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Report

The Hay Wells Syndrome-Derived TAp63αQ540L Mutant Has Impaired Transcriptional and Cell Growth Regulatory Activity

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KEY WORDS

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ABSTRACT

p63 mutations have been associated with several human hereditary disorders characterized by ectodermal dysplasia such as EEC (ectrodactyly, ectodermal dysplasia, clefting) syndrome, ADULT (acro, dermato, ungual, lacrimal, tooth) syndrome and AEC (ankyloblepharon, ectodermal dysplasia, clefting) syndrome (also called Hay-Wells syndrome). The location and functional effects of the mutations that underlie these syndromes reveal a striking genotype-phenotype correlation. Unlike EEC and ADULT that result from missense mutations in the DNA-binding domain of p63, AEC is solely caused by missense mutations in the SAM domain of p63. In this paper we report a study on the TAp63α isoform, the first to be expressed during development of the embryonic epithelia, and on its naturally occurring Q540L mutant derived from an AEC patient. To assess the effects of the Q540L mutation, we generated stable cell lines expressing TAp63α wt, ΔNp63α or the TAp63α-Q540L mutant protein and used them to systematically compare the cell growth regulatory activity of the mutant and wt p63 proteins and to generate, by microarray analysis, a comprehensive profile of differential gene expression. We found that the Q540L substitution impairs the transcriptional activity of TAp63α and causes misregulation of genes involved in the control of cell growth and epidermal differentiation.

INTRODUCTION

The p63 protein is a transcription factor homolog of the p53 tumor suppressor. Unlike p53, p63 functions primarily in epidermal-mesenchymal development during embryogenesis. Mice in which p63 was inactivated displayed a fundamental defect in epithelial lineage development and failed to develop stratified epithelia and epithelial appendages, such as teeth, hair follicles and mammary glands.[1,2] The role of p63 in the development and differentiation of stratified epithelia, remains controversial. Indeed, recent studies indicate that p63 may act as a molecular switch required for initiation of epithelial stratification, or for maintaining the proliferative potential of basal keratinocytes in the mature epidermis.[3-5] It also seems to play a substantial role in the induction of apoptosis and chemosensitivity.[6-8]

The p63 gene displays a high sequence and structural homology to p53.[9] Like p53, the p63 protein contains a transcriptional activator domain (TA) to induce transcription of target genes, a DNA-binding (DB) domain and an oligomerization domain (OD), used to form tetramers.[10] In contrast to p53, multiple protein products are produced by the p63 gene. Two promoters are present at the 5' end of the gene. The first produces TA-p63 proteins, while transcription from the second creates ΔN-p63 products lacking the aminoterminal TA domain. In addition three alternative splicing routes at the 3' end generate proteins with different C-termini, denoted α, β and γ. The TA and ΔNp63α isoforms alone contain a Sterile Alpha Motif (SAM) domain absent in p53: this is the most remarkable structural difference between p63 and p53. This domain is a 65–70 amino acid residue sequence found in many proteins, from yeast to human, whose functions range from signal transduction to transcriptional repression.[11] It is a protein-protein interaction domain also found in p73, another member of the p53 gene family, as well as in other developmentally important proteins, such as several Eph receptor tyrosine kinases.[12] Recent studies have identified a transcriptional inhibitory (TI) domain located between the SAM domain and the C-terminus of p63α isoforms that is believed to be responsible for the lack of transactivation ability of TAα compared to TAβ and γ on several different p53 target promoters.[13] The analysis of the functions of the multiple p63 isotypes are complicated by the existence of several mechanisms regulating their expression levels. Such mechanisms are still under investigation.[14-19]

A broad spectrum of mutations found in several ectodermal, craniofacial and limb syndromes, namely EEC (ectrodactyly, ectodermal dysplasia, clefting), AEC (ankyloblepharon, ectodermal dysplasia, clefting), LMS (limb-mammary syndrome), ADULT (acro-dermato-ungual-lacrimal-tooth) and SHFM (split-hand/split-foot malformation) have been mapped in distinct p63 domains.[20]

The location of mutations in the p63 protein domains and their functional implications reveal a striking genotype-phenotype correlation: EEC and ADULT result from missense mutations in the DB domain and SHFM from mutations in either the DB or the C-terminal domain,[21] whereas AEC is solely caused by missense mutations in the SAM domain. Unlike the other ectodermal dysplasia syndromes, AEC does not comprise ectrodactyly or other major limb defects, but has ankyloblepharon and severe scalp dermatitis as its distinguishing features.[22]

A p63 SAM-domain model structure has been used to divide the naturally occurring AEC mutations into in two groups. The first (L518V, I541T, C526W) includes mutations in amino acids that are predicted to be buried inside the protein and are believed to affect its overall structure and stability. The second (G534V, T537P and Q540L), whose direct effect on the protein is less obvious, contains all the other amino acids that have a larger solvent accessible surface and are not predicted to cause gross conformational changes.[22] These AEC mutations may disrupt the structural integrity of the SAM domain or interfere with particular protein-protein interactions.[12] They have, in fact, already been shown to disrupt the interaction of p63 with the Apobec-1 binding protein-1(ABPP1)[23] and thus alter the splicing mechanism of fibroblast growth factor receptor-2, FGFR2.[11]

Here, we report data from a study on the TAp63α isoform, which is the first to be expressed during the development of embryonic epithelia,[3] and on the AEC-derived TAp63αQ540L (1607 A to T) mutant protein. This was described by Hay and Wells in their case no. 5.[24] It is located within exon 13 and is predicted do not destroy the overall structure of the SAM domain.[22]

To study the effects of the Q540L mutation on p63 functions we generated stable cell lines that express wild-type (wt) TAp63α, ΔNp63α or the TAp63αQ540L mutant under the control of a TET-inducible promoter and used them to compare the effects of the mutant and wt p63 proteins on cell proliferation and generate, by microarray analysis, a comprehensive profile of differential gene expression. We found that the Q540L substitution affects the transcriptional activity of TAp63α and causes misregulation of genes involved in the control of cell growth and epidermal differentiation.

MATERIALS AND METHODS

Plasmids. Wt p63α in pcDNA3-His expression vector has been described.[15] Mutation Q540L was created by PCR, using the NdeI site-containing upstream primer, p63NdeI_FW (CCA TCT TCA TAT GGT AAC AGC TCC CCA CCT C) and the downstream primer p63NcoI_RW (ATC ATC CAT GGA GTA ATG CTC AAT CAG ATA GA) containing the NcoI site and the substitution A → T that introduces the mutation. The mutated fragment was replaced in the TAp63α wt sequence,[15] digested with NdeI/NcoI to generate the TAp63αQ540L sequence and then cloned in pcDNA3.

