UNIVERSITÀ DEGLI STUDI DI NAPOLI

"FEDERICO II"

Scuola di Dottorato in Medicina Molecolare

Dottorato di Ricerca in Genetica e Medicina Molecolare

Analysis of the p63 function in cell proliferation and differentiation through the study of the mechanisms regulating p63 protein stability and transcriptional activity.

Coordinatore:
Prof. Carmelo Bruno Bruni

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Dip. Biologia Strutturale e Funzionale, Università degli Studi di Napoli "Federico II"
Complesso Universitario Monte S. Angelo

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Sede amministrativa:
Dipartimento di Biologia e Patologia Cellulare e Molecolare "Luigi Califano"

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XX ciclo

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Candidata: Antonella Di Costanzo

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Introduction

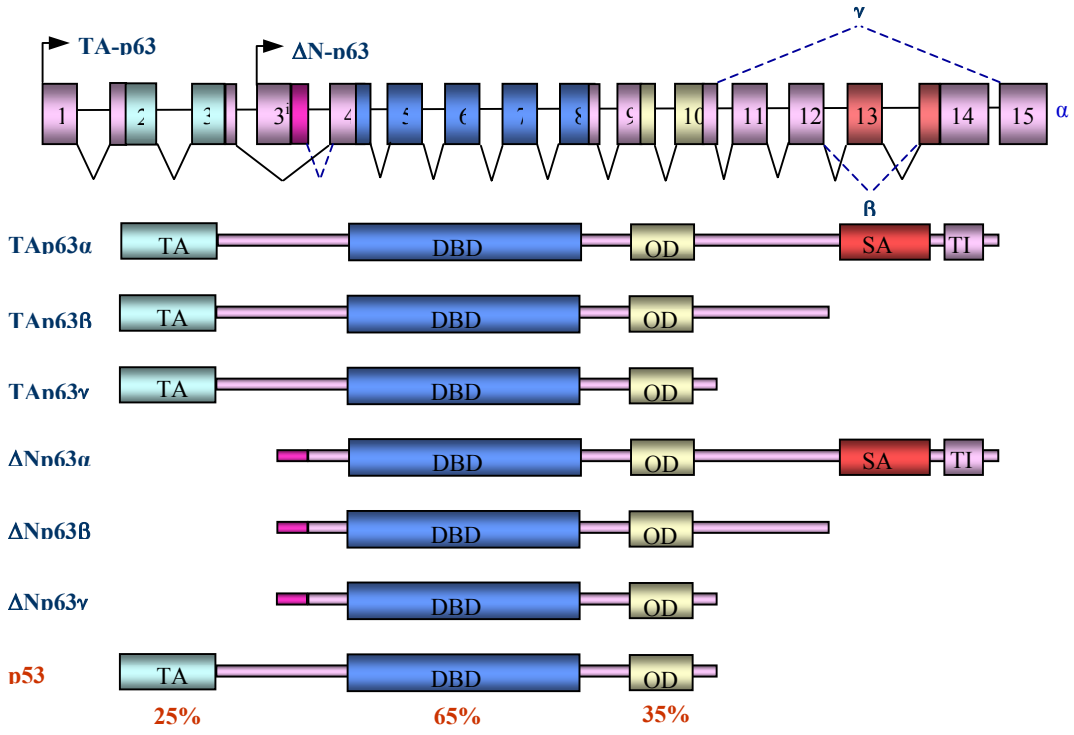
p53 family membersAt the end of the last century, the discovery of p53 protein homologues named p63 and p73, engendered the concept of a new family of p53-like transcription factors. The research focus expanded from a single protein named the " guardian of the genome" to a family of transcription factors that likely have distinct roles through the diverse collection of genes they regulate. p53 was discovered 25 years ago as a protein interacting with the oncogenic T antigen from SV40 virus. P53 transcription factor is the product of a pivotal tumor-suppressor gene, whose inactivaction is the most frequent single gene event in human cancer, and germline mutation in human p53 gene are cause of enhanced risk of developing cancer (Li-Fraumeni syndrome) . The p53 gene encodes a protein with a central DNA binding domain, flanked by an N-terminal transactivation domain, and a C-terminal tetramerization domain (Levine et al;1997). The active form of p53 is a tetramer and, consistent with its tetrameric state, p53 binds DNA sites that contain four repeats of the pentamer sequence motif 5'-Pu-Pu-Pu-C-A/T-3'. Until now, the p53 gene structure was considerate much simpler, with only one promoter and transcribing three mRNA splicing variants encoding, respectively, full-length p53, p53i9 (Flaman et al;1996), and Δ40p53 (Courtois et al.2002; Ghosh et al.2004). Recent studies have proposed for the p53 gene a structure more complex than has been previously thought. The p53 gene, as the other two family members p63 and p73, contains two promoters and can generate six different mRNAs, that encode at least six p53 isoforms (Bourdon et al. 2005). P53 isoforms are expressed in several normal human tissue. The functions of p53 are primarily the regulation of cell cycle

checkpoints, apoptosis, and genome stability. A substantial number of genes, involved in cell cycle arrest or in induction of apoptosis, are activated by p53. These include MDM2, p21WAF, GADD45, bax and IGF-BP3. p53 has also been reported to negatively regulate the transcription of a number of genes such as presenilin 1, topoisomerase IIα, bcl2 and hsp70. The transcriptional activity and stability of p53 are highly regulated by posttranslational mechanisms involving protein-protein interaction, phosphorylation, acetylation, ubiquitination, and sumoylation.

MDM2 is an ubiquitin ligase and a p53 transcriptional target; it binds to the p53 transactivation domain, and inhibits its transcriptional activity. MDM2 shuttles p53 out of the nucleus targeting the protein for ubiquitin-mediated proteolysis (Vogelstein et al. 2000). MDM2, thus, is assumed to be the principale regulatorof p53 protein levels. The p14ARF tumor suppressor protein, one of the alternative products of the INK4A locus, antagonizes MDM2 activity leading to p53 stabilization. Several mechanisms have been postulated to inactivate p53 such as amplification of MDM2 gene, deletion of ARF gene, expression of some viral oncogenes that stimulates p53 degradation or missense mutation in DNA binding domain that disrupt the DNA binding capability of p53.

