Anti-Apoptotic NF-κB and “Gain of Function” mutp53 in Concert Act Pro-Apoptotic in Response to UVB + IL-1 via Enhanced TNF Production

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In response to genotoxic stress, including UVB radiation, transcription factors NF-κB and p53 inevitably influence the cellular fate. Loss of p53 function has been attributed to malignant transformation and interferes with therapeutic interventions, whereas “gain of function” mutants even enhance tumor promotion. Constitutive NF-κB activation is linked to tumor maintenance and resistance against chemotherapy. The cross talk between p53 and NF-κB, however, is still under debate. Using the non-transformed keratinocyte cell line HaCaT, we shed light on the interplay between p53 and NF-κB by providing clear evidence that chronically activated NF-κB together with designated “gain of function” mutp53 promotes apoptosis via cooperative tumor necrosis factor (TNF) production in response to UVB + IL-1. Performing chromatin immunoprecipitation analysis we demonstrate that both transcription factors bind to the TNF promoter, whereas UVB-induced inhibition of Ser-Thr-phosphatase protein phosphatase 2A facilitates prolonged phosphorylation of NF-κB and the transcriptional cofactor cAMP response element–binding protein, both being required for extended TNF transcription. Thus, two major anti-apoptotic factors, NF-κB and mutp53, in concert may generate pro-apoptotic responses. As human skin is constantly exposed to UVB, causing IL-1 production as well, we hypothesize that the remarkable amount of hotspot p53 mutations within the epidermis (4%) may serve a protective function to eliminate precancerous cells at an early stage.

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INTRODUCTION

In response to cellular stresses, the cross talk between pro- and anti-apoptotic factors determines the cellular fate. Whereas activation of the tumor-suppressor p53 causes upregulation of pro-apoptotic genes following genotoxic stress (Seitz et al., 2010), activation of the transcription factor nuclear factor κB (NF-κB) is mainly associated with anti-apoptotic responses (Fujioka et al., 2012; Almeida et al., 2014). Inactivation of p53 is observed in most human cancers, with mutations in p53 occurring in about 50% of all tumors (Soussi and Wiman, 2007), reaching up to 90% in non-melanoma skin cancer (Gervin et al., 2003). Intriguingly, in addition to “loss of function” mutants that lack the tumor-suppressive function, “gain of function” mutp53 variants lose the sequence-specific DNA binding but exert complex DNA interactions instead, thereby modifying the set of target genes expressed (Goehler et al., 2005; Kim and Deppert, 2007). Accordingly, contact (p53R248W) and structural (p53R175H) mutations are enabled in order to promote a large spectrum of cancer phenotypes and contribute to drug resistance (Moll et al., 2005; Deppert, 2007; Vousten and Lane, 2007). In contrast to wt53, both mutants were shown to confer a selective advantage during tumor development, although the individual mechanisms are still under debate (Mello and Attardi, 2013).

NF-κB is mainly triggered in response to pro-inflammatory cytokines, including IL-1. Receptor-driven activation of a downstream kinase cascade involving IκB-kinase β (IKKβ) causes proteosomal degradation of the inhibitor of κBα (IκBα), allowing for nuclear translocation of NF-κB. Because constitutive activation of NF-κB has been linked to transformation, proliferation, and anti-apoptosis (Aggarwal, 2004), limitation of its activity is warranted by negative feedback regulation involving NF-κB-dependent transcription of IκBα (Delhase et al., 1999). More recently, increasing evidence has been found that additionally attribute pro-apoptotic functions to NF-κB when exposed to DNA-damaging agents (Delhase et al., 1999; Campbell et al., 2004; Liu et al., 2006; Szoltysek et al., 2008; O’Prey et al., 2010; Narayanan et al., 2014). Accordingly, previous work from our lab showed co-stimulation with IL-1 to enhance UVB-induced apoptosis in epithelial cells by accelerated NF-κB-dependent transcription of tumor necrosis factor (TNF). Released TNF triggered TNF-R1 in an autocrine

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Abbreviations: CREB, cAMP response element–binding protein; IκBα, inhibitor of κBα; IκBα-SR, IκBα super-repressor; PP2A, protein phosphatase 2A; TNF, tumor necrosis factor

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manner to additively promote apoptosis (Poppelmann et al., 2005; Strozky et al., 2006; Barisic et al., 2008; Witt et al., 2009; Barisic et al., 2010).

