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The FoxO3a gene is a key negative target of canonical Notch signalling in the keratinocyte UVB response

Anna Mandinova[1,5], Karine Lefort[2,5], Alice Tommasi di Vignano[1], Wesley Stonely[1], Paola Ostano[3], Giovanna Chiorino[3], Haruhi Iwaki[4], Jotaro Nakanishi[4] and G Paolo Dotto[1,2,*]

[1]Cutaneous Biology Research Center, Massachusetts General Hospital, Charlestown, MA, USA, [2]Department of Biochemistry, University of Lausanne, Epalinges, Switzerland, [3]Laboratory of Cancer Pharmacogenomics, Fondo 'Edo Tempia', Biella, Italy and [4]Shiseido Life Science Research Center, Fukuura, Kanazawa-ku, Yokohama, Japan

Notch signalling has an important role in skin homeostasis, promoting keratinocyte differentiation and suppressing tumorigenesis. Here we show that this pathway also has an essential anti-apoptotic function in the keratinocyte UVB response. Notch1 expression and activity are significantly induced, in a p53-dependent manner, by UVB exposure of primary keratinocytes as well as intact epidermis of both mouse and human origin. The apoptotic response to UVB is increased by deletion of the Notch1 gene or down-modulation of Notch signalling by pharmacological inhibition or genetic suppression of 'canonical' Notch/CSL/MAML1-dependent transcription. Conversely, Notch activation protects keratinocytes against apoptosis through a mechanism that is not linked to Notch-induced cell cycle withdrawal or NF-κB activation. Rather, transcription of FoxO3a, a key pro-apoptotic gene, is under direct negative control of Notch/HERP transcription in keratinocytes, and upregulation of this gene accounts for the increased susceptibility to UVB of cells with suppressed Notch signalling. Thus, the canonical Notch/HERP pathway functions as a protective anti-apoptotic mechanism in keratinocytes through negative control of FoxO3a expression.

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Introduction

Notch cell surface receptors and their ligands belonging to the Delta and Serrate/Jagged families have a crucial role in cell-fate determination and differentiation, functioning in a cell- and context-specific manner (Bray, 2006). The best-characterized 'canonical' pathway of Notch activation involves proteolytic cleavage and translocation of the cytoplasmic domain of the receptor to the nucleus, where it associates with the DNA binding protein CSL converting it from a repressor into an activator of transcription (Mumm and Kopan, 2000; Lai, 2002). However, direct binding of Notch to a second ancillary protein, Mastermind-like 1–3, is required for elevated levels of CSL-dependent transcriptional activation through recruitment of further transcriptional co-activators such as p300 (Petcherski and Kimble, 2000; Wu *et al*, 2000; Oswald *et al*, 2001). Transcriptional repressors of the HES (Hairy Enhancer of Split)/HERP family are well-characterized direct targets of Notch/CSL activation (Iso *et al*, 2003).

In mammalian cells, Notch activation is generally thought to maintain stem cell potential and inhibit differentiation, thereby promoting carcinogenesis (Ellisen *et al*, 1991; Weijzen *et al*, 2002; Pece *et al*, 2004; Weng *et al*, 2004; Balint *et al*, 2005; Hopfer *et al*, 2005; Ayyanan *et al*, 2006). However, in specific cell types such as keratinocytes, increased Notch activity causes exit from the cell cycle and commitment to differentiation, whereas down-modulation or loss of Notch1 function promotes carcinogenesis (Rangarajan *et al*, 2001; Nicolas *et al*, 2003; Devgan *et al*, 2005; Lefort *et al*, 2007). In mouse keratinocytes, the gene for the cyclin/CDK inhibitor $p21^{WAF1/Cip1}$ is also induced by the Notch/CSL complex through both a direct and indirect mechanism, with $p21^{WAF1/Cip1}$ functioning downstream of Notch (Rangarajan *et al*, 2001; Devgan *et al*, 2005; Mammucari *et al*, 2005). In human keratinocytes, Notch activation has more long-term effects, restricting keratinocyte stem cell potential through down-modulation of p63 and Rho/Cdc42 effectors (Nguyen *et al*, 2006; Lefort *et al*, 2007). Notch activation also impinges on other pathways important for keratinocyte growth, differentiation and tumour development such as NF-κB (Nguyen *et al*, 2006; Shin *et al*, 2006), AP-1 (Talora *et al*, 2002) and Wnt signalling (Devgan *et al*, 2005).

Besides its role in growth and differentiation, Notch signalling has also been shown to have a pro- or anti-apoptotic function depending on the context and/or cell type (Weng *et al*, 2003; Nefedova *et al*, 2004; Sade *et al*, 2004; Yang *et al*, 2004; Zweidler-McKay *et al*, 2005; Mungamuri *et al*, 2006; Lewis *et al*, 2007). In keratinocytes, recent evidence has established Notch1 as a key p53 target gene in tumour suppression, which is induced upon UVB exposure (Lefort *et al*, 2007; Yugawa *et al*, 2007). However, the pro- or anti-apoptotic function of the Notch pathway in the keratinocyte UVB response and the underlying mechanisms have not been investigated. This is an important question, considering the impact of UV light on normal skin homeostasis and carcinogenesis.

*Corresponding author. Department of Biochemistry, University of Lausanne, Chemin de Boveresses 155, Epalinges 1066, Switzerland. Tel.: +41 21 692 5720; Fax: +41 21 692 5705;
E-mail: Gian-Paolo.Dotto@unil.ch
[5]These authors contributed equally to this work

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FoxO3a is a prominent member of the FoxO family, which, similar to other transcription factors with a Fox domain, binds DNA and activates transcription as a monomer (Kaestner *et al*, 2000). FoxO3a-dependent transcription has been variously linked with control of the cell cycle, apoptosis and differentiation (Accili and Arden, 2004). Notably, the FoxO3a gene is often lost in tumours, consistent with its possible tumour-suppressing function (Galili *et al*, 1993). FoxO3a activity has been shown to be regulated at the post-transcriptional level, with phosphorylation by the Akt and related SGK kinases keeping this factor in an inactive state in the cytoplasm, whereas its phosphorylation by JNK brings it to the nucleus and activates transcription (Brunet *et al*, 2001; Quevedo *et al*, 2007). Additional regulation of FoxO3a function is provided by the tight control of its acetylation state by members of the Sir2 family of protein deacetylases including SIRT1 (Brunet *et al*, 2004). By de-acetylating FoxO3a, SIRT1 appears to prevent its ability to induce apoptosis (Motta *et al*, 2004). Surprisingly, little or no information exists on transcriptional control of the FoxO3a gene. We show here that this gene is a negative transcriptional target of Notch/HERP in keratinocytes, with the canonical Notch pathway exerting a protective function in the UVB response of these cells through down-modulation of FoxO3a expression.

Results

UVB exposure induces Notch1 expression and activity in keratinocytes and skin of both mouse and human origin

The role of Notch signalling in the UVB response of keratinocytes remains to be established. UVB irradiation of mouse primary keratinocytes caused a dose-dependent activation of Notch signaling, as assessed by the activity of a synthetic Notch-responsive promoter (Figure 1A). Concomitantly, quantitative RT–PCR analysis showed that expression of the endogenous HES1 gene, a 'canonical' Notch target, is induced by UVB treatment of these cells *in vitro* (Figure 1B) as well as *in vivo*, in the intact epidermis (Figure 1C). A similar induction of HES1 and of the related HERP1, as well as of the Notch1 gene itself, was also triggered by UVB treatment of human primary keratinocytes (Figure 1D). Such induction was blocked by siRNA-mediated knock-down of p53 expression, consistent with the recent finding that Notch1 is a direct p53 target gene in keratinocytes (Lefort *et al*, 2007; Yugawa *et al*, 2007) (Figure 1D and E). In parallel with increased Notch1 expression and activity, UVB exposure of proliferating keratinocytes also induced differentiation marker expression (keratin 1, involucrin), and induction of these markers, for example induction of keratin 1, was p53-dependent (Figure 1F).