P63 and p73 are genes structurally related to p53. In fact, also p63 and p73 proteins contain an N-terminal transactivation domain, which shares 25% homology with N-terminal part of p53, the DNA binding domain, which shares 65% of homology with the corresponding p53 domain, and the tetramerization domain, which shares 35% of homology with the oligomerization domain of p53. The p63 and p73 genes are

transcribed from two distinct promoters, giving rise to proteins that either contain (TA isoforms) or lack (ΔN isoforms) the N-terminal transactivating domain. In addition, both p63 and p73 genes, are subject to alternative splicing event that generate three (α, β, γ) and seven (α, β, γ, δ, ε, ζ,η) different C-termini respectively for p63 and p73 encoded proteins (Yang and McKeon; 2002). The α isoforms contain a sterile α motif (SAM) and a transactivation inhibitory domain (TID). The SAM domain, which is absent in p53, is a protein-protein interaction domain also found in other developmentally important protein, such as several Eph receptor tyrosine kinase (Schultz et al; 1997).



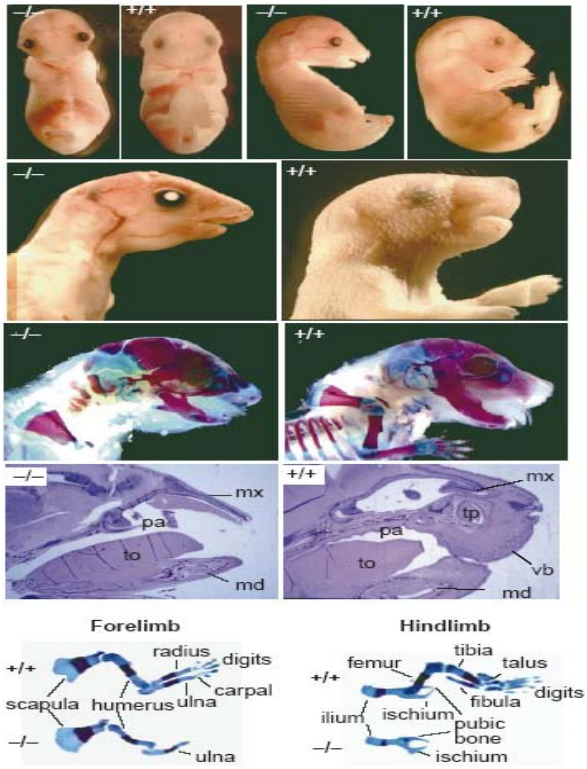
Structure of both TP63 and major protein isotypes. TP63 uses several transcription initiation sites and extensive alternative splicing, to generate different mRNA. Several protein domains can be distinguished; of these, the TA domains, the DBD, and the OD domain are highly homologous to the corresponding domain in p53. The SAM domain and the TID are not contained in the p53 protein.

The Transactivation inhibitory domain of p63 binds to the N-terminal TA domain masking residues that are important for transactivation (Serber et al; 2002). In fact, p63 isoforms that contain the γ and β C-termini are associated with higher transactivation competency that ones with α terminus (Shimada et al; 1999). The lack of TA domain in ΔNp63 isoforms suggest that they are transcriptionally competent. Since ΔNp63 isoforms retain the oligomerization and DNA binding domains, it is plausible that they act as dominant negative inhibitorsof p53 and TA-containing p53 family members (Yang et al;1998. Westfall et al; 2003) . Indeed, numerous studies show that co-expression of ΔNp63 with either TAp63, TAp73, or p53 has inhibitory effect on TAp63-mediated transcription. A plausible mechanism is the formation of transcriptionally inactive ΔN-TA heterotypic or homotypic tetramers (composed of either all-TA or all-ΔN monomers) that compete for the same DNA binding sites. Despite the well-documented role of ΔNp63 as a dominant negative transcriptional repressor, several studies have shown that ΔNp63 isoforms directly transactivated a set of genes including Hsp70 and p57Kip (Beretta et al; 2005; Wu et al; 2005; King et al; 2003). This is possible thanks to existence of two cryptic transactivation domains in ΔNp63 isoforms: a region encompassing the first 26 N-terminal amino acids named TA2 domain and a prolin rich sequence corresponding to exon 11/12 present in p63 β and α isoforms (Ghioni et al; 2002). Surveillance of cellular integrity might be achieved throught a network of these p53-like tumor suppressors. This speculation was, further, fueled by the observation that the p73 gene is localized to chromosome 1p36.3, a region that is frequently lost in

neuroblastomas and in other types of cancers (Kaghad et al; 1997; Takahashi et al; 1998), while the p63 chromosomal location, 3q27-29, is deleted in some bladder cancers and amplified in some cervical, ovarian, lung , and squamous cell carcinoma (Yang et al; 1998) where ΔNp63 was the predominant isoform expressed at protein level (Cui R et al; 2005). Moreover, both p73 and p63 can bind to p53 DNA-binding sites and activate transcription of genes that mediates cell cycle arrest or apoptosis in vivo. Despite all this circumstantial evidence, however, only a very few mutation in p63 and in p73 have been found in human tumors, and a direct link to carcinogenesis similar to that for p53 has so far not established (Ichimiya et al; 2001; Nomoto et al; 1998). Moreover the analysis of p63 and p73 deficient mice and studies on human tumors have often led to conflicting results with regards to its role in tumorigenesis (Westfall et al; 2004; Flores et al; 2005; Keyes et al; 2006).

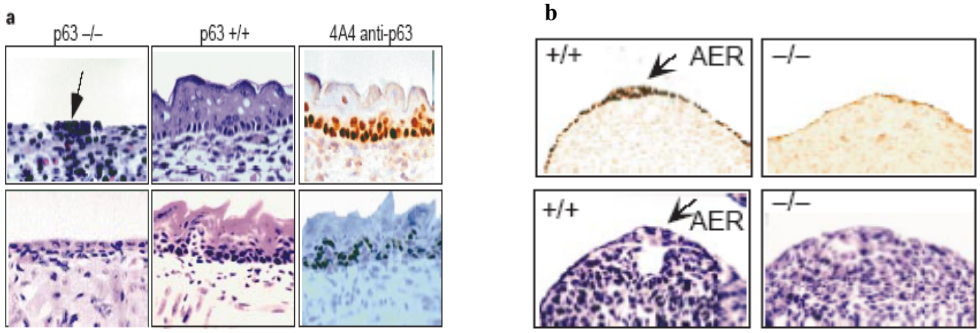
p63 knock-out and transgenic mouse models: toward a comprehension of p63 physiological function.