A versatile cross talk between p53 and NF-κB seems to exist especially in tumor cells, whereas in most cases mutual inhibition has been proposed (Webster and Perkins, 1999; Tergaonkar et al., 2002; Bohuslav et al., 2004; Tergaonkar, 2009). Also the competition of both transcription factors for identical cofactors like CREB (cAMP response element–binding protein) was shown to limit the relative activation status of p53 versus NF-κB (Ma et al., 2008), or direct protein–protein interaction (Schneider et al., 2010). Less, however, is known about the interplay between ‘gain of function’ mutp53 and NF-κB in response to genotoxic stress.

Utilizing the non-transformed keratinocyte cell line HaCaT stably knocked down for p53 and/or stably inhibiting NF-κB activation, we demonstrate that mutp53R248W and mutp53R175H but not wt cooperate with NF-κB through direct binding to the TNF promoter. Consequently, accelerated TNF production enhances apoptosis in response to UVB + IL-1. We further provide evidence that UVB-induced inhibition of Ser-Thr-phosphatase protein phosphatase 2A (PP2A) plays a crucial role in sustaining phosphorylation of NF-κB-p65 and CREB to maintain both factors in an active state over time. Hence, we report that two initially anti-apoptotic factors, which are commonly present in various cancers, in concert may promote pro-apoptotic responses.

RESULTS

Co-stimulation with IL-1 enhances UVB-induced apoptosis in an NF-κB-dependent manner

In accordance with previous findings (Poppelmann et al., 2005; Strozky et al., 2006; Barisic et al., 2008), co-stimulation of HaCaT keratinocytes with IL-1 significantly enhanced UVB-induced apoptosis (Figure 1a). As this enhancement could be blocked with an antagonistic TNF-R1 antibody, TNF-dependent TNF-R1 activation seemed to affirm the underlying mechanism as reported before (Poppelmann et al., 2005; Barisic et al., 2008). Thus, enhancement of apoptosis was also evident by pronounced processing of caspase-3 and poly(ADP-ribose)-polymerase (PARP) (Figure 1b) and coincided with considerable TNF release (Figure 1c). Similar to epithelial

![Figure 1](image-url)

Figure 1. IL-1 enhances UVB-induced apoptosis due to pronounced tumor necrosis factor (TNF) release. (a) Cells were stimulated with IL-1, TNF, UVB, or a combination of IL-1 + UVB or TNF + UVB, or pretreated for 30 minutes with an antagonistic anti-TNF-R1 antibody. Apoptosis was determined with Cell Death Detection ELISA. (b) Cleavage of caspase-3 and poly(ADP-ribose)-polymerase (PARP) was documented by Western-blotting. (c) TNF secretion was analyzed with TNF ELISA. (d) Cells were stimulated with IL-1 or UVB for 30 minutes as indicated. Cytosolic and nuclear fractions were analyzed for IκBα depletion (cyt) by Western-blotting and NF-κB (nuc) by electrophoretic mobility shift assay. (e) Cells were stimulated as in (d) and TNF expression determined by reverse transcriptase-PCR. (f) IκBα status of HaCaT-IκBα SR super-repressor (IκBα SR) cells was documented by Western-blotting. (g) HaCaT-mock and -IκBα SR cells were treated as in (a), apoptosis was determined with Cell Death Detection ELISA, and (h) TNF release was documented with TNF ELISA. *P<0.05; **P<0.001. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
cells, TNF production in keratinocytes could be attributed to disruption of the negative feedback regulation of NF-κB.

