Cell Death and Differentiation (2009) 16, 253–263

www.nature.com/cdd

p63 regulates the caspase-8-FLIP apoptotic pathway in epidermis

S Borrelli[1], E Candi[2], D Alotto[3], C Castagnoli[3], G Melino[2,4], MA Viganò[1] and R Mantovani*[,1]

The transcription factor p63, member of the p53 family, is crucial for epithelial development. An RNAi screening identified the apoptotic gene Procaspase-8 as a target activated by p63. The caspase-8 inhibitor FLIP is also under p63 control. We analysed and detailed the direct transactivation through the use of RNAi, reporter assays, ChIPs, western blots, confocal studies in HaCat, as well as in primary human keratinocytes. The direct ΔNp63 regulation of these targets was confirmed *in vivo* using transgenic ΔNp63 mice under the K5 promoter, as compared with p63 knockout mice, and *in vitro* in normal human primary keratinocytes following UV irradiation. Lowering the steady state of p63 protein levels changes the relative *ratio* of FLIP isoforms, causing the activation of the expressed, inactive Procaspase-8, into the active isoform thus triggering the proapoptotic cascade. Therefore, p63 fine-tunes the Procaspase-8-FLIP pro- and antiapoptotic pathway in keratinocytes.
Cell Death and Differentiation (2009) **16,** 253–263; doi:10.1038/cdd.2008.147; published online 17 October 2008

p63 is a transcription factor homologous to p53 and p73.[1] p53 is a crucial transcription factor in response to DNA damage by impinging on cell-cycle control and proapoptotic pathways,[2] whereas p63 is involved in epithelial development. Six p63 proteins have been described, resulting from two distinct promoters (TAp63 and ΔNp63 isoforms) and from alternative mRNA splicing (isoforms α, β, γ). TAp63 contain a transcriptional activation (TA) domain that is missing in the N-terminally deleted isoforms (ΔNp63); differential splicing generates isoforms with or without a sterile α motif domain, allegedly implicated in the protein–protein interactions. The relative properties and function of these isoforms are still not fully evident, but the major isoform present in keratinocytes and multilayered epithelia ΔNp63α is essential for ectodermal development in zebrafish, as well as in mammals.[3,4] Indeed, the importance of p63 in skin development is shown by mice lacking p63 that die soon after birth with severe defects in limb, craniofacial and skin development.[4] In humans, several syndromes showing abnormalities in limbs, skin and epithelial annexes are caused by mutations in the p63 gene.[5] p63 is crucial for the activation of the epithelial cell adhesion programme.[6] Recently, p63 has been demonstrated to play a major role in maintaining the proliferative potential of stem cells of the multilayered epithelia.[1,7] As for p53, the role of p63 in apoptosis has been investigated extensively. Although several reports have assigned a proapoptotic role to TAp63, ΔNp63α might exert a dominant-negative effect on the TAp63 isoforms, resulting in an antiapoptotic effect.[8–10]

The identification of p63 target genes is key to our understanding of its biological role. Through the use of RNAi inactivation coupled to gene expression profiling and ChIP on chip, several labs have recently identified hundreds of p63 targets.[6,11–14] Specifically, procaspase-8 emerged in the RNAi profiling of human HaCat cells as a gene regulated by p63.[14]

Caspases are an evolutionarily conserved family of aspartate-specific cystein-dependent proteases involved in apoptosis, as well as in inflammation.[15] Caspase-8 and -10 possess large prodomains containing related homotypic oligomerization motifs, such as the death effector domain (DED). Apoptotic stimuli triggers formation of the (death-inducing signalling complex) DISC,[16,17] in which the procaspase-8, the inactive uncleaved form, is bound to the adaptor molecule FADD by two DED.[18] This association is necessary for processing of procaspase-8 into the active p10–p18 forms by proteolytic cleavage. These are released to the cytosol and mediate the activation of 'effectors' Caspases-3 and -7, which are predominantly responsible for the limited proteolysis characterizing the apoptotic destruction of the cell. The major control of procaspase 8 cleavage is exerted by c-FLIP, a molecule sharing homology to caspase-8, but lacking a functional protease domain. At the protein level, c-FLIP has three different isoforms – FLIPl, FLIPr and FLIPs – in humans, and two – FLIPl and FLIPr – in mice.[19–24]

In this report, we demonstrate that p63 activates the genes of both procaspase-8 and of its negative regulator, c-FLIP.

[1]Dipartimento di Scienze Biomolecolari e Biotecnologie, Università degli Studi di Milano, Via Celoria 26, Milano 20133, Italy; [2]Biochemistry IDI-IRCCS laboratory, C/o University of Rome 'Tor Vergata', Via Montpellier 1, Rome 00133, Italy; [3]Dipartimento di Chirurgia Plastica, Banca della Cute, Ospedale CTO, Torino, Italy and [4]Medical Research Council, Toxicology Unit, Hodgkin Building, Leicester University, Lancaster Road, P.O. Box 138, Leicester LE1 9HN, UK
*Corresponding author: R Mantovani, Dipartimento di Scienze Biomolecolari e Biotecnologie, Università degli Studi di Milano, Via Celoria 26, Milano 20133, Italy. Tel: +39 02 5031 5005; Fax: +39 02 5031 5044; E-mail: mantor@unimi.it
Keywords: p63; keratinocytes; caspase 8; FLIP; apoptosis
Abbreviations: TAp63, amino-terminal transcriptional activation domain-containing p63; ΔNp63, transcriptional activation domain-deleted p63; CHIP, chromatin immunoprecipitation; RNAi, RNA interference; DED, death effector domain; DISC, death-inducing signalling complex; FLIP, flice inibitory protein; NHEK, normal human epidermal keratinocytes; BCoR, BCL-6 interacting corepressor gene; K, keratin; tg, transgenic mice; KO, knockout mice
Received 06.2.08; revised 26.6.08; accepted 28.8.08; Edited by RA Knight; published online 17.10.08

Results

The Caspase-8 gene is directly regulated by p63 in keratinocytes. By performing Affimetrix microarray analysis in HaCat cells silenced for p63 by siRNA, we identified genes regulated, directly or indirectly, by p63.[14] As caspase-8 emerged as a gene activated by p63, we validated it as a target by RT-PCR in HaCat and primary normal human epidermal keratinocytes – NHEK – following p63 RNAi: a concomitant decrease of p63 and caspase-8 was seen. Another p63 target – BI-1 – was also dramatically reduced,[13] compared with the housekeeping genes GAPDH and β-actin (Figure 1a). Western Blot analysis confirmed the substantial decrease of ΔNp63α at the protein level (Figure 1a, bottom panels). As ΔNp63α is the most expressed p63 isoform in NHEK, we overexpressed this isoform in NHEK and analysed endogenous mRNA levels by semiquantitative RT-PCR (Figure 1b): caspase-8 is indeed induced, whereas caspase-3 and caspase-9 are not. We conclude that caspase-8 is regulated by p63. To assess whether it is a direct target, we performed ChIP assays using anti-p63 and control antibodies on the promoter region. We selected amplicons in the core promoter area where conservation between human and mouse was stronger: strong positivity with the anti-p63 antibody in both HaCat and NHEK chromatin was observed (Figure 1c). As a control, we checked a region of the human BCoR gene,[25] which was not regulated by p63 and was devoid of any p63 binding (Figure 1c, lower panel).

Finally, we tested the transcriptional activity of the p63 isoforms in transient transfection assays with a caspase-8 promoter (-1 Kb to $+100$) fused to a CAT reporter in NHEK cells. As shown in Figure 1d, all p63 isoforms are able to transactivate the promoter, with different levels of efficiency: maximal for the TAp63 and minimal for the ΔNp63 isoforms. Altogether, these data indicate that caspase-8 is under direct regulation of p63 in human keratinocytes.

Caspase 8 expression in skin. We analysed the expression of caspase-8 in the skin of 19.5-day-old mouse embryos, using an antibody recognizing the procaspase form. Caspase-8 is expressed in the cytoplasm of all epidermal layers and staining is more intense in the basal and spinous layers, where p63 is also expressed (Figure 2, upper panel). p63 null mice have only rare, scattered skin patches, identified by staining with anti-K14, a marker of the basal epidermis (Figure 2, middle panels). In these areas, we detected very low expression of caspase-8 (Figure 2, middle panel), not proportional to the relative levels of K14 and far less than in normal animals. Finally, we analysed transgenic mice expressing ΔNp63α under the control of the Keratin 5 promoter, genetically complemented into p63-null background; these mice possess larger areas of epithelialization and re-express several differentiation markers.[26] As shown in Figure 2 (lower panels), these mice display strong caspase-8 expression in p63-positive cells, correctly localized in the cytoplasm, indicating that ΔNp63 positively influences caspase-8 expression in a genetically clean mouse model. Taken together, these data suggest that p63 is a major regulator of procaspase-8 expression.

