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Report

Connecting p63 to Cellular Proliferation

The Example of the Adenosine Deaminase Target Gene

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ABSTRACT

An unresolved issue regards the role of p73 and p63, the two homologs of the p53 oncosuppressor gene, in normal cells and in tumor development. Specific target genes for each protein need to be identified and characterized in order to understand the specific role of each protein in tumor initiation and progression as well as in oncosuppression and development. We tested whether p63 is implicated in transcriptional events related to sustaining cell proliferation by transactivation of antiapoptotic and cell survival target genes such as Adenosine Deaminase (ADA), an important gene involved in cell proliferation. We demonstrate that ADA is a direct target gene of p63 isoforms. In human keratinocytes, the rate of proliferation and the high level of ADA transcript diminished upon elimination of p63 by small interfering RNA. Reporter assays and chromatin immunoprecipitation experiments indicate a physical interaction of p63 with the two putative p53 binding sites we identified in the ADA gene. Moreover, in response to p53 stabilization and ΔNp63 downregulation in normal keratinocytes after U.V. treatment, we found a change in the transcriptional pattern of the p53 family target genes, consistent with the different roles played by p53 and p63 in tumor suppression and cellular proliferation. In fact p53 upregulation determined an increase in p21, which in turn mediated the cell cycle arrest, while the downregulation of ΔNp63 determined a marked decrease in ADA transcript. The experiments reported here support the hypothesis that TAp63 and ΔNp63 might contribute to tumor genesis not exclusively by antagonizing p53, but by conferring a proliferative potential on cancer cells through the transactivation of target genes indispensable for cell division, such as the Adenosine Deaminase gene.

INTRODUCTION

Correct cellular proliferation reflects a balance between cell growth and cell division. Elaborate mechanisms prevent uncontrolled cell proliferation and the p53 family members (p53, p63 and p73) are crucial players in this context. p53 is a sequence-specific DNA binding protein that induces, after a genotoxic stress, the expression of target genes involved principally in cell growth suppression, DNA repair and apoptosis. The two p53 homologs, p63 and p73, were initially speculated to overlap p53 functions, as they were able to bind the p53 consensus sequence and to transactivate some p53 target genes. However, differences between family members emerged rapidly. Live-born p53 knockout mice develop normally, on the contrary p73 and p63 knockout mice demonstrate the important role of these two proteins during development.[1,2] Some of the differences between the three protein functions might be explained by the fact that p53 predominantly exists as a single isoform, while both p63 and p73 undergo alternative splicing at their C-termini, resulting in three p63 isorforms (α–γ) and seven p73 isoforms (α–η), called TA variants, which appear to have some overlapping functions with p53, at least when overexpressed. A second promoter located in intron 3 of both the p63 and p73 genes generates ΔN variants, which may interfere with several of the p53 activities. In particular ΔNp63 isoforms have been shown to exhibit dominant-negative effects towards both p53 and TAp63 and are thought to have oncogenic properties.[3-5] A second important difference between p53, p63 and p73 is the pattern of expression. p53 is widely expressed at low levels. In human, p73 has been detected in the brain, kidney, placenta, colon, heart, liver and spleen. p63 also follows a restricted pattern of expression in normal tissues and functions to maintain progenitor cell populations in stratified epithelia. In particular, ΔNp63 isoforms are expressed in the basal layer of all stratified epithelia, with a gradual decrease in the terminally differentiated cell layers.[6,7]

All three family members seem to be implicated in cancer. The study of mouse and human cancer genetics underlines the importance of p53 inactivation in carcinogenesis, while the p63 and p73 genes are rarely mutated in human cancer and the link of these two homologs to cancer and their effect on p53 tumor suppression are under investigation. Although it was recently reported that $p63^{+/-}$ and $p73^{+/-}$ mice develop spontaneous tumors and loss of p63 and p73 can cooperate with p53 loss in tumor development,[8] p73 and p63 do not appear to adhere to Knudson's classical model of tumor suppressor genes. A number of tumors (e.g., neuroblastoma, breast carcinoma and ovarian cancer) have been reported to express higher levels of p73 compared with surrounding normal tissues.[9] Other cancers derived from squamous epithelia overexpress ΔNp63 isoforms in particular. Moreover, it has been reported that the overexpression of ΔNp63 in rat cells 1a increased colony growth in soft agar and xenograft tumor formation in nude mice, supporting the view that p63 acts as an oncogene.[10] It is possible that modulation of p73 and p63 expression contribute to cancer initiation or progression under certain conditions.

In order to understand the specific role of each protein in cell cycle progression, as well as tumor suppression and development, the p53 family target genes and in particular the unique or the preferential target genes for each protein need to be identified and characterized. Some specific target genes have already been identified, for example, AQP3, a glycerol and water transporter is induced by p73, but weakly by p53.[11] The notch receptor ligands, JAG1 and JAG2, are also induced by p63 and p73, but not by p53.[12]

The goal of the current study was to determine whether p63 is implicated in transcriptional events related to sustaining cellular proliferation by transactivating target genes involved in cellular growth. Since we recently found in our previous work that the Adenosine Deaminase (ADA) gene, which codes for an important enzyme involved in DNA synthesis and repair, is induced by p73 but not by p53 in the physiological conditions tested,[13] we investigate whether ADA gene is also a direct target of p63. The experiments described here show that in human keratinocytes, the suppression of p63 by small interfering RNA causes a decrease in the proliferation rate and ADA gene expression. Moreover, reporter assays and chromatin immunoprecipitation experiments demonstrate that the ADA gene is a direct TAp63α and ΔNp63α target. We further analyzed the effect of U.V. treatment in normal keratinocytes on the transcriptional changes of specific p53 family target genes in response to a decrease in ΔNp63 and an increase in p53.[14,15] The results we obtained are consistent with the hypothesis that ΔNp63 has a major role in cell growth and p53 in tumor suppression.

Our experiments well support the hypothesis that the essential role of p63 in maintaining the proliferative capacity of the basal cells of stratified epithelia and of the tumor cells might be due to the transactivation of genes necessary for cell division such as the ADA gene.