Consistent with the above findings, immunohistochemistry with an antibody recognizing the cleaved form of Notch1 revealed a significant increase of Notch1 activation also in the intact skin of volunteers at various times of UVB exposure *in vivo* (Figure 1G).

Notch signalling has a pro-survival function in the UVB and DNA damage response of keratinocytes

Previous work suggested that differentiating keratinocytes are more resistant to UVB-induced apoptosis than cells of the proliferative compartment (Chaturvedi *et al*, 2004). To verify this conclusion, mouse keratinocytes under growing conditions and at 24 h after calcium-induced differentiation were subjected to UVB light exposure. TUNEL assays showed a substantially lower apoptotic response of differentiating keratinocytes at both 8 and 24 h of UVB treatment (Figure 2A). To assess whether the higher UVB resistance of differentiating cells is Notch-dependent, primary keratinocytes from mice with the Notch1 gene flanked by loxP sites were infected with a Cre-expressing adenovirus (AdCre) for deletion of this gene. Relative to parallel cultures infected with a control GFP-expressing adenovirus (AdGFP), keratinocytes with deletion of the Notch1 gene exhibited a significantly enhanced apoptotic response to UVB under both growing and differentiating conditions (Figure 2B). Besides TUNEL assays, the increased susceptibility of keratinocytes with Notch1 deletion to UVB-induced apoptosis was confirmed by immunoblot analysis with antibodies against the caspase 3 cleaved form of PARP (Figure 2C). Efficient loss of Notch1 expression upon infection with AdCre was confirmed by immunoblot analysis with an anti-Notch1 antibody (Figure 2C). *In vivo*, mice with a conditional keratinocyte-specific deletion of the Notch1 gene were exposed to UVB in parallel with corresponding controls. Even in this case, deletion of the Notch1 gene resulted in enhanced sensitivity to UVB-induced apoptosis (Figure 2D).

To assess whether increased Notch signalling exerts a converse anti-apoptotic function, mouse primary keratinocytes were infected with adenoviruses expressing a constitutive active form of Notch1 (AdNIC) in parallel with AdGFP. As shown in Figure 2E, expression of constitutive active Notch1 rendered keratinocytes more resistant to UVB-induced apoptosis, mirroring the increased sensitivity of cells with Notch1 deletion. These protective effects were not due to cell cycle arrest caused by activated Notch1 expression, as they were not observed after similar adenovirus-mediated expression of CDK inhibitors such as $p21^{WAF1/Cip1}$, $p27^{Kip1}$ and $p16^{Ink4a}$ (Figure 2F). As NF-κB activity is induced by Notch activation (Nguyen *et al*, 2006), an attractive possibility was that the pro-survival effect of Notch activation in keratinocytes depends on NF-κB. However, this does not appear to be the case, as the anti-apoptotic effects of activated Notch1 were counteracted to a very limited extent by NF-κB inhibition as achieved by the concomitant expression of an IκB super-repressor (Wang *et al*, 1999) (Figure 2G).

To evaluate whether the Notch pathway has a similar function in human keratinocytes, we relied on two complementary approaches. Treatment with γ-secretase inhibitors, for example DAPT, blocks activation of endogenous Notch receptors (Geling *et al*, 2002; Morohashi *et al*, 2006), whereas expression of a 51-amino-acid peptide corresponding to the amino terminus of the MAML1 protein (MAM51) provides an effective method to suppress canonical Notch/CSL/MAML-dependent transcription (Weng *et al*, 2003). For the first approach, we established a dose–response curve of primary human keratinocytes treated with increasing concentrations of the DAPT plus/minus UVB exposure. Expression levels of the HERP1 gene were used to assess the inhibitory effects on endogenous Notch signalling (Figure 3A). Parallel TUNEL assays showed a good correlation between the extent of inhibition of HERP1 expression by increasing DAPT concentrations and the level of UVB-induced apoptosis (Figure 3B). For the second approach, keratinocytes were infected with a

