the FEBS Journal

Regulation of ΔNp63α by tumor necrosis factor-α in epithelial homeostasis

Hae-ock Lee[1], Jung-Hwa Lee[1], Tae-You Kim[2] and Hyunsook Lee[1]

1 Department of Biological Sciences and Research Center for Functional Cellulomics, Seoul National University, Korea
2 Department of Internal Medicine, Cancer Research Institute, Seoul National University College of Medicine, Korea

Keywords
apoptosis; ΔNp63α; NF-κB; TNF-α; ubiquitin-dependent proteolysis

Correspondence
H. Lee, Department of Biological Sciences and Research Center for Functional Cellulomics, Seoul National University, San56-1 Shillim-dong, Gwanak-ku, Seoul 151-742, Korea
Fax: +82 2 886 4335
Tel: +82 2 880 9121
E-mail: HL212@snu.ac.kr

(Received 18 July 2007, revised 1 October 2007, accepted 26 October 2007)

doi:10.1111/j.1742-4658.2007.06168.x

A dominant negative form of p63, ΔNp63α, is critical for maintaining the proliferative potential of epidermal stem cells and progenitor cells. The expression of ΔNp63α also confers a selective advantage for cancer cell survival, underscoring the importance of ΔNp63α in both normal and neoplastic stratified epithelia. Regulation of ΔNp63α can be achieved at the transcriptional and post-translational levels, the latter being greatly influenced by external stimuli such as UV irradiation. In this study, we have found that tumor necrosis factor-α (TNF-α), a multifunctional cytokine that has been implicated in epidermal homeostasis during normal and pathophysiologic conditions, also triggers the degradation of ΔNp63α in immortalized keratinocytes and cervical cancer cells. Conversely, down-regulation of ΔNp63α sensitized cancer cells to TNF-α-induced apoptosis, suggesting a counteractive interaction between TNF-α and ΔNp63α in the regulation of epithelial cell death. The degradation of ΔNp63α by TNF-α was delayed when cells were treated with nuclear factor-κB inhibitors, whereas the induction of apoptosis by TNF-α was accompanied by the dramatic upregulation of the proapoptotic gene *Puma*. These observations further elucidate the relationship between TNF-α and ΔNp63α, two well-known mediators of epidermal homeostasis, and further suggest crosstalk between the two molecules in normal and pathophysiologic epidermis.

p63 (*TP63/AIS/KET/CUSP/p40/p51/p73L*), a recently identified *p53* homolog, is essential for epidermal development. Mice lacking a functional copy of this gene have deficiencies in all stratified epithelia and its derivatives [1,2]. *p63* knockout mice also have defects in limb and craniofacial development, probably due to a failure in maintaining the specialized epithelia of the apical ectodermal ridge and the branchial arches. *p63* mutations in humans also cause a number of malformation syndromes, manifesting as skin defects and limb and craniofacial abnormalities [3]. *p63* encodes two types of protein with opposing functions in transcription control by using two different promoters: the transcription-activating domain containing gene, *TAp63*, is transcribed from the 5′-promoter; and *ΔNp63*, which lacks the N-terminal transcription-activating domain, is transcribed from the intronic internal promoter. At the C-terminus, alternative splice variants are generated, making multiple isoforms in combination [4]. Among these isoforms, *ΔNp63α* is the predominant isoform expressed during embryogenesis and in adult epidermal tissues, and is responsible for epidermal proliferation [4,5]. The ΔNp63α protein lacks most of the N-terminal transcription-activating domain but does contain the C-terminal sterile α-motif and transcription inhibition domain. It functions as

Abbreviations
BHK, baby hamster kidney; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Iκ-Bα, inhibitor of kappa B; JNK, c-jun N-terminal kinase; NF-κB, nuclear factor-κB; si, small interfering; TA, transactivating; TNF-α, tumor necrosis factor-α; 7AAD, 7-amino-actinomycin D.

dominant negative towards p53 and TA (transactivating) isoforms of p63 and p73 (TAp63 and TAp73) [4–7]. In addition to its p53-dominant negative function, ΔNp63α is also able to activate epidermal specific genes [8].

In zebrafish, ΔNp63α was shown to be required for the proliferation of epidermal cells by inhibiting p53 activity during embryogenesis [5]. In mammals, the epidermis consists of basal stem cell layers and differentiated upper layers, which act as a barrier [9]. Remarkably, the expression of ΔNp63α is restricted to the proliferating stem cell compartment, and the levels of ΔNp63α rapidly decline upon differentiation of the isolated keratinocytes [2,10–12]. Together, these studies support the critical function of ΔNp63α in the proliferation and maintenance of epidermal stem cells and suggest that tight control of ΔNp63α levels is necessary. Both the transcriptional regulation and post-translational regulation of ΔNp63α have been investigated. For the transcriptional control of ΔNp63α, a long-range enhancer element and transcription factors involved – including activator protein-2 and p63 – have been identified [13,14]. At the protein level, it has been proposed that ΔNp63α may undergo ubiquitin-mediated proteasomal degradation or caspase-dependent degradation. Overexpression of p53 induces caspase-dependent cleavage of ΔNp63α [15] by an unknown mechanism. Ubiquitination, by comparison, occurs at steady state and increases following UV irradiation [16,17] or treatment with other genotoxic stimuli (our unpublished data). The ubiquitin–proteasome pathway allows for the rapid adjustment of protein levels and is therefore critical for the response to acute damage.

Epidermal homeostasis requires a balance between proliferative signals and differentiation/death signals. Given the critical function of ΔNp63α for epidermal stem cell proliferation, we were interested to know whether factors involved in maintaining epidermal homeostasis affect ΔNp63α expression. We were particularly interested in tumor necrosis factor-α (TNF-α), as this pleiotropic cytokine influences epidermal proliferation, differentiation and death during wound healing, chronic inflammation, and cancer [18–20]. TNF-α exerts its biological effects by binding to the receptors TNFRI and TNFRII (although epidermal keratinocytes predominantly express TNFRI) [21,22]. Ligand-bound TNFRI transmits downstream signals through procaspase 8, nuclear factor-κB (NF-κB) and c-jun N-terminal kinase (JNK) [23]. The imbalance of TNF-α signaling either towards the JNK or the NF-κB pathway has been shown to cause epidermal hyperplasia or hypoplasia, respectively [24,25]. In this study, we have investigated the relationship between TNF-α and ΔNp63α. We have found that TNF-α destabilizes ΔNp63α by both proteasomal and caspase-dependent degradation pathways. The degradation of ΔNp63α by TNF-α was attenuated by inhibition of NF-κB, suggesting that activation of NF-κB may be involved in the regulation of the degradation of ΔNp63α. Interestingly, knockdown of *ΔNp63α* expression in *ΔNp63α*-expressing cancer cells resulted in TNF-α-mediated apoptosis, with a concomitant induction of the pro-apoptotic gene *Puma*. These results indicate that *ΔNp63α* expression may provide a selective advantage for cell survival under inflammatory conditions. Taken together, ΔNp63α and TNF-α appear to provide mutual regulation, and may work together to maintain epidermal homeostasis.