MATERIALS AND METHODS

Cell cultures. The human embryonic kidney Flp-In T-Rex-293 cell line from Invitrogen was used to generate stable TAp63α and ΔNp63α expression cell lines. The Flp-In T-Rex-293 cell line contains two stably, independently integrated, plasmids which exhibit the following features: (1) the pcDNA6/TR plasmid, which stably expresses the Tet repressor gene under the control of the constitutive human cytomegalovirus (CMV) immediate-early enhancer promoter; (2) the pFRT/lacZeo plasmid, which introduces a single Flp Recombination Target (FRT) site into the genome and stably expresses the lacZ-zeocin fusion gene under the control of the SV40 early promoter. The FRT site encodes a 34 bp sequence that serves as the binding and cleavage site for Flp recombinase. These plasmids are integrated into transcriptionally active regions of the genome.

The cDNAs corresponding to wild type TAp63α and ΔNp63α were cloned into the expression vector pcDNA5/FRT/TO designed for use with the Flp-In T-rex cells. The expression of our cDNAs from pcDNA5/FRT/TO plasmid was controlled by the strong cytomegalovirus (CMV) immediate early enhancer/promoter into which two copies of the tet operator ($TetO_2$) sequence were inserted in tandem. These $TetO_2$ sequences confer regulation by tetracycline to the promoter. The expression of our cDNAs was induced by the addition of tetracycline to the culture medium. The pcDNA5/FRT/TO plasmid contains a single FRT site for Flp recombinase-mediated integration. We generated stable Flp-In T-rex 293 cell lines expressing our cDNAs by the cotransfection of pcDNA5/FRT/TO expression constructs and the pOG44 plasmid, which encodes a site-specific recombinase, under the control of the human CMV promoter. The Flp recombinase mediates a site-specific recombination reaction between interacting DNA molecules via pairing of interacting FRT sites. Following the integration of the pcDNA5/FRT/TO construct, the Flp-In T-Rex-293 cell line becomes sensitive to Zeocin and resistant to hygromycin.

The MCF-7, H1299 and HaCat cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) plus 10% foetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C, 5% CO_2.

Human epidermal Keratinocytes (NHEK) were isolated from neonatal foreskin specimens derived from a normal human Caucasian (PromoCell). To allow proliferation, passages 2–3 of NHEK were maintained in Keratinocyte Growth Medium 2 (PromoCell) supplemented with 0.4% Bovine Pituitary extract, 0.125 ng/ml Epidermal Growth Factor, 5μg/ml Insulin, 0.33 μg/ml Hydrocortison, 10 μg/ml Transferrin, 0.39 μg/ml Epinephrine, 0.15 mM $CaCl_2$. U.V. treatment consisted in a 40 J/m^2 performed in a CL-1000 Ultraviolet Crosslinker (UVP).

Colony suppression assay. HaCat cells (5×10^3) in 2 cm plates were transfected with 160 pmol of scramble or siRNAp63 (Dharmacom) using Lipofectamine™ Reagent (Invitrogen). Fourty-eight hours later, cells were rinsed with PBS, fixed with methanol for 30 min. at room temperature and stained with Giemsa.

Cell proliferation assay by MTT reduction. HaCat cells (5×10^3) in 2 cm plates were transfected with 160 pmol of scramble or siRNAp63 (Dharmacom) using Lipofectamine™ Reagent (Invitrogen). Fourty-eight hours later, 200 μl of MTT solution (5mg/ml) was added to the cells and incubated for 4 h at 37°C. Then the supernatant was removed and the blue formazan crystals were resuspended in isopropanol prior to reading the absorbance to 580 nm.

Transfections. MCF cells were cultured in 10 cm culture dishes 24 h before transfection (about 60–80% confluency). 5 μg of pcDNA3-TAp63α or pcDNA3-ΔNp63α was incubated in 200 μl of serum free medium plus 15 μl of Fugene (Roche) for 15 min at room temperature and subsequently added to the cell cultures. Then the cells were cultured in DMEM plus serum for 24 h.

HaCat cells were cultured without antibiotics in 6-well plates 24 h before transfection (about 60–80% confluency). One-hundred sixty pmol of Scramble or p63 small interfering RNAs were diluted in 100 μl D-MEM without serum plus 16 μl of Plus™ Reagent (Invitrogen) and incubated at room temperature for 15 min. Six microliters of Lipofectamine™ Reagent (Invitrogen) were diluted in 100 μl D-MEM without serum in a second tube. Precomplexed DNA and diluted Lipofectamine™ Reagent (Invitrogen) were mixed and incubated for 15 min at room temperature and subsequently added to the cell cultures. The cells were cultured in DMEM plus serum without antibiotics for 24 h. Then the medium containing the complexes was replaced with fresh complete medium and the cells were cultured for an additional 24 h.

CAT assays. The fragments containing the p53-RE in the promoter (Target A) and in Intron 1 (Target B) of the ADA gene (GenBank Accession N°:M13792) were amplified from the human genome DNA and respectively cloned upstream of the reporter CAT gene in the promoter-less pBLCAT3