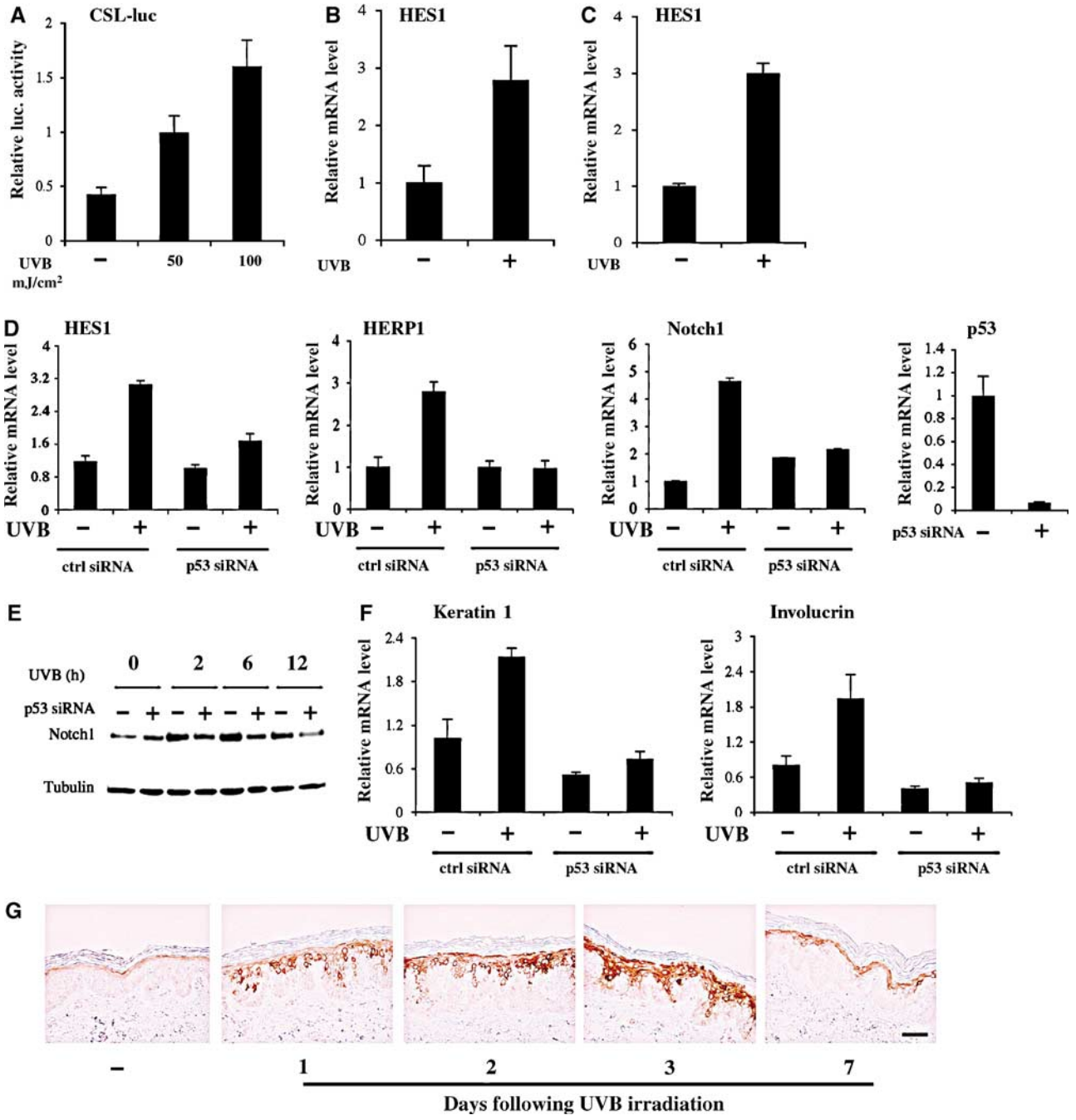


Figure 1 Increased Notch1 signalling and expression in the keratinocyte UVB response. (**A**) Primary mouse keratinocytes were transfected with a synthetic Notch-responsive promoter consisting of six CSL binding sites in front of the luciferase gene (CSL-luc), followed, 24 h later, by UVB irradiation (50 or 100 mJ/cm^2). Promoter activity was measured 24 h after irradiation by luciferase assays, using a *Renilla* reporter with a minimal promoter for internal value normalization. (**B**) Primary mouse keratinocytes were irradiated with UVB (50 mJ/cm^2), followed, 2 h later, by measurement of HES1 mRNA levels by real-time RT–PCR. Values are expressed as relative units after internal normalization for GAPDH mRNA levels. (**C**) Back skin of 3 days old mice was irradiated with UVB (220 mJ/cm^2). Eight hours later, the epidermis was separated from the underlying dermis by a brief heat treatment (Nguyen *et al*, 2006) followed by total RNA preparation and analysis of HES1 mRNA expression by real-time RT–PCR, using GAPDH mRNA for normalization. (**D**) Primary human keratinocytes transfected with siRNAs for p53 or scrambled siRNA controls for 48 h were irradiated with UVB (50 mJ/cm^2) followed, 2 h later, by measurement of HES1, HERP1 and Notch1 expression levels by real-time RT–PCR, with 36B4 mRNA for normalization. Suppression of endogenous p53 was verified by real-time RT–PCR. (**E**) Primary human keratinocytes were treated as before and Notch1 protein levels were analysed at various times (hours) after irradiation by immunoblotting with the corresponding antibody, using blotting for γ-tubulin as an equal loading control. (**F**) RNA samples from the same cells as in panel D were analysed for levels of keratin 1 and involucrin differentiation marker expression by real-time RT–PCR with the corresponding specific primers. (**G**) Mid-back skin of healthy male volunteers, with their informed consent, was irradiated with an FL20S-E Lamp (290–320 nm) (Toshiba, Tokyo). Biopsies taken at the indicated times (days) were processed for immunostaining with antibodies specific for cleaved from of Notch1 (Val1744). Bar, 30 μm.

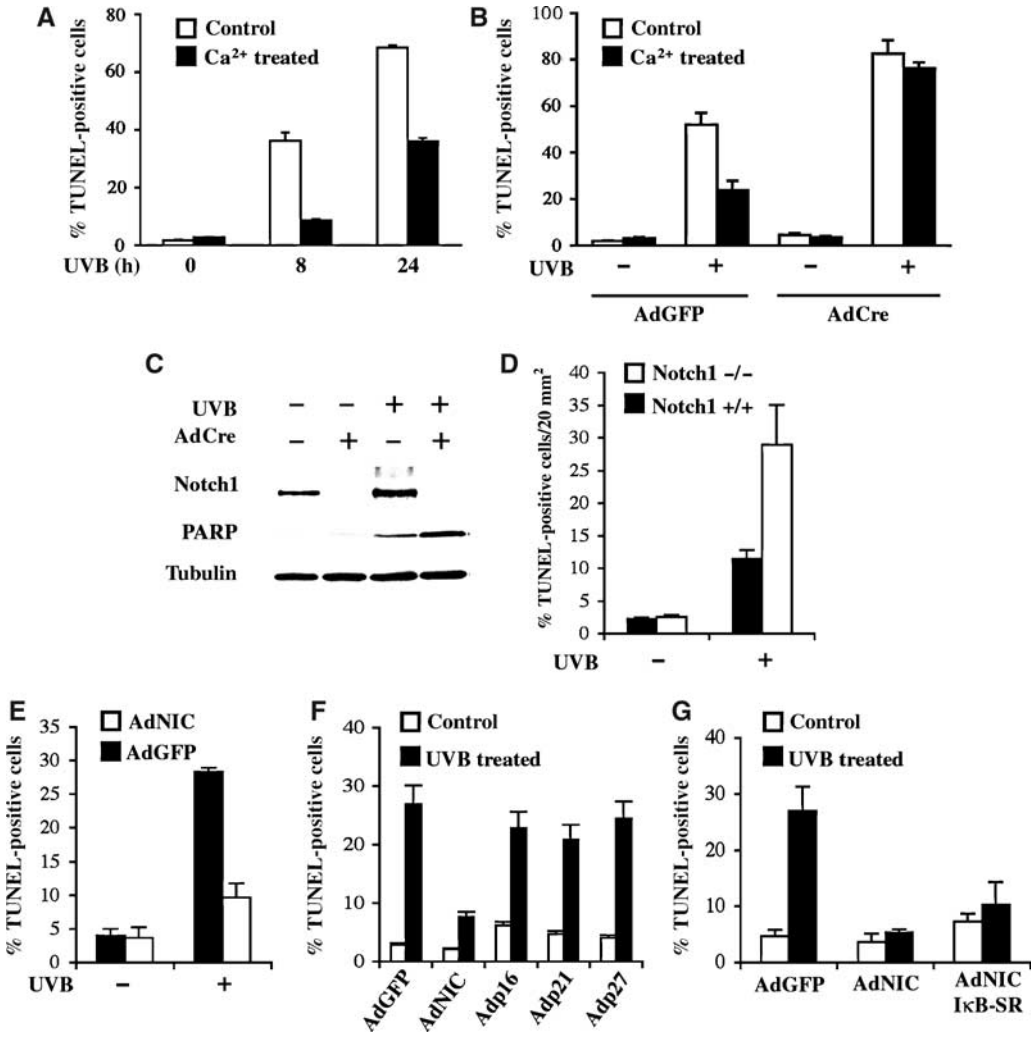


Figure 2 Protective function of Notch signalling in the UVB response of mouse keratinocytes and skin. (**A**) Primary mouse keratinocytes under growing conditions and at 24 h of calcium-induced differentiation (Missero *et al*, 1996) were treated with UVB (50 mJ/cm^2) followed, 8 and 24 h later, by determination of the apoptotic response by TUNEL assays. (**B**) Primary keratinocytes from mice homozygous for the Notch1 gene flanked by loxP sites (Notch1$^{loxP/loxP}$) were infected with an AdCre, to induce deletion of the Notch1 gene, or AdGFP control. At 72 h after infection, part of the cells was induced to differentiate by calcium treatment. After 24 h, keratinocytes under either growing or differentiating conditions were treated with UVB (50 mJ/cm^2). The apoptotic response was determined 12 h later by TUNEL assays. (**C**) Primary mouse keratinocytes plus/minus deletion of the Notch1 gene as in the previous panel were analysed, under growing conditions, by immunoblotting with antibodies specific for the caspase 3 cleaved form of PARP in parallel with antibodies against Notch1. (**D**) Mice with Cre-induced keratinocyte-specific deletion of the Notch1 gene (Notch1$^{loxP/loxP}$ × K5-Cre-PR1) in parallel with their Cre-negative littermates (Notch1$^{loxP/loxP}$) (Rangarajan *et al*, 2001) were irradiated with UVB (140 mJ/cm^2) on their back skin, at 8 weeks of age. Apoptosis was measured 12 h later by TUNEL assays. (**E**) Primary mouse keratinocytes were infected with a recombinant adenovirus expressing a constitutive active form of Notch1 (AdNIC) or AdGFP control. After16 h, cells were UVB irradiated (50 mJ/cm^2) and the apoptotic response was evaluated 8 h later by TUNEL assays. (**F**) Primary mouse keratinocytes were infected with AdNIC and AdGFP in parallel with recombinant adenoviruses expressing the CDK inhibitors p16^{INK4a}, p21$^{WAF1/Cip1}$ and p27^{Kip1}. After 16 h, cells were UVB irradiated (50 mJ/cm^2) and the apoptotic response was evaluated 8 h later by TUNEL assays. (**G**) Primary mouse keratinocytes were infected with AdGFP and with the AdNIC virus individually and in combination with an adenovirus expressing a stabilized super-repressor mutant form of IκBα (IκB-SR) (Wang *et al*, 1999). Cells were subsequently irradiated and analysed by TUNEL assays as in the previous panels.

retrovirus expressing the MAM51 dominant-negative peptide fused to GFP, in parallel with a control retrovirus expressing GFP alone. As shown in Figure 3C, cells expressing the MAM51 peptide exhibited a significantly higher apoptotic response to UVB exposure than the controls. Conversely, activation of endogenous Notch signalling by co-culture of human primary keratinocytes with fibroblasts expressing the Notch ligand Delta decreased significantly UVB-induced apoptosis (Figure 3D).