IL-1 stimulation caused transient NF-κB activation with maximum degradation of IκBα after 30 minutes (Figure 1d). Rapid NF-κB-dependent resynthesis entailed replenishment of IκBα after 1–2 hours, which terminated nuclear NF-κB activity. In contrast, co-stimulation with UVB + IL-1 completely abrogated the reappearance of IκBα up to 6 hours, coinciding with elevated nuclear NF-κB level (Figure 1d). Consequently, prolonged transcription of TNF was facilitated upon UVB + IL-1 treatment (Figure 1e), being a prerequisite for enhanced TNF secretion (Barisic et al., 2008). The notion that inhibition of negative feedback regulation drives NF-κB-dependent TNF production was confirmed in HaCaT cells stably overexpressing an IκBα super-repressor (IκBα-SR; Figure 1f). Overall, apoptosis was slightly enhanced, but more importantly, the enhancing effect of IL-1 on UVB-induced apoptosis was abrogated (Figure 1g) because of significantly reduced TNF secretion (Figure 1h). Still, some residual TNF production remained, indicating that additional factors might contribute to elevated TNF production.

The intensity of DNA damage correlates with the apoptosis rate induced and the amount of TNF released

Irradiation of cells with increasing doses of UVB caused gradual enhancement of apoptosis (Figure 2a), which correlated with increasing amounts of cyclobutane-pyrimidine dimer formation (Figure 2c). At a constant IL-1 level also the amount of TNF secretion gradually increased (Figure 2b), implying a link between DNA damage and TNF production. Conclusively, we assumed an additional transcription factor to become activated in response to UVB-driven DNA damage that may cooperate with NF-κB for full TNF production in response to UVB + IL-1.

p53 contributes to TNF-mediated enhancement of UVB-induced apoptosis

The transcription factor p53 plays a crucial role in mediating DNA damage responses. HaCaT cells are known to harbor mutated p53, carrying heterozygous C→T transitions (Lehman et al., 1993), changing it into a “gain of function”-like phenotype. As “gain of function” mutations of p53 are known to exert an anti-apoptotic phenotype, stable knockdown of p53 sensitized three different HaCaT-p53i clones to UVB-induced apoptosis (Figure 3a and b), whereas the IL-1-mediated upregulation (n-fold) appeared to be impaired in HaCaT-p53i cells and also yielded less TNF (Figure 3d). The protein level of other p53 family members, p63 and p73, remained unaffected by loss of p53 (Figure 3c), indicating that solely p53 might contribute to accelerated apoptosis and TNF production in response to UVB + IL-1. Accordingly, additional inhibition of NF-κB by stably overexpressing IκBα-SR in HaCaT-p53i cells (Figure 3e) minimized both, apoptosis enhancement (Figure 3f) and TNF release (Figure 3g). Conclusively, data implied that both transcription factors may collectively amplify the pro-apoptotic effect in UVB + IL-1-treated cells.

NF-κB and p53 additively promote TNF transcription in response to UVB + IL-1

Activation of the NF-κB subunit p65 is facilitated by Ser536 phosphorylation, whereas Ser15 phosphorylation is required for p53 activation (Graham and Gibson, 2005; Ray et al., 2012). Treatment of HaCaT cells with IL-1 caused transient phosphorylation of p65, being terminated after 2 hours, the time point of IκBα resynthesis. Phosphorylation of the transcriptional coactivator CREB at Ser133 (Friedrich et al., 2010) followed the identical pattern, indicating that both proteins are required for proper IL-1-induced transcription. The DNA damage–responsive transcription factor p53 remained inactive following IL-1 stimulation, as anticipated. In response to UVB, instead, p65 stayed inactive, whereas phosphorylation of p53 rapidly occurred and lasted for at least 4 hours. Activation of p53 also coincided with CREB phosphorylation, confirming CREB to be an important cofactor for both transcription factors (Huang et al., 2007). Finally, co-stimulation with UVB + IL-1 allowed phosphorylation of all three components. Whereas the phosphorylation pattern of p53 and CREB remained unchanged compared with UVB-treated cells, p65 phosphorylation was extended (Figure 4a; Barisic et al., 2008)). Interestingly, knockdown of p53 did not influence the phosphorylation pattern of p65 and CREB (Figure 4b), implying that NF-κB and p53 act additively but independently to enhance TNF transcription. In accordance with this assumption,
co-immunoprecipitation revealed that p65 and p53 do not directly interact at the protein level (Figure 4c and d).

