-}$;ΔN;TA complemented mice, as well as $p63^{-/-}$;TA mice, show higher levels of IKKα expression in vivo compared with $p63^{-/-}$ mice. As loading control we detected actin. Lower panel shows the normalisation of the induction of IKKα to actin protein level.

cells (Fig. 3A, lanes 1, 2-4 for TAp63; lanes 1, 5-7 for ΔNp63) and NHEK (data not shown).

By real-time PCR analysis (Fig. 3B) we confirmed that TAp63 and IKKα mRNA increase during keratinocyte differentiation, whereas, as expected during epidermal differentiation (26), ΔNp63 and K14 mRNA levels decrease. The increase of IKKα mRNA was also confirmed by western blot (Fig. 3C) in differentiating keratinocytes. Here, protein levels increase ~threefold after 3 days of treatment with Ca^{2+} (Fig. 3D).

Indirect regulation of IKKα by p63 via GATA-3

To determine additional potential indirect mechanisms of p63-mediated regulation of IKKα, we analysed the human IKKα promoter. The IKKα proximal promoter contains several binding sites for transcription factors which play important roles during skin development and differentiation (see supplementary material Table S1 and Fig. S1). Beside Ets-1, we decide to investigate the possible role of GATA-3, an important mediator of skin and hair development (Kaufman et al., 2003), in regulating IKKα expression, based on the observation that GATA-3 contains a p53-like consensus binding site in its proximal promoter region (–242 to –262 bp). Unlike the IKKα promoter, both TAp63α and ΔNp63α significantly increased luciferase activity of the GATA-3 promoter (Fig. 4A, lanes 2, 5). Interestingly, p63β and p63 γ isoforms are less efficient in transactivating this promoter. Using the inducible HA-tagged TAp63α and ΔNp63α Saos-2 cell lines, we demonstrated that both p63 isoforms (p53 included as positive control), directly bind to the putative p53-like responsive element in GATA-3 promoter as demonstrated by the ChIP experiments (Fig. 4B). Immunoprecipitation, using anti-HA antibodies, of protein-DNA complexes containing HA-tagged TAp63 and ΔNp63, yielded DNA fragments containing GATA-3 sequences. No GATA-3 sequence was amplified from immunoprecipitates prepared with the control anti-K5 antibody (compare Not Sp-IP with Sp-IP). As a positive control we also performed ChIP experiments using p21 (Fig. 4B).

Finally, using a luciferase assay using the IKKα promoter, we observed that GATA-3 transactivates IKKα in a dose-dependent manner (Fig. 4C, lanes 1, 6-7). Moreover, promoter activity increases when GATA-3 is co-transfected with p63 (Fig. 4C, lanes 8-9).

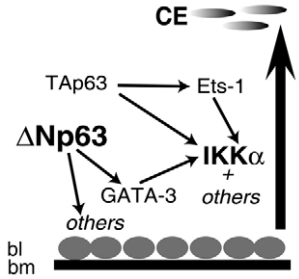

Fig. 5. Proposed mechanism of action of p63 upstream of IKKα. TAp63α and ΔNp63α have different, synergistic functions in the formation of the epidermis; TAp63 exerts its effects, at least in part, by acting directly upstream of IKKα (which is also indirectly transactivated via Ets-1 and GATA-3), whereas ΔNp63 can only activate IKKα indirectly, at least partly via GATA-3. bm, basal membrane; bl, basal layer of keratinocytes; CE, cornified envelope.

IKKα mediates the function of p63 in genetically complemented mice

To elucidate the individual role of p63 isoforms in the development of the epidermis, we generated transgenic mice expressing either TAp63α and/or ΔNp63α under the control of a keratinocyte specific promoter (keratin 5); we then crossed these mice into a $p63^{-/-}$ background, generating single ($p63^{-/-}$;TA and $p63^{-/-}$;ΔN) and double complemented ($p63^{-/-}$;ΔN;TA) mice (Candi et al., 2006). Supplementary material Fig. S2 shows the histology of the epidermis both in

the wild type, knockout and genetically complemented mice. The $p63^{-/-}$;ΔN as well as the $p63^{-/-}$;ΔN;TA complemented mice showed greater, though still not normal, formation of the epidermis (Candi et al., 2006). We therefore took advantage of these mice to evaluate the in vivo relationship between p63 and IKKα.