Target gene disruption studies in mice have established an important role for p63 in development and differentiation. Mice lacking p63 are born alive but have striking developmental defects. Their limbs are absent or truncated, defects that are caused by a failure of the apical ectodermal ridge (AER) to differentiate.



p63-/- mice on postnatal day have hypoplastic upper and lower jaws, and have no eyelids, whisker pads, skin and related appendages, including vibrissae, pelage follicles and hair shaft. Homozygous mutants lack distal components of the forelimb, including the radius, carpals and digits, as well as all components of the hindlimb.

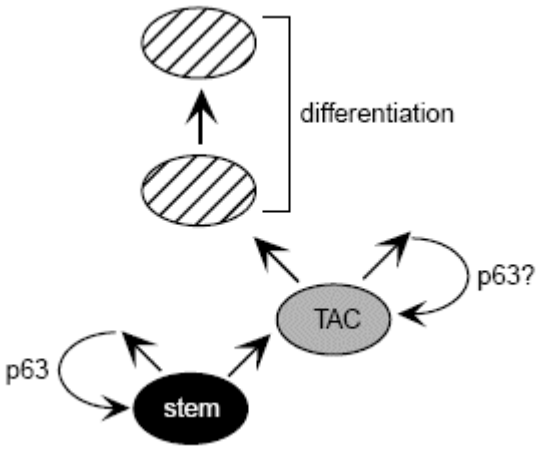
The lack of a proper AER in p63 -/- limb buds results from a failure of the ectoderm to undergo growth and differentiation that give rise to this stratified epithelium (Mills et al; 1999). The skin of the p63 deficient mice does not progress past an early developmental stage: it lacks stratification and does not express differentiation markers .



a)Defects in stratified epithelial differentiation in p63-deficient mice. p63-/- mice lacking squamous stratification in the epidermis (top) and tongue epithelium (bottom)

.b) Immunohistochemical staining with the 4A4 anti-p63 antibody, showing p63 protein expression in the apical ectodermal ridge. The AER is absent in the p63-/-.

At birth, p63-deficient mice have striking and visible skin defects, in fact, they die within a day of birth from dehydration. Structures dependent upon epidermal-mesenchymal interactions during embryonic development, such as hair follicles, teeth and mammary glands, are also absent in p63 deficient mice. The surface of the skin is covered by a single layer of flattened cells, without the spinosum, granulosum and stratum corneum. Two contrasting models have been advanced to explain the absence of stratified epithelia (McKeon et al; 2004). One model posits that p63 is required for simple epithelial cells to commit to a stratified epithelial lineage during development (Mills et al; 1999). The second model argues that the primary defect resides not in the acquisition of stratified epithelial cell fate but rather in an inability of epidermal stem cells to sustain epidermal self-renewal (Yang et al; 1999).



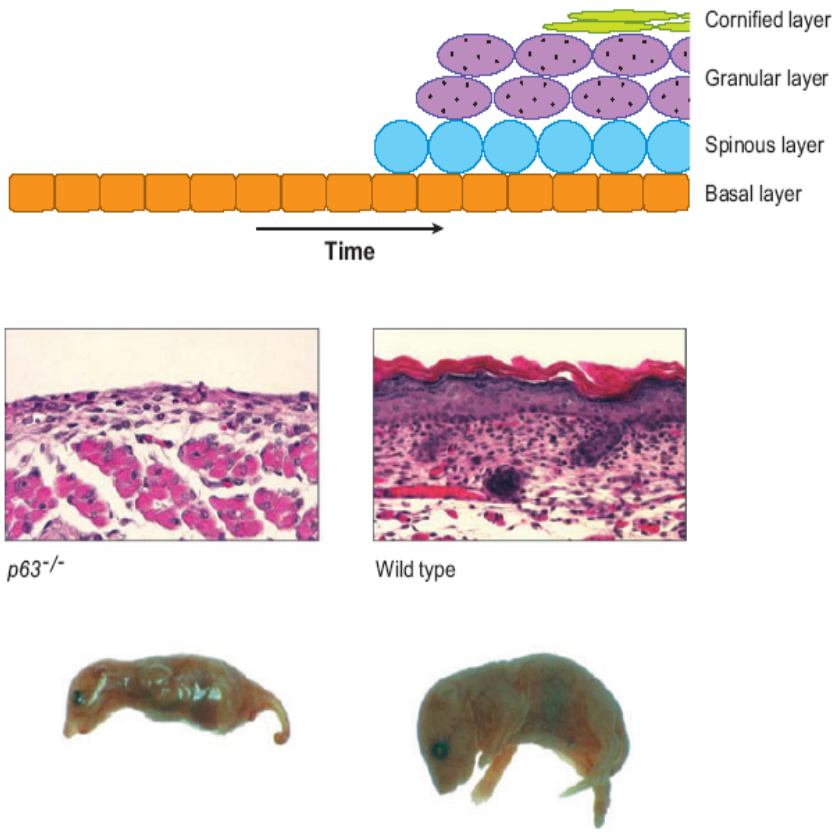
Model for p63 in maintaining the proliferative capacity of epithelial progenitor cells. Stem cells in the basal layer of stratified squamous epithelia express high levels of p63 and undergo asymmetric division to enable both selfrenewal and progression to transient amplifying cells (TACs). TACs, which may express less p63, are also capable of limited proliferation and self-renewal, but are ultimately destined for terminal differentiation. The absence of p63 results in the failure to maintain a basal cell population, suggesting a requirement for p63 in the regenerative aspect of stem cell division.

The finding that p63 is specifically expressed in epithelial cells that have adopted an epidermal fate suggested that p63 is involved in development of the embryonic basal layer, the first layer of embryonic epidermis.