From previous studies, we knew that UVB-mediated inhibition of catalytic subunit of PP2A (PP2Ac) crucially contributes to abrogation of negative feedback regulation for NF-κB through chronic IKKβ activation (Barisic et al., 2008, 2010). We here discover an additional role for PP2A in tuning NF-κB activity. In control cells, only treatment with UVB + IL-1 caused p65 phosphorylation, whereas p53 and CREB were activated by UVB and UVB + IL-1, respectively. Inhibition of PP2Ac by small interfering RNA-mediated knockdown and addition of calyculin A had no effect on p53 phosphorylation but significantly stabilized phosphorylation of p65 and CREB (Figure 4e). These results disclose two ways of PP2A-mediated NF-κB regulation: indirectly by dephosphorylating IKKβ to terminate nuclear localization of NF-κB and directly by dephosphorylating p65 and CREB to terminate transcription (Figure 4f). Activation of p53, instead, is exclusively driven in a DNA damage-responsive manner and remains unaffected by PP2A.

Only mutp53 but not wt p53 binds to the TNF promoter to enhance NF-κB-driven TNF transcription

According to our findings so far, we assumed that p65 and p53 may cooperatively bind to the TNF promoter in response to UVB + IL-1. This idea was strengthened by the finding that, besides p65 and CREB consensus elements, a putative (partial) p53 binding motive also exists within the TNF promoter sequence ([Kuo and Leiden, 1999], Supplementary Figure S1 online). As expected, chromatin immunoprecipitation analysis of p65 consolidated the prolonged association of NF-κB with the TNF promoter in response to UVB + IL-1 in semiquantitative (Figure 5a) and quantitative PCR analyses (Figure 5b). In order to dissect the binding properties of different p53 variants, we overexpressed wt p53 as well as mutp53R248W and mutp53R175H in HaCaT-p53wt cells (Figure 5c). As assumed, endogenous mutp53 bound to the TNF promoter for at least 2 hours following UVB + IL-1 treatment (Figure 5d semiquantitative and 5e quantitative). Interestingly, wt p53 was shown to hardly associate with the TNF promoter compared to mutp53R248W and mutp53R175H (Figure 5d and e). Taken together, binding of NF-κB and only “gain of function” mutp53 was significantly intensified over time, whereas binding of wt p53 was negligible. Concordantly, we could identify different effects of wt p53 versus mutp53 expression on apoptosis as well as TNF release (Figure 5f and g). IL-1-mediated enhancement of UVB-induced apoptosis was stronger in cells harboring mutp53—i.e. normal HaCaT cells as well as mutp53R248W and mutp53R175H expressing HaCaT-

Figure 3. Knockdown of p53 sensitizes HaCaT cells to UVB. (a) Three stable HaCaT-p53i clones were stimulated with IL-1, UVB, or both, and apoptosis was determined using Cell Death Detection ELISA. (b) Western-blot analysis documenting p53 knockdown. (c) Western-blot analysis of p63 and p73 expression level of HaCaT-mock versus -p53i cells. (d) HaCaT-mock and HaCaT-p53i (clone 2) cells were stimulated with IL-1, UVB, or both. Tumor necrosis factor (TNF) release was determined with TNF ELISA. N-fold increase of apoptosis is indicated. (e) Western blot analysis documenting IkBa and p53 status of HaCaT-p53i/IκBα super-repressor (IkBa-SR) cells. (f) HaCaT-mock, -p53i, -IkBα-SR, and -p53i/IκBα-SR cells were stimulated as in d. Apoptosis was determined with Cell Death Detection ELISA (n-fold enhancement of apoptosis is indicated) and (g) TNF release with TNF ELISA. *P<0.01; **P<0.001. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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p53i cells, and remained only marginal in HaCaT-p53i- and -wtp53-expressing cells (Figure 5f). Of note, the apoptotic responses correlated well with the amount of TNF released by the individual cell lines. Data confirmed accelerated TNF release to closely correlate with pronounced enhancement of apoptosis and strengthens the view that this effect is most pronounced in mutp53-expressing cells (Figure 5g).