DNA damage is a main direct consequence of UVB exposure, which triggers apoptosis and which has been recently reported to induce Notch1 expression (Yugawa *et al*, 2007). As after UVB treatment, human keratinocytes with suppressed Notch signalling, by either DAPT treatment or MAM51 expression, exhibited a similarly increased apoptotic reaction in response to DNA-damaging agents such as MMS (methyl methanesulphonate) or 4-NQO (4-quinoline-1-oxide) (Figure 3E).

Canonical notch signalling protects keratinocytes against UVB-induced apoptosis through negative regulation of FoxO3a expression

Our finding that the expression of the MAM51 dominant-negative peptide increases the sensitivity of keratinocytes to

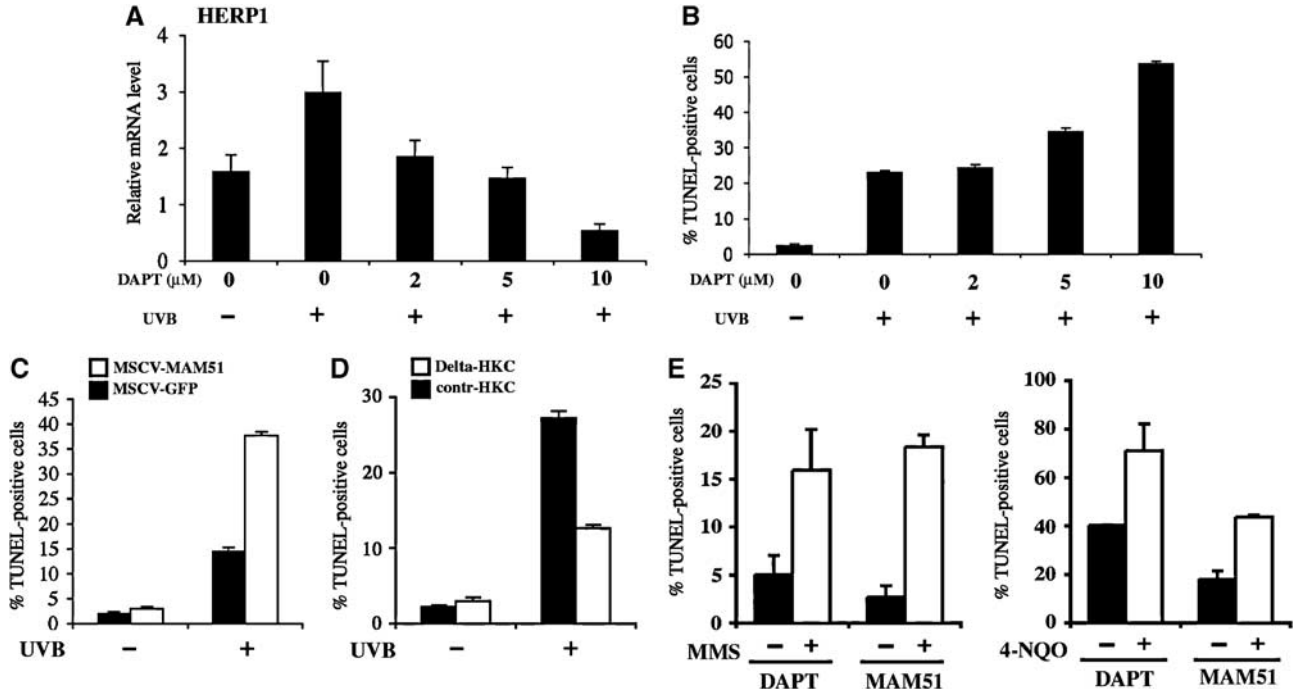


Figure 3 Protective function of Notch signalling in human keratinocytes against UVB exposure or pharmacologically induced DNA damage. (**A**) Primary human keratinocytes were treated with DMSO vehicle or increasing concentrations of DAPT for 16 h, followed by UVB irradiation (50 mJ/cm^2). After 8 h, cells were analysed for HERP1 mRNA expression levels by real-time RT–PCR analysis. (**B**) Cultures treated in parallel as in panel A were analysed for their apoptotic response by TUNEL assays. (**C**) Primary human keratinocytes infected with a retrovirus expressing the MAM51 peptide (MSCV-MAM51) or GFP control (MSCV-GFP) were irradiated with UVB (50 mJ/cm^2) and the apoptotic response was measured by TUNEL assays 8 h later. (**D**) Human primary keratinocytes with stable GFP expression (by retroviral infection) were co-cultured with control mouse NIH3T3 fibroblasts (contr-HKC) or fibroblasts stably expressing full-length Delta 1 (Delta-HKC) for 48 h, followed by UVB irradiation (50 mJ/cm^2). Eight hours later, the apoptotic response of the GFP-labelled keratinocytes was measured by TUNEL assays. Values are expressed as a percentage of GFP-labelled cells that were TUNEL positive. (**E**) Primary human keratinocytes treated with DAPT (+) or DMSO (−) or infected with retroviruses expressing the MAM51 peptide (+) or GFP control (−) were treated with MMS (100 μg/ml) for 4 h or 4-NQO (2 mg/ml) for 1 h. The apoptotic response was measured by TUNEL assays 12 h later.