Results

ΔNp63α turnover rate is determined by ubiquitin–proteasomal degradation

The levels of ΔNp63α are critical for controlling epithelial cell fate. Therefore, understanding the mechanism for ΔNp63α turnover is of great importance. Previous studies have shown that ΔNp63α is ubiquitinated and subject to proteasomal degradation [16,17,26]. We confirmed that ΔNp63α was ubiquitinated by immunoprecipitation and western blotting after transfection of overexpressing Myc-tagged ΔNp63α- and HA-ubiquitin-encoding plasmids into cells (Fig. 1A). The polyubiquitination of ΔNp63α suggested that the ubiquitin–proteasome pathway is one way to control the turnover of ΔNp63α. In order to test whether the half-life of ΔNp63α is regulated by ubiquitin-dependent proteasomal degradation, we utilized a CHO cell line (ts20) that harbors a temperature-sensitive E1 ubiquitin-activating enzyme [27]. In ts20 cells, the thermolabile ubiquitin-activating enzyme E1 is irreversibly inactivated at the nonpermissive temperature of 40 °C, leading to the disruption of ubiquitination. The half-life of ΔNp63α was less than 2 h at the permissive temperature (34 °C) in ts20 cells. In contrast, a temperature shift to the nonpermissive temperature stabilized ΔNp63α, and significant levels of ΔNp63α persisted until 4 h later (Fig. 1B). These results indicate that ΔNp63α is degraded by polyubiquitination-mediated proteolysis.

TNF-α induces degradation of ΔNp63α

During epidermal stratification, the basal stem cells in the basal layer just above the underlying dermis give rise to the differentiated upper layers, finally forming

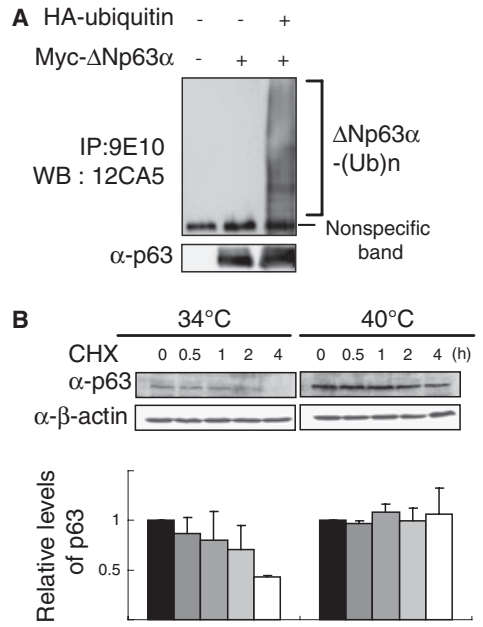

Fig. 1. The ubiquitin–proteasome pathway regulates the half-life of ΔNp63α. (A) BHK21 cells transfected with MycΔNp63α- and HA-ubiquitin-encoding plasmids were subjected to immunoprecipitation (IP) with the α-Myc monoclonal antibody, 9E10 and western blotting with the α-HA monoclonal antibody 12CA5. Immunoprecipitated ΔNp63α was detected by the 4A4 p63 antibody. (B) ts20 cells with a thermolabile E1 enzyme were transfected with ΔNp63α. After 48 h, the cells were incubated at 34 °C or 40 °C for 18 h, and then treated with 20 ng·mL^{-1} cycloheximide (CHX) for the indicated times. Blots were reprobed with an α-β-actin antibody as loading control. The bar graph represents average values of two independent experiments.

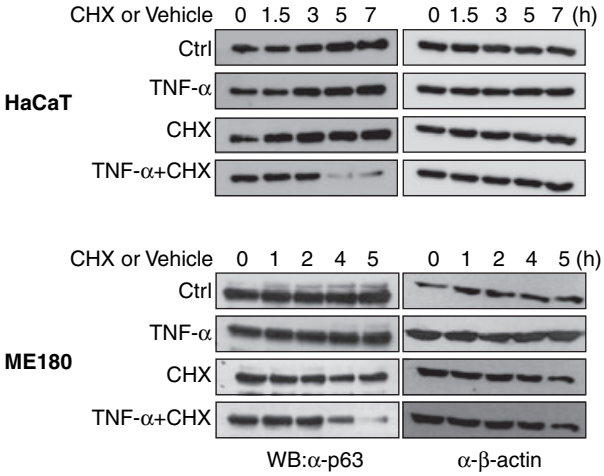

Fig. 2. TNF-α and cycloheximide treatment induce ΔNp63α degradation. HaCaT cells (upper panel) and ME180 cells (lower panel) expressing endogenous ΔNp63α were treated with TNF-α (10 ng·mL^{-1} or 20 ng·mL^{-1}, respectively) for 18 h, and then the cells were treated with or without cycloheximide (20 ng·mL^{-1}, CHX) for the indicated time points (in hours) before lysis. Whole cell lysates were analyzed by western blot analysis using the 4A4 p63 antibody. The blots were reprobed with an antibody against β-actin as loading control.

the terminally differentiated stratum corneum at the outermost layer [28]. The expression of ΔNp63α is restricted to proliferative cells in the basal layer, and the rapid and complete disappearance of ΔNp63α in the differentiated stratified epithelia suggests that both transcriptional repression and degradation of ΔNp63α might occur. Previously, we and others have reported that UVB irradiation – a well-known external stimulus triggering keratinocyte differentiation, death, and premature aging of the skin – stimulates ΔNp63α degradation in a proteasome-dependent manner [16,17]. This suggests that factors influencing epidermal homeostasis may also modulate the level of ΔNp63α. Although the regulation of ΔNp63α by external UV irradiation has been well characterized, the cellular factors regulating epidermal homeostasis and ΔNp63α stability have not been described.

We were interested in TNF-α in particular, as this pleiotrophic cytokine is known to induce keratinocyte differentiation [29], in addition to cell death, and its downstream signaling molecule, NF-κB, is implicated in epidermal homeostasis [24,25]. Therefore, we investigated whether TNF-α affects the stability of ΔNp63α. In immortalized HaCaT keratinocytes and the ME180 cervical cancer cell line, ΔNp63α was highly expressed (Fig. 2, Ctrl). Treatment of these cells with TNF-α alone did not alter the level of ΔNp63α. However, combined treatment with TNF-α and cycloheximide (to avoid *de novo* synthesis) resulted in the degradation of ΔNp63α. The mRNA level of *ΔNp63α* was not significantly altered by TNF-α treatment (see Fig. 6A below). These results demonstrate that TNF-α signaling induces degradation of ΔNp63α in both immortalized keratinocytes and transformed cell lines.

We next tested whether TNF-α-induced ΔNp63α degradation was dependent on proteasome or caspase. We examined these pathways in particular because they have both been implicated in regulating ΔNp63α stability [15–17]. TNF-α-mediated degradation of ΔNp63α was blocked by the addition of the proteasome inhibitor MG-132 to the culture (Fig. 3A), suggesting a role for the ubiquitin–proteasome pathway. In addition, the pan-caspase inhibitor Z-VAD-fmk also prevented TNF-α-induced ΔNp63α degradation (Fig. 3A), suggesting that caspases regulate ΔNp63α stability as well. Collectively, TNF-α induces ΔNp63α degradation through polyubiquitination and caspase-dependent pathways.