The epidermis is an example of stratified epithelium. It functions as a barrier protecting the organism from dehydration, mechanical trauma, and microbial insults. This barrier function is established during embryogenesis through a complex and tightly controlled stratification program. The epidermis, the outermost component of the skin, is the primary barrier that protects the body from dehydration, mechanical trauma, and microbial insults. The epidermis is separated from the underlying dermis by the basement membrane, which consists of proteins secreted by epidermal keratinocytes and by dermal fibroblasts (McMillan et al. 2003). The two compartments of the skin, the dermis and the epidermis, function cooperatively and

together are responsible for the development of epidermal appendages, including hair follicles and mammary glands (Chuong et al; 1998). Therefore, a failure to properly develop either the dermis or the epidermis may result in defects in appendage development. This is, for example, illustrated by ectodermal dysplasias, in which primary defects in epidermal development are the cause of subsequent defects in epidermal appendages (Koster and Roop; 2004; Priolo et al; 2000).

The barrier function of the epidermis is established during embryogenesis and is the result of a complex and precisely coordinated stratification program. In mice, the execution of this program occurs in a period of approximately 10 days, between E8.5 and E18.5, and initiates when cells of the surface ectoderm commit to an epidermal fate.

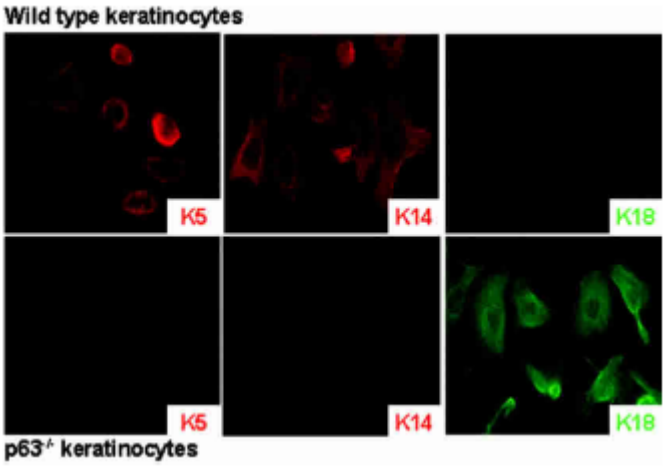


(*a*) Schematic illustrating epidermal morphogenesis. During epidermal morphogenesis, the single-layered surface ectoderm that initially covers the developing embryo initiates a stratification program culminating in the formation of the epidermal barrier. (*b*) In wild-type mice, epidermal stratification and barrier formation are completed by birth. The surface ectoderm of mice lacking the transcription factor p63 fails to adopt an epidermal fate, and therefore stratification and barrier formation do not occur. As a consequence, mice lacking p63 are born with a single layer of ectodermal cells covering their bodies and die shortly after birth due to dehydration. (c) Images of *p63-/-* and wild-type mice.

After this initial commitment step, keratinocytes in the newly established embryonic basal layer give rise to a second layer of cells, the periderm (M'Boneko and Merker; 1988). The periderm is shed before birth in conjunction with the acquisition of epidermal barrier function (Hardman et al. 1998). The next layer of the epidermis to form is the intermediate cell layer, which develops between the basal layer and the periderm (Smart et al; 1970). Development of this layer is associated with asymmetric cell division of embryonic basal keratinocytes (Lechler and Fuchs; 2005, Smart et al; 1970).

Like basal keratinocytes, intermediate cells undergo proliferation, and the loss of this proliferative capacity is associated with the maturation of intermediate cells into spinous cells (Koster et al. 2007, Smart 1970).

Spinous cells subsequently undergo further maturation into granular and cornified cells. The morphological changes that are a hallmark of epidermal stratification are associated with changes in the expression of keratin differentiation markers (Koster and Roop; 2004).



Primary p63−/− surface epithelial cells are blocked in their commitment to a stratified epithelial lineage. Differentiation markers K5 and K14, which are expressed in epithelia that have committed
to a stratification program, are not expressed in primary p63−/− cells. These cells do, however, express K18, a marker for singlelayered epithelia

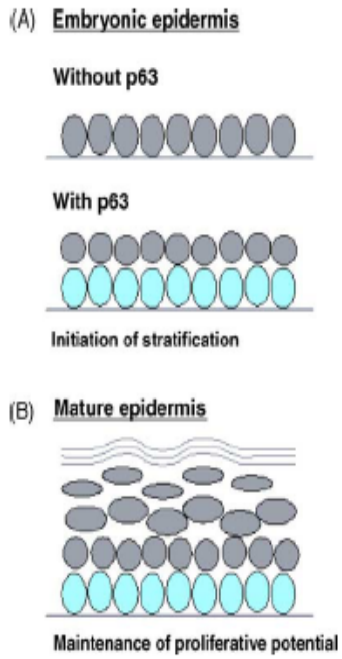
For example, whereas the uncommitted surface ectoderm expresses keratins K8 and K18 (Moll et al. 1982), K5 and K14 are induced as these cells commit to an epidermal fate (Byrne et al. 1994). Subsequently, the initiation of terminal differentiation results in the induction of K1 and K10 expression in the newly formed suprabasal keratinocytes (Bickenbach et al. 1995, Fuchs and Green; 1980).

The final step in epidermal stratification involves the formation of the epidermal barrier. During normal development, barrier acquisition is patterned and initiates at the dorsal surface, spreading laterally to the ventral surface in approximately one day (Hardman et al; 1998). The process of barrier formation is characterized by the formation of cornified cell envelopes, composed of proteins crosslinked into a rigid scaffold and of lipids covalently attached to the exterior surface (Rice and Green; 1977, Steven and Steinert; 1994). Although established in utero, the barrier function of the epidermis is maintained during postnatal life owing to the continuous selfrenewal of the epidermis, a process mediated by epidermal stem cells (Blanpain and Fuchs; 2006). Under homeostatic conditions, the epidermal stem cells that are located in the interfollicular epidermis are responsible for the maintenance of this structure (Ito et al. 2005).