In the single complemented mice, selective reintroduction of both TAp63α and ΔNp63α was associated with increased IKKα expression compared with $p63^{-/-}$ mice, although the increase was greater with TAp63 complementation (tenfold with TAp63 versus 3.5-fold with ΔNp63; Fig. 4D, lanes 2 and 3). As expected, IKKα expression was also enhanced in the double complemented mice (Fig. 4D, lane 4).

The function of p63 in epithelial development is mediated by IKKα

TP63 is expressed very early in keratinocyte differentiation (Mills et al., 1999; Yang et al., 1999). The ΔNp63 protein is expressed in the basal layer of the human epidermis (Laurikkala et al., 2006; Nylander et al., 2002) and, in both zebra fish and mice, determines the outgrowth of the epidermis (Laurikkala et al., 2006; Lee and Kimelman, 2002). Recently, a distinct contribution of the TAp63 protein has also been demonstrated in mice (Candi et al., 2006). As yet, however, relatively few downstream targets of p63 are known (Candi et al., 2006; Ihrie et al., 2005; Laurikkala et al., 2006) although one of these is fos, a member of the AP1 complex crucial for skin differentiation (Wu et al., 2003) and another is PERP, crucial for epithelial stratification, being localised in desmosomes (Ihrie et al., 2005). Here, we demonstrate that TAp63 and ΔNp63 regulate expression of the gene encoding IKKα, which is also crucial for formation of the epidermis. This regulation is both direct, through one of the three p53 consensus motifs in the IKKα promoter, and indirect, (1) through TAp63 induction of Ets-1, and (2) through TAp63 and ΔNp63 induction of GATA-3, which subsequently drives IKKα expression (Fig. 5). However, as suggested by recent studies (Ihrie et al., 2005; Koster et al., 2004), and by the limited reversion of the p63-null epidermal phenotype by selective TAp63 and ΔNp63 complementation (Candi et al., 2006), other genes must contribute to the proliferative and differentiation potential of the epidermis.

Conclusion

Within the limits of our experimental model, our overall conclusion is that the p63/IKKα pathway is essential for epidermal development. These data are important in understanding the molecular mechanisms of development and maintenance of epithelia and thus have important clinical implications (McKeon, 2004).

Materials and Methods

Cell culture

Primary keratinocytes were isolated as described (Yuspa et al., 1989) from the skin of E19.5 transgenic neonates. Saos-2 cells with doxycycline (Dox)-inducible expression of HA-TAp63a, -b, -g and HA-DNp63a, -b, -g were generated as described previously (Gressner et al., 2005).

Mice

The recombination vector contained the K5 promoter (from Manfred Blessing, Joannes Gutemberg University, Mainz, Germany), the $PolyA^+$ signal and N-tagged (hemagglutinin antigen, HA) mouse TAp63a or DNp63a (Breuhahn et al., 2000). Transgenic mice on a C57/B6 background were backcrossed with $p63^{+/-}$ mice (Yang et al., 1999) to generate genetically complemented mice (Candi et al., 2006). Histology was performed according to standard procedures.

Western blots

HaCaT cells were differentiated by addition of Ca^{2+} and collected after 1 and 3 days of treatment. Saos-2-inducible cells were cultured and treated as described previously (Gressner et al., 2005). Cells were lysed as previously reported (Candi et al., 2006) and after extraction, 50 mg protein were separated by SDS-PAGE and transferred onto PVDF membranes; blots were performed using standard procedures. Primary antibody dilutions were: monoclonal anti-p63 (1:200), polyclonal anti-HA (Y-11, Santa Cruz, 1:100), polyclonal anti-IKKα (1:200), polyclonal anti-K14 (1:300). Normalisation was achieved with a polyclonal anti-tubulin (H-235, Santa Cruz, 1:1000 dilution) or with a goat anti-actin antibody (C-11, Santa Cruz, 1:1000 dilution). Proteins were detected using the ECL method.

