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Original Article

Transcriptional control of late differentiation in human keratinocytes by TAp63 and Notch

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Abstract: We previously showed that in cervical carcinoma cells, the TAp63β isoform of the p63 transcription factor is negatively interfering with the carcinogenic pathways promoting anchorage-independent growth. In this study, we have defined the mechanisms underlying the effects of TAp63β through a transcriptome analysis of human keratinocytes overexpressing this protein. TAp63β modulated expression of 1203 genes (944 activated and 259 repressed; *P*-value <0.05), notably genes involved in epithelial development and keratinocyte differentiation. In comparison, while TAp63γ acts similarly to TAp63β to transactivate a selected panel of target genes, other p63 isoforms, including ΔNp63α, which is highly expressed in keratinocytes, are inactive. Upon induction of differentiation of primary human keratinocytes, we observed endogenous expression of TAp63β and γ isoforms, along with transcriptional activation of selected target genes. Intriguingly, our data also indicated that TAp63β activates transcription of members of the Notch pathway, which is known to promote keratinocyte differentiation. By inhibiting and activating the Notch pathway, we revealed a subset of TAp63β-activated genes that were co-dependent on Notch for their expression. Our work demonstrates that the shorter TAp63 isoforms (TAp63β/γ) are specifically induced in human keratinocytes and cooperate with Notch signalling to activate transcription of late differentiation genes supporting their role as putative tumor suppressors in HPV-associated tumorigenesis.

Key words: human keratinocyte differentiation – TAp63 – transcriptional activation

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Introduction

The p53 family of transcription factors also includes p63, which is a master regulator of skin homeostasis. p63 exists as six isoforms that can be broadly subdivided into two groups: transcription from a distal promoter gives rise to the TAp63 isoforms, while an alternative promoter in the first exon drives production of the truncated ΔNp63 isoforms. Alternative splicing of TAp63 and ΔNp63 finally generates either the full length α or truncated β and γ versions of the protein. Mice lacking p63 show defects in epithelial development and die at birth (1,2), illustrating p63's importance for correct development, stratification and maintenance of the epidermis (3). Previous studies have shown that knocking down all p63 transcripts causes loss of proliferative potential in epithelial stem cells (4), while knock-down of TAp63 uncovered its role in maintaining the epidermal stem cell population to avoid cellular senescence and prevent premature ageing of the epidermis (5). ΔNp63 isoforms are required to maintain the basement membrane and initiate keratinocyte differentiation through the regulation of epidermal genes *FRAS1* and *IKK*α (6) and induce *ALOX12* for barrier formation (7). Recently, TAp63 have additionally been described as specifically required for terminal differentiation of keratinocytes in appendages in zebrafish (8).

However, most studies have not addressed the roles of the individual isoforms in human skin, despite evidence of distinct transcriptional activity (9,10). Bearing in mind the importance of p63 for homeostasis of the epithelium, we find it particularly interesting that the TAp63β isoform is specifically degraded by the E6 oncoprotein of human papillomavirus (11), which causes cervical carcinoma. Moreover, our previous work indicated that TAp63β is the best expressed TAp63 isoform in several cervical carcinoma cell lines thus raising the question of its role in normal skin development.

One of p63's known functions is regulation of Notch1-dependent transcription (12), which is important for activation of cellular differentiation (13). Ligation of the Notch receptor (Notch-1 to Notch-4 in mammals) induces a series of proteolytic cleavages to release the Notch intra-cellular domain (NICD) that translocates into the nucleus and enables binding of transcriptional coactivators to target genes (14,15). Notch signalling is required for keratinocyte terminal differentiation (13,16), specifically for induction of the epidermal spinous layer (17). Notch has also been reported to induce important differentiation markers of the suprabasal layers, including *KRT1*, *KRT10* and *IVL*, and key maturation pathways involving NF-κB and PPARγ (18,19). While the importance of both p63 and Notch in the epidermis is evident, how Notch interacts with the TAp63 isoforms is unknown.

In this study, we sought to define the specific activity of TAp63 isoforms and their interaction with the Notch signalling pathway in human keratinocytes. Keratinocytes overexpressing the β and γ isoforms of TAp63, but not the TAp63α isoform, exhibited activation of late keratinocyte differentiation genes. Accordingly, in primary human keratinocytes differentiated *in vitro*, the endogenous levels of TAp63β and γ increased during differentiation. We also uncovered a functional link between TAp63β and Notch signalling that results in transcription of a subset of genes involved in keratinocyte terminal differentiation.

Materials and methods

Infection of keratinocytes with recombinant adenoviruses

Recombinant adenoviruses expressing TAp63β, TAp63γ and ΔNp63β isoforms were prepared from the pIRES expression plasmids containing these genes followed by the IRES sequence and the GFP gene as previously described (11). Recombinant adenoviruses expressing the TAp63α and ΔNp63α were a kind gift from Barry Trink (11,20).

Cells were infected with purified adenoviruses at a m.o.i. of 50 PFU/cell for 24 h.