Mutp53 prevents clonogenic outgrowth of UVB + IL-1-treated cells
Three weeks following UVB or UVB + IL-1 stimulation, clonogenic outgrowth of HaCaT-mock compared with HaCaT-p53i cells was determined. Whereas cell density of UVB + IL-1-treated HaCaT-p53i cells reached an average 89% compared with UVB-treated cells, only 77% cell density was achieved for HaCaT-mock cells (Figure 6a and b). This supported the concept that cell survival might be impaired following UVB + IL-1 treatment if p53 is mutated.

Taken together, we here disclose the cooperation of “gain of function” mutp53 and NF-κB at the TNF promoter and provide evidence that two initially anti-apoptotic proteins in concert may generate pro-apoptotic responses.

DISCUSSION
Dysregulation of both p53 and NF-κB confers a selective advantage during tumor development and conveys therapy

Figure 4. Phosphorylation pattern of p65 and cAMP response element–binding protein (CREB) depends on PP2A. (a) HaCaT-mock and (b) HaCaT-p53i cells were treated with IL-1, UVB, or both. At the indicated time points, the status of IκBα, pSer536-p65, p65, pSer15-p53, p53, pSer133-CREB, and CREB was determined by Western-blot analysis. (c) Cells were stimulated with IL-1 or UVB + IL-1 for 2 h; p53 or (d) p65 was immunoprecipitated, and co-precipitation of p65 and p53, respectively, was documented by Western-blot analysis. (e) Catalytic subunit of PP2A (PP2Ac) was knocked down and residual activity blocked with calcyulin A. After 72 hours, cells were stimulated with IL-1, UVB, or both as indicated. After 2 hours, the status of pSer536-p65, p65, pSer15-p53, p53, pSer133-CREB, and CREB was determined by Western-blot analysis. (f) Scheme displaying the processes of accelerated tumor necrosis factor expression. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IKKβ, IκB-kinase β; SN, supernatant.
Figure 5. p53 and p65 cooperate to enhance transcription of tumor necrosis factor (TNF).

(a) HaCaT cells were treated with IL-1 or UVB + IL-1 as indicated. Reverse transcriptase–PCR (RT-PCR) analysis of NF-κB-p65 chromatin immunoprecipitation (ChIP) is shown. (b) N-fold expression calculated by quantitative PCR (qPCR) of three independent experiments. (c) Western-blot analysis documenting expression of wt p53, mut p53R175H, and mut p53R248W in HaCaT-p53i cells.

(d) HaCaT-mock, -p53i, -p53i-wt p53, -p53i-mut p53R248W, and -p53i-mut p53R175H cells were treated as in a. RT-PCR analysis of p53 ChIP is shown. (e) N-fold expression calculated by qPCR of three independent experiments. (f) HaCaT-mock, -p53i, -p53i-wt p53, -p53i-mut p53R248W, and -p53i-mut p53R175H cells were treated with IL-1, UVB, or both. After 16 hours, apoptosis was determined with Cell Death Detection ELISA (n-fold enhancement of apoptosis is indicated) and (g) TNF release with TNF ELISA. *P<0.05; **P<0.01. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
resistance. A versatile cross talk between NF-κB and p53 exists at multiple levels; however, the balance between synergistic and antagonistic processes may rely on the cellular context. In the present study, we confirm the molecular mechanism by which chronic NF-κB activation contributes to enhancement of UVB-induced apoptosis via upregulation of TNF in keratinocytes (Poppelmann et al., 2005; Barisic et al., 2008).

Intriguingly, at a constant IL-1 concentration required for NF-κB activation, increasing UVB exposure linked TNF release to the intensity of DNA damage induced, pointing at p53 to support TNF transcription. Hence, TNF release and thereby IL-1-mediated enhancement of UVB-induced apoptosis was minimized in HaCaT-p53i-κBz-SR cells.