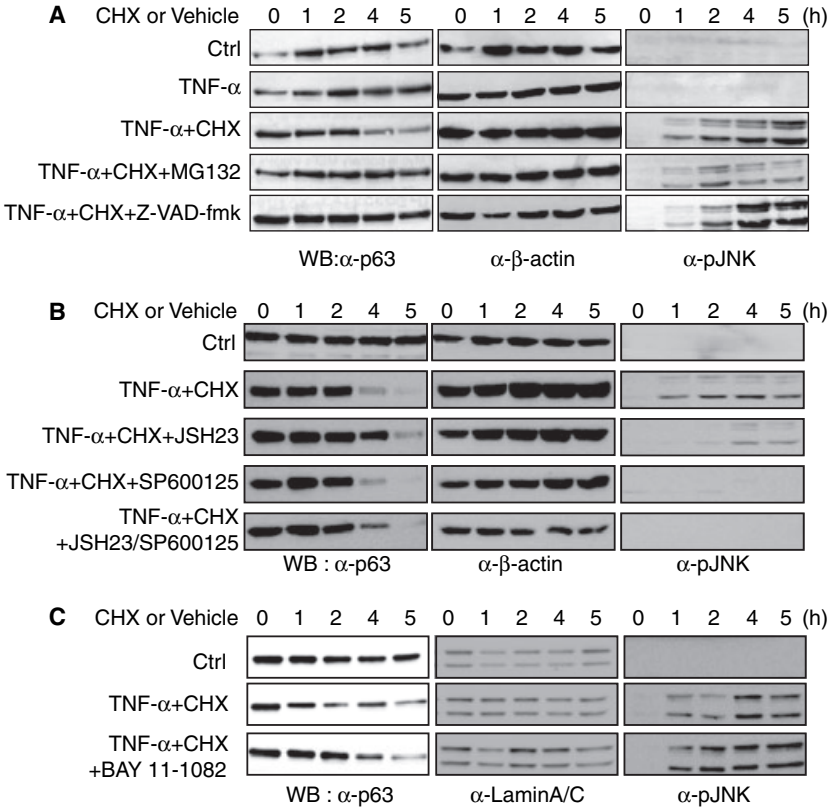

Fig. 3. Both ubiquitin-dependent and caspase-dependent proteolysis regulate TNF-α-mediated ΔNp63α degradation, and may require activation of the NF-κB pathway. (A) ME180 cells were treated with TNF-α (10 ng·mL^{-1}) for 18 h with or without the various reagents indicated, to assess which proteolytic pathway was involved in ΔNp63α degradation. Cycloheximide (20 ng·mL^{-1}) was added to the cultures along with MG132 (10 μM) or Z-VAD-fmk (10 μM) as indicated. At the indicated time points, whole cell lysates were analyzed by western blot analysis using antibodies specific for p63 and β-actin. The same blot was reprobed with anti-phospho-JNK (α-pJNK) to assess the activation of JNK upon TNF-α treatment. (B) ME180 cells were treated with TNF-α (10 ng·mL^{-1}) in the presence of the NF-κB inhibitor JSH23 (20 μM) or the JNK inhibitor SP600126 (30 μM). Eighteen hours after TNF-α treatment, cycloheximide was added at the indicated time points before lysis, and whole cell lysates were analyzed by western blot with 4A4 (α-p63). Reprobing the blot with α-phospho-JNK antibody shows the autophosphorylation state of JNK. The same blot reprobed with α-actin shows that similar amounts of total cell lysates were employed for western blot analysis. (C) Experiments were performed as in (B) except for the use of a different NF-κB inhibitor, BAY 11-1082 (10 μM). The same blot was reprobed for western blot analysis with anti-laminA/C as loading control.

NF-κB inhibitors attenuate the degradation of ΔNP63α

TNF-α exerts its biological effects by binding to its receptors, TNFRI and TNFRII [23]. Ligand-bound TNFRI can recruit the TRADD–TRAF–RIP complex and activate NF-κB or the TRADD–FADD–procaspase 8 complex and activate the apoptotic signaling cascade. TNFRI can also activate other signaling cascades, including the JNK pathway. To determine whether NF-κB or JNK signaling is involved in the degradation of ΔNp63α, we utilized inhibitors of these molecules. JSH23 is known to block the nuclear translocation of p65, a subunit of NF-κB [30], and SP600125 is an ATP competitive inhibitor for JNK1, JNK2 and JNK3 [31]. As shown in Fig. 3B, pretreatment of ME180 cells with JSH23 resulted in a delay of ΔNp63α degradation after TNF-α treatment. In contrast, the JNK inhibitor SP600125 had no effect, despite its ability to block JNK autophosphorylation (Fig. 3B, right panel). The involvement of the NF-κB pathway in the degradation of ΔNp63α was further supported by use of another NF-κB inhibitor, BAY 11-1082 (Fig. 3C). Together, these data suggest that TNF-α may trigger ΔNp63α degradation, and that activation of the NF-κB pathway may be involved. This is consistent with previous findings demonstrating a role for NF-κB in antagonizing keratinocyte proliferation and regulating epithelial cell differentiation [24,25].

In our experiments, TNF-α alone was insufficient to induce the degradation of ΔNp63α, but cotreatment with cycloheximide was required. As we found that

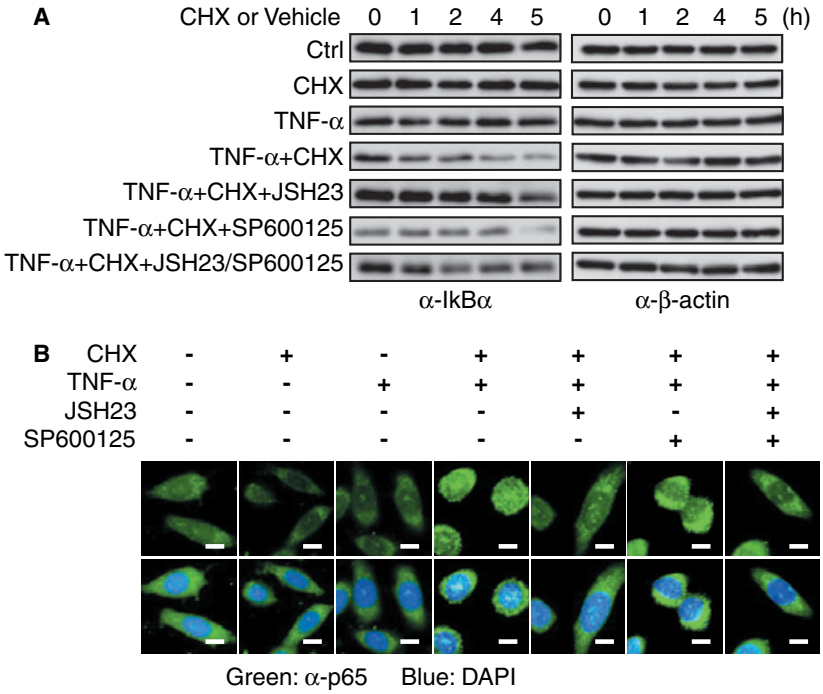

Fig. 4. TNF-α and cycloheximide (CHX) cooperate to induce IκBα degradation and nuclear translocation of NF-κB.(A) ME180 cells were treated as in Fig. 3B, and whole cell lysates were analyzed by western blot with antibodies to IκBα. Control groups were treated with vehicles only. IκBα degradation occurred after combined treatment with TNF-α and CHX, and was blocked by the NF-κB inhibitor JSH23. The same blot was reprobed with antibodies to β-actin as loading control. (B) ME180 cells grown on a coverglass were treated as in (A) and fixed 5 h after CHX addition. Cells were then immunostained with antibody to p65. 4′-6-diamidino-2-phenylindole (DAPI) staining is visualized in blue and perinuclear translocation of p65 is shown in green only after combined treatment of TNF-α and CHX. White scale bars represent 10 μm.