Cell culture, treatments and transfections

N/Tert-1 cells, a gift of James Rheinwald (Harvard) (21), are normal human keratinocytes immortalized by transduction of the hTERT gene and were cultured in keratinocyte-SFM medium with L-glutamine, EGF, BPE, 3 mM $CaCl_2$ and 1% penicillin–streptomycin. Primary human epidermal keratinocytes (pooled), from CELLnTEC Advanced Cell Systems, were cultured in CnT-57 medium containing supplements A, B, C and BPE. NIH 3T3 control, and 3T3-Jagged2 cell lines, a gift from Jon Aster (Harvard), were grown in high-glucose Dulbecco's modified Eagle's medium (Invitrogen Waltham, Massachusetts, U.S.A.) with 10% heat-inactivated foetal bovine serum and 1% penicillin–streptomycin.

All cells were grown using standard procedures in a 37°C humidified incubator with 5% CO_2.

For co-culture experiments, the N/Tert-1 cells were infected with adenoviruses expressing GFP and TAp63β before addition of NIH 3T3 control and NIH 3T3-Jagged2 cells for 48 h, followed by RNA extraction. Notch inhibitors, γ-secretase inhibitors XXI, Compound E (Merck Millipore, Billerica, MA, USA) and DAPT (Sigma-Aldrich St. Louis, MO, U.S.A.) were used at 1 μM and 10 μM, respectively, for 40 h.

Microarray hybridization and analyses

Microarrays were performed by Biopolis Shared Facilities (BSF), Singapore, using the Illumina platform. Illumina Bead Array is a genomewide microarray containing 47 000 probes, including about 30 000 genes. Data were analysed using Partek® Genomics Suite software and DAVID Bioinformatics Resources (22,23).

RNA isolation and quantitative RT-PCR

Total RNA was extracted using RNAEasy Mini Kit (Qiagen Venlo, Limburg, Netherlands) before 2.5 μg of RNA was reverse transcribed with Superscript II (Invitrogen) according to the manufacturer's instructions. 1/100th of the resulting single-stranded cDNA was used for each real-time PCR in the presence of a 0.1 μM of specific primers (Data S1) and Syber Green master mix (Applied Biosystems Waltham, Massachusetts, U.S.A.). Quantitative PCR was performed with an MX3005P sequence detection system (Stratagene California, U.S.A.). Levels of each cDNA were normalized to those of histone deacetylase 1 (HDAC1) and glyceraldehyde-3-phosphate dehydrogenase (GADPH). PCR was performed in duplicate and fitted to standard curves, providing mean cycle threshold values that were translated into arbitrary units corresponding to mRNA levels. Data were analysed with MxPro v.4.00 software (Stratagene). RT-PCR data were calculated as a mean of three independent experiments, ±standard deviation. Student's paired *t*-test was used to calculate significance, $*P < 0.05$, $**P < 0.01$.

Western blot

Transduced cells were collected 24 h postinfection and protein extracts subjected to electrophoresis before transfer onto nitrocellulose membranes. The membranes were then incubated with antibodies specific for TAp63 (Poly 6189; BioLegend, California, U.S.A.), ΔNp63 (Poly 6190; BioLegend) or beta-actin (A2066; Sigma-Aldrich); and either mouse or rabbit secondary antibodies coupled to peroxidase before visualization with the Amersham ECL plus kit (GE Healthcare Life Sciences Little Chalfont, U.K.).

Immunofluorescence

Cells grown on coverslips were fixed at 4°C for 30 min with 2% paraformaldehyde (v/v). The cells were permeabilized for 30 min at room temperature with 0.1% Triton in PBS, 2% serum. After washing with PBS, cells were incubated with primary antibodies for 2 h at room temperature then washed three times with PBS, 2% serum, followed by incubation with secondary antibodies for 1 h at room temperature. Cell nuclei were stained with DAPI (Invitrogen). Primary antibodies used were specific for: paxillin (clone 5H11; ab3127; Abcam, Cambridge, U.K.), vinculin (clone hVIN-1; (Sigma-Aldrich)) or Involucrin (SY5; Novocastra Germany). Secondary antibodies were fused to Alexa fluor 568 (Invitrogen). Images were captured with a Zeiss Axio Imager (Germany).

Results

TAp63β regulates human keratinocyte late terminal differentiation markers

To identify genes specifically regulated by the TAp63β isoform, we performed microarray analyses of human N/Tert-1 keratinocytes transduced with recombinant adenoviruses expressing TAp63β.

TAp63β-overexpression in N/Tert-1 cells induced modulated expression of 1203 genes, of which 944 were activated and 259 were repressed, relative to control GFP-overexpressing cells (Table S1, S2). Modulated genes included known p63 targets such as *KRT15, IGFBP3, IL1B, IRF6, PVRL4* (24–27), as well as the known TAp63-specific target genes, *p57/KIP2 (CDKN1C)* and *AP-2γ (TFAP2C)* (5,28). Some shared p53 target genes were also identified, including *APAF1, MDM2, NOXA (PMAIP1), PCDD, TP53AIP1, TP53I13* and *TP53I3* (Table S1, S3).