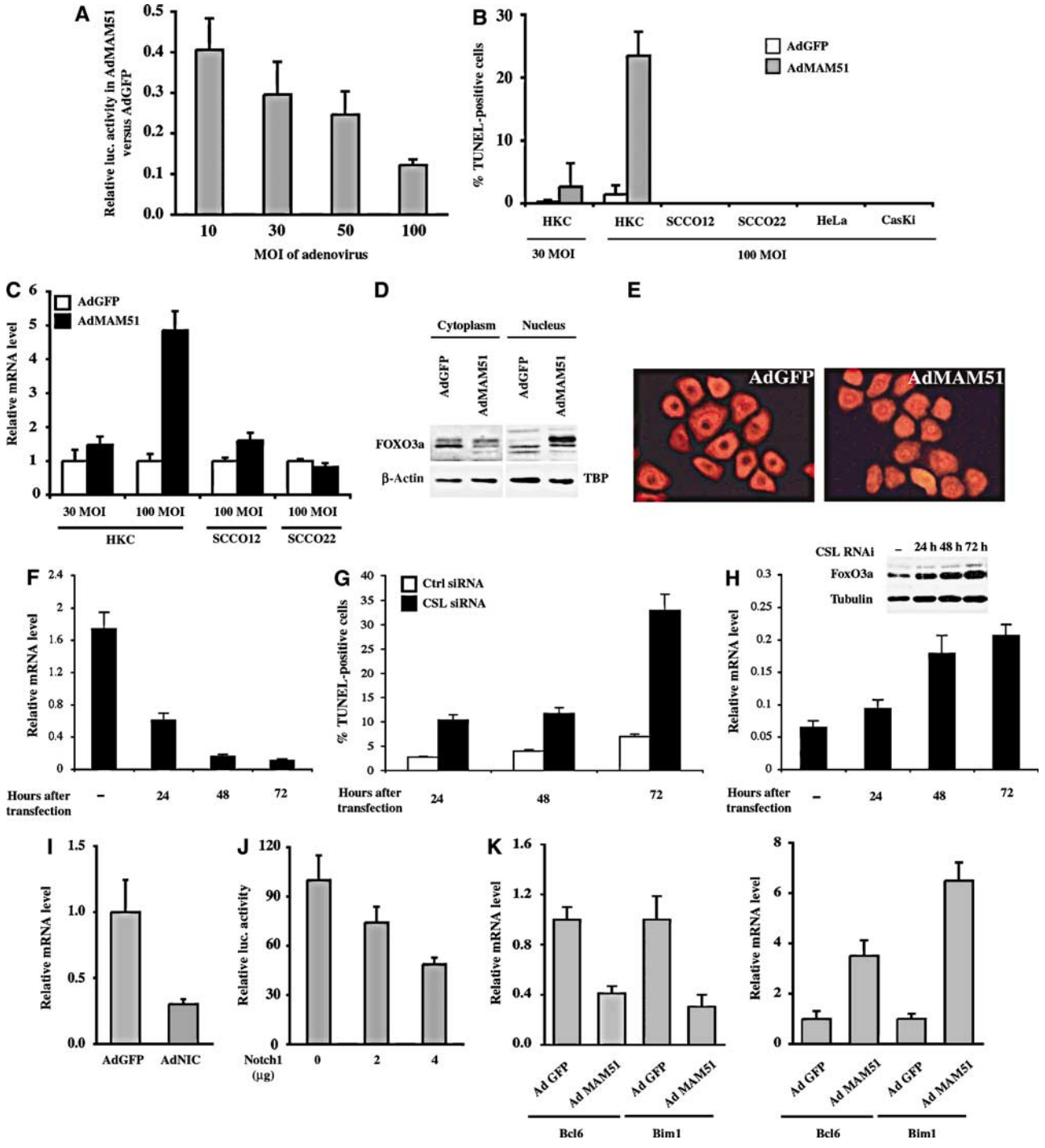
UVB-induced apoptosis pointed to canonical Notch/CSL/MAML-dependent transcription as the likely underlying mechanism. For further insights, we set up an adenoviral delivery system for expression of the MAM51 peptide in increasing amounts as a function of multiplicity of infection (MOI). Activity of Notch signalling, as assayed by a Notch/CSL promoter reporter assay, was strongly suppressed by infection of primary human keratinocytes with the MAM51-expressing adenovirus (AdMAM51) at high MOI, whereas a progressively lesser suppression was observed after infection with lower viral amounts (Figure 4A). Biologically, greater suppression of Notch signalling by MAM51 expression was already sufficient to induce apoptosis in a large fraction of human primary keratinocytes without any UVB exposure, whereas lower Notch suppression caused a lesser apoptotic response (Figure 4B). The pro-apoptotic effects of elevated MAM51 expression do not reflect aspecific toxicity, as they were not observed in a number of keratinocyte-derived cancer cell lines, including SCCO12, SCCO22, HeLa and CasKi (Figure 4B). Global analysis of gene expression was used to identify molecular targets of Notch/MAML-dependent transcription that may be responsible for its protective anti-apoptotic function. We focused on genes with selectively increased expression in human primary keratinocytes by high versus low levels of MAM51 expression and with opposite modulation by activated Notch1. A restricted number of genes matched these criteria, including FoxO3a, a transcription factor with a key pro-apoptotic function (Essafi *et al*, 2005; You *et al*, 2006) (Supplementary Table 1).

Real-time RT–PCR analysis confirmed elevated induction of the FoxO3a gene in human primary keratinocytes with high versus low levels of MAM51 expression, with little or no induction in SCCO12 and SCCO22 cancer cell lines (Figure 4C). The induction of FoxO3a gene expression was accompanied by an increased nuclear localization of the protein (Figure 4D and E).

To assess whether other means to suppress Notch signalling, and more specifically Notch/CSL-dependent transcription, elicit the same effects as high doses of MAM51 expression, we transfected human primary keratinocytes with siRNAs specific for the CSL gene in parallel with scrambled siRNA controls. Real-time RT–PCR analysis showed that a progressive suppression of CSL gene expression over time (Figure 4F) was associated with an increasing fraction of cells spontaneously undergoing apoptosis (Figure 4G) and a parallel increase in FoxO3a mRNA and protein expression (Figure 4H).

Mirroring the above findings, expression of activated Notch1 caused down-modulation of FoxO3a gene expression (Figure 4I) in parallel with suppression of FoxO3a activity, as assessed by transient transfection promoter activity assays with a FoxO3a-responsive reporter (Figure 4J) and measurement of the endogenous FoxO3a target genes Bcl6 and Bim1 (Tang *et al*, 2002; Essafi *et al*, 2005) (Figure 4K).

HES/HERP proteins are well-established mediators of the suppressive effects of Notch activation on gene expression. To assess whether increased expression of one or more of these proteins can reproduce the effects of activated Notch1, human keratinocytes were infected with recombinant adenoviruses expressing the HES1, HERP1 and HERP2 proteins. Strong down-modulation of FoxO3a gene expression was caused by increased HERP1 expression, a lesser down-modulation by HES1 and little or no effects by HERP2 (Figure 5A). Sequence analysis of the FoxO3a promoter region revealed the presence of two HES/HERP binding sites around position −1.2 kb (specifically −1.21 and −1.17 kb from the transcriptional start site) and one at position −0.8 kb (Figure 5B). Binding to these regions was assessed by chromatin immunoprecipitation (ChIP) assays with antibodies against the adenovirally expressed epitope-tagged proteins. Paralleling the differential effects on the endogenous gene, we found very effective binding of HERP1 to the −1.2 kb region of the FoxO3a promoter, with lesser binding to the −0.8 kb site and no binding to an upstream region devoid of HES/HERP sites (−4.7 kb) (Figure 5C). By contrast, there was no detectable binding with HERP2, and the HES1 protein bound to the same promoter regions but with lower efficiency (note the difference of scale in the HERP1 versus HES1 graphs) (Figure 5C



and D). Antibodies suitable for immunoprecipitation of endogenous HES/HERP proteins are not available. However, HES/HERP proteins bind to a specific transcriptional corepressor, Tle-1 (Stifani *et al*, 1992), which can be readily immunoprecipitated with the existing antibodies (Nuthall *et al*, 2004; Mammucari *et al*, 2005). ChIP assays with these antibodies showed that UVB exposure of human primary keratinocytes induced association of the Tle-1 protein to the same −1.2 kb region of the FoxO3a promoter bound by the HERP1/HES1 proteins (Figure 5E).

A major form of FoxO3a regulation is Akt-dependent phosphorylation (Brunet *et al*, 1999). The induction of FoxO3a expression by MAM51 overexpression was unaffected by inhibition of the PI3K/Akt pathway by wortmannin treatment (Figure 6A). Human keratinocytes with lower levels of MAM51 expression (as achieved by retroviral transduction) also exhibited induction of FoxO3a protein expression upon UVB exposure, with no changes in its Akt phosphorylation (Figure 6B). Upregulation of FoxO3a expression at the mRNA level was also observed in these cells (Figure 6C), as well as after UVB irradiation of mouse keratinocytes with Cre-mediated deletion of the Notch1 gene (Figure 6D). FoxO3a mRNA levels were also increased in human keratinocytes concomitantly treated with UVB and DAPT with actinomycin D treatment, showing that the induction of FoxO3a expression by UVB irradiation and Notch suppression is not due to increased message stability (Figure 6E).

To assess the functional significance of FoxO3a upregulation in keratinocytes with concomitant UVB treatment and Notch suppression, we employed an siRNA-mediated knock-down approach. Control and MAM51-expressing keratinocytes were transfected with FoxO3a-specific siRNAs in parallel with scrambled siRNA control, followed by real-time RT–PCR at 48 h after transfection to determine the efficiency of gene knock-down (Figure 6F). When challenged with UVB, the increased apoptotic response of the MAM51-expressing keratinocytes, as assessed by both TUNEL assays and immunoblotting for activated caspase 3, was counteracted by the FoxO3a knock-down (Figure 6G and H). Similar results were observed with DAPT-treated cells plus/minus FoxO3a knock-down upon UVB treatment (Figure 6I).