To create the plasmids encoding the p63 proteins under the control of the rtTA responsive promoter, the cDNA fragments were extracted from pcDNA using Hind III and Xba I, blunted and cloned into the pBIG-βgal Not I site. The pTet-On, pTK-Hyg, and pBIG-βgal constructs were provided by Clontech. The Bp100CAT and p21/WAF/CAT reporter plasmids have already been described.[15,25]

Cells, transfection and reporter assays. Human lung carcinoma H1299 cells (p53 null, no p63 expression) were obtained from the American Tissue Culture Collection and grown at 37°C in humidified 5% CO_2 in DMEM supplemented with 10% fetal calf serum. H1299 cells (5 x 10^4) were plated and transfected by calcium-phosphate precipitation with 20 μg of pTet-On plasmid (Clontech). Fourty-eight hours later, the cells were selected by adding G418 (100 μg/ml) to the medium. After four weeks, single G418-resistant clones (H1299-rtTA) were picked up and expanded. The presence of the rtTA regulator in these clones was checked by performing a β-galactosidase assay on H1299-rtTA cell extracts after transient transfection with the pBGI-βgal empty construct, with or without doxycycline (Sigma-Aldrich) (1 μg/ml).

Tet-On/TAp63α, Tet-On/ΔNp63β, Tet-On/TAp63αQ540L cell lines were produced as follows:

7 x 10^4 H1299-rtTA cells were cotransfected with each of the pBIG-p63 constructs and the pTK-Hygro vector (20 to 1 rate) by calcium-phosphate precipitation. Twenty-four hours later, each 100 mm plate was split (1:2) and cells were selected by adding 800 μg/ml hygromycin (Sigma-Aldrich). After 4 weeks, single hygro-resistant clones were picked up and expanded, and their p63-inducible gene expression was determined by Western blot and specific immunodetection. p63 stable clones were maintained in DMEM supplemented with 10% Tet-Approved serum (Hyclone). CAT assays with the WAFCAT or BP100CAT reporter plasmids were performed as previously described.[16]

Growth rate determination. Approximately 6 x 10^4 cells were seeded in 60 mm-diameter plates in the presence or absence of doxycycline (1 μg/ml) for five days to regulate exogenous protein expression. Medium was replaced every 48 hrs. At the indicated time points, two plates were rinsed twice with PBS to remove dead cells and debris. Live cells on the plates were trypsinized and collected separately. Cells from each plate were counted three times in a Burker chamber. The average number from two plates was used to determine the growth rate.

DNA histogram analysis. Cells were counted and seeded at 2 x 10^5/ 100 mm plate with or without doxycycline (1 μg/ml). At the indicated time points, live cells on the plates were trypsinized and both floating dead cells in the medium and trypsinized live cells were centrifuged and washed twice with PBS. Approximately 10^6 cells were incubated in 1 ml of 0.1% Na citrate, 50 μg/ml propidium iodide (Sigma Chemical Co., St. Louis, MO, USA), 20 μg/ml RNase A and 0.1% Nonidet P-40. Cells were incubated for 40' at RT in a dark box. Stained cells were analysed in a fluorescence-activated cytometer (FACSCalibur-BD, Menlo Park, CA, USA) within 1 hr. Data on DNA cell-content were acquired with the CellQuest program (Beckton-Dickinson) on 20,000 events at a rate of 150 ± 50 events/second and the percentages of cells in the $SubG_1$, G_0–G_1, S and G_2–M phases were quantified with the ModFit software (Beckton-Dickinson). The percentage of dead cells/total cells was determined by trypan blue dye staining. Briefly, aliquots of cells were mixed with an equal volume of 0.4% trypan blue dye solution (Sigma Chemical Co., St. Louis, MO, USA) and incubated for 15' at RT. Stained (dead) and unstained (live) cells were counted with a hemocytometer and the percentage of dead cells/total cells was determined by scoring an average of over 300 cells, twice per plate.

Subcellular immunolocalization assay. Immunolocalization was performed on doxycycline (1 μg/ml) induced or uninduced Tet-On cells, 10^5 cells/ 35 mm plate were grown on micro cover glasses (BDH). Fourty-eight hours later, cells were washed with cold phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (Sigma-Aldrich), for 15' at 4°C. After a rinse with PBS, fixed cells were permeabilized with ice-cold 0.1% Triton X-100 for 10' and rinsed again with PBS. Cells were than incubated with DAPI (4,6-diamidino-2-phenylindole; 10 mg/ml Sigma-Aldrich) for 3' and washed again with PBS. Lastly, the glasses were mounted with Moviol (Sigma-Aldrich) and cells were examined under a fluorescence microscope (Nikon). To detect p63 protein the H137 (Santa Cruz) and the CyTM 3-conjugated anti-rabbit IgG (ImmunoResearch Laboratories) antibodies were used at RT for 30'. Images were digitally processed with Adobe Photoshop software.

Western immunoblot analysis. At the indicated time after transfection, cells were lysed in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5%

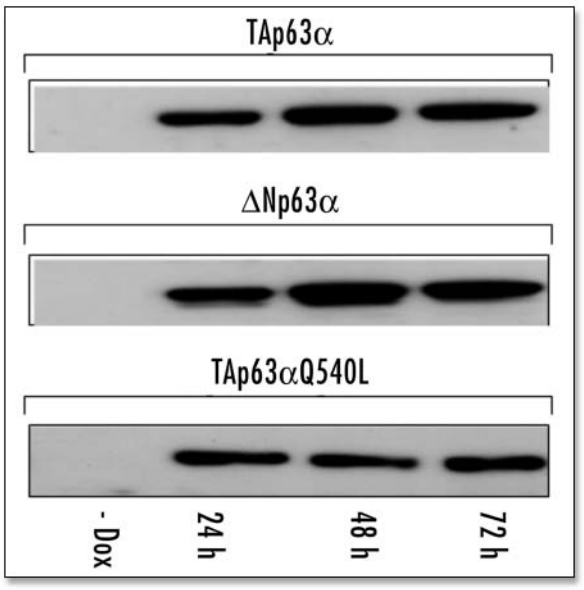


Figure 1. Expression of p63 isoforms in H1299 stable clones. Western blot analysis for detection of the p63 protein expression levels in Dox-inducible H1299 cells. Cells were harvested at the indicated time points after induction with 1 μg/ml doxycycline. Equal amounts of soluble lysates (30 μg) from uninduced and induced clones expressing wild-type TAp63α, ΔNp63α or the mutant TAp63αQ540L protein were evaluated for p63 protein levels by Western blotting using an anti-p63 antibody (4A4; Santa Cruz Biotechnology).

Nonidet-P40, 10 mM glycerol, 5 mM EDTA, 0.5% NaDOC and 1 mM PMSF. Proteins were separated on 8% SDS-PAGE and blotted onto PVDF membrane. Filters were incubated with the following primary antibodies MDM2 (SC-965; Santa Cruz); p63 (H137; Santa Cruz); p63 (4A4; Santa Cruz); anti-p21/WAF1 (Ab-11, CP74; Neomarkers), actin (1-19; Santa Cruz); anti-goat IgG/HRP (Santa Cruz); anti-mouse IgG/HRP (Amersham); anti-rabbit IgG/HRP (Bio-Rad).