In addition, we observed changes in expression of late differentiation genes that encode cornified envelope precursor proteins, which have not been previously reported as p63 targets (Table S2). These included the *SPRR* (small proline rich-proteins) and *LCE* (late cornified envelope) genes of the epidermal differentiation complex (EDC) that contains over 30 genes encoding epidermal cornification and S100 proteins (Fig. S1a) (29).

Biological process analysis of the microarray data according to Gene Ontology (GO) representation revealed that TAp63β-modulated genes were predominantly associated with epidermal and keratinocyte development and differentiation thus extending the functions of TAp63 (Fig. 1a).

TAp63β and TAp63γ transactivate the same genes in human keratinocytes and induce comparable changes in morphology

To understand the transcriptional role of TAp63β in the context of the other p63 isoforms, we transduced N/Tert-1 cells with recombinant adenoviruses expressing either TAp63α, β or γ, or ΔNp63α or β, then measured expression of a subset of TAp63β novel target genes involved in terminal differentiation. Protein expression of the p63 isoforms in transduced cells was confirmed by Western blotting and was higher than the endogenously expressed ΔNp63 isoform α (shown by an asterisk in Fig. 1b). The

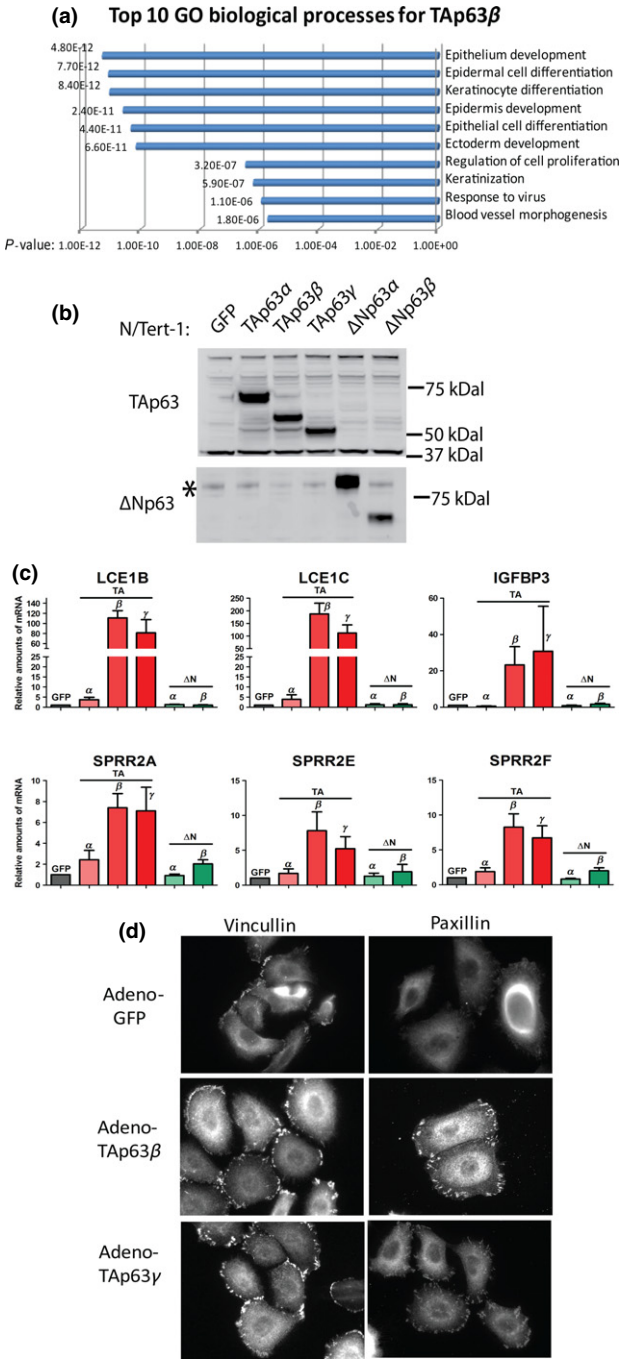

Figure 1. TAp63β regulates epithelial development by direct transcriptional activation of genes involved in terminal differentiation. (a) DAVID Gene Top 10 GO biological processes associated with the 1203 cellular genes modulated by TAp63β-overexpression in N/Tert-1 cells. (b) Western blot analyses of N/Tert-1 cells transduced with adenoviruses expressing GFP, TAp63α, TAp63β, TAp63γ, ΔNp63α or ΔNp63β, probed with specific TAp63 and ΔNp63 antibodies, as shown. *indicates the endogenous ΔNp63α. (c) N/Tert-1 cells transduced with recombinant adenoviruses expressing GFP, TAp63α, TAp63β, TAp63γ, ΔNp63α or ΔNp63β were subjected to real-time PCR analyses 24 h postinfection. Measurements were made of mRNA levels of six cellular genes associated with keratinocyte terminal differentiation selected from the microarray data, as indicated on the graphs. Experiments were performed three times, and results are given as mean $\pm$ standard deviation. (d) Primary human keratinocytes grown on coverslips were transduced with adenoviruses expressing GFP, TAp63β or TAp63γ before labelling with antibodies specific for vinculin and paxillin to visualize focal adhesions.

newly identified TAp63β target genes *LCE1B/C*, *IGFBP3* and *SPRR2A/E/F*, involved in keratinocyte terminal differentiation, were similarly activated by the two shorter TAp63 isoforms, TAp63β and γ, whereas TAp63α, ΔNp63α and ΔNp63β were either weak or non-activators of these genes (Fig. 1c). This is not unexpected, as the longer TAp63α isoform contains an inhibitory domain at its C-terminus that represses the transactivation domain (30) while the truncated ΔNp63 isoforms lack the N-terminal transactivation domain present in TAp63β and TAp63γ.