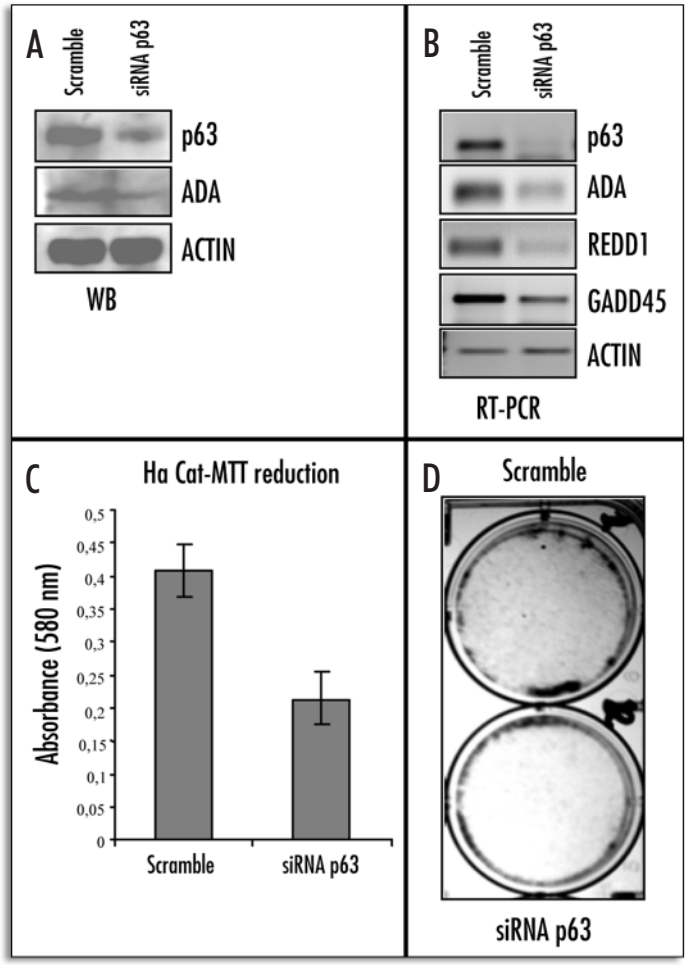

Figure 1. Suppression of p63 expression reduces cell growth and abolishes ADA gene expression. HaCat cells were transfected with scramble or siRNA p63. After 48 h, the expression of p63 and ADA were evaluated at the protein and RNA level by Western blotting (WB) (A) and RT-PCR (B). The expression of Actin, REDD1 and GADD45 were evaluated as controls. Cell growth was measured by MTT reduction (C) and colony suppression assays (D).

vector and in the pBLCAT2 vector. pBLCAT2 contains the Herpes Simplex virus tk promoter that permits the analysis of the effects of putative regulatory elements on a heterologous eukaryotic promoter. The resulting recombinant plasmids are indicated pBLCAT3-target A and pBLCAT2-target B. The two mutated pcDNA$_3$TAp63αR279Q and pcDNA$_3$ΔNp63αR279Q constructs were generated using Quick Change Site-Directed Mutagenesis Kit (Stratagene). 2.5 x 10^5 of human p53-null H1299 cells were cultured in 6 cm culture dishes 24 h before transfection (about 60–80% confluency). 2.5 μg of pBLCAT-p21 or pBLCAT recombinant vectors containing ADA-Target A or ADA-Target B alone or with 0.3 μg of an expression vector containing wt TAp63α or ΔNp63α or their R279Q mutant versions plus 0.1 μg of pSVβ-gal (Stratagene) was incubated in 100 μl of serum free medium and 8 μl of Fugene (Roche) for 15 min at room temperature and subsequently added to the cell cultures. Then the cells were cultured in DMEM plus serum for 48 h. Transfection efficacy was determined by β-galactosidase activity (β-gal assay, Roche). CAT expression was determined in duplicate using a CAT-ELISA (Roche) and calculated as ng CAT protein/mU β-gal. The data reported represent the average of at least three independent experiments and are shown with their standard deviations.

Western blot analysis. Cells were lysed with RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholat, 0.1% SDS, protease inhibitors cocktail tablets (Roche)) for 1 h on ice. Then the lysates were clarified by centrifuged at 13,600 x g at 4°C, aliquoted and stored at -20°C. Fifty micrograms of the total proteins in 2X SDS-PAGE sample buffer were heated at 95°C for 2 min and submitted to 12% SDS-PAGE. Separated proteins were electroblotted onto Nitrocellulose PROTRAN^R BA85 (Schleicher & Schuell). Blots were exposed for 60 min at room temperature to the following primary antibodies: CM1 (anti-p53 polyclonal), Abp63 4A4 (Santa Cruz), AbADA C-20 (Santa Cruz). Bound primary antibodies were visualized using ECL Western Blotting detection reagents (Amersham Pharmacia biotech).

RNA extraction and RT-PCR. Total RNA from different cell lines was extracted using an RNasy mini kit (Qiagen). Reverse transcription reactions were carried out for 50 min at 42°C and contained 1.5 μg of total RNA, 250 ng of oligo(dT), 0,5 mM dNTP mix, RNase Inhibitor, 1X RT buffer, 5 mM $MgCl_2$, 10mM DTT and 200 U of SuperScript II RT (Invitrogen) in a total volume of 20 μl.

Two microliters of each cDNA, 200 μM dNTP, 20 pmol of sense and antisense primers, 1X PCR buffer, 1.5 mM $MgCl_2$, and 2.5 units of Taq (Invitrogen) were used in the PCR reactions.

The following primers were used: TAp63 : For: TGT TCA GTT CAG CCC ATT GAC T; Rev: AGT CCT GCA TGC GGA TAC AGT ; ΔNp63 : For: TGT ACC TGG AAA ACA ATG CCC A

Rev: GAC GAG GAG CCG TTC TGA ATC T; NOXA : For: GGG AAG AAG GCG GCA AGA A Rev: AGG TTC CTG AGC TGA AGA GTT T ; REDD1 : For: GGG ACC GCT TCT CGT CGT CGT Rev: CTT TGC CCA CCT GGC TTA CCA A; ADA :For: GGA TCG CCT ATG AGT TTG TAG AG Rev: CCC AAG ACC TCT TTT ACT ACT TCG; GADD45 : For: CCC GAT AAC GTG GTG TTG TGC C Rev: ACC AGC ACG CAG TGC AGG TCC; MDM2 : For: AAC CAC CTC ACA GAT TCC AG Rev: TCA AGG TGA CAC CTG TTC TC.

Flow-cytometric analysis. 1-1.5 x 10^6 T-Rex-293-TAp63α T-Rex-293-ΔNp63α and NHEK cells were seeded into a 100 mm diameter Petri dish. The total population, including floating and adherent cells un-induced and after the TAp63α and ΔNp63α overexpression or U.V. irradiation at the indicated times, were harvested and fixed in 75% ethanol for at least 2 h at -20°C. The cells were then rehydrated in cold PBS, pelleted and treated with 150 mg/ml RNase A for 30 min at 37°C and stained with 5 mg/ml propidium iodide (PI) for 45 min. The cells were analysed in a FACScalibur; cell cycle and apoptosis analyses were performed using ModFit analysis software (Becton Dickinson).