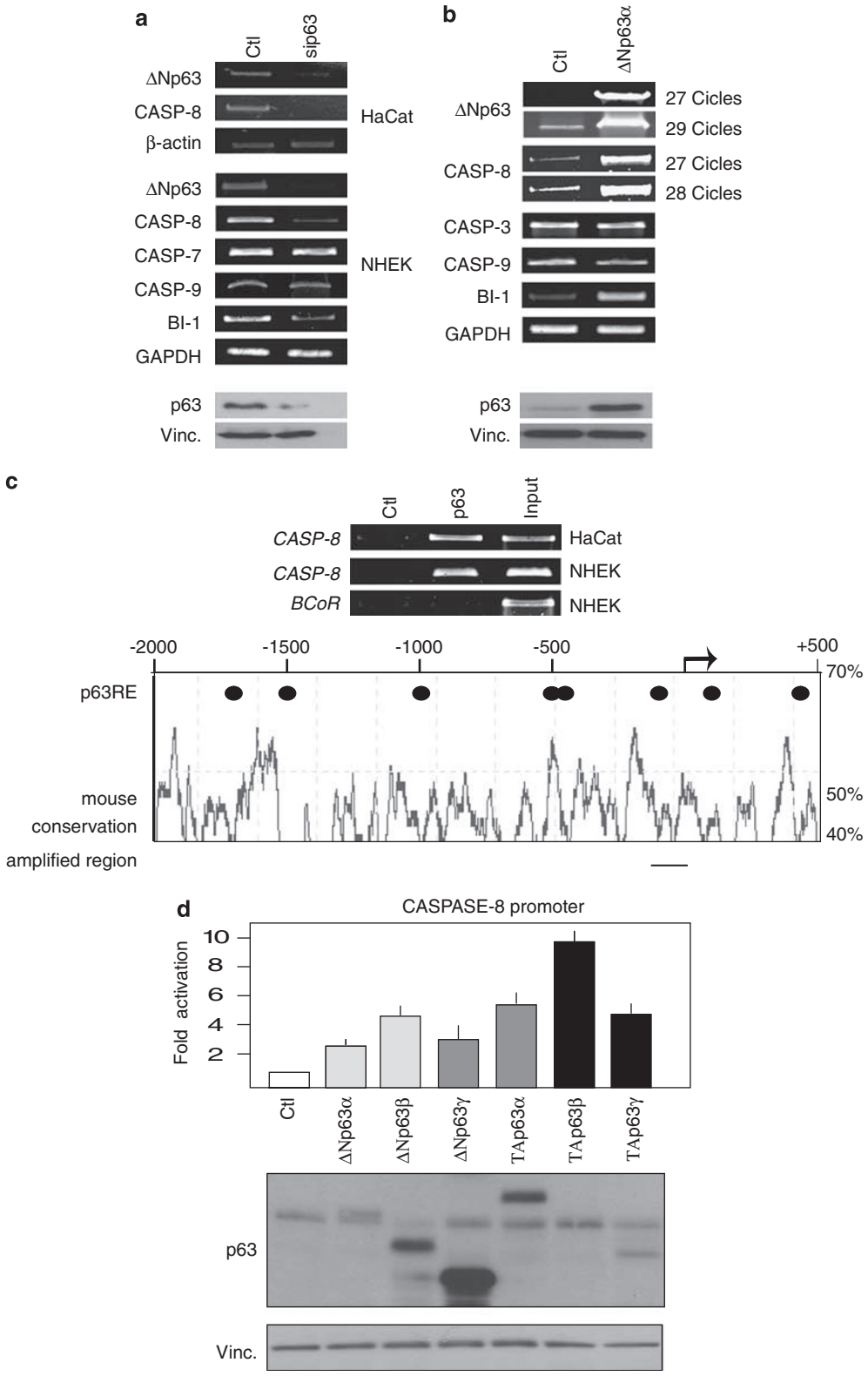
p63 and caspase-8 in response to UV irradiation. As caspase-8 is involved in apoptosis, including UV irradiation, we examined its activity after exposure to increasing doses of UVB and UVC in NHEK. We performed immunofluorescence on irradiated NHEK, using an antibody specific for the cleaved, active form of caspase-8, caspase-3 and caspase-9. Caspase-8 is not activated 12 h after exposure to 50, 100 and 200 J/m^2 of UVC, unlike caspase-9 and -3 (Supplementary Figure 1). After 12 h exposure to 250 and 400 J/m^2 of UVB light, NHEK display activated caspase-8 and -3, but not caspase-9, suggesting that the intrinsic pathway is active after UVC, whereas caspase-8 activation is observed only after UVB irradiation.

The levels of p63 were shown to decrease upon UVB irradiation in keratinocytes.[27,28] We investigated whether p63 levels might impact on caspase-8 activation, functionally inactivating p63 in NHEK cells by shRNA: (Figure 3b, and data not shown). Reduction of p63 significantly increased caspase-8 activation, compared with the levels in control cells, as shown in confocal microscopy of irradiated keratinocytes with antibodies against cleaved caspase-8 (Figure 3a and c). Cleaved caspase-8 is cytoplasmic and correlates to the highest level of p63 downregulation (Figure 3a, red arrow in the merge panel). In cells in which p63 downregulation is less efficient, cleaved caspase-8 staining is very weak (white arrow). Taken together, these results suggest that p63 elimination sensitizes NHEK to UVB-induced caspase-8 activation.

Next, we performed the reverse experiment, namely we assayed caspase-8 activation in UVB-irradiated NHEK after ΔNp63α overexpression. p63 levels were first checked by RT-PCR and western blots (Supplementary Figure 2), and no effect was observed on caspase-8 activation in normal

Figure 1 Validation of Caspase-8 as a target of p63. (**a**) Expression analysis of the indicated genes in HaCat and NHEK cells, treated with siRNA p63 or scramble oligo (Ctl). Upper panels, RT-PCR with RNA extracted from HaCat; β-actin was used to normalize cDNA. Lower panel, RT-PCR with RNA extracted from NHEK; GAPDH was used to normalize. The bottom panels show western blot of p63 in control and silenced cells. Vinculin was used as loading control. (**b**) p63 overexpression in NHEK. RT-PCR analysis of indicated genes with RNA extracted from NHEK transiently transfected with a ΔNp63α expression plasmid for 48 h. The normalization of the cDNAs was performed with GAPDH gene. The bottom panels show western blot of p63 in control and p63 overexpressing cells. Vinculin was used as loading control. (**c**) Chromatin immunoprecipitation analysis of HaCat (upper panel) and NHEK (middle and bottom panel) cells with α-p63 and control α-Flag antibodies on the caspase-8 promoter. As a control, the BCoR promoter was used in NHEK (bottom panel). The lower chart is a schematic representation of the caspase-8 promoter locus, with black dots indicating *in silico* p63 sites in the appropriate position. Displayed is also the mouse conservation from CONSITE. The black bar indicates the region amplified in the ChIP experiments. (**d**) Transactivation assay in NHEK cells. Transcription activity of the different p63 isoforms cotransfected with the human caspase-8 promoter. Bars represent the CAT activity ($+/-$S.D.), expressed as fold activation over the activity of the promoter transfected with an empty vector control construct (Ctl). Western blot of the various p63 isoforms overexpressed is shown (bottom panel): note the higher levels of ΔN isoforms as previously shown by us and others[13]

conditions (Supplementary Figure 2, lower panels). However, a marked decrease of cleaved caspase-8 upon UVB treatment was evident both by immunofluorescence and western blot analysis, in cells showing p63 overexpression (Figure 4a). Cleaved caspase-8 and p63 stainings appear mutually exclusive, as in cells without p63 overexpression caspase-8 was activated (Figure 4a, white arrow). Figure 4b shows western blot analysis of p63 and cleaved caspase-8:



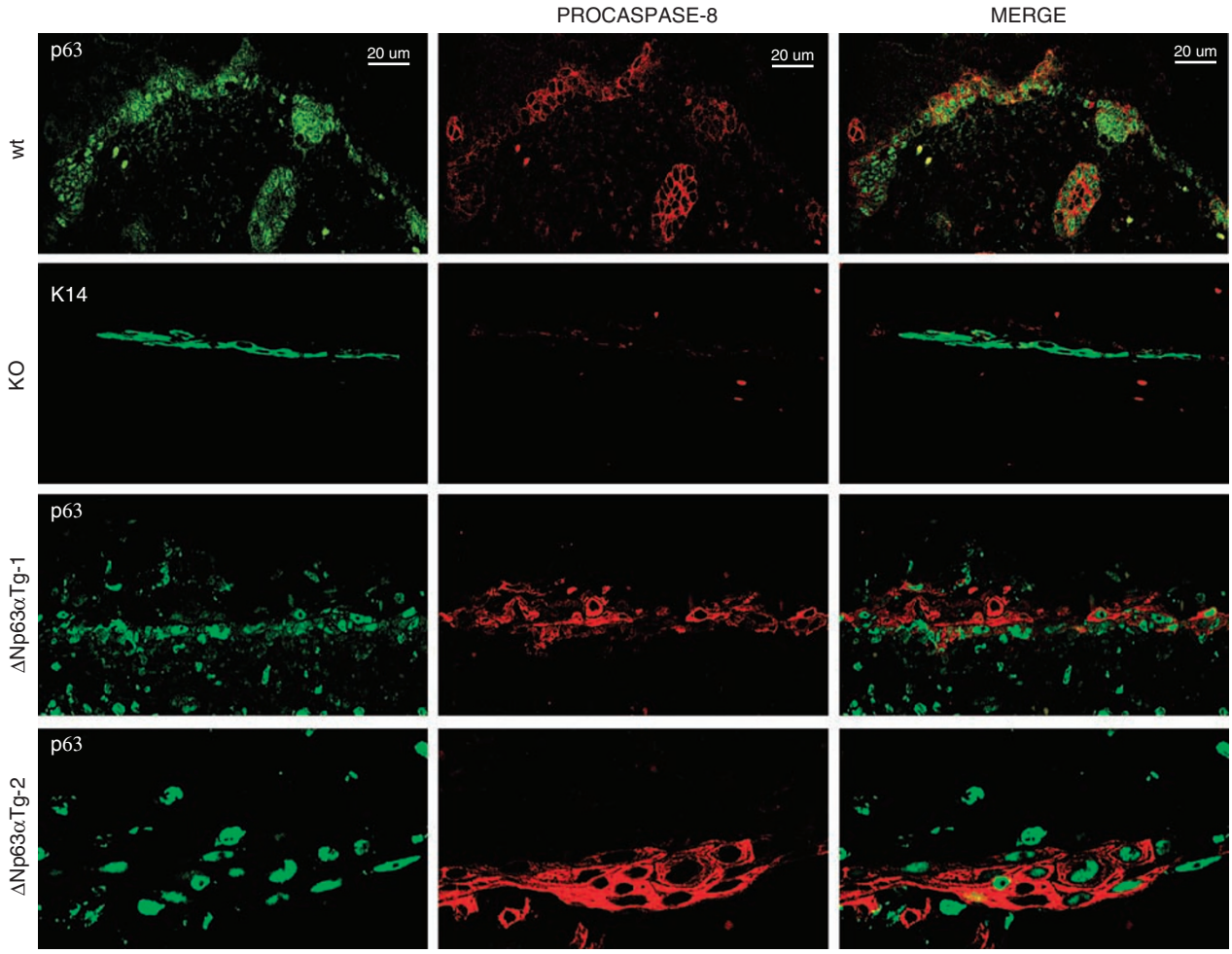


Figure 2 Immunostaining of procaspase-8 in mouse skin. Upper panels, confocal images of mouse wt skin immunostained with p63 (left) and procaspase-8 (middle). Middle panels, p63$^{-/-}$ mouse skin immunostained with K14 (left) and procaspase-8 (middle). Lower panels, ΔNp63-Tg mice immunostained with p63 (left) and procaspase-8 (middle). The right panels in each condition represent the merge of the costaining. The bottom panels show a second line of ΔNp63-Tg analysed at higher magnification

as expected, following exposure to UVB, NHEK show caspase-8 active products p45 and p18 (Figure 4b, lane 2). p63 overexpression has no effect on caspase-8 activation in non irradiated cells (Figure 4b, lane 3), but blocks caspase-8 activation, as shown by the absence of p18, and a reduction of p45 products in UVB-irradiated cells (Figure 4b, lane 4). We further confirmed this result by using a quantitative colorimetric assay for caspase-8 activation (Figure 4c). These findings suggest a role for p63 not only in caspase-8 expression, but also in caspase-8 activation. Thus, we conclude that ΔNp63α protects against exposure to UVB irradiation, and that this counter correlates with caspase-8 activation.

Activation of caspase-8 in p63 KO. To assess whether caspase-8 is activated in epithelial cells of p63 KO and transgenic mice, we stained embryo skin sections from these mice with the cleaved caspase-8 antibody: as shown in Figure 5a, cleaved caspase-8 is not detectable in wt mice (upper panels), but is present in the Keratin 14^{+} epithelial cells of p63 KO and ΔNp63α-tg mice (middle and lower panels). Compared with the data of Figure 2, obtained with the anti-procaspase-8 antibody, these results suggest that, in p63 knockout (KO) mice a significant amount of caspase-8 is cleaved, whereas the vast staining of procaspase-8 in the ΔNp63α-tg mice is not paralleled by a concomitant increase in active, cleaved caspase-8, as in the wt mice. We also performed TUNEL assays on the wt, KO and transgenic mice, to analyse the apoptotic state of mouse skin. As expected, in wt mice apoptotic events occur rarely, and only in cells of the subcorneum layer (Figure 5b); KO mice display copious apoptotic epithelial cells. Finally, ΔNp63-Tg show only some TUNEL-positive cells, a clear reduction with respect to KO animals. In conclusion, p63 elimination led to lower levels of caspase-8 expression, but higher apoptosis, and re-expression of ΔNp63α restores high levels of caspase-8, but lowers apoptosis. The same results were obtained by evaluating procaspase-8 and cleaved caspase-8 levels in human interfollicular epidermis (Supplementary Figure 3). Taken together, these results indicate that ΔNp63 regulate genes controlling not only caspase-8 transcription, but also its activation.