Real-time PCR assay

RNA was extracted from control and Dox-induced SaOs-2 Tet-on HA-tagged TAp63 and –DNp63 clones, using Trizol (Invitrogen, Carlsbad, CA). Real-time PCR was performed as previously described (Candi et al., 2006). Primer sequences are available upon request. Relative quantification of gene expression was performed using ABI 7500 SDS software v 1.31.

ChIP and transcription assay

This was performed according to a previous published protocol (Munarriz et al., 2004). Samples (1.5×10^6 cells) were incubated with 2 mg monoclonal anti-HA (16B12, Covance) or monoclonal anti-p63 (Ab4, Neomarkers). The negative control was incubated with 2 mg mouse anti-K5 (Santa Cruz). DNA samples were then analysed with 38 cycles of PCR to amplify IKKa promoter sequences (94°C for 30 seconds, 58°C for 40 seconds, 72°C for 40 seconds). For IKKa amplification we used specific forward and reverse primers (5′-GTGGTTCCGTTCAGCCCT-3′ and 5′-TGCTCGCGCGTCTTTG-3′). The resulting product was 188 bp and contained the last two putative p53 consensus sequences as well as the Ets-1 binding motif in the IKKa promoter. The following specific oligos were used to determine p53/p63 binding sites of p21 and GATA-3 genes: p21 site F(5′-ATGTATAGGAGCGAAG-GTGCA-3′); p21 site R(5′-CCTCCTTTCTGTGCCTGAAACA-3′); GATA-3 site F(5′-GAATTCCCTCCTGCCTGTCC-3′); GATA-3 site R(5′-CTTCACCTCCAC-CCCCATCC-3′). For luciferase assay in Saos and HaCaT cells, we used the reporter plasmid containing the luciferase gene under the control of IKKα promoter and the expression vectors encoding for TAp63α, β, γ and ΔNp63α, β, γ (see legend figures for ratios). When needed, the pcDNA empty vector (Invitrogen) was added to reach the total amount of DNA (400 ng) used in each transfection. In all cases, 10 ng of *Renilla* luciferase vector (pRL-CMV; Promega, Madison, WI) were co-transfected, as a control of transfection efficiency. Luciferase activities of cellular extracts were measured, by using a Dual Luciferase Reporter Assay System (Promega); light emission was measured over 10 seconds using an OPTOCOMP I luminometer. Efficiency of transfection was normalised using *Renilla* luciferase activity. For β-galactosidase assay, cells were transiently transfected with a reporter construct containing GATA-3 promoter (–308pLacZ, kindly provided by J. D. Engel, University of Michigan Medical School, MI) and expression vectors encoding p53, p63 isoforms and pcDNA3.1 (see legend figures for ratios). Each transfection experiment was done in triplicate. 48 hours after transfection, cells were washed with PBS and harvested with 100 μl of lysis buffer (5 mM DTT, 250 mM Tris-HCl pH 8.1 containing protease inhibitors). A total of 100 μg of total cell lysate was incubating with 800 μl substrate solution (2 mg/ml ONPG (Pierce) in 0.1 M Na_2HPO_4, 1 M NaH_2PO_4, 1 M KCl, 1 M $MgCl_2$ with 20% β-mercaptoethanol) for 40 minutes at 37°C. The reaction was stopped by addition of 1 M Na_2CO_3 and the absorbance was measured at 420 nm.