Chromatin immunoprecipitation assay. T-Rex-293-TAp63α and T-Rex-293-ΔNp63α cells were cultured in 15 cm culture dishes for 12 h after induction of the two proteins. Proteins were cross-linked to DNA in living nuclei by adding formaldehyde directly to the cell culture medium to a final concentration of 1%. Cross-linking was allowed to proceed for 10 min at 37°C. Cross-linked cells were washed with phosphate-buffered saline, scraped off the plates and resuspended in 20mM Tris-chloride pH = 8.3, 3 mM $MgCl_2$, 20 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin. Nuclei were pelleted by microcentrifugation and lysed by incubation in nuclear lysis buffer (1% sodium dodecyl sulphate, 10 mM EDTA, 50 mM Tris-chloride pH = 8.1, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin). The resulting chromatin solution was sonicated for twenty sets of 10 second pulses at 40 watts to generate 300–2000 bp DNA fragments. After microcentrifugation, the supernatant was diluted 1:10 with dilution buffer (0.01% sodium dodecyl sulphate, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-chloride pH = 8.1, 167 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1μg/ml leupeptin, 1 μg/ml aprotinin) and precleared with protein A-Agarose/salmon sperm DNA and divided into aliquots. Five micrograms of p63 antibody (Ab p63-H137 -Santa Cruz) or any antibody (as negative control), was added to the chromatin solution and incubated on a rotating platform for 12–16 hours at 4°C. Antibody-protein-DNA complexes were precipitated with protein A-Agarose/salmon sperm DNA. After centrifugation, the beads were washed and the protein-DNA complexes were eluted with 1% sodium dodecyl sulphate, 100 mM Sodium Carbonate. DNA-protein cross-links were reversed by heating at 65°C for 4 hours and DNA was phenol extracted and ethanol precipitated. DNA fragments were analyzed by PCR using primers

specific for the ADA-Target A, ADA-Target B and the Interleukin-10 promoter, which was used as a negative control.

RESULTS

siRNA-mediated knockdown of p63 decreases cell growth and ADA gene expression. We tested whether a decrease in p63 at mRNA and protein levels was capable of exerting growth suppression and whether p63 is implicated in transcriptional events related to sustaining cell proliferation through the transactivation of target genes involved in cell proliferation, such as Adenosine Deaminase. To this end we suppressed the expression of p63 in HaCat cells by using p63siRNA. The p63siRNA, we used, is designed to target all the known p63 variants, without targeting related gene family members. Cells were transfected with scramble RNA (control) or p63siRNA for 48 h. As shown in (Fig. 1A and B), Western Blot and RT-PCR analyses demonstrated that the cells transfected with p63siRNA displayed a decrease in p63 protein and mRNA levels. Quantification of cytoproliferation was measured by MTT reduction and by Giemsa-staining of the attached cells. As shown in (Fig. 1C and D), a significant suppression of proliferation was achieved upon treatment with p63siRNA with respect to the control. Interestingly, in cells that underwent suppression of p63, a marked decrease was observed for ADA mRNA and protein levels (Fig. 1A and B). The suppression of p63 activity determines a decrease in the mRNA levels of well known p63 target genes, such as GADD45 and REDD1 used as controls. Therefore, these results indicate that in keratinocytes the depletion of p63 determines the inhibition of proliferation, which is correlated with a marked decrease in ADA gene expression.

p63 transactivates the two p53-responsive elements in the ADA gene. As the p63 siRNA knockdown experiments showed a decrease of ADA expression, we tested whether the ADA gene could be a p63 direct target gene by performing a reporter assay. Using PatSearch and DNAfan algorithms,[16,17] we identified two p53 family responsive elements (REs) in silico within the ADA gene (GenBank Accession N°: M13792), which we have called ADA-target A in the promoter region (3483 bp upstream the transcription start site) and ADA-target B in the first intron (13991-14048) (Fig. 2A).