EMSA. EMSA experiments were performed as already described.[26] P63 proteins were translated in vitro by using TnT reticulocytes from Promega with 0.5 μg of p63 plasmid DNA. Next, 10 μl of the individual reactions was used for the binding reaction and for Western blot analysis. The probe is a radiolabeled oligonucleotide duplex containing a p53-binding site present in the p21 promoter (p21.1 described in ref. 27). A 100-fold molar excess of the same cold oligonucleotide or an oligonucleotide containing a consensus binding site for E2F1 were used for competition experiments. For the supershift anti-p63 antibodies (4A4; SantaCruz) or unrelated polyclonal anti-p21 antibodies (C-19; SantaCruz) were added to the sample prior to the binding reaction (30' in ice).

Microarray sample preparation. Total RNA (ttlRNA) was extracted and purified from stably transfected H1299 cell lines with the Concert Cytoplasmic RNA Purification Reagent (Invitrogen, Carlsbad, CA), as suggested by the manufacturer. ttlRNAs were then quantified and inspected with a Bioanalyzer (Agilent Technologies). cRNAs were generated and hybridized on 12 HGU133plus2 Affymetrix DNA chips according to the Affymetrix protocol, ttlRNA (8 μg) was used to prepare double-stranded cDNA with the one-cycle cDNA synthesis kit (Affymetrix, USA). The cDNA was then used as a template to synthesize a biotinylated cRNA (16 hr, 37°C) with the IVT kit (Affymetrix, USA). In vitro transcription products were purified with the IVT cleanup module and approximately 35 μg of cRNA were treated with the fragmentation buffer (35' at 94°C). Affymetrix 12 HGU133plus2 array chips were hybridized with biotinylated cRNA (20 μg/chip, 16 hr, 45°C using the hybridization buffer and control provided by the manufacturer (Affymetrix Inc.). GeneChip Fluidics station 400 (Affymetrix Inc.) was used to wash and stain the arrays. The standard protocol suggested by the manufacturer was used to detect the hybridized biotinylated cRNA. The chips were then scanned with a specific scanner (Affymetrix Inc.) to generate digitized image data (DAT) files.

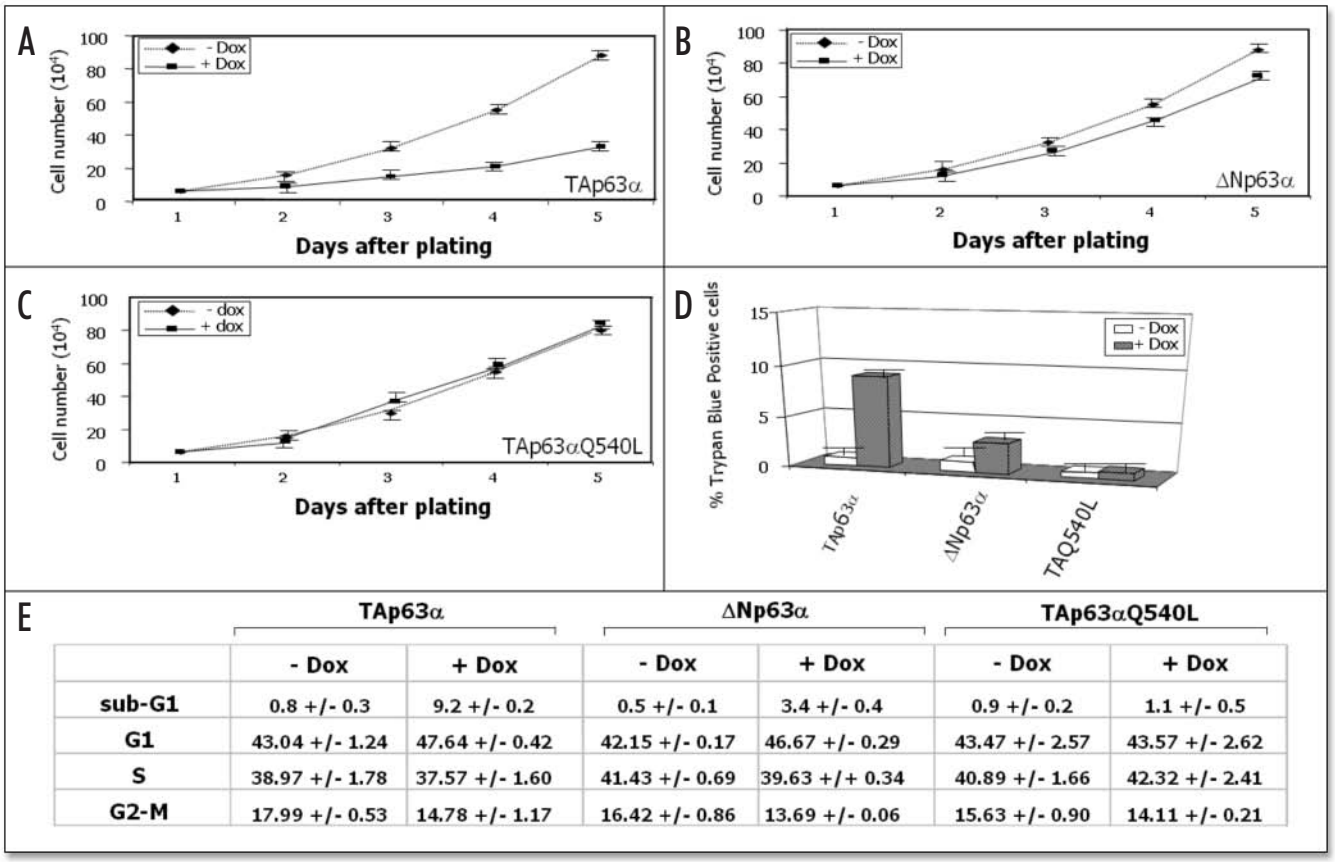
Microarray data analysis. DAT files generated for the four prototypic situations (TAp63α wt without induction, TAp63α wt with induction, TAp63αQ540L without induction, TAp63αQ540L with induction) were analyzed by GCOS (Affymetrix, USA) to generate background-normalized image data (CEL files). The presence of hybridization/construction artifacts was evaluated with the fitPLM function (Bioconductor package affyPLM). The probes (PM) intensity distribution was evaluated using hist function (Bioconductor package affy). Only one array from the TAp63αQ540L with induction group was characterized by a narrow distribution of PM intensities and was discarded.