Previous studies have described mutp53 to augment TNF-induced NF-κB activity (Weisz et al., 2007) or have shown that NF-κB-p65 is required for TNF-induced upregulation of p53 target genes in different mouse tissues (Wang et al., 2009; O’Prey et al., 2010). However, a collaboration of the two transcription factors at the human TNF promoter has never been reported. Even though NF-κB-p53 protein–protein interactions have been proposed at responsive promoter elements (Gurzov et al., 2010) or at NF-κB-responsive reporter constructs (Kawauchi et al., 2008), we could not co-immunoprecipitate p53 and p65 in UVB-IL-1-exposed cells. This indicates that both factors independently but additively trigger the TNF promoter. The activation kinetics for p65, p53, and the joint coactivator CREB clearly showed UVB to activate p53 and also to extend IL-1-induced activation for p65 and CREB. Although p53 phosphorylation follows an established ataxia telangiectasia mutated-dependent (ATM) mechanism (Zhang et al., 2011), we here deciphered a PP2A-dependent mechanism that drives prolonged p65 and CREB phosphorylation. On the basis of current and former data, we propose the following scenario to underlie activation of p53 and hyperactivation of NF-κB at the TNF promoter.

IL-1 causes canonical activation of NF-κB, whereas UVB activates p53. In parallel, UVB-induced inhibition of PP2Ac allows for nuclear persistence of NF-κB (Barisic et al., 2008; Witt et al., 2009; Barisic et al., 2010; Zhang et al., 2011; Konrath et al., 2014) and warrants prolonged activation of p65 and CREB. The fact that NF-κB activity still vanishes at later times point can be attributed to other IκB members, such as IκBε, that take over NF-κB inhibition in a delayed and more linear manner (Hoffmann et al., 2002).

Thus, the present study is of high patho-physiological relevance, because IL-1 is constantly produced from UVB-exposed keratinocytes within human skin (Feldmeyer et al., 2007). UVB radiation may serve as a carcinogen by activation of skin oncogenes, inactivation of tumor suppressors, and repression of cell-based immune responses that are generally able to eliminate highly antigenic skin tumors (Yarosh and Kripke, 1996). At the same time, UVB-induced DNA damage is a prerequisite for execution of apoptosis, leading to elimination of cancer-prone cells (Kulms and Schwarz, 2000; Murphy et al., 2001). The molecular switch, however, has not yet been identified. As p53 carries hotspots for UVB-induced mutations, numerous studies exist postulating 4% of all keratinocytes to bare p53 mutations; however, less squamous cell carcinoma develop (Murphy et al., 2001; Klein et al., 2010). It is further common knowledge that up to 50% of all tumors and up to 90% of non-melanoma skin cancers bear p53 mutations (Murphy et al., 2001; Gervin et al., 2003), clearly supporting that mutp53 selectively promotes tumor progression once the oncogenic pressure exceeds a certain threshold. Still, we hypothesize that at sub-oncogenic stresses and concomitant IL-1 production, provided e.g. by keratinocytes from the microenvironment, expression of mutp53 may even be beneficial for mildly damaged cancer-prone cells to enforce their elimination via TNF-dependent apoptosis.

In addition, the present study sheds light on the ongoing discussions about the p53–NF-κB interplay in cancer and may have even broader implications regarding cancer treatment, where currently a lot of effort is made to reconstitute wt p53 and/or inhibit NF-κB in cancer cells (Lin and Karin, 2003; Muller and Vousden, 2013).

MATERIALS AND METHODS

Results of quantitative PCR, Cell Death Detection--, and TNF-ELISA are presented as mean±SD of three independently performed experiments. Reverse transcriptase-PCR, electrophoretic mobility shift assay, and western blot analyses represent one out of three independently performed experiments. For statistical analysis, Student’s t-test was performed.

Cells and reagents

The human keratinocyte cell line HaCaT (Fusenig et al., 1990) was cultured in DMEM, 10% fetal calf serum, and 1% PenStrep (Thermo Scientific, Waltham, MA) and irradiated with UVB (300 J m⁻²) using TL12 fluorescent bulbs (290–320 nm, Philips; Eindhoven, the Netherlands) or stimulated with recombinant human IL-1β (10 ng ml⁻¹) or TNF (100 ng ml⁻¹; both R&D Systems, Minneapolis, MN). TNF-R1 was blocked with an antagonistic antibody H-398 (40 µg ml⁻¹) kindly provided by Dr Peter Scheurich, University of