As TAp63β and γ similarly regulated the selected keratinocyte differentiation genes (Fig. 1c), we asked whether TAp63γ might also induce the increase in focal adhesion, as a marker of cell adhesion and differentiation that we have previously documented in TAp63β-expressing cells (11). Keratinocytes transduced with adenoviruses expressing either TAp63β or γ underwent comparable changes in cell morphology consistent with increased adhesion, and this was confirmed by clear vinculin and paxillin labelling at the cell surface (Fig. 1d). TAp63γ shorter isoform has previously been reported as a p53-like activator (10), and we detected elevated expression of a series of p53 target genes involved in cell death, as TAp63β targets in our microarrays. Thus, we compared the activation of four of these known p53 target genes by the various TAp63 isoforms in keratinocytes. Transcription of *AEN;* apoptosis enhancing nuclease, *APAF1;* apoptotic peptidase activating factor 1, *MDM2;* mouse double minute two homolg (p53 E3 ubiquitin ligase) and *NOXA* or *PMAIP1*, phorbol-12-myristate-13-acetate-induced protein1 were induced by both TAp63β and γ (Fig. S1b), in agreement with previous studies on the TAp63γ isoform (10). However, as shown in Fig. 1a, the main biological process modulated by TAp63β in keratinocytes is not the p53 pathway but rather keratinocyte differentiation.

In summary, TAp63β and TAp63γ activate the same set of genes, including those involved in keratinocyte terminal differentiation. Consistent with this, they also induce a similar phenotype upon overexpression by stimulating cell adhesion.

Overexpression of ΔNp63α regulates a subset of TAp63β target genes

As ΔNp63α is the best endogenously expressed p63 isoform in keratinocytes, it might strongly influence gene regulation by other p63 isoforms. Therefore, we compared the genes regulated by overexpressed TAp63β with those regulated when ΔNp63α is overexpressed to determine their regulatory link. N/Tert-1 cells transduced with ΔNp63α recombinant adenovirus expressed at least 10-fold more ΔNp63α than control, endogenously expressing cells (Fig. 1b). ΔNp63α overexpression induced significant changes in expression of 120 genes (Table S4). The top ten Gene Ontology biological processes regulated by ΔNp63α included chaperone proteins that are induced in response to stress, as well as negative regulators of apoptosis (Fig. 2a). Real-time PCR confirmed that overexpression of ΔNp63α specifically activated a group of genes encoding the heat shock proteins, *CRYAB*, *DNAJA4*, *HSPA1B*, *HSPA1B* and *HSP1H* (Fig. S1c). Interestingly, of the 1203 genes regulated by TAp63β, only 36 were also regulated by ΔNp63α (Fig. 2b, c). The common genes represent only 3% of TAp63β's target genes but a larger 30% of ΔNp63α's target genes. Common targets include genes involved in the immune response, such as *IL-8,* which is activated by both TAp63β and ΔNp63α, and *IL1F9* which is activated by TAp63β but repressed by ΔNp63α (Table S4).