These stem cells represent a small proportion of basal keratinocytes and, through asymmetric cell division, give rise to a daughter stem cell and a transit-amplifying cell, which ultimately undergoes terminal differentiation (Dunnwald et al. 2003, Potten andMorris; 1988, Schneider et al. 2003).

Attempts to establish unequivocally the role of p63 in epithelial development are complicated by the fact that this protein exists in multiple isoforms with different, often contradictory, biological activities. Koster and Roop reported that TAp63 was expressed earlier than ΔNp63 (Koster et al; 2004) during epidermal development. Thus, TAp63, but not ΔNp63α, was proposed to be required for the initiation of epidermal stratification. TAp63 was reputed to be the molecular switch responsible for epithelial stratification while ΔNp63 was believed to counteract the TAp63 isoform allowing keratinocytes terminal differentiation(Koster et al; 2006). Accordingly, studies on transgenic mice, demonstrate that upregulated TAp63α expression resulted in skin hyperplasia and a failure of keratinocytes to properly differentiate (Candi et al; 2006).

These data are in contrast with other works where ΔNp63 was reported to be the only isoform expressed in epidermal development, until E13 embryonal stage (Mikkola et al; 2007. Laurikkala et al; 2006), and that ΔNp63 is expressed in the basal undifferentiated layer of the skin, in particular in the stem cell compartment, and was rapidly degraded when keratinocytes are induced to differentiate (Yang et al; 1998. Pellegrini et al; 2001. Rossi et al; 2006).



The down-regulation of ΔNp63 in suprabasal keratinocytes was recently shown to be mediated, at least in part, by signalling through Notch, which induces terminal differentiation of keratinocytes (Nguyen et al; 2006). In vitro studies suggested that, in basal keratinocytes, ΔNp63α induces proliferation and prevents premature entry into terminal differentiation (King et al; 2003. King et al; 2006). Consistent with this hypothesis, many currently identified ΔNp63 target genes are involved in proliferation or in preventing differentiation (Westfall et al; 2003. Nguyen et al; 2006. Wu et al; 2005. Sbisa et al; 2006). In particular ΔNp63α was reported to repress the expression of p21 and 14-3-3σ, two genes induced during epidermal terminal differentiation, and the expression of genes required for cell cycle progression including cyclin B2 and cdc2 (Testoni et al; 2006).

In addition to maintaining the proliferative state of a subset of basal keratinocytes ΔNp63α may also maintain the expression of the basal keratins K5 and K14. (Westfall et al; 2003. Nguyen et al; 2006. Missero et al; 1995).

Later one, data from different groups have demonstrated coexpression of ΔNp63 and TAp63α isotypes during embryonic development and differentiation of developmentally mature keratinocytes. Karin Nylander, using antibodies directed against specific p63 proteins, detected TAp63 in normal stratified epidermis (Nylander et al; 2002). Paul Khavari's group, using a siRNA approach directed against p63 in regenerating human epidermis, demonstrated that ΔNp63 isoforms downregulation was responsible for the majority of the skin defects while TAp63 isoforms appeared to be relevant for late differentiation (Truong et al; 2006). Our group has recently demonstrated that TAp63 starts to be expressed, together with ΔNp63, at stage 11.5 of mouse embryo development in the skin of anterior and posterior limbs. Remarkably, the Dlx3 homeobox gene, a gene specifically expressed in the granular layer of the epidermis, was found regulated by p63 during ectoderm development. Further underlying, the role of TAp63 in the epidermal stratification program.

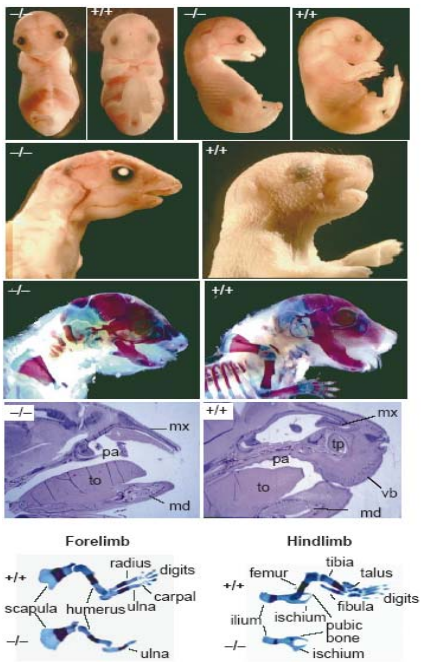
Two recent studies (Carroll et al; 2006. Ihrie et al; 2005) have revealed a role for p63 in the transcriptional regulation of adhesion programs affecting both cell-cell and cell matrix interaction suggesting that p63 may direct the development of stratified epithelia by orchestrating a series of different programs important for tissue integrity and function. The first epithelial cell-cell adhesion protein identified as a direct target

of p63 transcriptional control was *Perp*. Perp knockout mice exhibited postnatal lethality associated with the presence of epithelial blistering, particularly in the skin and oral cavity. Further investigation into the cause of the observed lethality and epithelial integrity defects in the Perp knockout mice demonstrated that Perp is highly expressed in stratified epithelia, where it is a constituent of desmosomes, and further, that marked structural defects were present in the desmosomes of Perp-/- mice. A connection between p63 and Perp was identified, as expression of Perp depends on p63 during the epidermal stratification program, and p63 can directly regulate Perp. All isoforms of p63 can transactivated Perp, and further, chromatin immunoprecipitation (ChIP) analyses demonstrated p63 occupancy of p53/p63 sites in the Perp gene *in vivo*, in the epidermis. These studies revealed that p63 controls aspects of cell-cell adhesion through induction of Perp expression. It has suggested that p63 can regulate the expression of an entire axis of cellular adhesion. Multiple genes, such as integrins 3,4, 5 and 6, laminin, two cadherins, plakoglobin and other can be, directly or indirectly, regulated by ΔNp63 or TA isoforms. Interestingly, ablation of both integrin α3 and α6 genes causes severe bilateral hypoplasia and limb malformation, resulting from a defect in the morphogenesis of the apical ectodermal ridge (AER) (De Arcangelis et al; 1999). The defects in limb formation and the AER observed in the double knockout model bear some resemblance to those seen in the p63 null mice. Since p63 can directly regulate the expression of both of these integrins (Carroll et al; 2006; Kurata et al; 2004), the failure to establish and

differentiate the AER in the p63 null mice could be at least partially caused by the reduction or loss of α3 and α6 integrin expression in these animals.