Discussion

UV light is a major aetiological agent of skin ageing and cancer (de Gruijl, 1999; Armstrong and Kricker, 2001). Keratinocytes in the outermost layer of the epidermis are the most exposed to the damaging effects of high-energy UVB but their differentiated state renders them more resistant than cells of the proliferative compartment (Chaturvedi *et al*, 2004; this communication: Figure 2A). In parallel with its key role in promoting differentiation, we have shown here that Notch signalling has an equally important function in protecting keratinocytes against UVB exposure, both *in vitro* and *in vivo*. This protective function is mediated by the 'canonical' Notch/HERP pathway through a novel mechanism involving transcriptional down-modulation of the FoxO3a gene.

p53 has a key role in the UV/DNA damage response of cells, controlling the decision between growth arrest and apoptosis by a number of alternative mechanisms (Latonen and Laiho, 2005; D'Errico *et al*, 2007). The recent finding that Notch1 is a p53 target gene in keratinocytes pointed to the possibility that this gene is also implicated in the UVB/DNA damage response of these cells (Lefort *et al*, 2007; Yugawa *et al*, 2007). In fact, we have found that the apoptotic response to UVB is increased in the skin of mice with deletion of the Notch1 gene as well as in keratinocytes with down-modulation of Notch signalling, whereas increased Notch signalling protects against this process. Consistent with a protective anti-apoptotic function of Notch in the skin, there is a recent report of spontaneously increased apoptosis in mice with double keratinocyte-specific deletion of the Notch1 and Notch2 genes (Lee *et al*, 2007).

As for its role in growth/differentiation control, Notch signalling can exert either a pro- or anti-apoptotic function in a manner that is highly cell- and context-dependent. For instance, in neuronal progenitor cells, Notch activation leads to apoptosis through increased nuclear accumulation of p53 and subsequent upregulation of its pro-apoptotic targets (Yang *et al*, 2004). Similarly, in B-cell leukaemia cells, activation of Notch signalling and induction of its downstream target HES1 lead to growth arrest and cell death (Zweidler-McKay *et al*, 2005). A variety of mechanisms have been

Figure 4 Concomitant induction of apoptosis and FoxO3a expression by dose-dependent suppression of canonical Notch signalling. (**A**) Primary human keratinocytes were transfected with the Notch-pGA reporter (pGA-Luc; 0.5 μg) together with the phRL-TK *Renilla* reporter and subsequently infected with an adenovirus expressing the MAM51 peptide or GFP control at the indicated MOI. Promoter activity was measured 24 h later. Results are expressed as a ratio of luciferase activity (after *Renilla* normalization) in cells infected with the AdMAM51 versus AdGFP viruses at the various MOIs. (**B**) Human primary keratinocytes, together with keratinocyte-derived cancer cell lines (SCCO12, SCCO22, HeLa and CasKi), were infected with AdGFP and AdMAM51 at the indicated MOI. The fraction of apoptotic cells was assessed 24 h later by TUNEL assays. (**C**) Human primary keratinocytes and the SCCO12 and SCCO22 cell lines were infected with the AdMAM51 and AdGFP viruses at the indicated MOI and analysed 24 h later for levels of FoxO3a mRNA by real-time RT–PCR. Values are expressed in arbitrary units after normalization for β-actin expression. (**D**) Human primary keratinocytes were infected with AdMAM51 and AdGFP at an MOI of 100, followed by nuclear and cytoplasmic fractionation and immunoblot analysis for the FoxO3a protein, using the β-actin and TBP proteins as equal loading controls for the cytoplasmic and nuclear fractions, respectively. (**E**) Human primary keratinocytes were infected with AdMAM51 and AdGFP at an MOI of 100 as before. Cells were fixed 24 h later and processed for immunofluorescence analysis with an antibody against FoxO3a. (**F**) Human primary keratinocytes were transfected with siRNA specific for CSL in parallel with scrambled siRNA control, followed by assessment of CSL expression at 24, 48 and 72 h after transfection by real-time RT–PCR. (**G**) Parallel cultures treated as in panel F were analysed by TUNEL assays at various times (hours) after CSL knock-down. (**H**) Keratinocytes with and without CSL knock-down as in the previous experiments were analysed for levels of FoxO3a expression by real-time RT–PCR and immunoblotting (inset). (**I**) Primary human keratinocytes were infected with adenoviruses expressing activated Notch1 or GFP control for 24 h and analysed for levels of FoxO3a mRNA by real-time RT–PCR. (**J**) Primary human keratinocytes were transfected with a FoxO3a-responsive reporter (FHRE-luc) plus increasing amounts of an expression vector for activated Notch1. Promoter activity was determined at 30 h after transfection by luciferase assays, using the phRL-TK *Renilla* reporter for internal normalization. (**K**) Primary human keratinocytes infected with adenoviruses expressing activated Notch1 or MAM51 in parallel with GFP controls were analysed by real-time RT–PCR for levels of expression of Bcl6 and Bim1, two well-established FoxO3a targets with pro-apoptotic function.