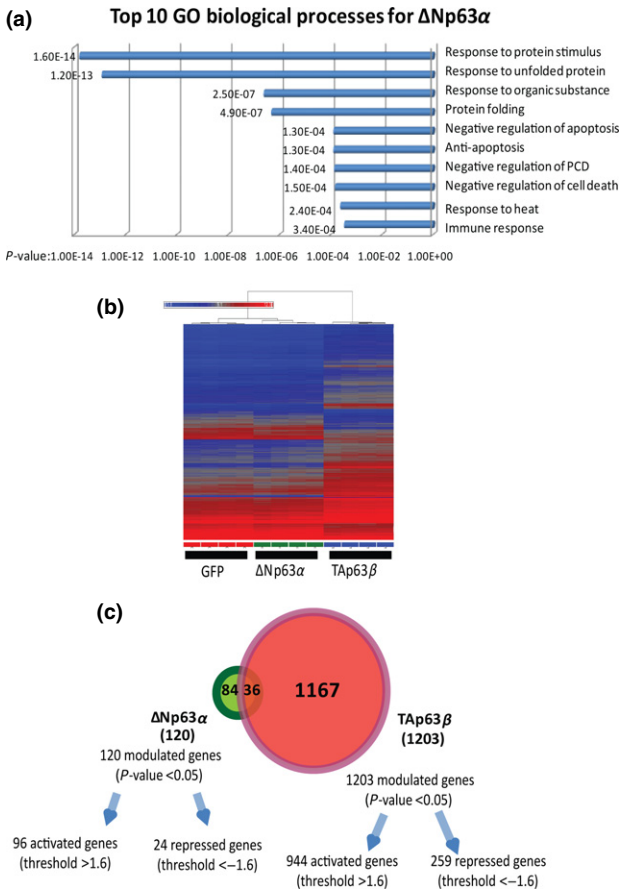

Figure 2. Comparative analyses of the ΔNp63α and TAp63β transcriptome in keratinocytes. (a) DAVID Gene Top 10 GO biological processes associated with the 120 cellular genes modulated by ΔNp63α-overexpression in N/Tert-1 cells. (b) Heatmap displaying the gene expression changes in N/Tert-1 cells transduced with adenoviruses expressing GFP, TAp63β or ΔNp63α as indicated. (c) Partek Genomics Software analyses revealed that in transduced N/Tert-1 cells TAp63β modulated 1203 genes, comprising 944 activated and 259 repressed genes (Table S1), while ΔNp63α modulated 120 genes with 96 activated and 24 repressed (Table S4), 36 of which were regulated by both p63 isoforms.

Direct comparison cannot be drawn between the ΔNp63α and TAp63β overexpression microarrays because, while there is very little endogenous TAp63β in N/Tert-1 cells, ΔNp63α is present at relatively higher levels, hence probably decreasing the number of genes modulated by its overexpression. However, some conclusions can still be drawn by comparing the ΔNp63α and TAp63β microarrays: (1) ΔNp63α and TAp63β have essentially distinct transcriptomes with some overlapping genes (3% of TAp63β's transcriptome and 30% of ΔNp63α's transcriptome) and (2) ΔNp63α and TAp63β regulate many of their common genes (25/36) similarly.

Overall, our experiments indicated that these two isoforms do not purely function in opposition to each other but that they probably cooperate in some aspects of epidermal development (24,31). However, TAp63 short isoforms show a distinct panel of target genes containing many genes involved in terminal differentiation that are not modulated by ΔNp63α. Interestingly, ΔNp63 has been shown to be activated in cervical carcinoma linking its presence to keratinocyte transformation and de-differentiation (32). Our data indicate that its role is probably more complex than being a direct antagonist of TAp63 (33,34).

In vitro differentiation of primary human keratinocytes induces TAp63β and γ expression and activation

To address whether differentiation can modulate the endogenous levels of TAp63 isoforms, we induced terminal differentiation of primary human keratinocytes *in vitro* and measured mRNA transcript levels of endogenous TAp63 and ΔNp63 isoforms alongside some of the target genes identified in our microarray.

Human primary keratinocytes (HPKs) were grown to confluence in the same plate for up to 12 days as previously described as an alternative method to calcium induction to efficiently trigger differentiation (35–37) (Fig. S2a). Terminal differentiation of the cells was confirmed by demonstration of gradual loss of proliferative potential (Fig. S2b) and increased expression of Involucrin, a terminal differentiation marker (Fig. S2c). Transcript levels of the differentiation markers *KRT1* and *KRT10* also increased during the 12 days culture period (Fig. 3a).

Interestingly, the levels of TAp63 transcripts increased as HPKs became increasingly more confluent and differentiated (Fig. 3b). By Western blot, we observed that TAp63β and more particularly TAp63γ protein levels were also substantially higher by day 10 compared to day 1 (Fig. 3c). At both transcripts and protein levels, the activation of TAp63 was not accompanied by a decrease in ΔNp63 indicating that relative expressions of the p63 isoforms are not correlated at least in the differentiation protocol used here. Real-time PCR analyses of two TAp63β target genes involved in keratinocyte terminal differentiation revealed that increases in their transcript levels closely correlated with the increase in TAp63 mRNA that accompanied progressive confluence (Fig. 3d).

Taken together, these experiments show for the first time that the shorter TAp63 isoforms and some of their differentiation target genes are spontaneously expressed and activated during *in vitro* differentiation of human keratinocytes obtained by confluence.

Notch signalling is involved in transcriptional regulation of TAp63 target genes

Our microarray identified several Notch target genes (*HEY1*; hes-related family bHLH transcription factor with YRPW motif 1 and *NRARP*; NOTCH-regulated ankyrin repeat protein) and genes of the Notch signalling pathway [*JAG2*; jagged 2, *DLL1*; delta-like 1 (drosophila), *LFNG*; LFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase] as activated by TAp63β (Table S1), which we confirmed by real-time PCR (Fig. 4a). As p63 is a known regulator of Notch1-dependent transcription (12), and Notch has also been reported as an activator of differentiation (13), we asked whether TAp63β and Notch might cooperate in their biological functions in promoting keratinocyte differentiation.