P63 human associated disorders

Mutations in the *p63* gene can cause at least five different syndromes: Ectrodactyly, Ectodermal dysplasia and Cleft lip/palate syndrome (EEC, OMIM 604292), Ankyloblepharon - Ectodermal defects - Cleft lip/palate syndrome (AEC, OMIM 106260), Limb Mammary Syndrome (LMS, OMIM 603543), Acro-Dermato-Ungual-Lacrimal-Tooth syndrome (ADULT, OMIM 103285) and Rapp-Hodgkin Syndrome (RHS, OMIM 129400). Furthermore, two non-syndromic human disorders are caused by *p63* mutations: isolated Split Hand/Foot Malformation (SHFM4, OMIM 605289) and recently non-syndromic cleft lip (Leoyklang et al; 2006). Human phenotypes caused by mutation in p63 gene resemble that of p63-knockout mice and are characterized by ectodermal dysplasia, split hand/foot malformation and orofacial clefting.



Lack of epidermis

Lack of mammary and prostate glands

Cleft lips and palate, lack of teeths, facial dysmorphism

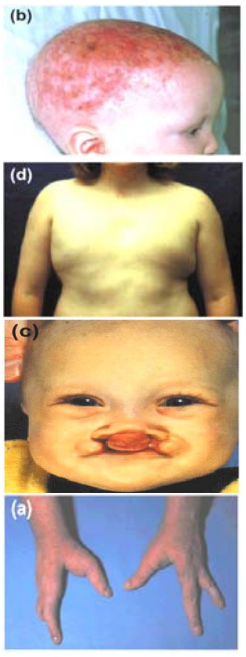
Truncated limbs

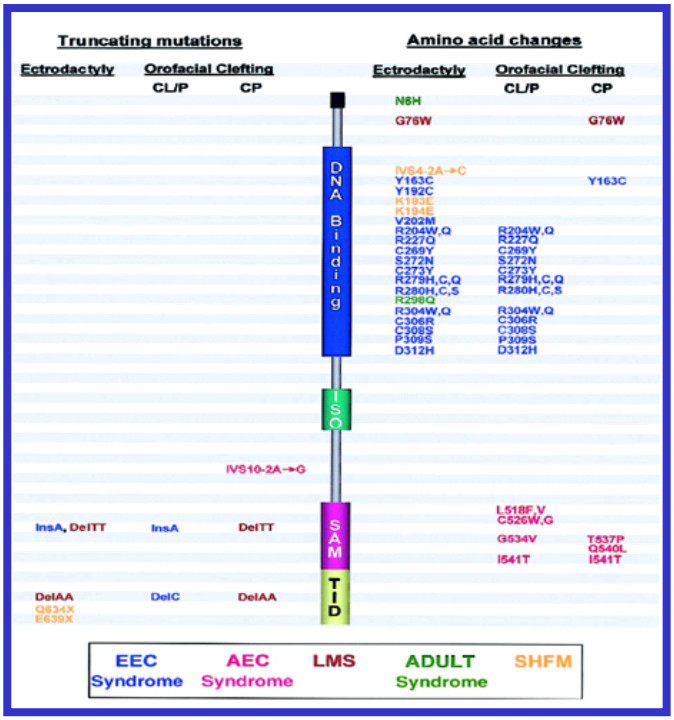
Ankyloblepharon, Ectodermal dysplasia, dystrophic nails, teeth and hair

Mammari gland hipoplasia

Cleft lip and palate, ipodontia

Ectrodactily or split hand and foot malformation





Distribution of mutation in p63, revealing a striking genotype-phenotype correlation. The approximate positions of truncating mutations and aminoacid correlation are indicated.

The localization and functional effects of the mutations that underlie these syndromes establish a striking genotype-phenotype correlation.

The prototype of the *p63* syndrome family is the ***EEC syndrome*** characterized by ectrodactyly dysplasia, ectodermal dysplasia and facial clefts. Ectodermal dysplasia affects the skin and other ectodermal derivates such as teeth, hair and nails. Clefting affects the lip, sometimes in combination with palate. Other symptoms are lacrimal-duct abnormalities, urogenitals problems and facial dysmorphism. EEC syndrome is mainly caused by point mutations in the DNA binding domain (DBD) of the *p63* gene. Altogether 34 different mutations have been reported, and 20 different amino acids are involved. Only two mutations are outside the DNA binding domain: one insertion (1572 InsA) and one point mutation (L563P) in the Sterile Alpha Motif domain (SAM). Five frequently mutated amino acids were found in the EEC

population: R204, R227, R279, R280 and R304, all located in CpG islands. The five p63 arginine hotspot mutations and probably also other DNA binding domain mutations that are found in EEC syndrome appear to affect the DNA binding capacity of p63, which results in impaired transactivation activity and altered regulation of transactivation. The autosomal dominant inheritance of EEC syndrome suggests that the EEC mutations have a dominant negative effect. However, recent genotype-phenotype analyses for the five hotspot mutations revealed significant differences between the corresponding phenotypes. For instance cleft lip/palate is present in the R304 mutation population (80%), whereas R227 patients seldom have cleft lip/palate. Syndactyly is completely absent in R227 population, while 30-60% of the other hotspot mutation population have syndactyly. It thus seems that these hotspot mutations exert specific effects. Such specificity might be brought about by different effects of these mutations on promoters for p63 transcriptional target genes. Alternatively, these hotspot mutations may exhibit gain-of-function effects, similar as for the p53 hotspot mutations.

AEC syndrome, which is also known as " Hay-Wells syndrome" has little or no limb involvement but instead includes ankyloblepharon, which is a partial or complete fusion of the eyelids that is very rare in other EEC- like syndrome. In addition to these major features, eroded skin at birth and recurrent scalp infection are important sign. The other Ectodermal Dysplasia symptoms, such as nail and teeth defects are present in more than 80% of patients, and hair defects and/or alopecia are almost constant features. AEC syndrome is caused by missense mutation within the SAM

domain of p63 (McGrath et al; 2001). These mutation are predicted to disrupt protein-protein interaction, by either destroying the compact globular structure of the SAM domain or substituting amino acids that are crucial for such interaction (McGrath et al; 2001).