Probe set intensities were obtained by means of GCRMA, a robust multiarray analysis method (http://www.biostat.jhsph.edu/~ririzarr/papers/p177-irizarry.pdf).[28] The full data set was normalized according to the quantiles method.[29] The HGU133plus2 54675 probe sets were filtered to provide an interquantile range (IQR) for each probe set greater then 0.25.[30] This filtering yielded 11857. Subsequently, "Significant analysis of microarrays" software (SAM-software)[31] was used to identify probe sets differentially expressed between wt and mutant p63 isoforms. Differentially expressed probe sets were initially identified with the multi class method (900 permutations, 50 false significants). This test requires one user-set parameter: a threshold value that can be adjusted to maximize the number of significant genes while minimizing the predicted false discovery rate. This analysis produced 4000 differentially expressed probe sets, which were then filtered to select those characterized by a fold change ≥ |2| between not-induced and induced cell lines in wt or mutant experiments. This filtering yielded 100 probe set ids (Additional information Table A): 87 were associated with 81 Entrez Gene identifiers (gene ID)[32] and the remaining 13 probe sets were not assigned to any gene ID. The IQR filtered data set was also analysed with two-class unpaired method, implemented in the SAM-software,[31] to highlight probe sets transactivated only by the wt or mutant isoforms. This test requires two user-set parameters: a minimal fold change value and a threshold value that can be adjusted to maximize the number of significant genes while minimizing the predicted false discovery rate. We conducted a blocked, two-class unpaired test using a 2-fold-change cut-off and a threshold allowing a false significant number of about 1. This analysis produced 18 differentially expressed probe sets for TAp63α wt (16 upmodulated and 2 downmodulated) and 7 probe sets for the Q540L mutant isoform (1 upmodulated and 6 downmodulated) (Fig. 1, additional information Table B). All these 18 probe sets included in sets identified as differentially expressed with the multi class method. Since a certain amount of leaking, at transcriptional level, of the tet-ON system was observed in our experiments (data not shown), differential expression between not-induced and induced cell lines could have been underestimated. Therefore, a two-class unpaired test (2-fold-change cut-off and false significant number about 1) between induced wt and mutant cell lines was also performed. The differentially expressed probe sets were 441 (Additional information Table C).

To generate a robust set of differentially expressed genes for further investigation, the intersection between the 100 probe sets derived from the multi class test and the 441 from the two-class test was selected. This intersection contains 45 probe sets linked to 36 annotated genes and 7 unmapped est (Fig. 3).

Overexpressed Gene Ontology Biological Process themes were searched with the Bioconductor GOstats package.[33] Gene annotation was performed by using the Bioconductor annaffy library and the HGU133plus2 annotation package (version 1.6.8).

Real-time RT quantitative PCR (qPCR) expression validation. Total RNA was reverse transcribed to cDNA with the Omniscript RT Kit following the manufacturer's instructions (QIAGEN GmbH, Hilden, Germany, EUROPE). The primer sequences are shown in Table 1. Primers were designed by using the sequence identified by the Affymetrix identifier and Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA). Real-time quantitative PCR, 20 μl contained 2 μl of the cDNA, 1X SYBR GREEN PCR Master Mix (PE Applied Biosystems, Foster City, CA, USA)



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	TAp63α		ΔNp63α		TAp63αQ540L	
	- Dox	+ Dox	- Dox	+ Dox	- Dox	+ Dox
sub-G1	0.8 +/- 0.3	9.2 +/- 0.2	0.5 +/- 0.1	3.4 +/- 0.4	0.9 +/- 0.2	1.1 +/- 0.5
G1	43.04 +/- 1.24	47.64 +/- 0.42	42.15 +/- 0.17	46.67 +/- 0.29	43.47 +/- 2.57	43.57 +/- 2.62
S	38.97 +/- 1.78	37.57 +/- 1.60	41.43 +/- 0.69	39.63 +/+ 0.34	40.89 +/- 1.66	42.32 +/- 2.41
G2-M	17.99 +/- 0.53	14.78 +/- 1.17	16.42 +/- 0.86	13.69 +/- 0.06	15.63 +/- 0.90	14.11 +/- 0.21

Figure 2. The Q540L amino acid substitution impairs the ability of wild-type TAp63α to induce a G_1 cell cycle arrest and cell death. Cell growth profiles of TAp63α (A), ΔNp63α (B) and TAp63αQ540L (C) stable cell lines under induced (+ Dox) or uninduced (- Dox) conditions. The growth rate was measured as described in Materials and methods. (D) TAp63α, ΔNp63α and TAp63αQ540L stable clones, induced (+ Dox) or uninduced (- Dox) to express the respective p63 proteins for 3 days, were analysed for the percentage of dead cells (blue cells/total cells) by trypan blue dye staining, as described in Materials and methods. (E) DNA content distribution of TAp63α, ΔNp63α and TAp63αQ540L cells, expressing (+ Dox) or not (- Dox) the respective p63 proteins, analysed for DNA content by propidium iodide staining of fixed cells. Data are the mean of three independent experiments. Standard deviations are also indicated.

and 150 μM of each primer were performed with an ABI PRISM 7900HT Sequence Detection System in the following cycle conditions: 50°C for 2 min, 95°C for 10 min., and 95°C for 15 s followed by 60°C for 1 min 40 cycles. 384 plates were assembled by QIAGEN 8000 BIOROBOT (QIAGEN GmbH, Hilden, Germany, EUROPE). Negative cDNA controls (no cDNA) were cycled in parallel with each run. Fluorescence data were analyzed with the SDS 2.1 software (Applied Biosystems, Foster City, CA, USA) and expressed as Ct, the number of cycles needed to generate a fluorescent signal above a predefined threshold. Target gene mRNA levels were expressed as $2^{-\Delta Ct}$, normalized for ACTB and POL2B, and fold changes were evaluates as $2^{-\Delta\Delta Ct}$ with the corresponding noninduced cell line as the calibrator according to Livak and Schmittgen.[34]

Additional informations. Probe sets intensities and additional information tables are available at http://www.bioinformatica.unito.it/bioinformatics/p63.

RESULTS

Production of TAp63α, TAp63αQ540L and ΔNp63α stable cell lines. To investigate the effects of the Q540L mutation on p63 protein functions, we generated stable H1299 cell lines, expressing TAp63α, ΔNp63α or TAp63αQ540L, under a tetracycline/doxycycline (Dox)-inducible promoter (see materials and methods). H1299 cells are p53 negative and show no detectable levels of p63 and/or p73 (and data not shown).[35]

We first analysed by Western blot the timing and level of expression of TAp63α wt, TAp63αQ540L and ΔNp63α proteins in our clones upon Dox induction. Results from representative cell lines for TAp63α wt, TAp63αQ540L and ΔNp63α are shown in Figure 1. Without Dox in the medium, p63 proteins were undetectable. Addition of 1 μg/ml Dox led to time-dependent induction of p63 proteins (Fig. 1). At 24 hrs of induction, p63 proteins were already abundant and their expression levels were comparable as shown by immunodetection of actin as a loading control (data not shown).

TAp63αQ540L is unable to induce a G_1 cell cycle arrest. We first decided to test the effects of wt TAp63α, ΔNp63α and mutant TAp63αQ540L protein on H1299 cell proliferation. TA and ΔNp63α were already known to induce H1299 cell cycle arrest and apoptosis, though to a different extent.[35] According to these data, H1299 cells, expressing wt TAp63α or ΔNp63α showed a reduction of cell growth rate while non-induced cells grew normally (Fig. 2A and B). The cell growth profile was completely unaffected by TAp63αQ540L expression (Fig. 2C). The trypan blue dye exclusion assay showed that TAp63α expression induced 9.2% of cell death and ΔNp63α 3.4 % 72 hr after induction, whereas TAp63αQ540L had completely lost this ability (Fig. 2D). Our p63-inducible clones were then examined by flow cytometry. In a Dox-free culture medium, the three p63 stable cell lines and the parental H1299 cell lines maintained a similar cell cycle phase distribution of DNA content (Fig. 2E and data not shown). At 48 hr, addition of 1 μg/ml of Dox resulted in G_1 cell cycle arrest by both wt TA and ΔNp63α with a parallel reduction in S and G_2/M phases and a significant increase of sub-G_1 events (Fig. 2E). In contrast, when the TAp63αQ540L mutant was induced, the percentage of cells in G_1 phase was unaffected and a slight increase in S phase with a corresponding decrease in G_2-M phase was observed (Fig. 2E).