We inhibited Notch signalling in TAp63β-overexpressing keratinocytes using the γ-secretase inhibitors, DAPT and Compound E (Cpd E), then measured expression of several TAp63β target genes. Transcriptional activation of the *SPRR2* group of genes (*SPRR2A/E/F*) was ~10- to 30-fold higher in TAp63β-overexpressing N/Tert-1 cells compared to controls, but this was significantly reduced in the presence of the two γ-secretase inhibitors (Fig. 4b, upper panel). Intriguingly, activation of other TAp63β target genes, including *LCE1B/C* and *IGFBP3*, was enhanced by inhibition of Notch signalling (Fig. 4b lower panels). This phenomenon

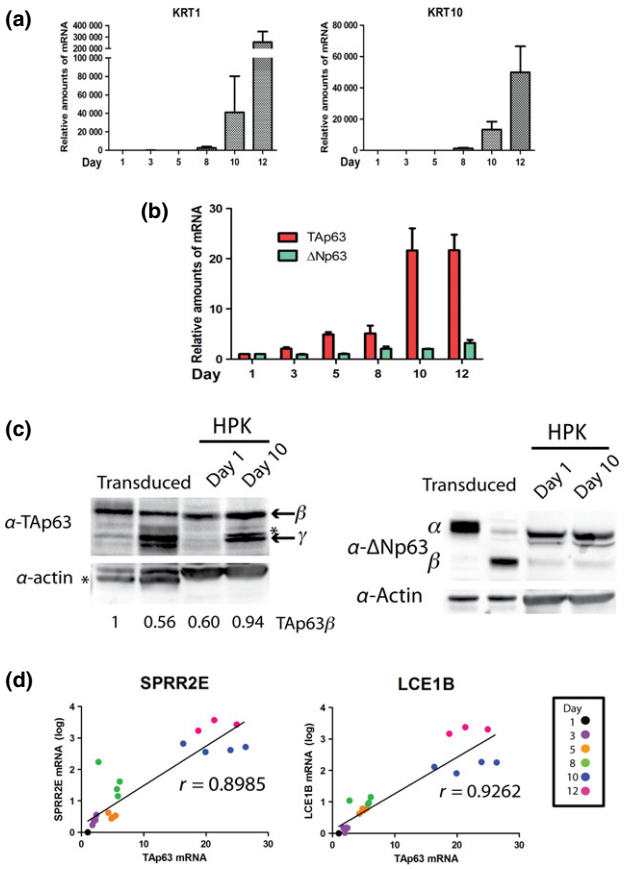

Figure 3. Differentiation induces TAp63 expression and activation in primary human keratinocytes. (a) Transcription of the differentiation markers, *KRT1* and *KRT10* was measured by real-time PCR at various time points of growth of the HPKs as indicated on the graphs. (b) Transcription of the TAp63 and ΔNp63 isoforms as in (b). Experiments in (a) and (b) were performed three times, and results are given as mean ± standard deviation. (c) Western blots of extracts of HPKs grown up to day 1 and day 10 were probed with antibodies against TAp63 (left panel) and ΔNp63 (right panel). N/Tert-1 cells transduced with adenoviruses expressing either TAp63 or ΔNp63 isoforms were used as controls for the expression of the various p63 isoforms. TAp63β and γ isoforms were marked out with arrows, and '*' denotes non-specific bands. Quantification levels of TAp63β were determined using ImageQuant TL Software (GE Healthcare Life Sciences), and Western blot images are given below. (d) Real-time PCR analyses of the amounts of TAp63 mRNA compared with amounts of its downstream target genes (log to the power 10), *LCE1B, LCE1C* and *SPRR2E* mRNA during keratinocyte differentiation. Data show a significant correlation between TAp63 and target gene mRNA levels according to Pearson's correlation test (r values given in graphs).