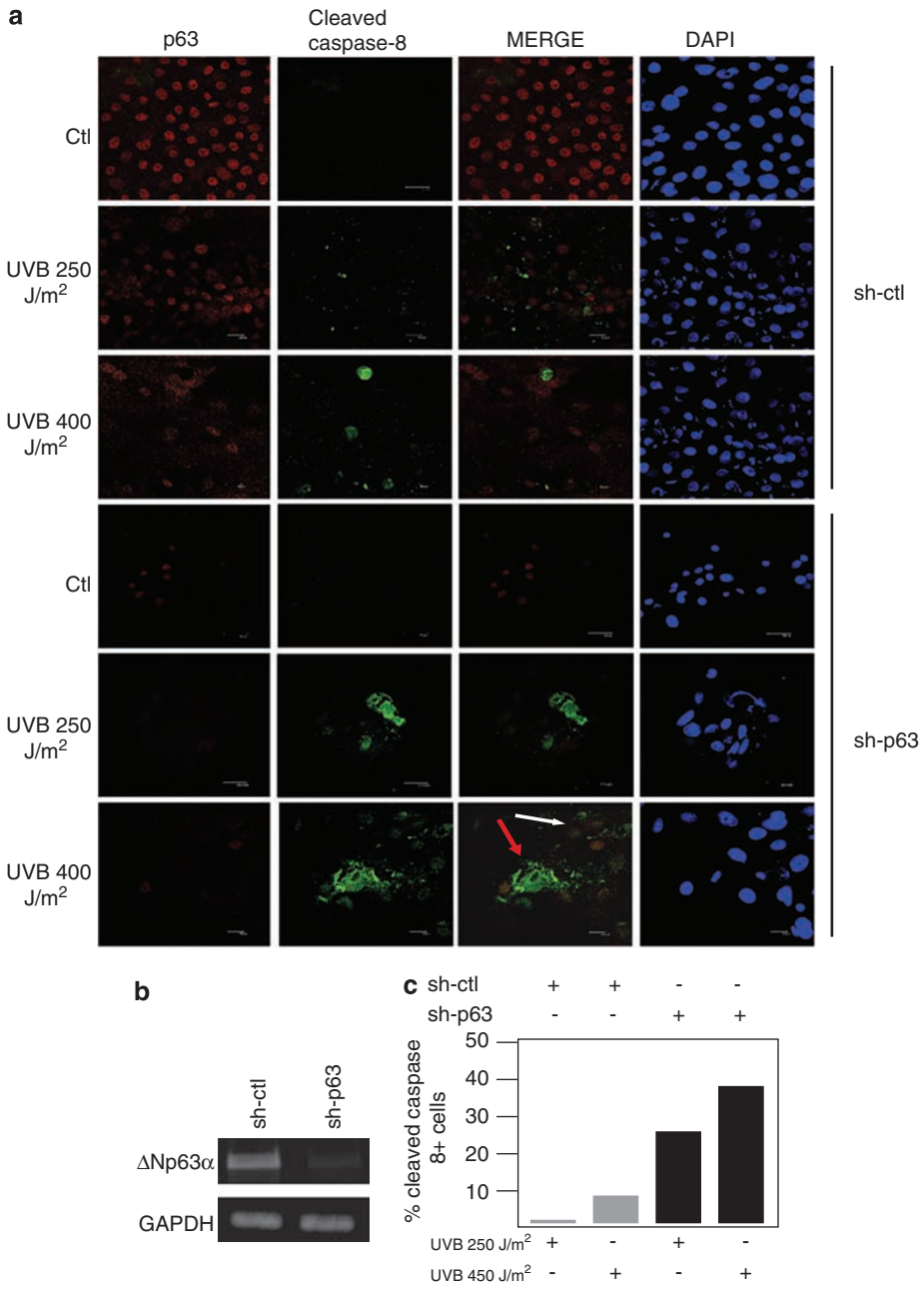


Figure 3 Caspase 8 activation after p63 silencing. (**a**) Confocal images of NHEK transfected with a scramble shRNA construct (upper panels, sh-Ctl) or a sh-p63 (lower panels, sh-p63) and treated with two doses of UVB (250 and 400 J/m^2). Keratinocytes were irradiated 96 h after transfection of the shRNA, the time point at which downregulation of p63 was most prominent. Immunostaining was performed with the antibodies indicated on top of the panels. Nuclear staining with DAPI is indicated on the right. The doses are indicated on the left of each row. Ctl refers to no UV treatment. The red arrow indicates a group of p63-silenced cells, positive for activated caspase-8. The white arrow indicates a group of p63-positive cells and -negative for caspase-8. (**b**) RT-PCR analysis of ΔNp63α expression in the NHEK used in A. cDNA was normalized with GAPDH. (**c**) Graphic representation of the percent of cells positive for activated caspase-8 in different microscopy fields containing comparable number of cells. Treatment are shown on top of the chart and UVB doses are at the bottom. Grey bars represent cells with a scramble sh-construct, whereas black bars are cells transfected with sh-p63

The caspase-8 inhibitor FLIP is a p63 target. One of the major inhibitor of procaspase-8 activation is FLIP. We therefore investigated whether p63 targets the c-FLIP gene: scanning the human c-FLIP promoter, we identified a cluster of potential p53/p63-binding sites 2.5 Kb upstream of the transcriptional start site, in a region showing considerable mouse–human homology (Figure 6a). We analysed whether p63 binds to the c-FLIP promoter in NHEK by ChIP assay; as shown in Figure 6a, p63 was bound to two regions of the FLIP promoter: one strong binding site at −1500, and a weaker one on the core promoter at −50.

Next, we verified whether FLIP expression is modulated upon p63 RNAi inactivation and overexpression experiments in NHEK. Western blots and RT-PCR analysis are shown in

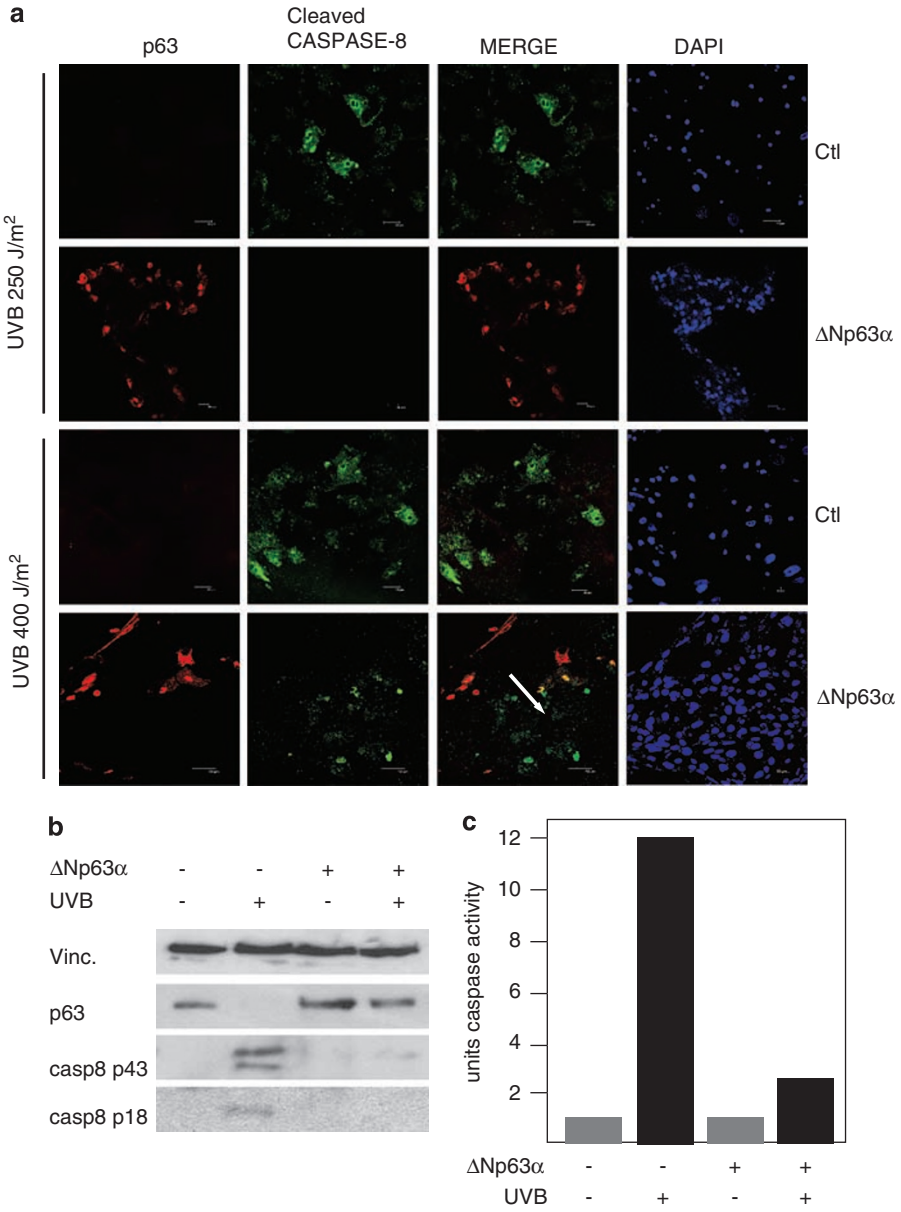


Figure 4 Caspase 8 activation after p63 overexpression in NHEK. (**a**) Confocal images of NHEK transfected with 2 μg of ΔNp63α expression construct and treated with different UVB doses (250 and 400 J/m^2). As for Figure 3a, the antibodies and DAPI stainings are indicated at the top of the panels. The treatment is indicated on the left and the transfection conditions are on the right. Ctl refers to NHEK transfected with 2 μg of empty vector. The white arrow indicates a group of cells not overexpressing ΔNp63α and positive for activated caspase-8. Note that to visualize p63 overexpressions, laser settings for image acquisition of ΔNp63α overexpressing cells were significantly lowered with respect to those shown in Figure 3a for endogenous p63: this is the reason why endogenous p63 expression was barely detectable here. (**b**) Western Blot analysis of p63 and activated caspase-8 of cell extracts from NHEK cells transfected with an empty vector (lanes 1 and 2), or ΔNp63α (lanes 3 and 4) and treated with 250 J/m^2 of UVB (lane 2 and 4). The antibodies are indicated on the left. αVinculin was used as a loading control. (**c**) Colorimetric assay of caspase-8 activity in NHEK transfected with a control (lane 2) and p63 (lane 4) vector, after UVB exposure (250 J/m^2). Bars represent units of caspase-8 activity after subtraction of the corresponding UVB-untreated control, either basal (lane 1) or overexpressing p63 (lane3). Assays were performed according to the manufacturer's protocol

Figure 6b and c: FLIPl and FLIPs do not show significant changes in mRNA, or protein levels. On the other hand, the mRNA levels of FLIPr are decreased after p63 interference, and conversely, increased by ΔNp63α overexpression, matching the protein levels tested by western blots. These findings indicate that p63 is able to positively regulate FLIP expression at the transcriptional level.

c-FLIP is downregulated upon UVB irradiation. We analysed the effect of UVB irradiation on the expression of FLIP. As shown in Figure 7a, UVB irradiation leads to a strong decrease of all FLIP variants; p63 levels are also decreased, in these conditions, as reported earlier.[27,28] As a control for this analysis, we verified the levels of Jun-B, whose expression levels are known to be increased,[29] and