NF-κB activation is involved in ΔNp63α degradation, we suspected that cycloheximide may be required for the efficient degradation of inhibitor of kappa B (IκBα) and hence activation of NF-κB [32]. Therefore, we examined the levels of IκBα as well as p65 translocation into the nucleus. TNF-α or cycloheximide treatment alone was insufficient to induce IκBα degradation in ME180 cells, but combined treatment with TNF-α and cycloheximide induced the degradation of IκBα (Fig. 4A). The nuclear translocation of p65, a subunit of NF-κB, also required both TNF-α and cycloheximide (Fig. 4B). Treatment of the NF-κB inhibitor JSH23 inhibited both IκBα degradation and p65 nuclear translocation. These data collectively suggest that TNF-α-induced ΔNp63α degradation requires IκBα degradation, and further suggest the involvement of the NF-κB pathway in the degradation of ΔNp63α.

The level of ΔNp63α determines cell fate after TNF-α treatment

During TNF-α treatment, a small percentage of ME180 cells undergo apoptosis (Fig. 5A). This indicates that ME180 cells are highly resistant to TNF-α-mediated apoptosis, despite their high expression levels of TNFRI (Fig. 5C). TNFRII expression was under the detection limit (data not shown). As ΔNp63α is overexpressed in ME180 cells, ΔNp63α may confer resistance to TNF-α-mediated apoptosis, as is the case with genotoxic stimuli [17,33]. To test this idea, we transfected cells with small interfering (si)RNA against p63, prior to TNF-α treatment. As ME180 cells express very low, if any, *TAp63* (data not shown), *p63* siRNA specifically interferes with *ΔNp63α* expression. We used these cells to determine how *ΔNp63α* expression levels affect cell survival. Cells undergoing apoptosis were stained with annexin V and 7-amino-actinomycin D (7AAD) vital dye, and measured by flow cytometry. We found that cells expressing a reduced amount of ΔNp63α were ~2.5 times more susceptible to TNF-α-induced cell death (Fig. 5A, 50% versus 20%). The levels of TNFRI were downregulated by TNF-α treatment, but silencing *p63* expression did not affect the surface expression of TNFRI (Fig. 5C). These data suggest that reducing *ΔNp63α* expression makes ME180 cancer cells susceptible to TNF-α-mediated cell death. Therefore, the overexpression of *ΔNp63α* may divert the cellular response after TNF-α treatment from cell death.

Next, we attempted to identify the apoptotic factor(s) that were regulated by ΔNp63α in response to TNF-α. TNF-α is known to trigger apoptosis by diverse mechanisms, including caspase activation and the mitochondrial death pathway [23]. ΔNp63α can antagonize p53 or TAp63/TAp73, and the silencing of ΔNp63α allows for the induction of the proapoptotic genes *Bax*, *Noxa*, and *Puma* [33]. Therefore, we employed real time RT-PCR to measure the levels of

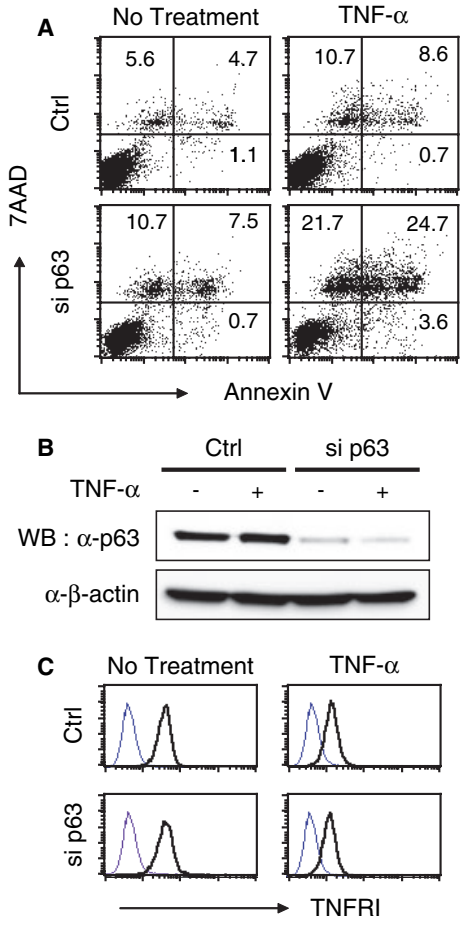

Fig. 5. Knockdown expression of *ΔNp63α* sensitizes ME180 cells to TNF-α-induced cell death. ME180 cells were transfected with *p63* siRNA duplex for 48 h and then treated with TNF-α for 24 h. (A) Cells were stained with annexin V–fluorescein isothiocyanate and 7AAD vital dye, and analyzed with a flow cytometer. The numbers indicate the percentages of apoptotic populations: complete death (upper left quadrant, annexin $V^-/7AAD^+$); early apoptotic (lower right quadrant, annexin $V^+/7AAD^-$); and late apoptotic (upper right quadrant, annexin $V^+/7AAD^+$). (B) To assess the level of silencing of *ΔNp63α* after transfection of duplex siRNAs (sip63), cells were lysed and subjected to western blotting with 4A4 and β-actin antibodies. As a control (Ctrl), siRNA for mouse p63 was employed. (C) To assess TNFRI expression, cells were stained with biotinylated α-TNFRI (thick line) or an α-trinitrophenyl control (thin line) antibody, and then treated with streptavidin–phycoerythrin. Samples were analyzed by flow cytometry. The graph represents three independent experiments with similar results.

proapoptotic gene expression. TNF-α treatment or *ΔNp63α* silencing alone did not significantly induce these proapoptotic genes (Fig. 6A). Notably, the proapoptotic gene *Puma* was upregulated more than 10-fold in cells transfected with *p63* siRNA and treated with TNF-α. In comparison, there were only slight changes in *Bax*, *Noxa* and the cell cycle inhibitor *p21* under similar conditions. The level of Puma was also elevated in TNF-α-treated cells only after silencing of *p63* expression (Fig. 6B). Furthermore, we found that the *Puma* promoter containing p53-responsive elements can be induced by all p53 members, especially TAp63γ and TAp73β (Fig. 6C). Transcriptional activation of *Puma* was susceptible to repression by the coexpression of ΔNp63α. Taken together, these data suggest that ΔNp63α antagonizes TNF-α-mediated epithelial cell apoptosis by inhibiting the expression of a proapoptotic gene, *Puma*.

Discussion

The present study illustrates the interaction between the epidermal transcription repressor ΔNp63α and the inflammatory cytokine TNF-α. TNF-α induced the degradation of ΔNp63α in both ME180 cervical cancer cells and HaCaT immortalized keratinocytes (in the presence of cycloheximide), and this degradation was delayed by inhibition of the NF-κB pathway. It is noteworthy that ΔNp63α expression is restricted to epidermal stem cells, progenitor cells, and cancer cells of epidermal origin. The level of ΔNp63α has been shown to be a critical determinant for cellular proliferation, differentiation and cell death in keratinocytes and cancer cells [17,33,34]. Our results suggest that TNF-α may regulate the homeostasis of the epidermal compartment through its modulation of ΔNp63α. Conversely, a reduction in *ΔNp63α* expression sensitized cells to undergo TNF-α-induced apoptosis in cancer cells. These observations imply that when treating epithelial cancer cells with TNF-α, the expression level of ΔNp63α should be taken into consideration.