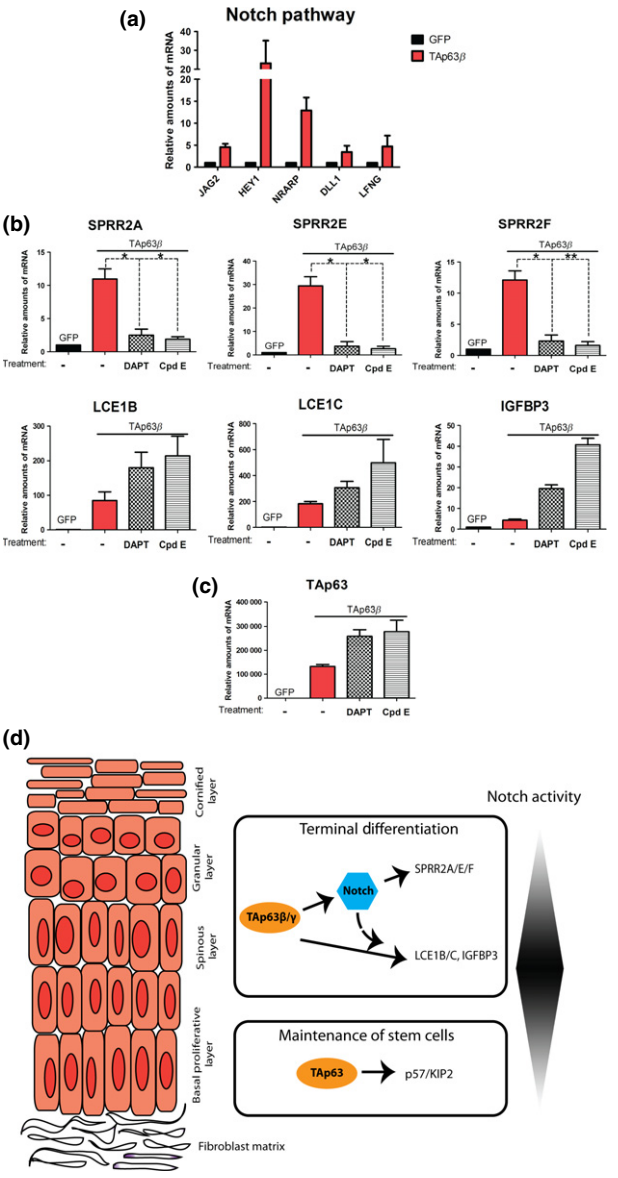

Figure 4. Notch signalling is implicated in the regulation of TAp63 target genes. (a) Real-time PCR confirmation of TAp63β activation of a series of genes selected from the microarrays that are associated with the Notch pathway, carried out in N/Tert-1 cells transduced with adenoviruses expressing GFP or TAp63β. (b) N/Tert-1 cells were treated with DMSO as control, or with the Notch inhibitors DAPT or Compound E, for 16 h before transduction with recombinant adenoviruses expressing GFP or TAp63β. The cells were then harvested 24 h post-transduction and real-time PCR analyses of selected TAp63β target genes involved in keratinocyte terminal differentiation were performed. Results are given as mean ± standard deviation; $*P < 0.05$, $**P < 0.01$. (c) Real-time PCR analyses of TAp63 mRNA expression in same conditions as in (b). Experiments in (a–c) were performed three times, and results are given as mean ± standard deviation. (d) A model for the roles of TAp63β/γ in the regulation of epithelial development. Notch expression is adapted from Blanpain *et al.* (17). We postulate that during keratinocyte differentiation (in the spinous and/or granular layer), TAp63β/γ and Notch cooperate in the activation of terminal differentiation genes in at least two different patterns; Notch is essential for activation of *SPRR2A/E/F,* whereas Notch is not absolutely essential for the activation of *LCE1B/C* and *IGFBP3*. Su *et al.* (5) have shown that TAp63 is important in the basal proliferative layer of the epidermis; TAp63 activates *p57/KIP2* to maintain a viable pool of stem cells by preventing hyperproliferation of these stem cells.

can be explained by activation of TAp63 transcription in the presence of the inhibitors (Fig 4c), which in turn would lead to activation of its target genes *LCE1B/C* and *IGFBP3*.

We then decided to perform the reverse experiment by activating the Notch pathway by co-culturing TAp63β- or GFP-overexpressing N/Tert-1 cells with NIH 3T3 cells stably expressing the secreted Notch ligand Jagged2. Notch activation in the absence of TAp63β overexpression induced three- to ninefold increases in *SPRR2* gene (*SPRR2A/E/F*) transcription); however, when TAp63β was overexpressed alongside Notch activation, transcription of these genes was dramatically increased (Fig. S3a, upper panels), thus confirming a strong cooperation between Notch and TAp63 to activate these genes. In contrast, *LCE1B/C* and *IGFBP3* were not activated by Notch signalling alone, while Notch activation alongside TAp63β expression had two types of effects as it did not further activate transcription of *LCE1B* but did increase abundance of *LCE1C* and *IGFBP3* mRNAs (Fig. S3a,

lower panels) independently of any increase in TAp63 transcripts.

Together, our data demonstrate that Notch signalling is indeed involved in the activation of several TAp63β target genes that can be separated in two distinct groups: the first group of *SPRR2* genes is dependent on Notch cooperation with TAp63 for full transcriptional activation, while the second group, illustrated by *LCE1B/C* and *IGFBP3,* is either not affected by Notch for *LCE1B* or exhibit some cooperation with TAp63 for *LCE1C* and *IGFBP3*. In the proposed model in Fig. 4d, TAp63 transcription factors are active in two different layers of the skin. As demonstrated previously by the activation of *p57/KIP2* (38), TAp63 is able to maintain the pool of stem cells in the basal layer while it is active in the upper layers with the help of Notch signalling either in a direct obligatory fashion (as shown for *SPRR2* genes) or in a more auxiliary fashion as shown for *LCE1C* and *IGFBP3*, to activate terminal differentiation genes.

Discussion

We report that overexpression of TAp63β in human keratinocytes modulates expression of an impressive group of 1203 cellular genes, with a significant bias towards genes involved in epidermal differentiation. The generation of specific TAp63 null mice has revealed unique functions of the p63 isoforms in developmental processes, including epidermal stem cell maintenance (5), glucose and lipid metabolism (39), protection of the fidelity of oocytes (40) and cardiac differentiation and development (41). Our transcriptional data strongly suggest an additional role for the short TAp63 isoforms in inducing terminal differentiation in human skin.