Figure 6. Mutp53 prevents clonogenic outgrowth of UVB + IL-1-treated cells. (a) Percentage of clonogenic outgrowth of HaCaT-mock versus -p53i cells 3 weeks after UVB and UVB + IL-1 stimulation, respectively. Outgrowth of UVB-only treated cells is set as 100% and the cell density of UVB + IL-1-treated cells calculated accordingly. **P<0.01. (b) Display of one representative experiment.
Stuttgart, Germany. TNF release was measured in a TNF ELISA (R&D Systems) and apoptosis in a Cell Death Detection-ELISA (Roche, Mannheim, Germany). For PP2Ac knockdown, 1.6 × 10^5 cells were transfected with 120 pmol scrambled 5′-gCggCUgCggAAAUUUACCTT-3′ or PP2Ac 5′-gAggUUGAgUGUCCAgUUATT-3′ small interfering RNA (MWG, Ebersberg, Germany) using Lipofectamine 2000 (Thermo Scientific). Calcyolin A (Cell Signaling, Beverly, MA) was added at 5 μM. Transfectants were generated by electroporating 6.5 × 10^5 cells in DMEM, 10% fetal calf serum, and 1.5% DMSO with 25 μg of plasmids encoding pRetroSuper-blasto-p53 (Zalcenstein et al., 2003), IκBz-32/36A (pBKCMV-IκB-SR), or mock (pcDNA3; EasyjetC-plus, PeqqLab, Erlangen, Germany) and stable clones selected with blasticidine (10 μg/mL; PAA, Colbe, Germany). Silent mutations were introduced into pCMV-neo-Bam-based expression constructs (p53wt, p53R175H, p53R248W) using a Quick Change Site-directed Mutagenesis Kit (Stratagene, Kirkland, WA) and the following primers: F: 5′-ATCACACTTggAAGATAgCTCCggCAACCTTCTAggACggAACAgC-3′; R: 5′-gCTgTTCCgTCCTAggCAACggAgCTCTtgATtgAT-3′.

Electromobility shift analysis

Cells were lysed in wash buffer (10 mM HEPES, pH 7.9; 10 mM KC1; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM dithiothreitol; 0.1 mM phenylmethylsulfonylfluoride, Complete; Roche) on ice for 20 minutes. Nuclear pellets were lysed in high-salt buffer (20 mM HEPES, pH 7.9; 400 mM NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM dithiothreitol; 0.1 mM phenylmethylsulfonylfluoride, Complete; Roche). The NF-κB consensus oligo-nucleotide (sc-2505; Santa Cruz, Heidelberg, Germany) was end-labeled using [γ-32]P ATP and T4 polynucleotide kinase (Thermo Scientific), and purified using a QiAquick Nucleotide Removal Kit (Qiagen, Minden, Germany). Binding reactions containing 15 μg protein extract, 4 μL of 5 × binding buffer (20 mM HEPES, pH 7.5; 50 mM KC1; 2.5 mM MgCl2; 20% (w/v) ficoll; 1 mM dithiothreitol), 2 μg poly(dIdC), 2 μg BSA, and 70,000 c.p.m. 32P-labeled NF-κB consensus nucleotide were incubated for 20 minutes at room temperature, separated on 4% native PAGE, and detected autoradiographically (Strozky et al., 2006).

Immunoprecipitation and western blot analysis

Cells were lysed in lysis buffer (50 mM HEPES, pH 7.5; 150 mM NaCl; 10% glycerol; 1% Triton X-100; 1.5 mM MgCl2; 1 mM EGTA; 100 mM NaF; 10 mM pyrophosphate; 0.01% NaN3; Complete, PhosSTOP, Roche), subjected to 7.5–15% SDS-PAGE, blotted, and incubated with antibodies (β-actin, #4970; caspase-3, #9665; CREB, #9197; phospho-CREB, #9198; GAPDH, #2118; IκBz #4814; phospho-p53, #9284; phospho-p65, #3033; PP2Ac, #2038—Cell Signaling; p53, 554293; poly(ADP-ribose)-polymerase, 551025—BD Biosciences, Heidelberg, Germany; p63, sc-843; p65, sc-8008—Santa Cruz; p73, PC385—Calbiochem, Darmstadt, Germany) and visualized by chemiluminescent detection (SuperSignal, Thermo Scientific). For immunoprecipitation, protein extracts were incubated with anti-p53 or -p65 antibody (#9282; #8242, Cell Signaling) and protein-A-Agarose (KPL, Gaithersburg, ML) beads overnight at 4°C. Proteins were eluted and subjected to western blot analysis.