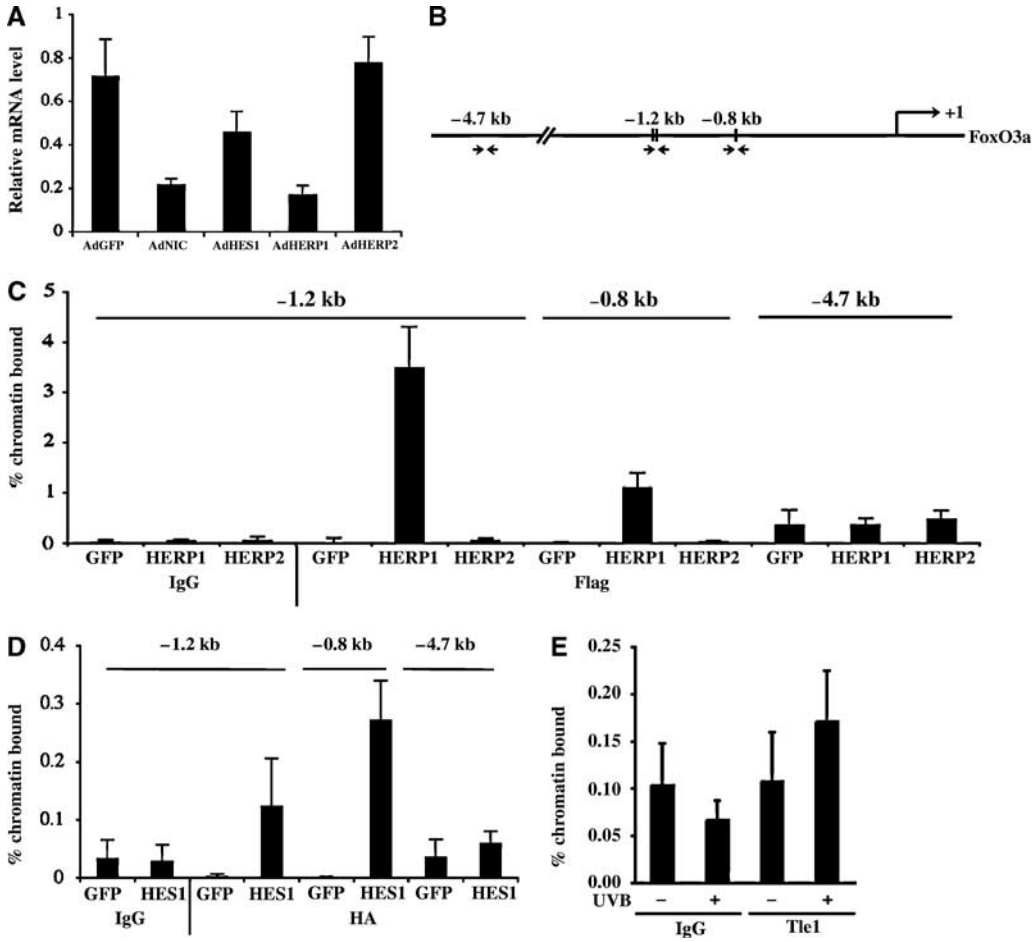


Figure 5 Differential control of the FoxO3a gene by HES/HERP family members. (**A**) Primary human keratinocytes were infected with adenoviruses expressing activated Notch1, HES1, HERP1, HERP2 or GFP control for 24 h and analysed for levels of FoxO3a mRNA by real-time RT–PCR. (**B**) The human FoxO3a promoter region was analysed by the MatInspector (Genomatix) software for analysis of transcription factor binding sites, and three CSL-binding sites at −1.21, −1.17 and −0.8 kb from the transcription initiation site were identified. Arrows refer to the position of the PCR primers utilized for the ChIP analysis of the following panels. (**C, D**) Primary human keratinocytes were infected with adenoviruses expressing HERP1 (Flag-tagged), HERP2 (Flag-tagged), HES1 (HA-tagged) or GFP. To minimize overexpression of the adenovirally encoded proteins, we used a low MOI for infection of ~30% of cells. Cells were processed for ChIP with antibodies against the Flag (C) or HA (D) tags and purified nonspecific IgGs as control. Real-time RT–PCR reactions for the −1.2 and −0.8 kb regions of the FoxO3a promoter were performed along with PCR of an upstream region located at −4.7 kb. The primers used in the RT–PCR reactions are listed in Supplementary Table 2. (**E**) Primary human keratinocytes treated or not with UVB (50 mJ/cm^2) were processed 6 h later for ChIP with an antibody against the HES/HERP-associated Tle1 protein (Stifani *et al*, 1992) or purified rabbit IgGs, followed by real-time PCR analysis of the −1.2 kb region of the FoxO3a promoter.

implicated in the Notch pro-survival function. These include, in T cells, the interaction with the orphan receptor protein Nur77 (Jehn *et al*, 1999); in myeloma cells, the induction of p21$^{WAF/Cip}$ expression; and, in cervical cancer cell lines, the induction of PI3K/Akt/mTOR signalling through as yet undefined mechanisms involving the 'non-canonical' Notch pathway (Nair *et al*, 2003; Sade *et al*, 2004). Other potential cell survival mechanisms in which Notch has been implicated include interference with JNK activation (Kim *et al*, 2005), upregulation of NF-κB activity (Oswald *et al*, 1998) and increased expression of proteins with direct anti-apoptotic function, such as Bcl-2 and Mcl-1 (MacKenzie *et al*, 2004; Oishi *et al*, 2004). We have shown here that in primary keratinocytes, but not in keratinocyte-derived cancer cell lines, 'canonical' Notch signalling exerts a pro-survival function that is linked to transcriptional down-modulation of the FoxO3a gene. This occurs without detectable changes in PI3K/Akt-dependent phosphorylation, which is a major form of FoxO3a regulation (Brunet *et al*, 1999). Rather, the 'canonical' Notch/HERP pathway is an important negative regulator of FoxO3a expression, through a mechanism involving binding of the HES/HERP/Tle transcription repressor complex to the FoxO3a promoter.

Like Notch, FoxO3a has multiple cellular functions, ranging from cell cycle control, differentiation, resistance to oxidative stress and apoptosis (Burgering and Kops, 2002). Target genes that mediate the pro-apoptotic function of this transcription factor include those for the Bcl-2 family members bNIP3, BCL2L11 and Bim1 (Greer and Brunet, 2005). FoxO3a can also induce apoptosis through more indirect mechanisms, such as induction of the transcriptional repressor Bcl6, which in turn down-modulates expression of the pro-survival Bcl-X(L) gene (Tang *et al*, 2002). In human keratinocytes, FoxO3a was recently found in a complex with Smad4 to control a subset of genes with cell cycle regulatory function (p21$^{WAF1/Cip1}$ and p15^{INK4b}), mediators