Rapp-Hodgkin syndrome (RHS) mimics AEC very much. Such AEC, even RHS is caused by mutation in SAM domain, and for this reason affecting only the α isoforms (Kantaputra et al; 2003). RHS has characterized by ankyloblepharon and the more severe skin phenotype in AEC (Rinne et al; 2006 Bertola et al; 2004). Other ED symptoms, such as orofacial clefting and the near absence of limb malformations are similar to AEC. Although, the severity of the skin phenotype is obvious and much more severe in AEC patients than in RHS patients, the strong overlap between AEC and RHS suggest, that they are variable manifestations of the same clinical entity.

LMS syndrome is caused by mutations located in the N- and C-terminus of the *p63* gene. A large LMS family (29 affected members) has a point mutation in the exon 4 (G76W) in the putative second transactivation domain (TA2) (vanBokhoven et al; 2002; Duijf et al; 2002). One other point mutation (S90W) is also located between the TA domain and DBD. Other LMS mutations are reported in the C-terminus: a TT deletion in the exon 13 and a AA deletion in exon 14. These will affect only the p63α protein isoforms, where they are predicted to cause a frame shift and a premature stop codon. Also a stop mutation in the transcription factor inhibitory domain (TI) (K632X) has been identified in a sporadic LMS patient.[11]

The LMS phenotype resembles the EEC syndrome phenotype, but the ectodermal manifestations are milder (vanBokhoven et al; 1999) A consistent feature of LMS is the mammary gland and/or nipple hypoplasia, moreover lacrimal duct obstruction and dystrophic nails are frequently observed. Hypohydrosis and teeth defects are detected, but other ectodermal defects such as hair and skin defects are rarely detected if at all. About 70% of LMS patients have similar limb malformations as in EEC syndrome, and about 30% orofacial clefting, notably always in form of cleft palate (Rinne et al; 2006).

ADULT syndrome phenotype is most similar to LMS syndrome, although clear differences can be seen when observing larger families or patient populations. The main difference is the absence of orofacial clefting and the presence of hair and skin defects in the ADULT syndrome. Teeth, skin and nail defects are constantly present in ADULT syndrome, but only rarely in LMS. Hair (53%) and lacrimal duct defects (67%) are observed in ADULT patients more frequently than in LMS.

ADULT syndrome is characterized by point mutation in exon 8, changing R298 in the DNA binding domain into either a glutamine or a glycine. While EEC syndrome mutations in the DNA binding domain impair the binding of p63 protein to DNA, arginine 298 is not located close to the DNA-binding interface, and mutation of this arginine does not affect DNA binding. Instead, earlier studies have shown a gain-of-function effect for the mutated ΔNp63γ isoform, which usually does not have a transactivation activity in assays using an optimized *p53*-responsive element (Duijf et al; 2002. Rinne et al; 2006).Two other mutations are located in the N-terminus:

N6H mutation affects only the ΔN-isoforms and in another isolated patient a missense mutation G134D is located just front of the DBD in exon 4 (Slavotinek et al; 2005).

Split Hand/Foot Malformation type 4 (SHFM4) is a "pure" limb malformation (ectrodactyly and syndactyly) condition, thus without orofacial clefting or ectodermal dysplasia. The non-syndromic SHFM4 is caused by several mutations, which are dispersed throughout the *p63* gene: a point mutation in the Transactivation domain (TA) (R58C), a splice-site mutation in front of exon 4, four missense mutations in the DNA binding domain (K193E, K194E, R280C, R280H), and two nonsense mutations in the TI-domain (Q634X, E639X). It is still unclear how these widely dispersed mutations cause the limb defect. Interestingly, several SHFM4 mutations are reported to cause alteration in the p63 protein activation and stability: Q634X and E639X are known to disrupt the sumoylation site, and therefore increase the stability and transcriptional activity of the p63alpha isoform (Huang et al; 2004. Ghioni et al; 2005). Furthermore, amino acids K193 and K194 are required for ubiquitin conjugation by E3 ubiquitin ligase (Itch) and naturally occurring mutations in those amino acids cause more stable p63 protein (Rossi M et al; 2006). Possibly, SHFM is caused by altered protein degradation, even though different degradation routes are involved.

P63 protein regulation

Very little is known of the molecular mechanisms underlying the regulation of p63 protein steady-state levels. In response to different extrinsic and intrinsic cell signals, sophisticated molecular mechanisms must operate in order to activate one or the other p53 family member and to switch on the particular isoform whose specific activity is required. These mechanisms are expected to finely operate keeping a right balance among these proteins either in a physiological or stress condition.

Following oncogenic and stress signals, p53 is stabilized and biochemically activated, leading to transcriptional upregulation or repression of a multitude of target genes. The principal regulator of p53 protein levels is assumed to be the E3 ubiquitin ligase MDM2. MDM2 binds to and ubiquitinates p53, driving it to degradation by the 26S proteasome. In addition to MDM2, other E3 ligases, such as Pirh2 and COP1, have been shown to regulate p53 levels. The p14ARF tumor suppressor protein antagonizes MDM2 activity leading to p53 stabilization.

Data from our lab and others show that p63 protein level is mainly regulated by Itch/AIP4, a HECT E3-ubiquitin ligase that drives p63 to lysosome-mediated degradation (Rossi et al; Cell Cycle 2006; Rossi et al; PNAs 2006). Furthermore, sumoylation of ΔNp63α affects protein stability and transcriptional activity (Ghioni et al; 2005). Recent data have indicated that PML protein interacts with p63 increasing its protein level as well as its transcriptional ability (Bernassola et al; 2005), such as SSRP1 and p300 that function as p63 co-activators (Zeng et al; 2002; MacPartlin et al; 2005).