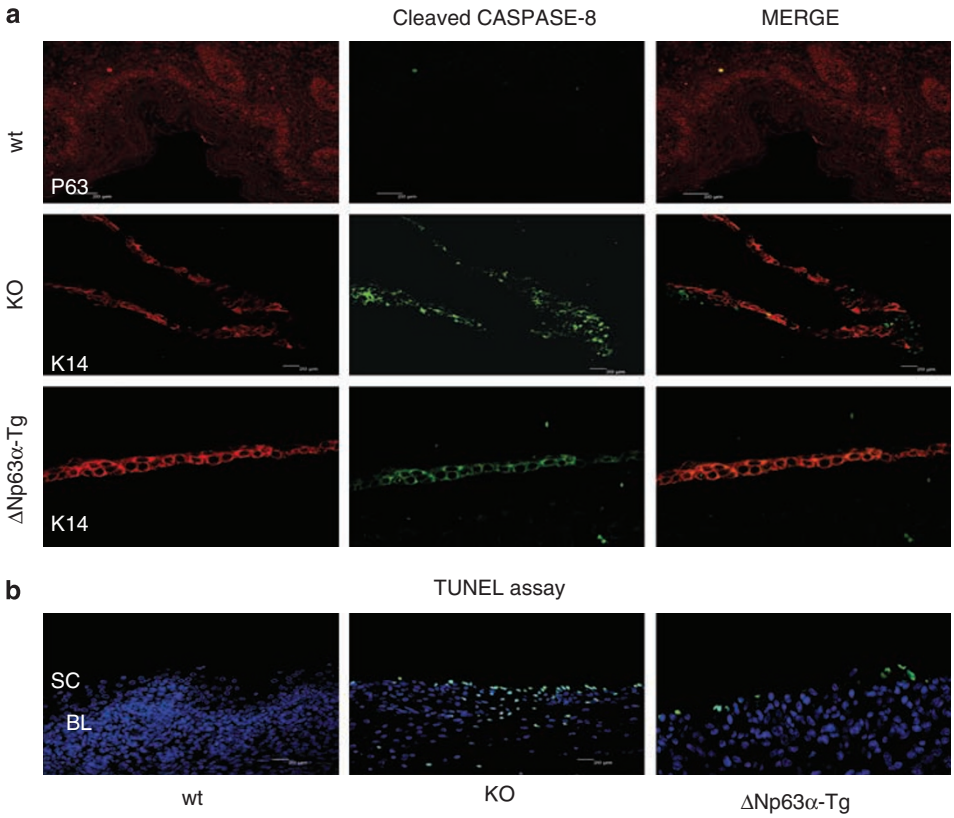


Figure 5 Caspase-8 activation in wt, p63−/− (KO) and transgenic (ΔNp63-tg) mice. (**a**) Upper panels, confocal images of wt skin immunostained with p63 and activated caspase-8. Middle panels, KO mice, and lower panels, transgenic mice (ΔNp63-tg) skin, stained for K14 and activated caspase-8. The right panels of each condition represents the merge of the costaining. (**b**) TUNEL assay by confocal fluorescence microscopy. Left panel: wt mice skin. Middle panel: $p63^{-/-}$ mice skin showing intense TUNEL staining (in green). Right panel: transgenic mice (ΔNp63-Tg) skin with few TUNEL-positive cells. BL (basal layer) and SC (subcorneum layer)

indeed they are (Figure 7a). We overexpressed ΔNp63α in NHEK, exposed them 24 h after transfection to UVB irradiation, and analysed FLIP by semiquantitative RT-PCR and western blots: we observed increased FLIPr levels, but not of the other two splicing isoforms, FLIPs and FLIPl. As shown in Figure 7b, p63 overexpression significantly prevented FLIPl downregulation after UVB irradiation, both at the mRNA and protein levels. The mRNAs of all FLIP isoforms dramatically drop after UV treatment, as expected from the decrease of p63. Upon overexpression of p63, mRNA levels increase robustly in the absence of UV, particularly of the antiapoptotic s and r isoforms. These isoforms are also increased upon UV irradiation, when compared with cells with normal levels of p63. The recovery is not complete, but the increase is actually more robust than in non UV-irradiated cells (compare lanes 1–3 and 2–4). In summary, all these results are in full agreement with a positive role of p63 in c-FLIP regulation.

FLIP expression in p63 KO mice. We investigated FLIP expression in the skin of the p63 mice models used above. As shown in Figure 8, FLIP shows a diffuse nuclear and cytoplasmic staining, in cells normally expressing p63, and a cytoplasmic staining in cells which do not express p63 (Figure 8, upper panels, white arrow). In p63 KO mice, we analysed FLIP and the cK14 marker: weaker staining is detected in $K14^{+}$ cells, compared to wt mice. Furthermore, KO mice show a cytoplasmic staining of FLIP. These *in vivo* data further substantiate the notion that FLIP is regulated by p63 in mouse skin.

Discussion

p63 and apoptosis. The role of p63 in apoptosis pathways is debated. Gressner *et al.*[9] described the role of TAp63α in activating apoptosis through death receptors and mitochondria by activating transcription of p53 targets.[14] ΔNp63 is believed to compete for, or form a transactivation incompetent heterocomplex with, p53 and TAp63.[8] Zebrafish studies confirmed the antagonistic roles of ΔNp63 and p53.[10] Thus, one could tentatively conclude that the antiapoptotic role of ΔNp63 reflects its negative impact on p53 functions. However, ΔNp63α RNAi led to DNA damage-induced apoptosis in cells carrying p53 mutant alleles, which have visibly lost proapoptotic behaviours, suggesting that additional antiapoptotic functions of ΔNp63α are crucially required.[7] This hypothesis is in agreement with the reported sensitivity of p53-mutated HaCat cells to UVB irradiation-mediated apoptosis.[30] In Figure 1, TA isoforms are more potent in Luciferase assays, especially the β and γ isoforms, but we think that this should not be taken as an indication that

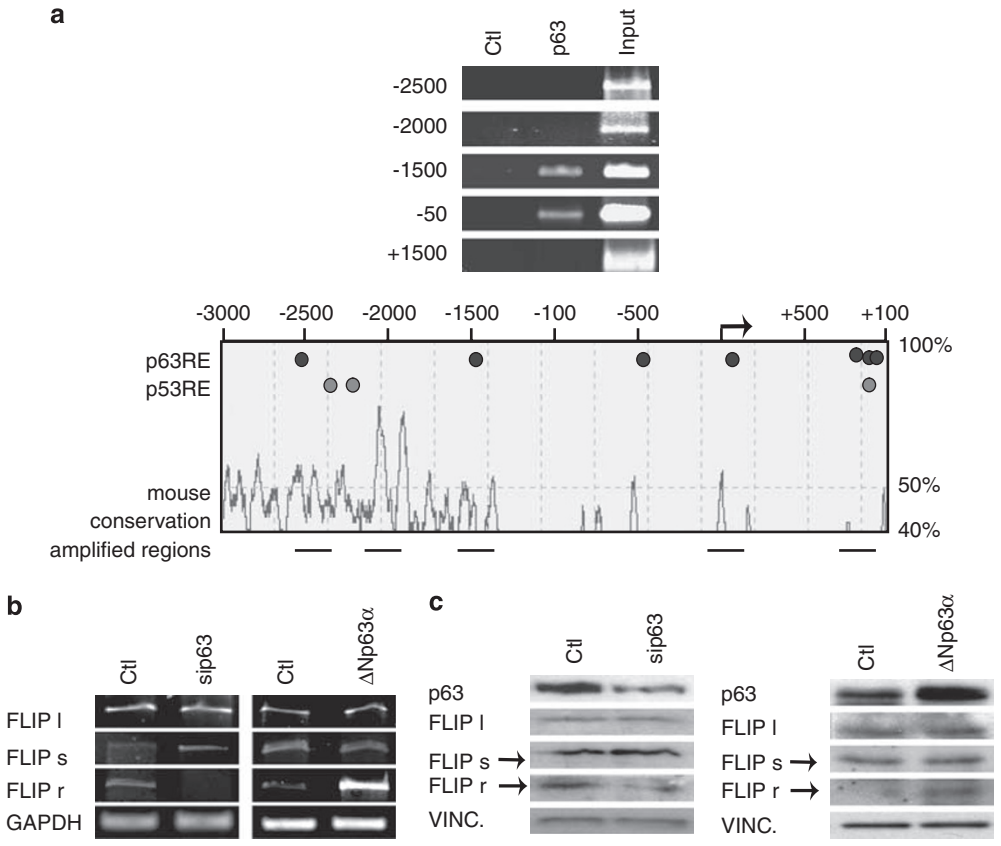


Figure 6 Identification of FLIP as a p63 target. (**a**) Chromatin Immunoprecipitation analysis of the c-FLIP promoter. ChIPs of NHEK cells with the α-p63 and control α-Flag antibodies. Different regions of c-FLIP promoter were amplified and the positions relative to the TSS are indicated on the left of each panel. The bottom chart is a schematic representation of the c-FLIP promoter with *in silico* p63 (black) and p53 (grey) sites indicated as dots at the appropriate position. Displayed is also mouse conservation (CONSITE) and black bars indicate the regions amplified in ChIP experiments. (**b**) RT-PCR analysis of different c-FLIP splice isoforms (FLIPl, r and s) with RNAs from NHEK cells silenced for p63 (left panels), or overexpressing ΔNp63α (right panels). GAPDH was used to normalize cDNAs. (**c**) Western Blot of NHEK total extracts in the same experimental conditions as in B, with α-FLIP and α-p63 antibodies. α-vinculin was used as a loading control

procaspase-8 is more of a target of TA than ΔN isoforms: in fact, we have noticed this behaviour routinely, even in promoters characterized as bona fide targets of ΔNp63α.[13,14] It is likely that this effect is due to the properties of the transient assays, which are presumably not including chromatin constraints.