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References

Breuhahn, K., Mann, A., Muller, G., Wilhelmi, A., Schirmacher, P., Enk, A. and Blessing, M. (2000). Epidermal overexpression of granulocyte-macrophage colony-stimulating factor induces both keratinocyte proliferation and apoptosis. *Cell Growth Differ.* **11**, 111-121.

Candi, E., Schmidt, R. and Melino, G. (2005). The cornified envelope: a model of cell death in the skin. *Nat. Rev. Mol. Cell Biol.* **6**, 328-340.

Candi, E., Rufini, A., Terrinoni, A., Dinsdale, D., Ranalli, M., Paradisi, A., De Laurenzi, V., Spagnoli, L. G., Catani, M. V., Ramadan, S. et al. (2006). Differential roles of p63 isoforms in epidermal development: selective genetic complementation in p63 null mice. *Cell Death Differ.* **13**, 1037-1047.

Celli, J., Duijf, P., Hamel, B. C., Bamshad, M., Kramer, B., Smits, A. P., Newbury-Ecob, R., Hennekam, R. C., Van Buggenhout, G., van Haeringen, A. et al. (1999). Heterozygous germline mutations in the p53 homolog p63 are the cause of EEC syndrome. *Cell* **99**, 143-153.

Fuchs, E. and Watt, F. M. (2003). Cell differentiation. Focus on epithelia. *Curr. Opin. Cell Biol.* **15**, 738-739.

Gressner, O., Schilling, T., Lorenz, K., Schulze Schleithoff, E., Koch, A., Schulze-Bergkamen, H., Maria Lena, A., Candi, E., Terrinoni, A., Valeria Catani, M. et al. (2005). TAp63alpha induces apoptosis by activating signaling via death receptors and mitochondria. *EMBO J.* **24**, 2458-2471.

Gu, L., Zhu, N., Findley, H. W., Woods, W. G. and Zhou, M. (2004). Identification and characterization of the IKKalpha promoter: positive and negative regulation by ETS-1 and p53, respectively. *J. Biol. Chem.* **279**, 52141-52149.

Hu, Y., Baud, V., Delhase, M., Zhang, P., Deerinck, T., Ellisman, M., Johnson, R. and Karin, M. (1999). Abnormal morphogenesis but intact IKK activation in mice lacking the IKKalpha subunit of IkappaB kinase. *Science* **284**, 316-320.

Hu, Y., Baud, V., Oga, T., Kim, K. I., Yoshida, K. and Karin, M. (2001). IKKalpha controls formation of the epidermis independently of NF-kappaB. *Nature* **410**, 710-714.

Ihrie, R. A., Marques, M. R., Nguyen, B. T., Horner, J. S., Papazoglu, C., Bronson, R. T., Mills, A. A. and Attardi, L. D. (2005). Perp is a p63-regulated gene essential for epithelial integrity. *Cell* **120**, 843-856.

Kaufman, C. K., Zhou, P., Pasolli, H. A., Rendl, M., Bolotin, D., Lim, K. C., Dai, X., Alegre, M. L. and Fuchs, E. (2003). GATA-3: an unexpected regulator of cell lineage determination in skin. *Genes Dev.* **17**, 2108-2122.

Koster, M. I., Kim, S., Mills, A. A., DeMayo, F. J. and Roop, D. R. (2004). p63 is the molecular switch for initiation of an epithelial stratification program. *Genes Dev.* **18**, 126-131.

Laurikkala, J., Mikkola, M. L., James, M., Tummers, M., Mills, A. A. and Thesleff, I. (2006). p63 regulates multiple signalling pathways required for ectodermal organogenesis and differentiation. *Development* **133**, 1553-1563.

Lee, H. and Kimelman, D. (2002). A dominant-negative form of p63 is required for epidermal proliferation in zebrafish. *Dev. Cell* **2**, 607-616.

Li, Q., Lu, Q., Hwang, J. Y., Buscher, D., Lee, K. F., Izpisua-Belmonte, J. C. and Verma, I. M. (1999). IKK1-deficient mice exhibit abnormal development of skin and skeleton. *Genes Dev.* **13**, 1322-1328.