Microarray and expression analysis. The finding that wt TAp63α inhibits cell proliferation and induces cell death, whereas the TAp63αQ540L mutant

Figure 3. Microarray data clustering. Hierarchical clustering, (parameters: Euclidean distance, average linkage clustering) was performed on average $\log_2$ fold change variation between induced and not-induced TAp63α wt and Q540L mutant stably transfected cell lines. (A) refers to genes which are not significantly modulated upon induction of TAp63α wt and are instead downmodulated by TAp63αQ540L expression. (B) includes only one gene which is downmodulated by TAp63α wt expression and not significantly modulated by TAp63αQ540L. (C) encloses genes not significantly modulated upon induction of TAp63α wt, but upmodulated by TAp63αQ540L. (D) refers to genes transactivated by TAp63α wt and characterized by a loss of regulation by TAp63αQ540L.

Table 1 **Primers designed to validate microarray data by qPCR**

ACTB_FW (Endogenous control)	GAGTCCGGCCCCTCCAT
ACTB_RW (Endogenous control)	GCAACTAAGTCATAGTCCGCCTAGA
ARX_FW	CTCGGAGCGGCAGTGTTC
ARX_RW	AAAAGAGCCTGCCGAATGC
BVES_FW	GGCATCTCCAAATACATTGAAAGTC
BVES_RW	CGTCTTGGTAACCTGAATTCTCTCT
CDKN1A_FW	CAGCGACCTTCCTCATCCA
CDKN1A_RW	GCTGCTAATCAAAGTGCAATGAA
CPM_FW	TGGGATTCCAGAGTTCAAATACG
CPM_RW	CAGCTCCCGCCCAACAG
CTCFL_FW	TGTACTTTTTCATAATGCCCAGTGA
CTCFL_RW	GAGGGTGGAAAAATCTTGTCAACT
FDXR_FW	TGGATGTGCCAGGCCTCTAC
FDXR_RW	TGGTTGTGGCTATGACACCTGTA
FGF5_FW	GCCCAGAATCAGCCCTACAA
FGF5_RW	GGAGGAAGGACAAGCTCATTCTT
GDF15_FW	AAACATGCACGCGCAGATC
GDF15_RW	CGGTCTTTTGAATGAGCACCAT
MDM2_FW	ACCACCTCACAGATTCCAGCTT
MDM2_RW	GCACCAACAGACTTTAATAACTTCAAA
PMAIP1_FW	TGAACTTCCGGCAGAAACTTC
PMAIP1_RW	GTTTTTGATGCAGTCAGGTTCCT
POLR2B_FW (Endogenous control)	CCTGATCATAACCAGTCCCCTAGA
POLR2B_RW (Endogenous control)	GTAAACTCCCATAGCCTGCTTACC
PTP4A1_FW	CCCTAGCATTAATTGAAGGTGGAA
PTP4A1_RW	CACGCCGCTTTTGTCTTATG

lacks these capabilities prompted us to generate a comprehensive profile of differential gene expression by microarray analysis. Four prototypic situations were evaluated: (a) TAp63α wt without induction (b) TAp63α wt with induction (c) TAp63αQ540L without induction (d) TAp63αQ540L with induction. The quality of the ttlRNA extracted from H1299 stable clones was assayed by Bioanalyzer (Agilent). Three biological replicas generated for all four situations were used to synthesize biotinylated cRNAs for hybridization on 12 HGU133plus2 arrays containing 54675 probesets. Microarray data show that there is a clear upmodulation of the wt and mutant p63α proteins upon induction with doxycycline (Fig. 3, additional tables A and B). Upmodulation of p63α was also confirmed by qPCR (data not shown). A total of 45 probe set ids (Fig. 3) were identified as differentially expressed and associated with 36 gene ids, whereas the other 7 have not been assigned.

QPCR validation was done for 11 out of 36 genes. Seven (Fig. 3, Y label) showed a perfect overlap between microarray and qPCR data, both qualitatively and quantitatively (i.e., same trend and similar fold change variation), three (Fig. 3, T label) were in agreement with microarray data, except that the fold change variation was lower, and one (Fig. 3, N label) could not be confirmed by qPCR.

A total of ten annotated genes transactivated upon induction of wt TAp63α (Fig. 3D) did not respond to TAp63αQ540L. Four (GDF15,

CDKNIA/p21/WAF, MDM2, ARX) were selected for qPCR to determine their responsiveness to TAp63α, ΔNp63α and TAp63αQ540L (Fig. 4). GDF15 and CDKNIA-p21/WAF were significantly transactivated by TAp63α only. GDF15 was already activated at 12 hr of induction (Fig. 4A) and p21/WAF after 24 hr (Fig. 4B). MDM2 and ARX were similarly transactivated by wt TA and ΔNp63α (Fig. 4C and D), but none of these four genes were modulated by TAp63α-Q540L (Fig. 4A and D).

We also compared the transactivation potential of TAp63α, ΔNp63α and TAp63αQ540L protein by CAT reporter assay in H1299 cells. A fixed amount of p21/WAF promoter-CAT construct was transiently transfected along with increasing amounts of plasmids encoding p63α proteins. As shown in Figure 5A, the Q540L mutation strongly affects the ability of TAp63α to induce the p21/WAF gene promoter, whereas ΔNp63α is a mild activator. Western blot analysis of the protein lysates used in this assay demonstrated that the mutant protein was expressed at equal, if not greater, levels than wt TAp63α These results suggest that the difference in activity between wt and mutated TAp63α protein was not due to differences in protein expression (data not shown).

The microarray data showed no increase of p21/WAF and MDM2 endogenous proteins in TAp63αQ540L stable cells upon induction (Fig. 5B). Moreover, the higher the expression of TAp63αQ540L protein in our stable clone, the lower was the level of endogenous p21/WAF and MDM2 proteins compared to noninduced cells (Fig. 5B). On the other hand, both wt TAp63α and ΔNp63α enhanced p21/WAF and MDM2 protein levels, though to a different extent (Fig. 5B). The same results were obtained when these experiments were repeated on two additional independent TAp63αQ540L stable clones isolated during our screening (data not shown).