In theory, p73 and p53 could also regulate the caspase-8-FLIP pathway, but several arguments specifically point toward p63 in keratinocytes under physiological conditions. (i) There is no genetic evidence linking p73 or p53 to significant developmental networks in keratinocytes, as KO mice have no overt skin alterations. (ii) Although the levels of p63 in human skin and keratinocytes are very robust, those of p73 – and p53 – are extremely low, if ever appreciable. (iii) We performed ChIPs in human keratinocytes with p73 antibodies and failed to obtain any results on any targets of p63 (S.B., not shown). Thus the role of p73 in the caspase-8-FLIP pathway should be explored in cellular systems where robust p73 levels are physiologically scored and in pathologic conditions, such as in tumours, where increased p73 levels – and mutated p53 – might well change the balance.

Transgenic mice overexpressing ΔNp63α display an impaired ability of epidermal keratinocytes to undergo apoptosis in response to UVB irradiation[27] and ΔNp63α expression is reduced in response to UVB, suggesting that p63 downregulation is necessary to allow apoptotic pathways. We find that overexpression of ΔNp63 protects primary human keratinocytes from apoptosis, whereas p63 inactivation dramatically enhances this phenomenon. We also confirm the decrease, but not the abolition of p63 expression upon UV irradiation. We note that this is specifically observed in UVB, as compared with UVC irradiation. Similarly, in mouse keratinocytes, p63 protects from apoptosis, as assessed by assays of caspase-3 cleavage.[28]

Mechanistically, the ΔNp63α ability to inhibit the proapoptotic function of TAp63 relies on the transcription inhibitory – TI – domain. It was demonstrated that p63 is itself a caspase target: cleavage of TAp63α by activated caspases lead to the loss of the TI domain, enhancing its transcriptional – and proapoptotic – activities.[31] In contrast, cleavage of ΔNp63α does not affect its transactivation, but remove its inhibitory effect on TAp63 isoforms. All these considerations lead to our surprise in finding a proapoptotic gene, caspase-8, among the targets of ΔNp63α, in unbiased RNAi profiling experiments.

p63 and caspase-8. Although caspase-8 post-transcriptional regulation has been studied in great detail, less is known about its transcriptional activation, which varies

significantly within different tissues and cells. Several transcription factors are capable to regulate the caspse-8 promoter.[32] p53 overexpression can induce transcription through an ETS-like element, even though an induction following DNA damage has not been reported. Overexpression, RNAi and transient transfections reporter experiments indicate that caspase-8 is activated by ΔNp63α.

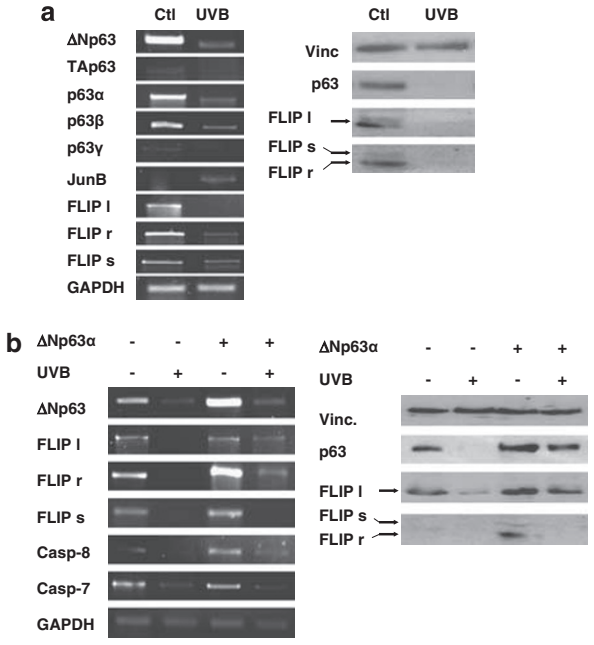


Figure 7 c-FLIP regulation upon UVB irradiation in NHEK. (**a**) RT-PCR (left panels) and western blot (right panels) analysis of the indicated genes with RNA and proteins extracted from irradiated (250 J/m^2 UVB), or untreated (Ctl) NHEK. The cDNAs were normalized with GAPDH. Protein loading was controlled with an α-Vinculin antibody. (**b**) RT-PCR (left panels) and western blot (right panels) analysis of the indicated genes with RNA and proteins extracted from control and p63 overexpressing NHEK, exposed to UVB irradiation. Transfections and UVB (250 J/m^2) treatments are indicated at the top of the panels. Normalization was done as in (**a**)

ChIP analysis, as well as studies in p63 KO and ΔNp63α-tg mice confirmed direct regulation of caspase-8, *in vivo*. Note that the overlap between p63 and procaspase 8 is not absolute in transgenics, as there are cells that are negative for the latter: therefore there are additional signals that limit procaspase-8 expression in selected cellular populations. The mRNA expression in NHEK fits with the levels of procaspase-8 protein, clearly detectable by IFs staining, both in human and mouse skin. Following UVB irradiation, both procaspase-8 and p63 transcription decreases, whereas proteolytic activation of caspase-8 activates apoptosis through inactivation of FLIP, the key negative regulator of procaspase-8.

p63 and FLIP. Caspase-8 activation is inhibited at the DISC by different FLIP splice variants at two different cleavage steps. Products released from the DISC upon receptor triggering depend on the ratios of procaspase-8 and FLIP at the DISC. Low levels of FLIP proteins allow processing of procaspase-8, giving rise to the active heterotetramer. High levels of $FLIP_L$ allow procaspase-8 recruitment at the DISC, but cleavage is blocked after the generation of the p43 product of caspase-8 and its role as an antiapoptotic molecule is still debated.[22,23] High levels of FLIPs and FLIPr lead to the recruitment at the DISC, but the cleavage is plugged and ineffective: hence, these isoforms are essentially antiapoptotic.[33] Our results suggest that p63 supports specifically FLIPr expression in non apoptotic conditions: following UVB exposure, FLIPr and FLIPs decrease, whereas FLIPl levels remain unaffected: the balance shifts in favour of the latter, resulting in the p41/p43 kDa products (Figure 7).

FLIP can be regulated at multiple levels and transcription is modulated by NF-κB, c-MYC, AR and AP1 family members,[34] whereas p53 can downregulate protein levels through the ubiquitin–proteasome system, in a transcription-independent manner.[35] Here, we describe two important findings: (i) direct

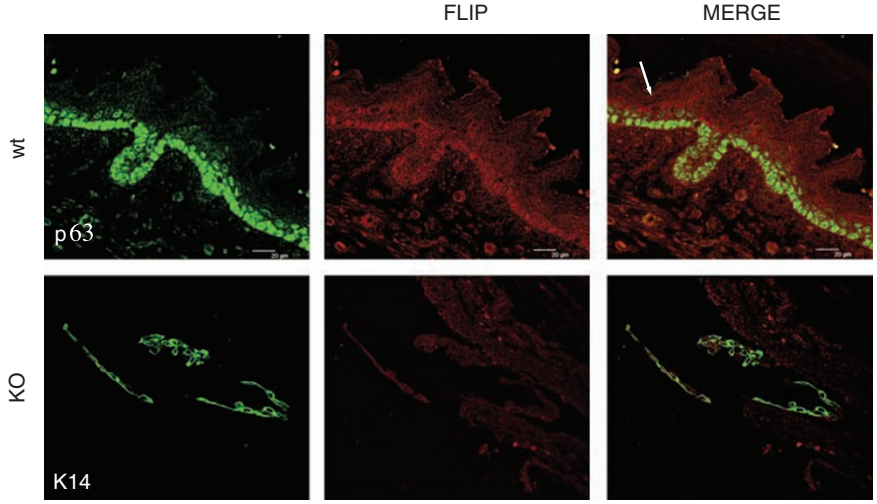


Figure 8 FLIP expression analysis in wt and p63−/− mice. Upper panels, Confocal images of wt mice skin immunostained with α-p63 and α-FLIP antibodies. Lower panels, KO mice staining performed with α-K14 and α-FLIP antibodies. The right panels of each condition represent the merge of the costaining. The white arrow indicates a group of cells not expressing p63, displaying FLIP cytoplasmic staining