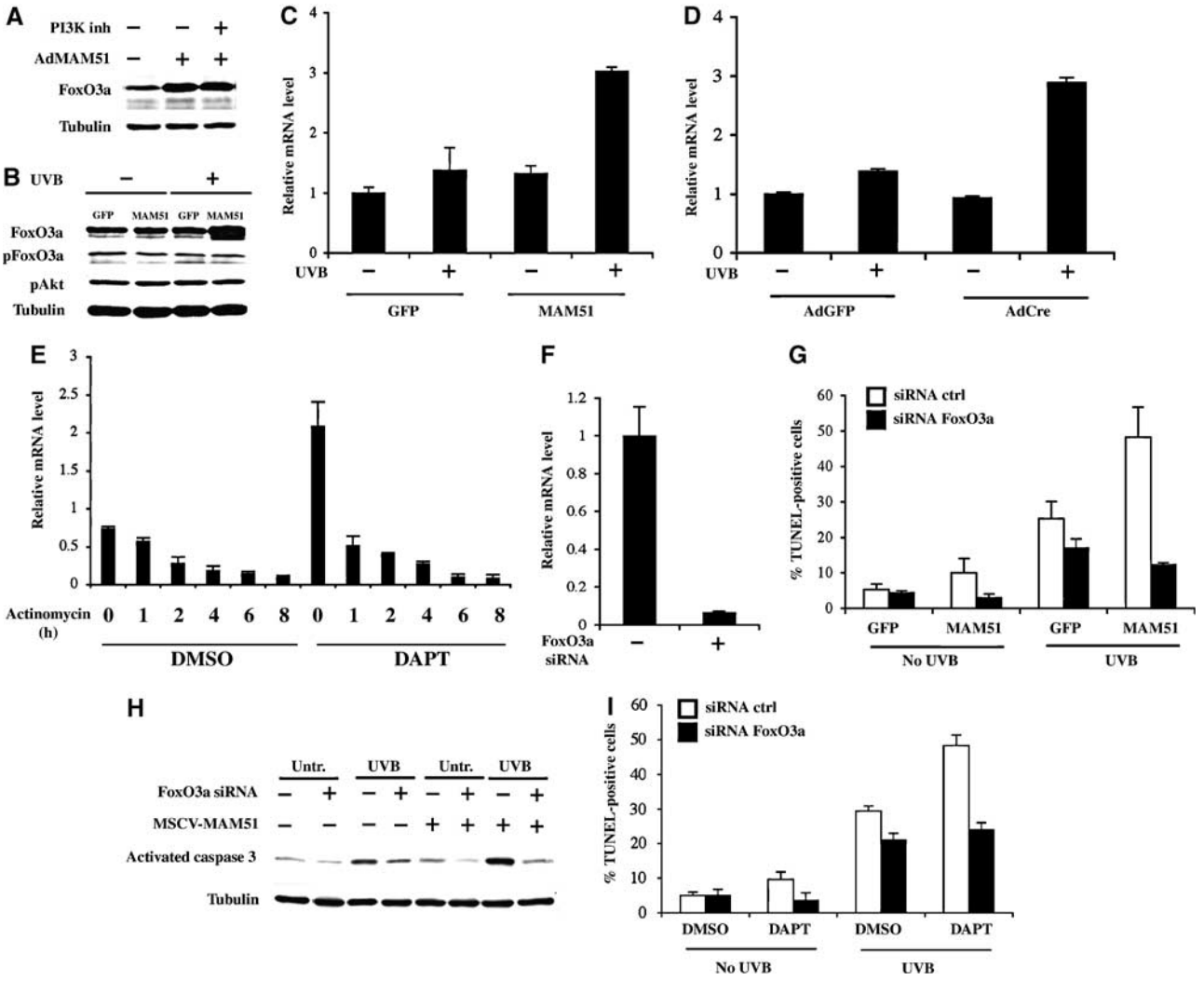


Figure 6 The Notch protective function in the keratinocyte UVB response through down-modulation of FoxO3a expression. (**A**) Primary human keratinocytes infected with AdMAM51 and GFP control at 100 MOI for 24 h were either untreated or treated with the PI3K inhibitor wortmannin (100 nM) for an additional 8 h, followed by analysis of FoxO3a protein levels by immunoblotting with a corresponding specific antibody. (**B**) Primary human keratinocytes were stably infected with a MAM51-expressing retrovirus in parallel with a GFP control virus and either untreated or treated with UVB (50 mJ/cm^2). Cells were analysed 24 h later for levels of total FoxO3a protein as well as protein phosphorylated at the critical Akt recognition site (Thr32) (pFoxO3a) by immunoblotting with the corresponding specific antibodies. The same extracts were also probed with antibodies against the phosphorylated active form of Akt (pAkt), in parallel with γ-tubulin as an equal loading control. (**C**) Similarly, retrovirally infected cells plus/minus UVB treatment as in panel B were analysed for FoxO3a mRNA levels by real-time RT–PCR. (**D**) Primary keratinocytes from Notch1$^{loxP/loxP}$ mice were infected with AdCre, to induce deletion of the Notch1 gene, or AdGFP control, as in Figure 1. Cells were subsequently treated with UVB (50 mJ/cm^2) followed, 30 h later, by measurement of FoxO3a mRNA levels by real-time RT–PCR. (**E**) Human primary keratinocytes were incubated for 16 h with DAPT (10 μM) or DMSO control, UVB-irradiated (50 mJ/cm^2) and, 8 h later, either collected or treated with actinomycin D (8 μg/ml) for the indicated times (hours). FoxO3a mRNA levels were assessed by real-time RT–PCR. (**F**) Human primary keratinocytes were transfected with validated siRNAs for FoxO3a or scrambled controls, followed by measurement of FoxO3a mRNA levels 48 h later by real-time RT–PCR. (**G**) Human keratinocytes stably infected with the MAM51- and GFP-expressing retroviruses were transfected with FoxO3a siRNAs or scrambled siRNA controls followed, 48 h later, by UVB irradiation (50 mJ/cm^2). The apoptotic response was determined 24 h after irradiation by TUNEL assays. (**H**) Similarly treated cells were analysed for levels of activated caspase 3, by immunoblotting with the corresponding antibodies, with γ-tubulin as an internal control. (**I**) Human primary keratinocytes were transfected with siRNAs for FoxO3a or scrambled controls and 24 h later treated with DAPT (10 μM) or DMSO control. Cells were UVB irradiated 24 h later and, after another 24 h, were analysed by TUNEL assays.

of stress responses (Gadd45α and Gadd45β) and adaptive cell signalling (Jagged1, CDC42EP3 and OVOL1) (Gomis *et al*, 2006), some of which are also under Notch control in these cells (Nguyen *et al*, 2006; our unpublished observations). Thus, an intriguing possibility is that FoxO3a participates in the already established cross-talk between Notch and Smad signalling in this cell type (Blokzijl *et al*, 2003). Irrespective of the detailed mechanism, this Notch1–FoxO3a connection may provide an opportunity for novel drug design approaches to maximize protection against the UVB/DNA damage response of the skin. It will also be interesting to explore whether regulation of FoxO3a by Notch signalling occurs in other cellular and developmental contexts where these important regulators of cell physiology have been implicated.

Materials and methods

Cell culture, viruses and plasmids

Primary mouse keratinocytes were prepared and cultured in minimal essential medium with 4% Chelex-treated fetal calf serum

(Hyclone), epidermal growth factor (10 ng/ml; BD Biosciences) and 0.05 mM $CaCl_2$ (low-calcium medium) as described previously (Missero *et al*, 1996). Primary human keratinocytes from adult skin were isolated and cultured as described previously (Nguyen *et al*, 2006). NIH fibroblasts expressing full-length Delta or empty vector control (Trifonova *et al*, 2004) were co-cultured with human primary keratinocytes stably expressing GFP for 48 h as described (Lowell *et al*, 2000). SCCO12 and SCCO22 cells were provided by Dr J Rocco (Massachusetts General Hospital, Boston, MA), whereas HeLa and CasKi cell lines were from ATCC. The MSCV-MAM51 (Weng *et al*, 2003), Ad-HES1 (Sriuranpong *et al*, 2002), Ad-IκB-SR (Wang *et al*, 1999), Ad-HERP1 (Mammucari *et al*, 2005), AdNIC, AdCre (Rangarajan *et al*, 2001), and Adp16, Adp21, Adp27 and AdGFP (Devgan *et al*, 2005) have been described previously. The recombinant adenovirus expressing truncated form of MAML1 (MAM51) was obtained by removing the insert cDNA (coding for amino acids 13–74 of the MAML1 protein fused to GFP; Weng *et al*, 2003) from MSCV-MAM51 by *Cla*I/*Bgl*II digestion and inserting it into the *Hin*dIII/*Bgl*II sites of the pAdTrack-CMV vector, followed by recombination into the adenoviral backbone plasmid pAdEasy-1 in bacteria (He *et al*, 1998). Recombinant adenoviruses were used at an MOI of 100, unless otherwise specified.

For siRNA experiments, cells were transfected as described previously (Nguyen *et al*, 2006) with validated Stealth-siRNAs (200 nM) for human p53, CSL (Invitrogen) and FoxO3a (Invitrogen) in parallel with corresponding nonspecific Stealth siRNA negative control Medium GC (Invitrogen).

Methyl methanesulphonate 4-nitroquinoline-1-oxide and wortmannin were from Sigma.

UVB irradiation

Fresh mouse or human keratinocytes were used for *in vitro* UVB treatment using a custom-made UVB irradiation apparatus with four photochemical lamps (RPR 3000, Southern N.E., Ultraviolet Co., Bradford, CT) as described previously (Wang *et al*, 2005). The derived UVB dose was measured each time by using a photometer (model IL 1400A, International Light Inc., Newburyport, MA). *In vivo* treatment of mice (8–9 weeks old female mice or 3 days old newborn as indicated), using the same UVB lamp and photometer as above, was as described (Wang *et al*, 2005). *In vivo* treatment of human volunteers, with their informed consent, was performed with an FL20S-E Lamp (290–320 nm) (Toshiba, Tokyo).

Promoter activity assays

The Notch- and FoxO3a-responsive promoters used in these experiments have been described previously (Taniguchi *et al*, 1998). Transient transfections for promoter activity assays were performed as described previously (Rangarajan *et al*, 2001) using cotransfection with a TK-*Renilla* reporter (Promega) for internal normalization. Total quantities of DNA were kept constant by adding appropriate amounts of empty vectors.

Analysis of gene expression and ChIP assays

mRNA expression was quantified by real-time RT–PCR as described previously (Mammucari *et al*, 2005). Each sample was tested in triplicate and results were normalized by real-time PCR of the same cDNA with GAPDH (for mouse samples) and 36B4 or β-actin (for human samples) primers. cDNA microarray analysis and ChIP assays were carried out as described previously (Devgan *et al*, 2005; Lefort *et al*, 2007). The sequence of the specific primers used for these experiments is provided in Supplementary Table 2.

Antibodies

We used the following antibodies: Notch1 (Santa Cruz, C-20), activated Notch1 (Cell Signaling), FoxO3a (Upstate), phospho-FoxO3a (Upstate), Akt (Cell Signaling), phospho-Akt (Cell Signaling), cleaved PARP (Cell Signaling), caspase 3 (Cell Signaling), activated caspase 3 (Cell Signaling), anti-Flag (Sigma), anti-HA (Sigma), γ-tubulin (Sigma), β-actin (Sigma), TATA box binding protein (Abcam), mouse and rabbit serum IgG (Sigma) and anti-Tle1 (Stifani *et al*, 1992).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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