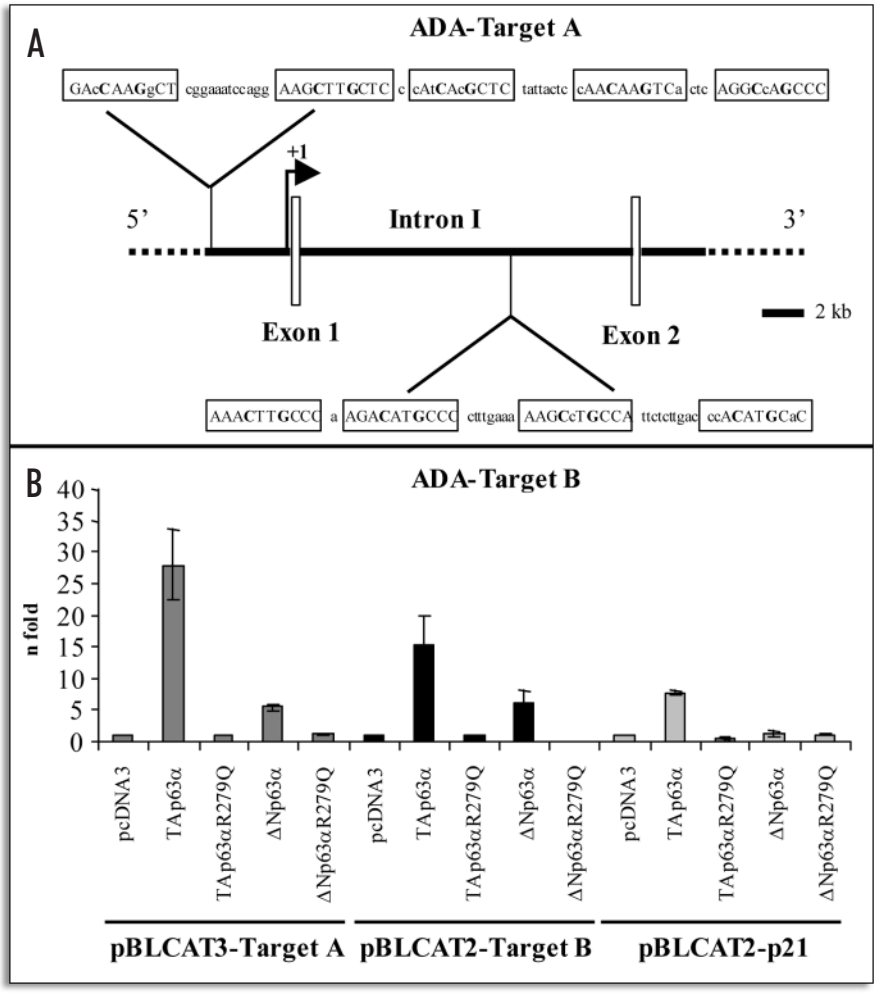

Figure 2. (A) Schematic map of the human Adenosine Deaminase genomic region containing the putative p53 responsive elements (ADA-Target A and ADA-Target B. The decamers are boxed and the nucleotides mismatched from the consensus sequences for p53 binding are in lower case. Target A contains five decamers, each spaced by a maximum of 12 nucleotides. Target B contains four decamers, each spaced by a maximum of 10 nucleotides. Two decamers are adjacent but for one base and show no mismatch. All decamers have fixed C4 and G7 positions and each double decamer contains a maximum of 3 mismatches. The start of transcription is indicated by an arrow. (B) Effect of TAp63α, ΔNp63α and their R279Q mutants on the transcriptional activity of ADA-Target A, ADA-Target B and p21. p53-null H1299 cells were cotransfected with pcDNA3-TAp63α or ΔNp63α wt or their mutated forms TAp63α-R279Q or ΔNp63α-R279Q, and CAT- reporter gene plasmids: pBLCAT3-Target A or pBLCAT2-Target B or pBLCAT2-p21 target. CAT expression was quantified by CAT-ELISA and normalized to β-galactosidase activity. The data represent the average of at least three independent experiments and are shown with the standard error.

To determine whether the two p53-REs in the ADA gene mediate transcriptional responses to TAp63α and ΔNp63α, we constructed CAT expression plasmids by cloning ADA-Target A in promoterless vector pBLCAT3 and ADA-Target B in pBLCAT2. pBLCAT 2 contains the Herpes Simplex virus tk promoter allowing the analysis of the effects of putative regulatory elements on a heterologous eukaryotic promoter. Moreover we mutagenized both the TAp63 and the ΔNp63 isoforms in residue 279 of the central domain, which is important for DNA binding and transactivtion.

Transactivation assays were performed by transfection, in p53-null H1299 cells, of the recombinant CAT plasmids (pBLCAT3-target A or pBLCAT2 -target B) with the empty vector $pCDNA_3$ (control) or with an expression vector containing either TAp63α ($pcDNA_3$TAp63α) or ΔNp63α ($pcDNA_3$ΔNp63α) or their R279Q mutated forms ($pcDNA_3$TAp63αR279Q, $pcDNA_3$ΔNp63αR279Q respectively).

Under these conditions TAp63α induced a 28 fold activation of ADA-target A and a 15 fold activation of target B compared to the control (Fig. 2B). ΔNp63α also induces a 5-6 fold activation of both ADA targets, although the activation was more modest than that induced by the TA isoform (Fig. 2B). The activation was dependent on functional p63 since transactivation with the defective mutants TAp63αR279Q or ΔNp63αR279Q was incapable of activating the reporter constructs. In order to control the specificity of the activities of p63, we used the pBLCAT2-p21 reporter vector (Fig. 2B). Interestingly the activation of the p21 reporter was mediated by TAp63α (8 fold activation compared to the control) but not by ΔNp63α (1.25 fold activation compared to the control), whereas the two ADA targets were activated by both TAp63α and ΔNp63α isoforms (Fig. 2B). TAp63α and ΔNp63α did not induce activation of ADA-Target A cloned in pBLCAT2 and ADA-Target B cloned in pBLCAT3, respectively(data not shown), demonstrating that ADA-Target A only shows promoter activity and ADA-Target B only enhancer activity.

These results indicate that the two p53-REs (ADA-target A and ADA-target B) identified in the ADA gene are sufficient to confer a positive transcriptional response on both TAp63α and ΔNp63α.