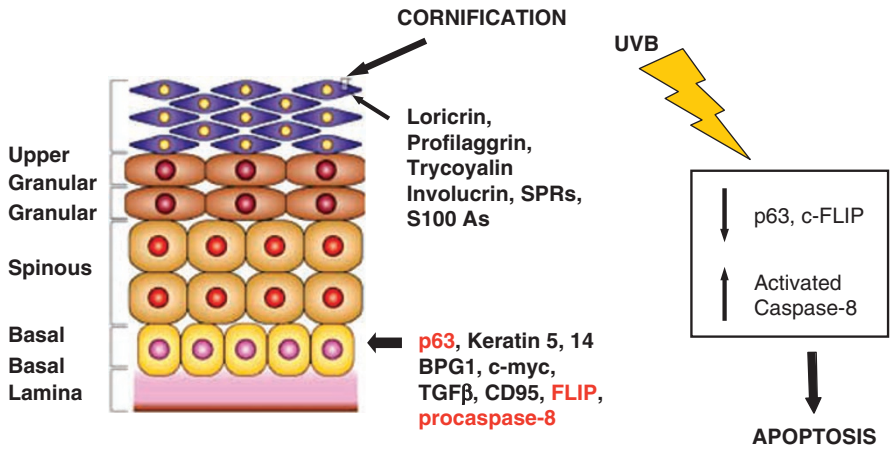


Figure 9 Proposed model of p63 regulation of Caspase-8-FLIP apoptotic pathway in skin

positive regulation of FLIP expression by p63, assessed by overexpression, RNAi, ChIP and p63 KO and transgenic mice experiments; (ii) modulation of the FLIP isoforms, which differentially impact on apoptosis. Transcription factors often control not only the overall rate of transcription initiation, but also the specific mRNA isoform produced (Reviewed by Fukazawa *et al.*[35]). Indeed, ΔNp63α was earlier reported to modulate splicing events of target genes;[36] the ability of p53 to regulate FLIP-protein levels[35] suggests antagonistic roles.

There are additional levels of common p63-FLIP regulation: one is represented by the the ubiquitin E3 ligase Itch, which specifically ubiquitylates FLIP and ΔNp63, inducing its proteosomal degradation.[37] Another level is represented by transcription factors known to regulate FLIP, which are also p63 targets: c-jun and Jun-B;[14] the former, in combination with Fos, represses c-FLIP expression.[34] NF-κB activates the FLIP promoter,[38–39] and its regulator IkB kinase α is induced by ΔNp63α.[40] The FLIP promoter is therefore at the centre of converging and interconnected survival pathways – p63 and NF-κB – as well as proapoptotic signals (c-jun). Hence, caspase-8 and FLIP are linked by a common fate, regulated at the transcriptional, post-transcriptional and post-translational levels.

Apoptosis in the skin. Two very important, completely unrelated processes of programmed cell death occur in the skin: apoptosis and cornification (Figure 9). Apoptosis is necessary for balancing keratinocyte responses to external toxic insults, such as UV, thus producing 'sun burn' cells; cornification is responsible for the formation of the stratum corneum. This layer is fundamental for the mechanical, impermeable and elastic barrier of our body, requiring a specific and sophisticated mechanism of differentiation.[26] In gross terms, apoptosis occurs in the lower layers of the epidermis, and seems to be repressed in the middle/upper layers, to allow differentiation, a process requiring the expression of toxic proteins – loricrin, involucrin, small proline-rich proteins – which would otherwise trigger apoptosis. Sun burn cells are typical of the basal layer, where the cornification genetic programme is inactive. In normal human epidermis, nine caspase genes are expressed in the lower layers – type-1, -2, -3, -4, -6, -7, -8, -9 and -10 – as part of the protective mechanism dealing with exposure to UV irradiation or DNA-damaging agents. In addition, Caspase-14 is implicated in terminal keratinocyte differentiation and cornification.[41] In summary, we employed several approaches to detail how ΔNp63α acts to sustain a proapoptotic pathway necessary to cope with an excess of dangerous environmental signals – UVB – while ensuring the efficient and fine-tuned expression of a dominant antiapoptotic gene. This is compatible with the hypothesis that, in actively proliferating cells of the lower epithelial layers, apoptosis is important for the response to toxic agents, placing p63 as a pivotal regulator of this balance.

Materials and Methods

Cells and culture conditions. HaCat were grown in DMEM in the presence of 10% fetal calf serum. First passage primary human keratinocytes – NHEK – were derived from the breast of healthy individuals and grown on a feeder-layer of lethally irradiated 3T3 cells in DMEM F12 added with insulin (5 μg/ml), EGF-R (10 ng/ml) hydrocortisone (0.4 μg/ml), T3 (2 nM), cholera toxin (0.1 nM) and transferrin (5 μg/ml).

RT-PCR and transfections. HaCat cells were transiently transfected using Oligofectamine (Gibco-BRL, USA) for 3 hours with 150 ng/cm^2 of human p63 siRNA oligonucleotide, which targets the central DNA-binding domain of p63.[14] After overnight incubation, transfection was repeated for 3 additional hours. 2.5×10^5 first passage NHEK were transfected with Nucleofector (Amaxa, Germany) using siRNA oligonucleotide at 0.5 nM. An off-target siRNA oligos mixture (5′-AUGAACGUGAAUUGCUCAA-3′, 5′-UAAGGCUAUGAAGAGAUAC-3′, 5′-AUGUAUUGGCCUGUAUUAG-3′, 5′-UAGCGACUAAACACAUCAA-3′; Dharmacon D-00181001) was used as control. NHEK cells silenced with a shRNA plasmid targeting all p63 isoforms (MISSION shRNA plasmid, Sigma) using Lipofectamine (Gibco-BRL); 5 μg of shRNA plasmid and a scramble sh control were used. RNA was extracted with RNA-Easy kit (Quiagen), 48 h after siRNA transfections and 96 h after shRNA transfections. For cDNA synthesis, 4 μg of RNA were retrotranscribed with M-MLV-RT kit (Invitrogen, USA). Semiquantitative PCR analysis was performed with primers listed in Supplementary Figure 4. Keratinocytes were irradiated 96 h after transfections. They were exposed to 250 and 400 J/m^2 UVB from lamps emitting light of wavelength from 275 to 400 nm, peaking at 315 nm. For transactivation experiments, 1×10^5 NHEK cells were transfected with Lipofectamine (Gibco-BRL) using 1.2 μg of reporter plasmids, 200 ng of p63 different splicing isoforms, and a carrier for a total DNA of 2 μg. Four independent transfections in duplicate were performed.

Chromatin immunoprecipitations. ChIP analysis were carried out as described earlier,[14] with an anti-p63 antibody, produced and purified in our laboratory, recognizing all isoforms of p63.[13] Casp-8 and c-FLIP promoters primers utilized are listed in Supplementary Figure 4.

Western blot and immunofluorescence. Total extract from NHEK were prepared by lysing cells in RIPA buffer (50 mM Tris-HCl PH 7.8, 150 mM NaCl, 10% glycerol, 1% sodiumdeoxicholate, 1% Triton X-100, 0.1% SDS, 5 mM EDTA, DTT 1 mM, 1 mM PMSF) followed by sonication. Western blot analysis was performed according to standard procedures. Immunofluorescence analysis was performed as in Viganò *et al.*,[13] with the following antibodies: p63 (4A4, DAKO, Diagenode), cleaved caspase-8, -9 and -3 (Cell Signalling), anti-FLIP (NF6, Alexis), anti-procaspase-8 (Active Motif).

TUNEL assay. Paraformaldehyde fixed, paraffin-embedded mouse embryo sections were deparaffinized and rehydrated in decreasing EtOH. Following a wash in distilled water, slides were treated with proteinase K working solution (10–20 ug/ml in 10 mM Tris/HCL, PH 7.4–8) for 15–30 min at 21–37°C, then washed in phosphate-buffered saline (PBS). Slides were then incubated with TUNEL reaction mixture (Roche) for 1 h at 37°C. This was followed by two washings in PBS, then the sections were stained with DAPI, mounted in ProLong Gold antifade reagent (Invitrogen) and analysed as above.

UVB irradiation and colorimetric assay. After irradiation, cells were rinsed two times with PBS, covered with PBS and exposed to 250 and 400 J/m^2 of UV light. Colorimetric assay for caspase-8 activity was performed with Apoalert Caspase Colorimetric Assay kits (Clontech) according to the manufacturer's instructions and calculation was done by subtracting the reading of the corresponding NHEK not irradiated.

Acknowledgements. We thank C Imbriano and M Romani for reagents, S Pozzi for help with human keratinocyte experiments. We thank S Rodeghiero and U Fascio at the CIMAINA facility for skilful assistance with confocal microscopy. MA Viganò was supported by UE-EPISTEM contract. This work was supported by Grants from Fondazione Cariplo to R.M. and UE-EPISTEM to R.M and G.M.