The involvement of TNF-α signaling in epidermal homeostasis has been previously demonstrated. TNF-α has been shown in many cell types to promote survival through NF-κB or cell death through caspase or JNK-mediated apoptotic signals [35,36]. However, in the skin, JNK drives proliferation and neoplastic outgrowth, and NF-κB induces growth arrest and differentiation [24,37,38]. NF-κB is localized in the cytoplasm of basal cells in the normal epidermis, but translocates into the nucleus of suprabasal cells [39]. The nuclear translocation or activation of NF-κB coincides with the disappearance of ΔNp63α upon keratinocyte differentiation [12], which suggests the involvement of NF-κB during the switch of epidermal cells from a proliferative to a differentiated state. Indeed, NF-κB/RelA(p65)-deficient skin derived from $rela^{-/-}$ mice displays hyperplasia [24,25]. This hyperplasia was accompanied by an increase in JNK

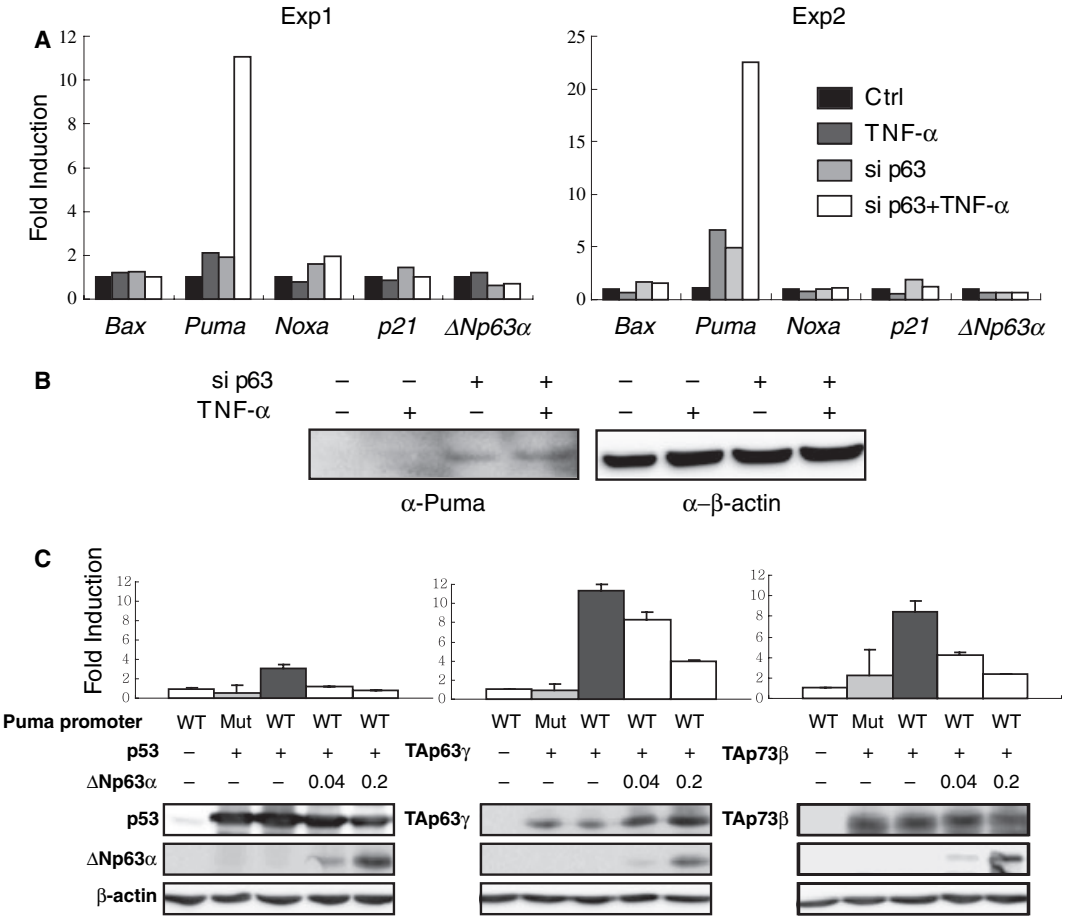

Fig. 6. Knockdown of *ΔNp63α* expression cooperates with TNF-α treatment and induces expression of the proapoptotic gene *Puma*. (A) RNA was isolated from ME180 cells prepared as in Fig. 5, and real-time RT-PCR was performed for candidate proapoptotic genes. Values shown on the *y*-axis are relative to GAPDH expression. Results from two independent experiments are shown. (B) ME180 cells were prepared as in Fig. 5, and protein lysates were obtained 18 h after TNF-α treatment. Western blotting shows the induction of Puma protein after knockdown expression of *p63*. Treatment with TNF-α in cells transfected with siRNA for *p63* further induces Puma. (C) BHK21 cells were transfected with *Puma Frag1* (WT) or *Puma Frag2* (Mut) luciferase reporter gene constructs to assess the inhibitory effects of ΔNp63α on *p53-*, *TAp63γ-* or *TAp73β*-mediated transcription activation. *pRL–TK–luc* was also transfected as a control plasmid. The *y*-axis shows the fold induction of firefly luciferase activity normalized to *Renilla* luciferase activity. Values are averages of duplicate transfections and represent two independent experiments. The protein levels of transfected plasmids determined by western analysis with the antibodies indicated are shown beneath.

activation that was abolished in cells that also lacked TNF-α or TNFRI [24,40]. Nonetheless, TNF-α or TNFRI deficiency does not cause epidermal defects during embryonic development; therefore, TNF-α is likely to regulate epidermal homeostasis postnatally and together with additional modulators.

In this study, we found that TNF-α can induce the degradation of ΔNp63α. This degradation seems to require activation of NF-κB, although this needs to be confirmed in NK-κB-deficient cells. At present, it remains unclear how NF-κB is involved in the degradation of ΔNp63α. In our experimental setting, the response of ΔNP63α proteolysis to TNF-α was not instant, as in many cases, but required much longer incubation times. Therefore, it is possible that *de novo* synthesis of factors involved in ΔNp63α degradation is required: upon TNF-α treatment, NF-κB may activate gene(s) responsible for degradation of ΔNp63α. Up to now, there have been no known p63-specific E3 ligases that are activated by NF-κB in the epidermis. Another mediator of TNF-α signaling, JNK, has been shown to phosphorylate and activate Itch [41] and 14-3-3σ [42]. These proteins can affect ΔNp63α stability [26,43]. However, we found that the JNK inhibitor failed to block TNF-α-induced ΔNp63α degradation, so it is unlikely that the JNK pathway is

directly involved. Therefore, the identification of downstream targets of NF-κB is likely to provide a key to understanding how ΔNp63α is degraded by TNF-α.

ME180 cervical cancer cells rarely undergo apoptosis after a single TNF-α treatment (Fig. 5A), despite their high expression level of TNFRI (Fig. 5C). Ligand-bound TNFRI recruits the TRADD–FADD–procaspase 8 complex, which results in the autocatalytic cleavage of caspase 8 [44]. Caspase 8, now in its active form, can cleave Bid, which results in the activation of the intrinsic mitochondrial death pathway [44]. Simultaneously, ligand-bound TNFRI may also recruit the TRADD–RIP1–TRAF2 complex, which can activate the NF-κB and JNK pathways [44]. JNK can process Bid, causing the release of Smac/DIABLO, which disrupts TRAF2–cIAP1/2 and allows for caspase 8 activation [45,46]. The activation of the NF-κB pathway usually promotes cell survival rather than cell death [23]. However, there are a few examples of NF-κB-dependent cell death during thymic development and following genotoxic agent treatment in cancer cells [47,48]. Despite the triggering of these proapoptotic signals, TNF-α treatment rarely results in apoptosis, probably due to its concurrent induction of prosurvival genes [23], so blocking of the synthesis of RNA or protein was required for cells to undergo apoptosis after TNF-α treatment [44]. In our study, knockdown expression of *ΔNp63α* resulted in the increase in *Puma* transcripts and sensitized cells to TNF-α-induced apoptosis. As ΔNp63α normally blocks the activation of p53 target genes, silencing *ΔNp63α* would cause the stimulation of many p53 targets. As ME180 cells are infected with human papilloma virus and p53 destabilized by human papilloma virus E6 protein [49], the p53 target gene induction might have been triggered by other p53 members. We and others [17,33] have found that TAp73 is a potent inducer of *Puma*, and thus may be a strong candidate. However, the involvement of TAp73 in TNF-α-mediated apoptosis was not directly assessed. Therefore, future investigation is warranted to determine whether TAp73 or an alternative member of the p63 gene family is involved in inducing *Puma* in response to TNF-α. Nonetheless, silencing *ΔNp63α* alone was not sufficient to trigger the activation of these genes, but treatment with TNF-α was required. These data suggest crosstalk between the TNF-α-mediated apoptotic pathway and the ΔNp63α-mediated antiapoptotic pathway. We speculate that the merging point of these two pathways is proapoptotic Puma.