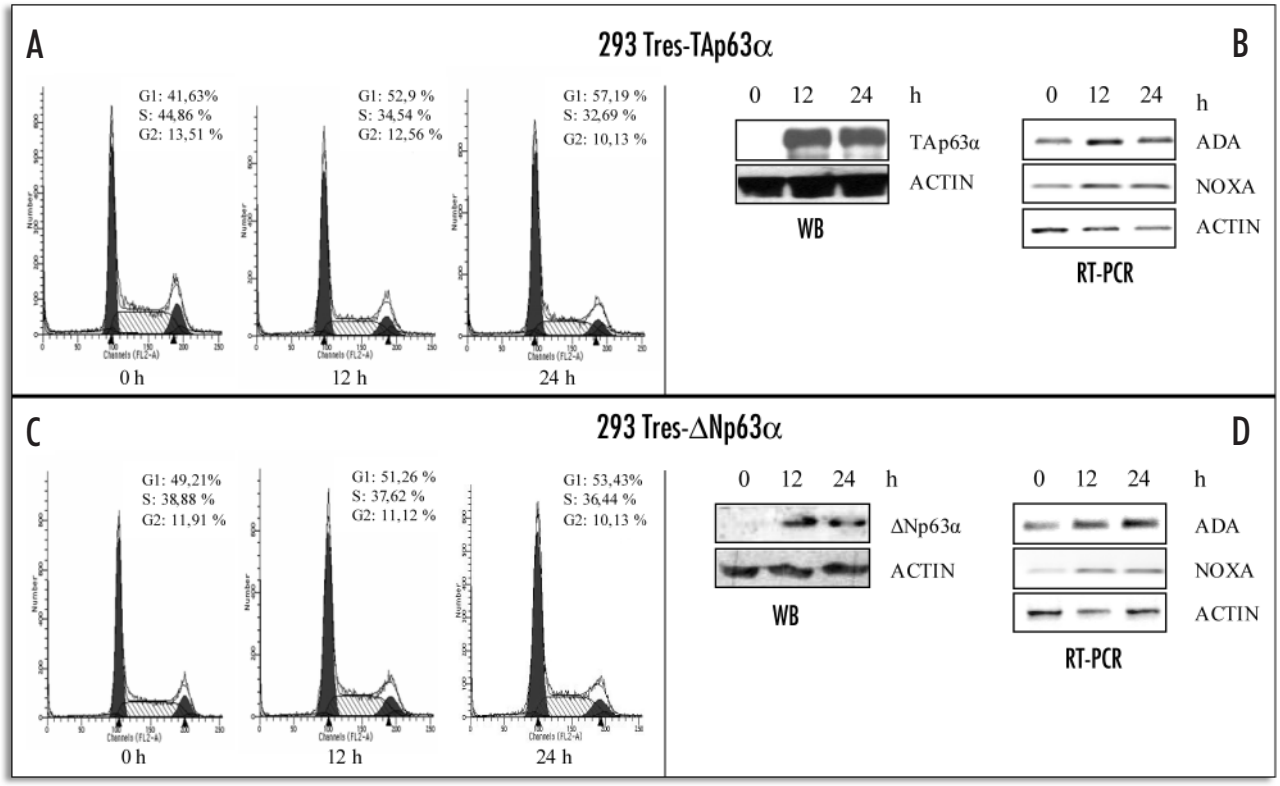

Figure 3. Effect of the TAp63α and ΔNp63α ectopic expression on cell cycle and on the ADA gene expression in T-Rex-293 isogenic cell lines. (A and C) The DNA content was quantified in flow cytometric analysis by propidium iodide staining of fixed T-Rex-293 cells that were un-induced or induced to express TAp63α (A) or ΔNp63α (C) for the indicated times and analysed in a FACScalibur with CellQuest analysis software (Becton Dickinson). The cell cycle analysis was performed with ModFit software (Becton Dickinson). (B) Western Blot and RT-PCR analyses of TAp63α and ADA genes in T-Rex-293 cell line stably transfected with pcDNA5/FRT/TO recombinant expression vector containing the TAp63α. (D) Western Blot and RT-PCR analyses of ΔNp63α and ADA genes in T-Rex-293 cell line stably transfected with pcDNA5/FRT/TO recombinant expression vector containing the ΔNp63α. The expression of Actin, and NOXA were evaluated as controls.

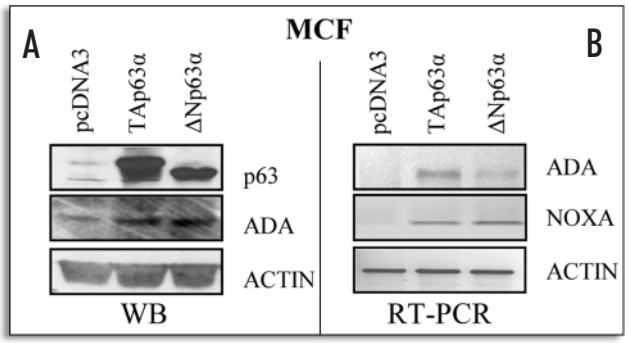

Figure 4. Effect of TAp63α and ΔNp63α ectopic expression on the ADA gene expression in MCF cells. Proteins and RNA were extracted from MCF cell line transiently transfected for 24h with empty expression vector $pcDNA_3$ or recombinant expression vector containing TAp63α or ΔNp63α. (A) Western Blot analysis of TAp63α, ΔNp63α and ADA. (B) RT-PCR analysis of ADA and NOXA mRNA expression. Western blot and RT-PCR of Actin were conducted as controls.

Effect of TAp63α and ΔNp63α ectopic expression on the *ADA* target gene. In order to examine the consequences of the TAp63α and ΔNp63α overexpression on intracellular ADA gene transcription in a comparable cellular context, we generated stable isogenic cell lines expressing the two isoforms under the control of a tetracycline inducible promoter. We used the human embryonic kidney Flp-In T-Rex-293 cell line (Invitrogen) to generate stable p63 expression cell lines. With this system we integrate the two cDNAs, cloned in the expression vector pcDNA5/FRT/TO, in the same genomic locus by a recombinase-mediated reaction, allowing the expression of the two isoforms to be absolutely comparable. We monitored the overexpression of TAp63α and ΔNp63α after 12 h and 24 h of induction by Western blot analysis (Fig. 3B and D). At the same times, the cell cycle profiles of the two cell lines T-rex-293-TAp63α and T-rex-293-ΔNp63α were also monitored by flow cytometric analysis (Fig. 3A and C). When TAp63α was expressed for 12 h and 24 h, a slight increase in G_1 DNA content and a decrease in S phase DNA content were detected (Fig. 3A). In contrast, at 12 h and 24 h, the cells maintained similar cell cycle phase distributions with no significant changes in G_2/M phases when ΔNp63α was expressed at the same times (Fig. 3C). We did not find an increase in the number of cells with sub-G_1 DNA content after the overexpression of both TAp63α and ΔNp63α for 12 h and 24 h, suggesting that under our conditions the cells were not in apoptosis.

Next we examined whether the TAp63α and ΔNp63α can regulate the expression of the endogenous ADA gene. Total RNA was extracted from T-Rex-293-TAp63α and T-Rex-293-ΔNp63α cells un-induced, at 12 h and 24h after induction of TAp63α and ΔNp63α expression, and analysed in RT-PCR experiments. We found that ADA mRNA was induced by both TAp63α and ΔNp63α (Fig. 3B and D). As a control of p63 activation, the same cDNAs were used for PCR amplification of one of the principal p63 target genes, NOXA, whereas the levels of actin were determined for use as quantity control.