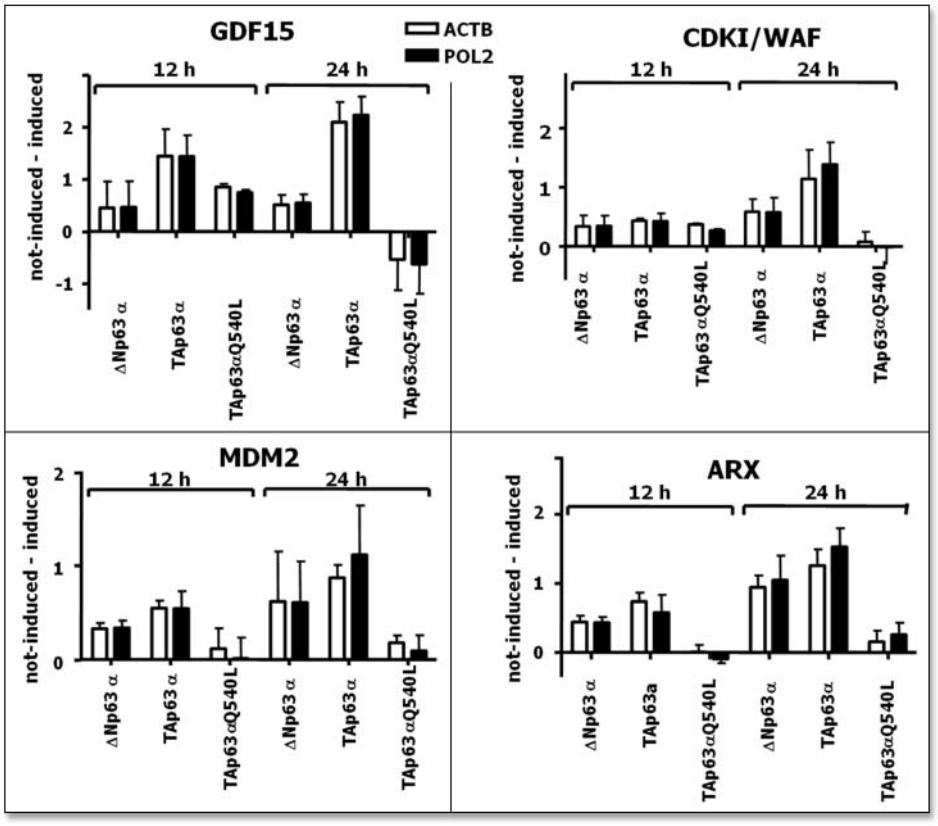

Figure 4. QPCR data related to four genes transactivated by TAp63α wt and characterized by a loss of control by the Q540L mutant. Gene expression was analyzed at 12 and 24 hours upon addition of doxycycline to ΔNp63α, TAp63α and TAp63αQ540L inducible cell lines. Target gene mRNA levels were normalized for ACTB (white bars) and POL2 (black bars) and expressed as -ΔΔCt (i.e., not-induced cell line—induced cell line Cts).

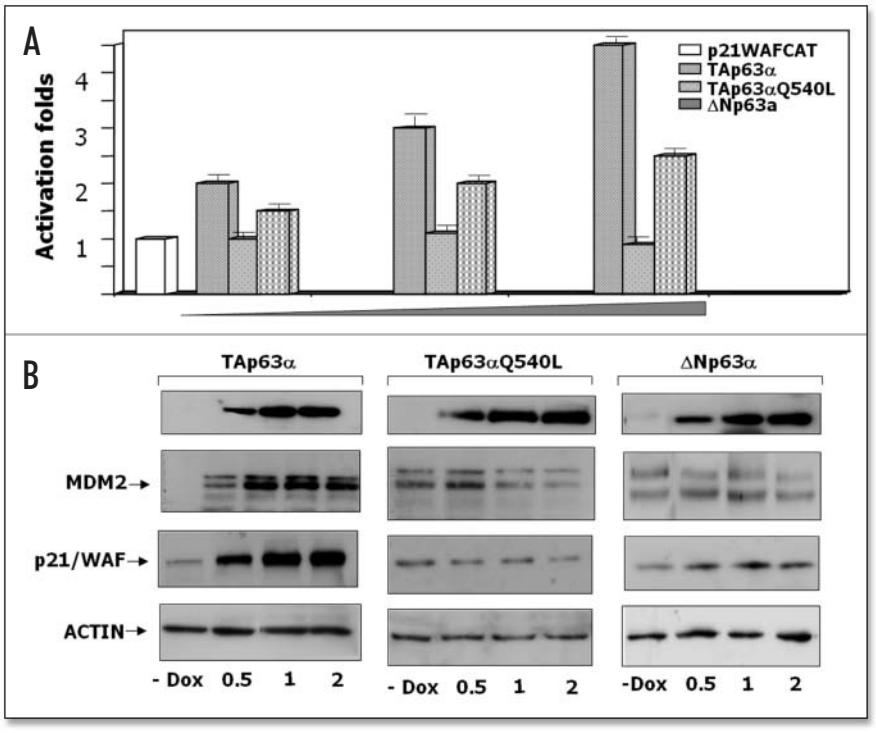

Figure 5. TAp63αQ540L has lost the ability to activate p21/WAF and MDM2 gene expression. (A) H1299 were transiently transfected with 2 μg of p21/WAF-CAT reporter plasmid/dish alone or with different amounts of each p63-expressing plasmid (1, 2 or 3 μg). After 48 h, cells were harvested, and CAT activity was determined. The basal activity of the reporter was set at 1. The data are presented as fold induction relative to the sample without effector (white bar). Each histogram bar represents the mean of triplicate assays from three independent experiments. Standard deviations are also indicated. (B) Western blot analysis showing expression of TAp63α wt, A p63αQ540L and ΔNp63α proteins in stable clones at 48 h upon induction with the indicated amounts of doxycycline. The expression of endogenous MDM2 and p21/WAF proteins was also evaluated by specific immunodetection. MDM2 and p21/WAF protein levels increase in parallel with TAp63α and ΔNp63α induction, but decrease upon TAp63αQ540L induction. β-Actin was used as a protein loading control.