We have demonstrated a functional interaction between TNF-α and ΔNp63α in this study. Although earlier studies have shown a correlation between these two signaling molecules, a direct relationship has never been demonstrated. We show here that TNF-α causes the degradation of ΔNp63α. Collectively, our results suggest that the balance between TNF-α-mediated signaling and ΔNp63α level regulate the homeostasis of epidermal cells.

Experimental procedures

Cell lines

BHK (baby hamster kidney) cells (ATCC, Manassas, VA) and ts20 (a gift from A. Ciechanover, Technion-Israel Institute of Technology, Israel) cells were cultured in DMEM supplemented with 10% v/v fetal bovine serum, 100 $U \cdot mL^{-1}$ penicillin and 100 $\mu g \cdot mL^{-1}$ streptomycin (Hyclone, Logan, UT). ME180 cervical cancer cells were cultured in RPMI-1640 with the same supplements. The HaCaT immortalized human keratinocyte line containing a p53 mutation (a gift from I. Kim, Cell & Matrix Research Institute, Kyungpook National University Medical School, Korea) was cultured in DMEM-F12 supplemented with 10% v/v fetal bovine serum, 100 $U \cdot mL^{-1}$ penicillin, 100 $\mu g \cdot mL^{-1}$ streptomycin, and 10 $\mu g \cdot mL^{-1}$ hydrocortisone (Sigma, St Louis, MO). All cells were maintained in 5% CO_2 at 37 °C, except for the ts20 cells, which were maintained at 34 °C.

Constructs and reagents

The p53, p63 and p73 expression plasmids and the antibodies to Myc (clone 9E10), p63 (clone 4A4), and laminA/C (clone IE4) were gifts from F. McKeon (Harvard Medical School, MA). *Puma Frag1–Luc*(WT) and *Frag2–Luc*(Mut) constructs [50], which contain two putative p53-binding sites or neither, respectively, were gifts from B. Vogelstein (Johns Hopkins University, MD). Monoclonal antibodies specific for β-actin (Sigma), phospho-JNK (Thr183/Tyr185; Cell Signaling, Dancers, MA), IkBα (Santa Cruz, Santa Cruz, CA), p65 (Santa Cruz) and Puma (Abcam, Cambridge, UK) were obtained commercially. Human recombinant TNF-α and the pan-caspase inhibitor Z-VAD-fmk were purchased from R&D Systems (Minneapolis, MN), and cycloheximide was obtained from Sigma. The protease inhibitor MG-132 and the NF-κB inhibitors JSH23 and BAY 11-1082 were obtained from Calbiochem (San Diego, CA). The JNK inhibitor SP600125 was purchased from BIOMOL (Exeter, UK).

Chemical treatments

For the half-life test, cells (5×10^5) were plated in 60 mm dishes for 24 h before the addition of TNF-α (10 $ng \cdot mL^{-1}$

for ME180 cells or 20 ng·mL^{-1} for HaCaT cells). TNF-α was added for 18 h, and then cyclohexamide (20 ng·mL^{-1}) was added for the indicated time in the presence of TNF-α. MG132 (10 μM) or Z-VAD-fmk (10 μM) was added along with cycloheximide before harvesting. To determine the signaling pathway required for TNF-α-dependent ΔNp63α degradation, cells were treated with 20 μM JSH23 or 10 μM BAY 11-1082 to inhibit NF-κB, or 30 μM SP600125 to inhibit JNK, for 1 h prior to the addition of TNF-α. Control groups for each chemical treatment received vehicle alone.

Immunoprecipitation

Cells were lysed in NETN buffer (150 mM NaCl, 20 mM Tris/Cl, pH 8.0, 0.5% v/v Nonidet P-40, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 1 μg·mL^{-1} aprotinin, 1 μg·mL^{-1} pepstatin A, 2 μg·mL^{-1} Na_3VO_4, 1 μg·mL^{-1} leupeptin, 10 mM *N*-ethylmaleimide). Lysates were immunoprecipitated at 4 °C overnight with the 9E10 α-Myc mAb. After incubation with the antibody, 30 μL of protein G (Upstate, Charlottesville, VA) was added to the reaction mixture, and mixed for 4 h at 4 °C. Immunoprecipitates were collected by centrifugation at 100 ***g*** for 5 min, and this was followed by three washes with NETN buffer. Following the final wash, samples were resuspended in 2 × SDS sample buffer, subjected to SDS/PAGE, and transferred to a nitrocellulose membrane. The immunoprecipitated proteins were then detected by a standard western blotting procedure.

Immunofluorescence

Cells on the coverglass were fixed in 4% paraformaldehyde (Sigma) for 15 min and permeabilized in 0.5% Triton X-100 (Sigma) for 15 min. Then, the cells were incubated with blocking solution (10% goat serum in NaCl/P_i containing 0.1% Triton X-100) for 30 min and rabbit anti-p65 O/N at 4 °C. After three washes in NaCl/P_i/0.1% Triton X-100, Alexa 488-conjugated goat anti-rabbit IgG was added for 2 h. Cells were washed 10 times with NaCl/P_i/0.1% Triton X-100 and mounted with Vectashield mouting medium containing DAPI (Vector Laboratory, Burlingame, CA). All incubations were performed at room temperature unless indicated otherwise. Images were acquired using a Zeiss Axiovert inverted microscope with a 40× oil lens (Carl Zeiss, Göttingen, Germany).

p63 gene silencing

p63 gene silencing was achieved by the transfection of siRNA duplex into ME180 cells. The sense and antisense siRNA (target sequences: 5′-CCACTGAACTGAAGAA ACT-3′; Samchullypham, Seoul, Korea) were annealed according to the manufacturer's recommendations. As an off-target control, siRNA generated against mouse *p63* gene (5′-GAGCACCCAGACAAGCGAG-3′) was used. ME180 cells (5×10^5) were plated on 60 mm dishes 24 h before transfection. The transfection of siRNA duplex was carried out using oligofectamine reagent (Invitrogen, Carlsbad, CA). The cells were incubated in the presence of TNF-α (10 ng·mL^{-1}) 48 h later. After 24 h in TNF-α, various assays were performed.

Apoptosis analysis and flow cytometry

Cells were stained with fluorescein-conjugated annexin V (Roche, Mannheim, Germany) and 7AAD (BD Pharmingen, San Diego, CA) according to the manufacturer's instructions, and analyzed with a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ) using CELLQUEST software. The expression of TNFRI was also measured by flow cytometry by treating cells with a biotinylated antibody to TNFRI and then labeling with streptavidin–phycoerythrin (BD Pharmingen).