McKeon, F. (2004). p63 and the epithelial stem cell: more than status quo? *Genes Dev.* **18**, 465-469.

Melino, G., De Laurenzi, V. and Vousden, K. H. (2002). p73: friend or foe in tumorigenesis. *Nat. Rev. Cancer* **2**, 605-615.

Melino, G., Lu, X., Gasco, M., Crook, T. and Knight, R. A. (2003). Functional regulation of p73 and p63: development and cancer. *Trends Biochem. Sci.* **28**, 663-670.

Mills, A. A., Zheng, B., Wang, X. J., Vogel, H., Roop, D. R. and Bradley, A. (1999). p63 is a p53 homologue required for limb and epidermal morphogenesis. *Nature* **398**, 708-713.

Munarriz, E., Barcaroli, D., Stephanou, A., Townsend, P. A., Maisse, C., Terrinoni, A., Neale, M. H., Martin, S. J., Latchman, D. S., Knight, R. A. et al. (2004). PIAS-1 is a checkpoint regulator which affects exit from G1 and G2 by sumoylation of p73. *Mol. Cell. Biol.* **24**, 10593-10610.

Nylander, K., Vojtesek, B., Nenutil, R., Lindgren, B., Roos, G., Zhanxiang, W., Sjostrom, B., Dahlqvist, A. and Coates, P. J. (2002). Differential expression of p63 isoforms in normal tissues and neoplastic cells. *J. Pathol.* **198**, 417-427.

Ohazama, A., Hu, Y., Schmidt-Ullrich, R., Cao, Y., Scheidereit, C., Karin, M. and Sharpe, P. T. (2004). A dual role for Ikk alpha in tooth development. *Dev. Cell* **6**, 219-227.

Owens, D. M. and Watt, F. M. (2003). Contribution of stem cells and differentiated cells to epidermal tumours. *Nat. Rev. Cancer* **3**, 444-451.

Rufini, A., Weil, M., McKeon, F., Barlattani, A., Melino, G. and Candi, E. (2006). p63 protein is essential for the embryonic development of vibrissae and teeth. *Biochem. Biophys. Res. Commun.* **340**, 737-741.

Sil, A. K., Maeda, S., Sano, Y., Roop, D. R. and Karin, M. (2004). IkappaB kinase-alpha acts in the epidermis to control skeletal and craniofacial morphogenesis. *Nature* **428**, 660-664.

Wu, G., Nomoto, S., Hoque, M. O., Dracheva, T., Osada, M., Lee, C. C., Dong, S. M., Guo, Z., Benoit, N., Cohen, Y. et al. (2003). DeltaNp63alpha and TAp63alpha regulate transcription of genes with distinct biological functions in cancer and development. *Cancer Res.* **63**, 2351-2357.

Yang, A. and McKeon, F. (2000). P63 and P73: P53 mimics, menaces and more. *Nat. Rev. Mol. Cell Biol.* **1**, 199-207.

Yang, A., Kaghad, M., Wang, Y., Gillett, E., Fleming, M. D., Dotsch, V., Andrews, N. C., Caput, D. and McKeon, F. (1998). p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Mol. Cell* **2**, 305-316.

Yang, A., Schweitzer, R., Sun, D., Kaghad, M., Walker, N., Bronson, R. T., Tabin, C., Sharpe, A., Caput, D., Crum, C. et al. (1999). p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature* **398**, 714-718.

Yang, A., Kaghad, M., Caput, D. and McKeon, F. (2002). On the shoulders of giants: p63, p73 and the rise of p53. *Trends Genet.* **18**, 90-95.

Yuspa, S. H., Kilkenny, A. E., Steinert, P. M. and Roop, D. R. (1989). Expression of murine epidermal differentiation markers is tightly regulated by restricted extracellular calcium concentrations in vitro. *J. Cell Biol.* **109**, 1207-1217.