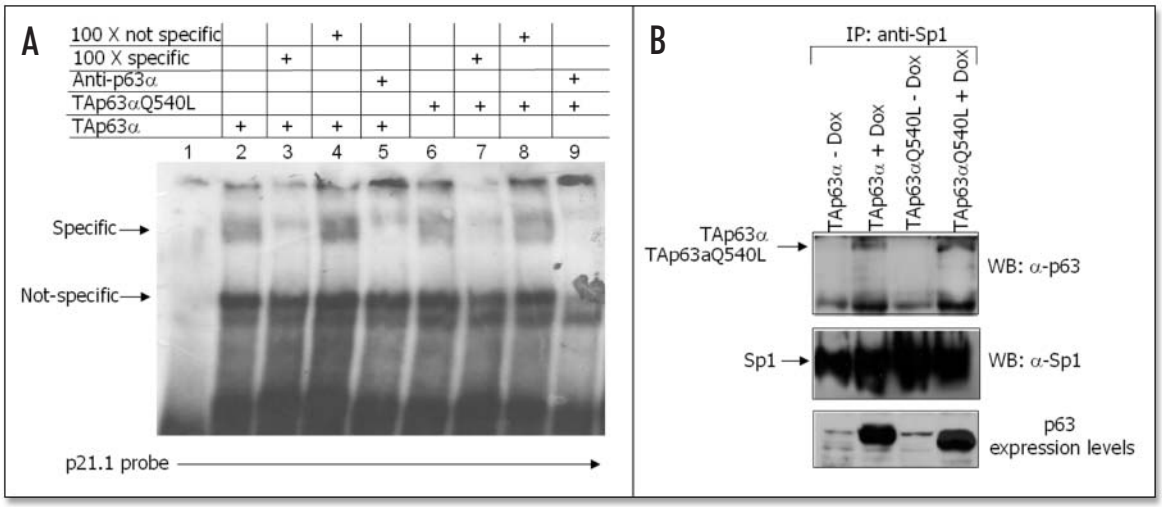

Figure 6. TAp63αQ540L binds to p53 consensus site in the p21/WAF target promoter and associates with the Sp1 transcription factor. (A) The indicated p63 proteins were in vitro translated by using TnT reticulocytes from Promega and 0.5 μ of each p63 plasmid DNA. Equal amounts of the individual reactions were subjected to EMSA using a ^{32}P-labeled oligo containing a p53-binding site present in the p21 promoter (p21.1 probe). Cold competition was performed with either the 100-fold molar excess of the same oligonucleotide (lanes 3 and 7) or an oligonucleotide containing a consensus binding site for E2F1 (lanes 4 and 8). For the supershift, anti-p63 antibodies (4A4; SantaCruz) were added to the sample prior to the binding reaction (lanes 5 and 9). (B) Coimmunoprecipitation in TAp63α and TAp63αQ540L expressing cell lines. Both wt and mutant TAp63α proteins were immunoprecipitated by a Sp1 polyclonal antibody only upon their induction with doxycycline.

TAp63α and ΔNp63α proteins are solely located in the nucleus where they act as transcriptional factors.[16] The lack of transcriptional activity of the TAp63αQ540L protein may stem from its inability to relocalate in the nucleus. Inspection of the subcellular location of wt TAp63α and TAp63αQ540L in our stable clones, by immunofluorescence showed that both proteins were uniformly distributed in the nucleus with nucleolar sparing. Therefore the Q540L amino acid substitution does not alter TAp63α subcellular distribution (data not shown).

TAp63αQ540L binds to p21 promoter sequences in vitro and vivo and interacts with SP1. The absolute inability of TAp63αQ540L to transactivate the p21/WAF promoter prompted us to find out whether it can still bind to the p53-consensus sequence of the p21/WAF promoter. We thus compared the DNA binding capacity of wt and mutant TAp63α isoforms by an in vitro DNA-binding assay. A radiolabeled duplex oligonucleotide representing a p53-binding site previously identified in the p21/WAF promoter was used as target DNA.[16] Incubation of this oligonucleotide with in vitro translated TAp63α or TAp63αQ540L mutant led to the formation of specific protein-DNA complexes (Fig. 6A, lanes 2 and 6). The specificity of the TAp63-DNA complexes was tested by a competition experiment: a 100-fold cold molar excess of the oligonucleotide completely abolished the binding, while an irrelevant control oligonucleotide had no effect (Fig. 6, lanes 3 and 4; 7 and 8). The identity of the TAp63α-DNA complexes was confirmed by a supershift experiment (Fig. 6A, lanes 5 and 9) in which the in vitro translated TAp63α proteins was incubated, prior to the binding reaction, with an antibody recognizing the p63 DNA-binding domain. Western blot analysis showed that the relative abundance of these proteins was comparable (data not shown). These observations indicate that wt TAp63α specifically binds to a p53 consensus sequence of the p21/WAF promoter and that the Q540L mutation does not affect this binding, at least in this in vitro assay.

The promoter of the human p21/WAF gene is characterized by a set of six proximal Sp1 binding sites located in the proximal region (nucleotides -120 to -40) and two distal p53 binding sites. These proximal sites have been shown to be essential for the activation of p21/WAF promoter by p53.[36] Concerning the MDM2 gene promoter, a series of five consecutive nnGGGGC repeats, bearing similarity to the Sp1 consensus, have been identified (nucleotides -415 to -318). These conserved GC elements contribute to the basal activity of the p53-inducible MDM2 promoter.[37] Since it has previously been reported that the γ isoform of TAp63α directly interacts with Sp1, we decided to determine whether TAp63α is also able to interact with Sp1 and, if so, the effect of the Q540L substitution. We performed coimmunoprecipitation experiments in TAp63α and TAp63αQ540L expressing cell lines, both Dox-induced and not. As shown in Figure 6B, both wt and mutant TAp63α proteins were immunoprecipitated by a Sp1 polyclonal antibody. Reciprocal immunoprecipitation, which detects Sp1 protein with the p63 monoclonal antibody, did not immunoprecipitate Sp1 (data not shown).

DISCUSSION

Epithelial development and differentiation in embryo rely on a set of temporally and spatially regulated molecular events. Recent observations designate p63 as a driving force of this process: the ΔNp63α isoform maintains the proliferative potential of basal keratinocytes in mature epidermis, whereas the TAp63α isoform, which is the first to be expressed in mouse embryo, is believed to act as a molecular switch required for commitment to epithelial stratification.[3] A broad spectrum of p63 mutations are responsible for several human ectodermal, craniofacial and limb malformations.[20] EEC and ADULT mutations are located in the DB domain of p63. They abolish p63 DNA-binding and produce highly stable, but transactivation-inert TAp63 proteins.[38] AEC mutations are confined to the SAM domain.[22] Their effects on p63 transcriptional functions are less predictable and they only affect the α isoforms.

The Q540L mutation impairs p63 transcriptional ability. Our study provides evidence that the Q540L amino acid substitution strongly impairs the transcriptional activity of TAp63α (Figs. 3 and 4). Our genome-wide transcriptional profiles comparing the transcriptional response induced by wt and TAp63αQ540L expression show that 14 out of 45 differentially modulated probe sets (ten annotated genes), are characterized by a loss of control (activation or repression) by the Q540L mutant (Fig. 3D). As demonstrated (see Fig. 2, qPCR and data not shown) the lack of transactivation ability of TAp63αQ540L cannot be attributed to a decrease in its expression, nor to alteration of its subcellular location. It is well documented

that, p63, like p73, can bind to the p53 consensus DNA-binding motif and activate a number of p53-regulated genes. In principle, the Q540L mutation, even though it is predicted to not destroy the overall structure of the SAM domain, could alter the DNA-binding affinity of the mutant protein. As regulation of p21/WAF was severely impaired in cells expressing the Q540L mutant, we tested whether the mutant protein was still capable of interacting with a p53-binding motif of the p21/WAF promoter. Our results indicate that both wt TAp63α and its Q540L mutant are equally active in binding to this sequence, at least in our in vitro system. On the other hand, several data argue that particular coactivators expressed in specific cell types and factors bound to the promoters confer specificity of gene regulation on the members of the p53 family and their isoforms.[39] For instance, ΔNp63α negatively regulates transcription of the hsp70 promoter through its interaction with the CCAAT-binding and NF-Y transcription factors,[40] while Sp1 cooperates with p53, p63 and p73 in synergistic transactivation of the p21/WAF promoter.[36] As a physical interaction between TAp63α and Sp1 takes place and is required for the regulation of EGFR gene expression,[41] we determined whether TAp63α also interacts with Sp1 and, if so, the effect of the Q540L substitution. Our data clearly indicate that TAp63Q540L is still able to interact with Sp1 (Fig. 6B). Other transcriptional factors may thus be crucial for p63-driven transcription and the Q540L amino acid substitution may affect the interaction between the p63 SAM domain and a still undefined factor.