Real-time PCR analysis

Total cellular RNA was extracted using TRIZOL (Invitrogen). cDNA was generated using SuperScript II reverse transcriptase (Invitrogen). The relative levels of *Bax*, *p21*, *Puma*, *Noxa* and *ΔNp63α* mRNAs were determined by real-time quantitative PCR with SYBR (Applied Biosystems, Foster City, CA) and normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) products. Primer sequences were as follows: *Puma* forward, 5′-ACGACCTCAACGC ACAGTACGAG-3′; *Puma* reverse, 5′-AGGAGTCCGCA TCTCCGTCAGTG-3′; *Noxa* forward, 5′-GAGATGCCTG GGAAGAAGG-3′; *Noxa* reverse, 5′-ACGTGCACCTCCT GAGAAAA-3′; *p21* forward, 5′-AAGACCATGTGGAC CTGT-3′; *p21* reverse, 5′-GGTAGAAATCTGTCATGC TG-3′; *Bax* forward, 5′-TGACATGTTTTCTGACGGCAA C-3′; *Bax* reverse, 5′-GGAGGCTTGAGGAGTCTCACC-3′; *ΔNp63α* forward, 5′-GGAAAACAATGCCCAGACTC-3′; *ΔNp63α* reverse, 5′-GTGGAATACGTCCAGGTGGC-3′; *GAPDH* forward, 5′-GAAGGTGAAGGTCGGAGTC-3′; *GAPDH* reverse, 5′-GAAGATGGTGATGGGATTTC-3′.

Luciferase reporter assays

BHK21 cells were transfected with 100 ng of the luciferase reporter plasmids *Puma Frag1–Luc*(WT) or *Frag2–Luc*(Mut) and with 1 μg of *Myc–p53*, *Myc–TAp63γ* or *TAp73β*. Some cells were also transfected with 0.04 or 0.2 μg of the *Myc–ΔNp63α* construct. The control vector *pRL–TK–luc* (100 ng) was also transfected into all cells. The amount of DNA for all transfections was equalized with the *pcDNA3–Myc* vector. Cells were lysed 48 h later, and luciferase activity was measured with the Luciferase Assay System (Promega, Madison, WI) and the Micro-

Lumat Plus LB 96 V luminometer (Berthold Technologies, Oak Ridge, TN). The protein levels of transfected plasmids were examined by western blotting of the remaining lysates.

Acknowledgements

We are grateful to Drs A. Ciechanover (Technion-Israel Institute of Technology, Israel), F. McKeon (Harvard Medical School, Boston), B. Vogelstein (Johns Hopkins University Medical School, Baltimore), and I. Kim (Kyungpook University, Korea) for ts20 cells, p63 antibodies, Puma reporter constructs, and HaCaT immortalized keratinocytes, respectively. This work was supported by grants from Biodiscovery Program (M10601000130-06N0100), 21C Frontier Functional Human Genome Project (M106KB010018-07K0201), National Cancer Center (0320250 and 0620070) and Basic Science program (R01-2006-000-11114-0) from the Ministry of Science and Technology in Korea.

References

1 Mills AA, Zheng B, Wang XJ, Vogel H, Roop DR & Bradley A (1999) p63 is a p53 homologue required for limb and epidermal morphogenesis. *Nature* **398**, 708–713.

2 Yang A, Schweitzer R, Sun D, Kaghad M, Walker N, Bronson RT, 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.

3 van Bokhoven H & McKeon F (2002) Mutations in the p53 homolog p63: allele–specific developmental syndromes in humans. *Trends Mol Med* **8**, 133–139.

4 Yang A, Kaghad M, Wang Y, Gillett E, Fleming MD, Dotsch V, Andrews NC, Caput D & 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.

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

6 Chi SW, Ayed A & Arrowsmith CH (1999) Solution structure of a conserved C-terminal domain of p73 with structural homology to the SAM domain. *EMBO J* **18**, 4438–4445.

7 Serber Z, Lai HC, Yang A, Ou HD, Sigal MS, Kelly AE, Darimont BD, Duijf PH, Van Bokhoven H, McKeon F *et al.* (2002) A C-terminal inhibitory domain controls the activity of p63 by an intramolecular mechanism. *Mol Cell Biol* **22**, 8601–8611.

8 Koster MI, Dai D, Marinari B, Sano Y, Costanzo A, Karin M & Roop DR (2007) p63 induces key target genes required for epidermal morphogenesis. *Proc Natl Acad Sci USA* **104**, 3255–3260.

9 Nemes Z & Steinert PM (1999) Bricks and mortar of the epidermal barrier. *Exp Mol Med* **31**, 5–19.

10 Bamberger C, Hafner A, Schmale H & Werner S (2005) Expression of different p63 variants in healing skin wounds suggests a role of p63 in reepithelialization and muscle repair. *Wound Repair Regen* **13**, 41–50.

11 De Laurenzi V, Rossi A, Terrinoni A, Barcaroli D, Levrero M, Costanzo A, Knight RA, Guerrieri P & Melino G (2000) p63 and p73 transactivate differentiation gene promoters in human keratinocytes. *Biochem Biophys Res Commun* **273**, 342–346.

12 Pellegrini G, Dellambra E, Golisano O, Martinelli E, Fantozzi I, Bondanza S, Ponzin D, McKeon F & De Luca M (2001) p63 identifies keratinocyte stem cells. *Proc Natl Acad Sci USA* **98**, 3156–3161.

13 Harmes DC, Bresnick E, Lubin EA, Watson JK, Heim KE, Curtin JC, Suskind AM, Lamb J & DiRenzo J (2003) Positive and negative regulation of deltaN-p63 promoter activity by p53 and deltaN-p63-alpha contributes to differential regulation of p53 target genes. *Oncogene* **22**, 7607–7616.

14 Antonini D, Rossi B, Han R, Minichiello A, Di Palma T, Corrado M, Banfi S, Zannini M, Brissette JL & Missero C (2006) An autoregulatory loop directs the tissue-specific expression of p63 through a long-range evolutionarily conserved enhancer. *Mol Cell Biol* **26**, 3308–3318.

15 Ratovitski EA, Patturajan M, Hibi K, Trink B, Yamaguchi K & Sidransky D (2001) p53 associates with and targets Delta Np63 into a protein degradation pathway. *Proc Natl Acad Sci USA* **98**, 1817–1822.

16 Westfall MD, Joyner AS, Barbieri CE, Livingstone M & Pietenpol JA (2005) Ultraviolet radiation induces phosphorylation and ubiquitin-mediated degradation of DeltaNp63alpha. *Cell Cycle* **4**, 710–716.

17 Lee HO, Lee JH, Choi E, Seol JY, Yun Y & Lee H (2006) A dominant negative form of p63 inhibits apoptosis in a p53-independent manner. *Biochem Biophys Res Commun* **344**, 166–172.

18 Tomic-Canic M, Komine M, Freedberg IM & Blumenberg M (1998) Epidermal signal transduction and transcription factor activation in activated keratinocytes. *J Dermatol Sci* **17**, 167–181.

19 Doger FK, Dikicioglu E, Ergin F, Unal E, Sendur N & Uslu M (2007) Nature of cell kinetics in psoriatic epidermis. *J Cutan Pathol* **34**, 257–263.

20 Lind MH, Rozell B, Wallin RP, van Hogerlinden M, Ljunggren HG, Toftgard R & Sur I (2004) Tumor necrosis factor receptor 1-mediated signaling is required for skin cancer development induced by NF-kappaB inhibition. *Proc Natl Acad Sci USA* **101**, 4972–4977.