Southwestern dot-blot analysis

Genomic DNA was extracted according to the manufacturer’s protocol (Qiagen). A volume of 2 μg DNA in denaturation buffer (1.5 M NaCl; 0.5 M NaOH) was vacuum dot-blotted onto a nylons− membrane, neutralized (1 μl Tris; 2 M NaCl), and fixed for 15 minutes at 80°C. Cyclobutane-pyrimidine dimers were detected with KIT53 antibody (Kamiya Biomedical, Thousand Oaks, CA) and equal loading was monitored with anti-adenosine (Research Plus Inc., Manasquan, NJ).

Semi-quantitative reverse transcriptase–PCR

RNA was extracted using GIT buffer and reverse-transcribed with an AMV Reverse Transcriptase kit (Thermo Scientific). Primers were used in a 20-μl reaction utilizing the RedTaq polymerase system (Sigma, St Louis, MO): GAPDH: F: 5′-gCCTCCtgCAGCACCAATgC-3′; R: 5′-CCTCCgAgCTgCTCTAgCC TCTT-3′; TNF: F: 5′-TgCTgTTCCCTAgCC TCTT-3′; R: 5′ATCCCAAgTAGACCTgCCC-3′.

Chromatin Immunoprecipitation

Cells were crosslinked in 1% formaldehyde, 125 mM Glycine added, and lysed in lysis buffer (10 mM Tris, pH 7.5; 10 mM NaCl; 3 mM MgCl2; 2.5% NP40; 10 μg MG-132; PhosSTOP®, Complete®, Roche). Pellet nuclei were resuspended in chromatin immunoprecipitation-buffer (10 mM Tris, pH 8.0; 0.5 mM EDTA pH 8.0; 0.5 mM EGTA pH 8.0; 10 μg MG-132; PhosSTOP® and Complete®, Roche), chromatin sheared (Covaris S2, Woburn, MA) at 20dc 8i 200 cps fs 600s, diluted in buffer (16.7 mM Tris, pH 8.0; 1.2 mM EDTA pH 8.0; 167 mM NaCl; 1.1% Triton X-100; 0.01% SDS; 10 μg MG-132; PhosSTOP®, Complete®, Roche), and precleared by adding Sepharose® CL-4B (Sigma). Complexes were precipitated with anti-p-p53 (#9284, Cell Signaling) or p65 (sc-372, Santa Cruz) antibody and Protein-A-Sepharose 4 Fast Flow (Thermo Scientific) overnight at 4°C. Precipitates were washed in low-salt buffer (20 mM Tris, pH 8.1; 150 mM NaCl; 2 mM EDTA; 1% Triton X-100; 0.1% SDS), high-salt buffer (20 mM Tris, pH 8.1; 500 mM NaCl; 2 mM EDTA; 1% Triton X-100; 0.1% SDS), wash buffer (10 mM Tris, pH 8.0; 1 mM EDTA; 1% deoxycholate; 1% NP40; 0.25 M LiCl), and Tris-EDTA buffer (10 mM Tris, pH 8.0, 1 mM EDTA). Complexes were eluted (0.1 M NaHCO3; 1% SDS) and crosslinking reversed in 0.2 M NaCl overnight at 65°C. After proteinase K treatment (55°C, 3 hours) and phenol–chloroform extraction, DNA fragments were analyzed using the DyNAmo ColorFlash SYBR Green qPCR kit in a PikoReal24ycler with PikoReal 2.2 software (Thermo Scientific): p65: F: 5′-CCACAgCAAAAggTAgAATgAg-3′; R: 5′-TCACgTAgACCTgCACCACAC-3′; p52: F: 5′-gCggCTgAAgATgAgg-3′; R: 5′-gAggTCAgAAgAggAggAgg-3′.

Clonogenic assay

A total of 50,000 cells were seeded in a 10-cm dish, stimulated, and medium changed every other day. After 3 weeks, cells were stained with crystal violet and dissolved in KH2PO4–ethanol, and absorption measured at 595 nm.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid
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