Furthermore, we also found probe sets that are specifically up or downregulated by the mutant protein alone (Fig. 3A and C). These apparently conflicting results, too, may be a consequence of a loss of transcriptional function, assuming that the above-mentioned genes are p63 secondary targets repressed or activated by p63 primary targets. Alternatively, the possibility that the Q540L aminoacid substitution confers new transcriptional and cell growth regulating properties on the TAp63α protein by altering its ability to interact with particular coactivators or corepressors cannot be ruled out.

This possibility needs investigated by comparing the activity of wt and mutant proteins directly on the promoters of these TAp63α-Q540L-regulated genes by means of transient reporter assays. Furthermore, other technologies, such as in vivo DNA-binding assays and mass spectrometry, will aid in the identification of key proteins involved in the regulation of p63's transcriptional activities.

Differentially expressed genes and their implication in AEC pathogenesis. An extensive search of the published literature to find links between the physiological functions of the deregulated genes and their role in AEC showed that, with the exception of p21/WAF and MDM2, they were the subject of very few publications and little was known about their functions. It was, however, found that GDF15, BVES, CLMN and CPM are involved in the mechanisms of cell differentiation,[42-45] while ARX and FGF5 are associated with embryonic development.[46-49] GDF15 is the murine ortholog of the human immunoregulatory cytokine macrophage inhibitory cytokine-1 (MIC-1) also known as PDF (prostate derived factor), a divergent TGF-β superfamily member. It has proapoptotic and antimitotic activities and is involved in the control of prostatic cell growth.[44]

Interestingly, the GDF15 promoter contains two putative p53 responsive elements and is upregulated by p53, though its expression in response to injury also appears to be induced p53-independently.[50,51] GDF15 seems to be a p63 target, specifically upregulated by TAp63α (Fig. 4D). This regulation is completely abolished by the Q540L amino acid substitution (Fig. 3D). The lack of GDF15 expression in epithelia may contribute to the abnormal differentiation of epithelia-derived structures observed in AEC patients.

Another gene closely involved in development is ARX.[47] Its expression profile is highly complex and dynamic in the mouse embryo brain, where it peaks at embryonic (E) day 9.5 just after the TAp63α expression peak (E. 8.5).[3] It is also a marker of adult neuronal stem cells.[47] Interestingly, both TAp63α and ARX transcripts decrease at E 13.5, which corresponds to the switch from TAp63α to ΔNp63α expression.[3] Our transcriptional profiling combined with the published data on ARX suggests that p63 and ARX may be linked in a common regulatory pathway. The information available, however, is not sufficient to allow a direct connection to be made between the function of p63 and ARX in AEC.

Calmin (CLMN) is a protein with calponin homology (CH domain) and transmembrane domains expressed in maturing spermatogenic cells. The cDNA encoding CLMN was isolated by RNA differential display applied to developing mouse skin. The region covering the CH domain showed a high level of homology with β-spectrin, α-actinin, and dystrophin. The CLMN transcript was detected in adult testis, liver, kidney, and large intestine; the expression in testis was by far the strongest.[42] CLMN is linked to skin development. In mice, its mRNA starts to be detectable in the epidermis at 15.5–16.5 dpc (days post-coitum) and its expression increases as the skin develops. The timing of CLMN gene expression corresponds to the switch from the TA to the ΔN isoform. CLMN is only transactivated by the Q540L mutant (Fig. 3C). Since induction of CLMN expression fits in nicely with the timing of the switch from TAp63α to ΔNp63α expression in the epithelial stratification program,[3] TAp63α may be supposed to act as a transcriptional repressor of this gene, with the result that expression of mutant TAp63α might improperly anticipate CLMN expression during skin development.

BVES/Pop1 is the prototype of a new class of cell adhesion molecules. It is expressed in the epithelial components of retina, lens and cornea,[52] during blood vessel development, in the gut endoderm and the epicardium and in all three germ layers during avian organogenesis.[53] BVES is transactivated by TAp63α and not modulated by the Q540L mutant, and hence may be required to promote cell adhesion and translocation during early embryogenesis.

Another interesting gene that is only transactivated by the mutant p63 is SERPINH1, also known as HSP47. Hsp47 protein is involved in skin wound regeneration and immunohistochemistry has demonstrated Hsp47-positive cells in the epidermal cell layer of fetal and neonatal rat skin. Hsp47 may be an important determinant of scar formation, since scarless healing of fetal skin wounds correlates with a lack of change in HSP47 expression.[54]

p21/WAF has long been known to arrest the cell cycle. In the epithelium it is involved in maintenance of the stem cell compartment:[55] p21 null mice are unable to limit the production of stem cells and their proliferative potential.[55] p21/WAF is strongly transactivated by the TAα wild-type isoform (Fig. 4A) and its promoter is not or only mildly responsive to the ΔNα isoform.[27] By inducing p21/WAF, TAp63α breaks the cell cycle by restraining stem cell proliferation: the overall system is committed to the formation of stratified epithelia. The parallel increase of ΔNα and decrease of TAp63α expression redirects the system to terminal differentiation.[27]

Our qPCR data (Fig. 4B) and expression studies (Figs. 3D and 5B) support this scenario since the p21/WAF promoter is strongly activated by the TAp63α wt isoform and p21/WAF upmodulation is reduced if ΔNp63α is expressed. Interestingly, while ΔNp63α seems less efficient than TAp63α as a p21/WAF activator (Figs. 4B and 5B), they both induce a similar G_1 cell cycle arrest. The aminoterminal-deleted isoform should not be generally defined as a

transactivation-defective isoform. Our and other published data indicate that ΔNp63α modulates transcription[40] and this ability is rather dependent on the specific gene promoter (see Fig. 4C and D). In conclusion, the difference in the growth rate profiles (Fig. 2A and B) of cells expressing either the TA or the ΔNp63α isoforms is likely to be the result of the relatively higher efficiency of TAp63α with respect to ΔNp63α in inducing cell death, as shown in Figure 2D.

The growth rate profiles and cell cycle distribution of cells expressing the AEC-derived TAp63α protein are undistinguishable from those of uninduced cells (Fig. 2C and E), indicating that the Q540L amino acid substitution affects both the cell cycle arrest and cell death inducing properties of p63. Finally, we suggest that deregulation of p21/WAF associated with the Q540L mutation will produce a defect in the process of commitment to epithelial stratification that simultaneously allows premature expression of skin differentiative markers. A defect of this kind would explain the skin fragility and chronic scalp erosions complicated by infections, which are a hallmark of AEC. In conclusion, further investigation of the differentially regulated genes identified in this study will result in a better understanding of the molecular mechanism underlying the AEC phenotype.

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