To further examine the regulation of ADA gene by TAp63α and ΔNp63α MCF-7 cells were transiently transfected with the empty expression vector $pcDNA_3$ (control) or with the vector containing the TAp63α or ΔNp63α variants (Fig. 4). The level of TAp63α and ΔNp63α isoforms in

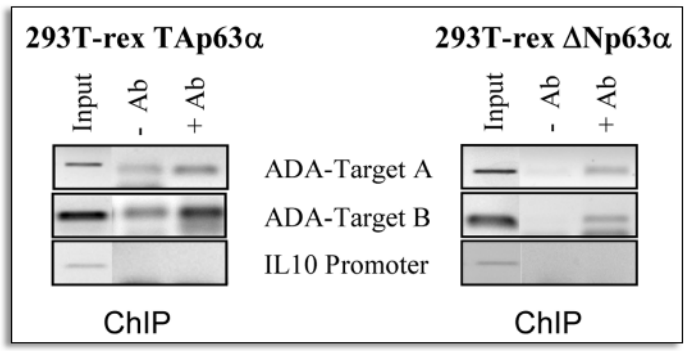

Figure 5. In vivo recruitment of TAp63α and ΔNp63α to ADA-Target A and ADA-Target B. In Chromatin Immunoprecipitation (ChIP), crosslinked chromatin was extracted from T-Rex-293 cell lines stably transfected with pcDNA5/FRT/TO recombinant expression vector containing the TAp63α, left panel) or ΔNp63α, right panel) and immunoprecipitated with anti-p63 antibody (p63H137). The immunoprecipitated material was amplified using primers specific for ADA-Target A or ADA-Target B, or for the unrelated Interleukin-10 promoter.

the cells were measured after transfection. Densitometric comparison of RT-PCR and Western Blot experiments of ADA mRNA and protein in the control cells and in MCF-7 cells transfected with TAp63α and ΔNp63α demonstrate that the ectopic expression of the two p63 isoforms induced endogenous ADA gene expression (Fig. 4A and B). The NOXA gene expression was used in the RT-PCR analysis as a control of p63 transactivation (Fig. 4B).

Chromatin immune precipitation (ChIP) assays were performed to determine whether TAp63α and ΔNp63α can directly bind in vivo, ADA REs (ADA-target A and ADA-target B). The cross-linked chromatin from T-Rex-293-TAp63α and T-Rex-293-ΔNp63α cells 12 h after the induction of the two proteins was immunoprecipitated with p63 antibody or without antibody as negative control. As shown in Figure 5, the DNA recovered from anti-p63 immunoprecipitation of both cell lines, was amplified with specific primers for ADA-Target A and ADA-Target B but not with primers specific for the Interleukin-10 promoter, which does not contain any p53/p63 RE, and which we used as control. These results provided further evidence that both TAp63α and ΔNp63α bind ADA targets in vivo in T-Rex-293 cell lines.

Downregulation of ΔNp63α and ADA in response to U.V. irradiation. Abundant genetic evidence indicates that p63 functions to maintain regenerative capacity of basal epithelia.[18] In particular, p63 is essential for normal epidermal development. Skin is continually exposed to the U.V.-B radiation in sunlight, which is the carcinogen responsible for most human skin cancers, such as basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and melanomas. Therefore, the skin has developed several defence mechanisms against the harmful effects of solar ultraviolet radiation. It was reported that the response to U.V. irradiation is characterized by induction of apoptosis primarily mediated by p53 through a decrease in p63 protein and mRNA levels,[14,15] while p73 protein levels remain constant in several cell types.[19] In other words, the three proteins show different responses in this physiological context, which we considered to be an ideal situation to discriminate some p53 specific target genes from those of the two homologues, in particular regarding p63, according to the different role they cover in normal growth or after a genotoxic stress induced by U.V. irradiation. To this aim normal human keratinocytes (NHEK) were exposed to 40 J/m^2 U.V. light and RNAs and proteins were extracted after 12 h. Cytofluorimetric analyses indicated that after this U.V. dose, the cells were arrested in G_1 (Fig. 6A) and western blot analyses showed a decrease in ΔNp63α and an increase in p53 protein levels, as previously reported (Fig. 6B). RT-PCR analyses demonstrated that the decrease in ΔNp63α also occurred at the mRNA level whereas the level of p53 transcript remained unaltered (Fig. 6C). In this scenario we examined the expression pattern of