In this report, we demonstrated that differentiation of human keratinocytes *in vitro* was accompanied by increased expression of the endogenous short TAp63 isoforms, both at the mRNA and protein levels, while no drastic change was observed in expression of ΔNp63 isoforms and no expression of the long TAp63α isoform was detected in any of our *in vitro* models. It is the first time that induction of TAp63 has been shown in human keratinocytes *in vitro,* and the next step is to progress to *ex vivo* detection of TAp63, to detect its expression in the upper differentiated layers. However, major technical challenges need to be solved before doing these experiments, as TAp63 protein levels in skin are likely to be low, the proteins being unstable and currently available antibodies have been proven unsuitable for detection in tissue.

The Notch pathway has both tumor-suppressive and differentiation-inducing functions in mammalian keratinocytes (13). It has also been reported to work in concert with p63 in epidermal development (12,42). Here, we demonstrated that Notch signalling cooperates with TAp63β to achieve full transcriptional activation of a panel of common target genes. Interestingly, p63-related tumor suppressor p53 has been reported as an upstream regulator of Notch and that both pathways share common transcriptional targets (13). Our findings extend this observation to TAp63β, which cooperates with Notch to activate a set of terminal differentiation genes. Nguyen et al. (12) previously proposed a cross-regulatory relationship between Notch and p63 in promoting keratinocyte differentiation whereby Notch activation suppresses ΔNp63α, hence allowing differentiation. ΔNp63 has also been described as a repressor of Notch (43). Similarly, Notch and ΔNp63 work antagonistically to specify the luminal or basal fate of mammary epithelial cells (44). Our data are in agreement with these studies as we show that cross-talk between the TAp63 short isoforms, but not ΔNp63, and Notch pathways are required for expression of several late differentiation genes. Interestingly, cross-talk between the TAp63 and Notch pathways has also been implicated in cochlear development, where TAp63-dependent activation of the Notch pathway components, *HES5* and *ATOH*, regulates the development of the organ of Corti (45). Besides the Notch pathway, cross-talk of p63 with the Wnt/β-catenin and Sonic hedgehog pathways that regulate epidermal homeostasis have also been reported (42,46), which would be exciting to further explore in our system.

It is interesting to note that TAp63 could be playing dual roles in epidermal development; in the maintenance of precursor cells (5), and in differentiation at the upper stratum of the epithelium as suggested by the present data. This dual function of TAp63 could be linked to its ability to negatively regulate cell proliferation and also to promote cell adhesion. The involvement of complementary pathways, such as Notch signalling, would help specify the layer-specific role of TAp63 in epithelial differentiation (Fig. 4d) as Notch signalling is only active in cells that are undergoing differentiation and not in basal precursors (17,47). Additionally, epidermal precursor cells can be controlled by other signalling pathway(s) from the stroma that could tweak the fate of the transcriptional control by TAp63. Lastly, another possible control is that TAp63 target genes are only accessible to the p63 transcription factor in the upper layers after chromatin remodelling (48–50).

Studies of TAp63 knockout mice did not reveal overt defects in their epithelium (51), but experiments with human keratinocytes have shown absence of stratum corneum in 3D organotypic cultures treated with siRNA TAp63 (24). The human skin is thicker than the mouse skin (52), and hence, it is plausible that TAp63β and γ may play a more significant role in terminal differentiation of the human skin.

Our previous findings demonstrated that the short β isoform of TAp63 is expressed in HPV-associated cervical carcinoma cell lines and targeted for degradation by the viral E6 oncogene (11). TAp63β therefore appeared as an oncogenic target together with p53 (53), and our aim in the present work was to determine the specific transcriptional function of this TAp63 isoform in comparison with other members of the p63 and p53 family. Interestingly, we also uncovered a new relationship between TAp63 and Notch, which has also been described as a target of the skin specific HPV E6 oncogene (54–56). As their specific function in skin appears to involve terminal differentiation and cell adhesion, it is probable that their degradation by the oncogenic HPV E6 protein is required for oncogenic transformation.

Our present report strongly suggest that TAp63β and γ isoforms are essential transcriptional switches in human skin terminal differentiation in cooperation with Notch thus playing a fundamental and specific role as tumor suppressors in HPV-associated carcinogenesis.

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Conflict of interests

The authors have declared no conflicting interests.

Supporting Information

Additional supporting data may be found in the supplementary information of this article.

Figure S1. (a) 6 TAp63β target genes extracted from the microarrays (top GO biological processes) that are specifically involved in keratinocyte and epithelium function.

Figure S2. (a) Phase contrast microscope images of human primary keratinocytes (HPKs) during growth to confluence (10× magnification).

Figure S3. (a) N/Tert-1 cells were transduced with adenoviruses expressing GFP or TAp63β and co-cultured with control NIH 3T3 or NIH 3T3-Jagged2 cells for 48 h before harvesting.

Table S1. Complete list of TAp63β regulated genes after overexpression in human keratinocytes.

Table S2. TAp63β target genes extracted from the top 6 GO biological processes that are specifically involved in keratinocyte and epithelium function.

Table S3. TAp63β target genes that were found modulated in the microarrays and have been previously reported by others as indicated (1–13).

Table S4. Complete list of ΔNp63α target genes after over expression in human keratinocytes.

Data S1. Supplementary Methods.

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