21 Trefzer U, Brockhaus M, Lotscher H, Parlow F, Budnik A, Grewe M, Christoph H, Kapp A, Schopf E,

Luger TA *et al.* (1993) The 55-kD tumor necrosis factor receptor on human keratinocytes is regulated by tumor necrosis factor-alpha and by ultraviolet B radiation. *J Clin Invest* **92**, 462–470.
22 Kristensen M, Chu CQ, Eedy DJ, Feldmann M, Brennan FM & Breathnach SM (1993) Localization of tumour necrosis factor alpha (TNF-alpha) and its receptors in normal and psoriatic skin: epidermal cells express the 55-kD but not the 75-kD TNF receptor. *Clin Exp Immunol* **94**, 354–362.
23 Papa S, Bubici C, Zazzeroni F, Pham CG, Kuntzen C, Knabb JR, Dean K & Franzoso G (2006) The NF-kappaB-mediated control of the JNK cascade in the antagonism of programmed cell death in health and disease. *Cell Death Differ* **13**, 712–729.
24 Zhang JY, Green CL, Tao S & Khavari PA (2004) NF-kappaB RelA opposes epidermal proliferation driven by TNFR1 and JNK. *Genes Dev* **18**, 17–22.
25 Gugasyan R, Voss A, Varigos G, Thomas T, Grumont RJ, Kaur P, Grigoriadis G & Gerondakis S (2004) The transcription factors c-rel and RelA control epidermal development and homeostasis in embryonic and adult skin via distinct mechanisms. *Mol Cell Biol* **24**, 5733–5745.
26 Fomenkov A, Zangen R, Huang YP, Osada M, Guo Z, Fomenkov T, Trink B, Sidransky D & Ratovitski EA (2004) RACK1 and stratifin target DeltaNp63alpha for a proteasome degradation in head and neck squamous cell carcinoma cells upon DNA damage. *Cell Cycle* **3**, 1285–1295.
27 Strous GJ, van Kerkhof P, Govers R, Ciechanover A & Schwartz AL (1996) The ubiquitin conjugation system is required for ligand-induced endocytosis and degradation of the growth hormone receptor. *EMBO J* **15**, 3806–3812.
28 McKeon F (2004) p63 and the epithelial stem cell: more than status quo? *Genes Dev* **18**, 465–469.
29 Basile JR, Zacny V & Munger K (2001) The cytokines tumor necrosis factor-alpha (TNF-alpha) and TNF-related apoptosis-inducing ligand differentially modulate proliferation and apoptotic pathways in human keratinocytes expressing the human papillomavirus-16, E7 oncoprotein. *J Biol Chem* **276**, 22522–22528.
30 Shin HM, Kim MH, Kim BH, Jung SH, Kim YS, Park HJ, Hong JT, Min KR & Kim Y (2004) Inhibitory action of novel aromatic diamine compound on lipopolysaccharide-induced nuclear translocation of NF-kappaB without affecting IkappaB degradation. *FEBS Lett* **571**, 50–54.
31 Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, Leisten JC, Motiwala A, Pierce S, Satoh Y *et al.* (2001) SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc Natl Acad Sci USA* **98**, 13681–13686.
32 Newton R, Adcock IM & Barnes PJ (1996) Superinduction of NF-kappa B by actinomycin D and cycloheximide in epithelial cells. *Biochem Biophys Res Commun* **218**, 518–523.
33 Rocco JW, Leong CO, Kuperwasser N, DeYoung MP & Ellisen LW (2006) p63 mediates survival in squamous cell carcinoma by suppression of p73-dependent apoptosis. *Cancer Cell* **9**, 45–56.
34 Parsa R, Yang A, McKeon F & Green H (1999) Association of p63 with proliferative potential in normal and neoplastic human keratinocytes. *J Invest Dermatol* **113**, 1099–1105.
35 Wullaert A, Heyninck K & Beyaert R (2006) Mechanisms of crosstalk between TNF-induced NF-kappaB and JNK activation in hepatocytes. *Biochem Pharmacol* **72**, 1090–1101.
36 Karin M & Lin A (2002) NF-kappaB at the crossroads of life and death. *Nat Immunol* **3**, 221–227.
37 Seitz CS, Deng H, Hinata K, Lin Q & Khavari PA (2000) Nuclear factor kappaB subunits induce epithelial cell growth arrest. *Cancer Res* **60**, 4085–4092.
38 Seitz CS, Freiberg RA, Hinata K & Khavari PA (2000) NF-kappaB determines localization and features of cell death in epidermis. *J Clin Invest* **105**, 253–260.
39 Seitz CS, Lin Q, Deng H & Khavari PA (1998) Alterations in NF-kappaB function in transgenic epithelial tissue demonstrate a growth inhibitory role for NF-kappaB. *Proc Natl Acad Sci USA* **95**, 2307–2312.
40 Alcamo E, Mizgerd JP, Horwitz BH, Bronson R, Beg AA, Scott M, Doerschuk CM, Hynes RO & Baltimore D (2001) Targeted mutation of TNF receptor I rescues the RelA-deficient mouse and reveals a critical role for NF-kappa B in leukocyte recruitment. *J Immunol* **167**, 1592–1600.
41 Chang L, Kamata H, Solinas G, Luo JL, Maeda S, Venuprasad K, Liu YC & Karin M (2006) The E3 ubiquitin ligase itch couples JNK activation to TNF-alpha-induced cell death by inducing c-FLIP (L) turnover. *Cell* **124**, 601–613.
42 Yoshida K, Yamaguchi T, Natsume T, Kufe D & Miki Y (2005) JNK phosphorylation of 14–3−3 proteins regulates nuclear targeting of c-Abl in the apoptotic response to DNA damage. *Nat Cell Biol* **7**, 278–285.
43 Rossi M, Aqeilan RI, Neale M, Candi E, Salomoni P, Knight RA, Croce CM & Melino G (2006) The E3 ubiquitin ligase Itch controls the protein stability of p63. *Proc Natl Acad Sci USA* **103**, 12753–12758.
44 Wajant H, Pfizenmaier K & Scheurich P (2003) Tumor necrosis factor signaling. *Cell Death Differ* **10**, 45–65.
45 Deng Y, Ren X, Yang L, Lin Y & Wu X (2003) A JNK-dependent pathway is required for TNFalpha-induced apoptosis. *Cell* **115**, 61–70.
46 Liu ZG (2003) Adding facets to TNF signaling. The JNK angle. *Mol Cell* **12**, 795–796.
47 Bian X, McAllister-Lucas LM, Shao F, Schumacher KR, Feng Z, Porter AG, Castle VP & Opipari AW Jr

(2001) NF-kappa B activation mediates doxorubicin-induced cell death in N-type neuroblastoma cells. *J Biol Chem* **276**, 48921–48929.

48 Hettmann T, DiDonato J, Karin M & Leiden JM (1999) An essential role for nuclear factor kappaB in promoting double positive thymocyte apoptosis. *J Exp Med* **189**, 145–158.

49 Srivastava S, Tong YA, Devadas K, Zou ZQ, Chen Y, Pirollo KF & Chang EH (1992) The status of the p53 gene in human papilloma virus positive or negative cervical carcinoma cell lines. *Carcinogenesis* **13**, 1273–1275.

50 Yu J, Zhang L, Hwang PM, Kinzler KW & Vogelstein B (2001) PUMA induces the rapid apoptosis of colorectal cancer cells. *Mol Cell* **7**, 673–682.