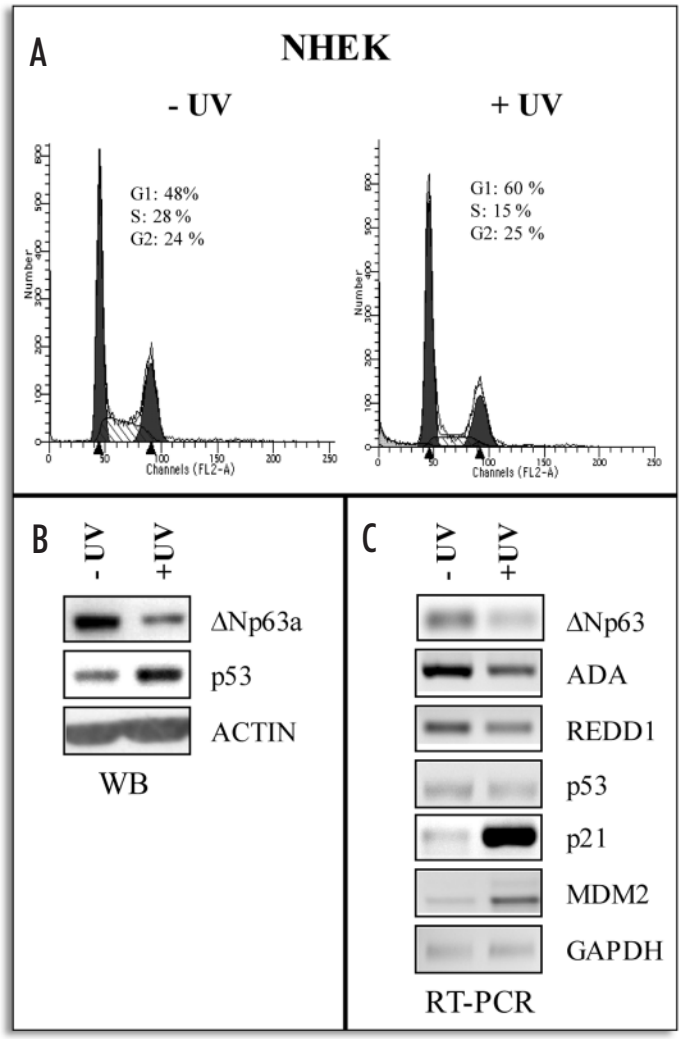

Figure 6. Effect of U.V. irradiation on ΔNp63α, p53 and ADA genes expression in normal human keratinocytes. NHEK cells untreated and 12 hours after U.V. irradiation were used. (A) Flow cytometric analysis of DNA content was quantified by propidium iodide staining of fixed cells and analysed in a FACScalibur using CellQuest analysis software (Becton Dickinson). The cell cycle analysis was performed with ModFit software (Becton Dickinson). (B) Western Blot analysis of ΔNp63α and p53. (C) RT-PCR analysis of ΔNp63α, p53, ADA, p21, MDM2, REDD1 mRNA expression. Western blot of Actin and RT-PCR of GAPDH were conducted as controls.

some p53 family target genes. RT-PCR analyses showed the increase of specific p53 targets, such as MDM2 and p21 (Fig. 6B). Indeed, it is clear that the cell cycle arrest is due to the p53 stabilization, which in turn determines the transactivation of the cyclin dependent kinase inhibitor p21. Interestingly we observed an evident decrease in ADA mRNA levels after U.V. treatment, which demonstrates once again that ADA gene is a specific p63 and not a p53 target. The REDD1 transcript, a specific p63 target, also decreased following ΔNp63α reduction (Fig. 6C).

DISCUSSION

The search for common and distinctive target genes of the three members of the p53 family is crucial to uncover the complex signalling pathway in which they are involved. In particular, while p53 is mutated in over 50% of all cancers and its pathway may also be affected in the remaining cases, the contribution of p73 and p63 to tumor

development is much less well established. It seems evident that p53 has evolved to protect cells from genotoxic insults and unprogrammed proliferation, whereas p63 may have evolved to perform tissue-specific functions distinct from those of p53.

The function of differently spliced p63 isoforms in normal and malignant epithelial cells is subject to active investigation. Recent reports show that ΔNp63α is the most abundant expressed isotype in tumor cells and it has been implicated in cell proliferation and oncogenic growth.[10,20] This overexpression can sometimes be the result of amplification of the p63 genomic locus.[10] These events are critical steps in the early development of certain cancers. Indeed ΔNp63α has been found to cause accumulation and signalling of β-catenin, supporting the oncogenic function of p63.[21] ΔNp63α is a target of the phosphoinositide-3-kinase (PI3K) pathway downstream of the epidermal growth factor receptor, implying a critical role for ΔNp63α in the survival and differentiation of squamous epithelia.[22] Moreover, it has been reported that ΔNp63α which was once considered inert, is capable of regulating gene expression.[23,24]

The principal aim of this study was to determine whether p63 is implicated in transcriptional events related to sustaining cellular proliferation and consequently whether p53 family members play different roles in cell cycle progression in response to genotoxic stress, in tumor genesis or suppression, by the transactivation of common but also of distinct specific target genes.

In particular, this study confirms and expands our earlier results showing that the Adenosine Deaminase gene is not a p53 target, but is a specific direct target of p63 as well as p73.[13] Considerable evidences indicate that the ADA protein is critical for the development and cell proliferation. Indeed, although the main function of ADA is the development of the immune system in humans, it also seems to be associated with the differentiation of epithelial cells and monocytes, neurotransmission and maintenance of gestation.[25] Moreover, increasing evidence indicates that maintaining high levels of ADA in mice placenta is essential for embryonic and fetal development.[26,27] We present data demonstrating that in epidermal keratinocytes, the p63 knockdown experiments with siRNA determine a significant suppression of proliferation which interestingly correlates with a decrease in ADA mRNA and protein levels (Fig. 1). We report the identification of two p53 REs within promoter and Intron 1 of the ADA gene, and we present data showing that these REs are capable of interacting with both TAp63α and ΔNp63α in vivo and are sufficient to confer transcriptional activation in response to overexpression of TAp63α and ΔNp63α (Figs. 5 and 2). Ectopic overexpression of TAp63α and ΔNp63α in the transiently transfected MCF and in stable 293-T-rex cell lines, which express TAp63α and ΔNp63α by using the tetracycline inducible expression system, demonstrate that ADA gene expression is regulated by both TAp63α and ΔNp63α (Figs. 4 and 3).

The balance between p53 and its negative regulator ΔNp63α may represent an important factor in determining the promotion of cell survival and proliferation or suppressing apoptosis. This balance is particularly important in keratinocytes when they are subject to genotoxic damage due to U.V. irradiation. We analyzed the effect of the U.V. treatment in normal keratinocytes on the transcriptional changes of specific p53 family target genes in response to a decrease in ΔNp63 and an increase in p53 (Fig. 6). Interestingly we found that after U.V. irradiation, the cells arrested in G_1. This cell cycle arrest is due to p53 stabilization, which in turn determines the transactivation of some specific targets we used, i.e., p21 and MDM2. On the contrary the levels of ΔNp63α transcripts and proteins decreased dramatically in correlation with its specific targets ADA and REDD1 transcripts, which are involved in cell proliferation and development.

It has been reported that during the asynchronous growth, p53 directs the expression of ΔNp63α in keratynocytes. In response to genotoxic stress, ΔNp63α protein is recruited on its own promoter downregulating its expression.[28] This finding is consistent with the prediction that loss of ΔNp63α in response to genotoxic stress would be permissive for p53-mediated cell cycle arrest or apoptosis.

Our experiments fully support the hypotheses that the differences between p53, p63 and p73 become evident when, under the same physiological conditions (cellular proliferation) or in response to the same stress (U.V.) they react, each according to their specific role, transactivating different targets and that p63 and in particular ΔNp63, in certain circumstances might contribute to tumorigenesis not exclusively by antagonizing p53, but by conferring a proliferative potential on cancer cells, allowing increased self-renewal by the transactivating target genes indispensable for cell division such as the Adenosine Deaminase gene.

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