1. McKeon F. p63 and the epithelial stem cell: more than *status quo*? *Genes Dev* 2004; **18**: 465–469.
2. Aylon Y, Oren M. Living with p53, dying of p53. *Cell* 2007; **130**: 597–600.
3. Mills AA, Zheng B, Wang XJ, Vogel H, Roop DR, Bradley A. p63 is a p53 homologue required for limb and epidermal morphogenesis. *Nature* 1999; **398**: 708–713.
4. Yang A, Schweitzer R, Sun D, Kaghad M, Walker N, Bronson RT *et al.* p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature* 1999; **398**: 714–718.
5. Rinne T, Hamel B, van Bokhoven H, Brunner HG. Pattern of p63 mutations and their phenotypes–update. *Am J Med Genet A* 2006; **140**: 1396–1406.
6. Carroll DK, Carroll JS, Leong CO, Cheng F, Brown M, Mills AA *et al.* p63 regulates an adhesion programme and cell survival in epithelial cells. *Nat Cell Biol* 2006; **8**: 551–561.
7. Senoo M, Pinto F, Crum CP, McKeon F. p63 Is essential for the proliferative potential of stem cells in stratified epithelia. *Cell* 2007; **129**: 523–536.
8. Yang A, Kaghad M, Wang Y, Gillett E, Fleming MD, Dotsch V *et al.* p63, a p53 homolog at 3q27–29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Mol Cell* 1998; **2**: 305–316.
9. Gressner O, Schilling T, Lorenz K, Schulze Schleithoff E, Koch A, Schulze-Bergkamen H *et al.* TAp63alpha induces apoptosis by activating signaling via death receptors and mitochondria. *EMBO J* 2005; **24**: 2458–2471.
10. Lee H, Kimelman D. A dominant-negative form of p63 is required for epidermal proliferation in zebrafish. *Dev Cell* 2002; **2**: 607–616.
11. Truong AB, Kretz M, Ridky TW, Kimmel R, Khavari PA. p63 regulates proliferation and differentiation of developmentally mature keratinocytes. *Genes Dev* 2006; **20**: 3185–3197.
12. Yang A, Zhu Z, Kapranov P, McKeon F, Church GM, Gingeras TR *et al.* Relationships between p63 binding, DNA sequence, transcription activity, and biological function in human cells. *Mol Cell* 2006; **24**: 593–602.
13. Viganò MA, Lamartine J, Testoni B, Merico D, Alotto D, Castagnoli C *et al.* New p63 targets in keratinocytes identified by a genome-wide approach. *EMBO J* 2006; **25**: 5105–5116.
14. Testoni B, Borrelli S, Tenedini E, Alotto D, Castagnoli C, Piccolo S *et al.* Identification of new p63 targets in human keratinocytes. *Cell Cycle* 2006; **5**: 2805–2811.
15. Lamkanfi M, Declercq W, Kalai M, Saelens X, Vandenabeele P. Alice in caspase land. A phylogenetic analysis of caspases from worm to man. *Cell Death Differ* 2002; **9**: 358–361.
16. Shi Y. Caspase activation: revisiting the induced proximity model. *Cell* 2004; **117**: 855–858.
17. Peter ME, Krammer PH. The CD95(APO-1/Fas) DISC and beyond. *Cell Death Differ* 2003; **10**: 26–35.
18. Medema JP, Scaffidi C, Kischkel FC, Shevchenko A, Mann M, Krammer PH *et al.* FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). *EMBO J* 1997; **16**: 2794–2804.
19. Golks A, Brenner D, Fritsch C, Krammer PH, Lavrik IN. c-FLIPR, a new regulator of death receptor-induced apoptosis. *J Biol Chem* 2005; **280**: 14507–14513.
20. Djerbi M, Darreh-Shori T, Zhivotovsky B, Grandien A. Characterization of the human FLICE-inhibitory protein locus and comparison of the anti-apoptotic activity of four different flip isoforms. *Scand J Immunol* 2001; **54**: 180–189.
21. Ueffing N, Keil E, Freund C, Kuhne R, Schulze-Osthoff K, Schmitz I. Mutational analyses of c-FLIP(R), the only murine short FLIP isoform, reveal requirements for DISC recruitment. *Cell Death Differ* 2008; **15**: 773–782. Jan 25.
22. Chang DW, Xing Z, Pan Y, Algeciras-Schimnich A, Barnhart BC, Yaish-Ohad S *et al.* c-FLIP(L) is a dual function regulator for caspase-8 activation and CD95-mediated apoptosis. *EMBO J* 2002; **21**: 3704–3714.
23. Micheau O, Thome M, Schneider P, Holler N, Tschopp J, Nicholson DW *et al.* The long form of FLIP is an activator of caspase-8 at the Fas death-inducing signaling complex. *J Biol Chem* 2002; **277**: 45162–45171.
24. Yeh WC, Itie A, Elia AJ, Ng M, Shu HB, Wakeham A *et al.* Requirement for Casper (c-FLIP) in regulation of death receptor-induced apoptosis and embryonic development. *Immunity* 2000; **12**: 633–642.
25. Huynh KD, Fischle W, Verdin E, Bardwell VJ. BCoR, a novel corepressor involved in BCL-6 repression. *Genes Dev* 2000; **14**: 1810–1823.
26. Candi E, Rufini A, Terrinoni A, Dinsdale D, Ranalli M, Paradisi A *et al.* Differential roles of p63 isoforms in epidermal development: selective genetic complementation in p63 null mice. *Cell Death Differ* 2006; **13**: 1037–1047.
27. Liefer KM, Koster MI, Wang XJ, Yang A, McKeon F, Roop DR. Down-regulation of p63 is required for epidermal UV-B-induced apoptosis. *Cancer Res* 2000; **60**: 4016–4020.
28. Ogawa E, Okuyama R, Ikawa S, Nagoshi H, Egawa T, Kurihara A *et al.* p51/p63 inhibits ultraviolet B-induced apoptosis via Akt activation. *Oncogene* 2008; **27**: 848–856.
29. Isoherranen K, Westermarck J, Kahari VM, Jansen C, Punnonen K. Differential regulation of the AP-1 family members by UV irradiation *in vitro* and *in vivo*. *Cell Signal* 1998; **10**: 191–195.
30. Takasawa R, Nakamura H, Mori T, Tanuma S. Differential apoptotic pathways in human keratinocyte HaCat cells exposed to UVB and UVC. *Apoptosis* 2005; **10**: 1121–1130.
31. Sayan BS, Sayan AE, Yang AL, Aqeilan RI, Candi E, Cohen GM *et al.* Cleavage of the transactivation-inhibitory domain of p63 by caspases enhances apoptosis. *Proc Natl Acad Sci USA* 2007; **104**: 10871–10876.
32. Liedtke C, Groger N, Manns MP, Trautwein C. The human caspase-8 promoter sustains basal activity through SP1 and ETS-like transcription factors and can be up-regulated by a p53-dependent mechanism. *J Biol Chem* 2003; **278**: 27593–27604.
33. Krueger A, Schmitz I, Baumann S, Krammer PH, Kirchhoff S. Cellular FLICE-inhibitory protein splice variants inhibit different steps of caspase-8 activation at the CD95 death-inducing signaling complex. *J Biol Chem* 2001; **276**: 20633–20640.
34. Li W, Zhang X, Olumi AF. MG-132 sensitizes TRAIL-resistant prostate cancer cells by activating c-Fos/c-Jun heterodimers and repressing c-FLIP(L). *Cancer Res* 2007; **67**: 2247–2255.
35. Fukazawa T, Fujiwara T, Uno F, Teraishi F, Kadowaki Y, Itoshima T *et al.* Accelerated degradation of cellular FLIP protein through the ubiquitin-proteasome pathway in p53-mediated apoptosis of human cancer cells. *Oncogene* 2001; **20**: 5225–5231.
36. Huang YP, Kim Y, Li Z, Fomenkov T, Fomenkov A, Ratovitski EA. AEC-associated p63 mutations lead to alternative splicing/protein stabilization of p63 and modulation of Notch signaling. *Cell Cycle* 2005; **4**: 1440–1447.
37. Melino G, Knight RA, Cesareni G. Degradation of p63 by Itch. *Cell Cycle* 2006; **5**: 1735–1739.
38. Kataoka T, Tschopp J. N-terminal fragment of c-FLIP(L) processed by caspase 8 specifically interacts with TRAF2 and induces activation of the NF-kappaB signaling pathway. *Mol Cell Biol* 2004; **24**: 2627–2636.
39. Wachter T, Sprick M, Hausmann D, Kerstan A, McPherson K, Stassi G *et al.* cFLIPL inhibits tumor necrosis factor-related apoptosis-inducing ligand-mediated NF-kappaB activation at the death-inducing signaling complex in human keratinocytes. *J Biol Chem* 2004; **279**: 52824–52834.
40. Koster MI, Dai D, Marinari B, Sano Y, Costanzo A, Karin M *et al.* p63 induces key target genes required for epidermal morphogenesis. *Proc Natl Acad Sci USA* 2007; **104**: 3255–3260.
41. Denecker G, Hoste E, Gilbert B, Hochepied T, Ovaere P, Lippens S *et al.* Caspase-14 protects against epidermal UVB photodamage and water loss. *Nat Cell Biol* 2007; **9**: 666–674.

Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)