Moreover, work in our lab has demonstrated that MDM2 and p14ARF, two components of the principal p53 regulatory pathway, are also involved in the control of p63 stability and activity, although their effects on p63 appear to be quite different (Calabrò et al; 2002; Calabrò et al; 2004). In particular, it has been shown that p14ARF physically associates with p63 (Calabrò et al; 2004) and, unexpectedly, it has a negative function on p63 activity, suggesting that p14ARF might not only stabilize p53 but also sequester and store p63, in an inactive complex. Thus, the molecular mechanisms regulating p53, and p63 appear to have largely diverged even though they share some components.

Preliminary data and aim of the thesis

Unequivocal establishment of the role of p63 in the pathogenesis of human ectodermal syndromes is complicated by the fact that this protein exists in multiple isoforms with different often contradictory biological activities. The roles of TA and ΔN isoforms in stratified epithelia have still remained enigmatic. Their spatial and temporal expression pattern suggests that the regulation of p63 function must be much more complex than that believed so far and that sophisticated molecular mechanisms must operate in order to switch on the particular isoform whose specific activity is required in a particular context and in the response to specific extrinsic and intrinsic cell signal.

The profile of identified p63 proteins partners, that may modulate its transcriptional activity, as well as its transcriptional target genes, is currently limited.

My research activity, during the last three years, has been focused on the clarification of the molecular mechanisms through which p63 exerts its function using two different but complementary approaches, i.e. the identification of p63 specific transcriptional targets and the study of the mechanisms regulating p63 protein transcriptional activity and stability.

1)As several developmental disorders associated to ectodermal dysplasia are caused by p63 mutation in humans I decided first to identify p63 specific transcriptional targets that were relevant for the pathogenesis of human AEC syndromes.

All the naturally occurring AEC mutations occur in the p63 SAM-domain and model structure has been used to divide AEC mutations 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 (Brunner et al; 2002). These AEC mutations may disrupt the structural integrity of the SAM domain or interfere with particular protein-protein interactions. They have, in fact, already been shown to disrupt the interaction of p63 with the Apobec-1 binding protein-1(ABPP1) and thus alter the splicing mechanism of fibroblast growth factor receptor-2, FGFR2(Fomenkov et al; 2003).

To gain information about the effects of the Q540L mutation on p63 functions I generated stable cell lines that express wild type TAp63α, ΔNp63α or the TAp63αQ540L mutant under the control of a TET inducible promoter. I used this experimental system to systematically compare the effects of the mutant and wild type p63 proteins on cell proliferation and to generate, by microarrays analysis, a comprehensive profile of differential gene expression. I found that the Q540L substitution affects the transcriptional activity of TAp63α and it causes misregulation of genes involved in the control of cell growth and epidermal differentiation.

2) Interestingly, our group has also demonstrated that AEC-derived TAp63 proteins exhibit an impaired ability to transactivate Dlx3, thus suggesting that the misregulation of the Dlx3 gene is involved in the pathogenesis of AEC.

Dlx3 is a transcription factor belonging to the ***Distal-less*** family of homeodomain proteins. It plays a central roles in embryonic pattern and regulation of different developmental processes. The Distal-less family of proteins are all related to Drosophila *Distalless* homeodomain gene.

Dlx3, in mouse, is expressed in terminally differentiated epidermal cells, and there is evidence to support an essential role as a transcriptional regulator of the terminal differentiation process. Dlx3 is activated in primary mouse keratinocytes cultured in vitro by increasing the level of extracellular Ca^{2+}.

Like p63, mutations in the DLX3 gene have been directly linked with human ectodermal dysplasias. Dlx3 molecular alteration causes tricho-dento osseous syndrome (***TDO***) and amelogenesis imperfecta hypoplastic-hypomaturation with taurodontism (***AIHHT***). TDO is characterized by defects in the development of hair and teeth, increased bone density in the cranium, and absence of overt limb malformations (Wright et al., 1997;Price et al., 1998). The mutation is due to a 4-bp deletion immediately downstream of the homeobox region (DNA binding domain), resulting in a truncated DLX3 protein C-terminus that can potentially still bind DNA but is functionally altered. In humans, the TDO mutation results in a dominant phenotype. It is interesting to note that p63 and Dlx3 mutations resulted in partial-overlapping phenotypes characterized by defects in the same structure, such as hair, teeth and bone, and by absence of limb malformation (Price et al; 1998). suggesting that these genes belong to a common signaling cascades regulating epidermal development.

In the attempt to clarify the relationship between Dlx3 and p63, I performed transient expression assays of Dlx3 in immortalized human HaCaT keratinocytes. The results clearly have shown a reduction of endogenous ΔNp63α protein. Such reduction was also observed on exogenously expressed p63 protein thus suggesting that p63 downregulation occurs at protein level. I have thus focused on the elucidation of the pathway and players through which Dlx3 regulates the p63 protein turnover.

3) Finally, as in our lab has been demonstrated that p63 directly interacts with p14ARF and that throught this interaction ARF inhibits p63-mediated transactivation and transrepression I decided to try to elucidate the mechanism through which p14ARF is able to inhibit p63 transcriptional activity.

Results

1) DIFFERENTIAL GENE EXPRESSION ANALYSIS TO IDENTIFY P63 SPECIFIC TRANSCRIPTIONAL TARGETS

1.1) Production and characterization of TAp63α, TAp63αQ540L and ΔNp63α stable cell lines.

To investigate on the effects of the Q540L mutation on p63 protein functions I generated stable H1299 cell lines, expressing TAp63α, ΔNp63α or TAp63αQ540L, under a tetracycline/doxycycline (dox)-inducible promoter using Tet-On Gene Expression System (see materials and methods). The Tet-On system has several advantages over other regulated gene expression systems that function in mammalian cells. In fact, Tet-On system assures no pleiotropic effects, extremely tight on/off regulation, high inducibility and fast response times.

I first analysed, by Western blot, the timing and level of expression of TAp63αwt, TAp63αQ540L and ΔNp63α proteins in stable clones upon dox induction. Results from representative cell lines for TAp63αwt, TAp63αQ540L and ΔNp63α are shown in Figure 1. Without dox in the medium p63 proteins were undetectable. Following addition of 1 μg/ml doxycycline in the culture medium, p63 proteins were induced in a time-dependent manner (fig. 1). At 24 hrs of induction, p63 proteins were already abundant and their expression levels were comparable .

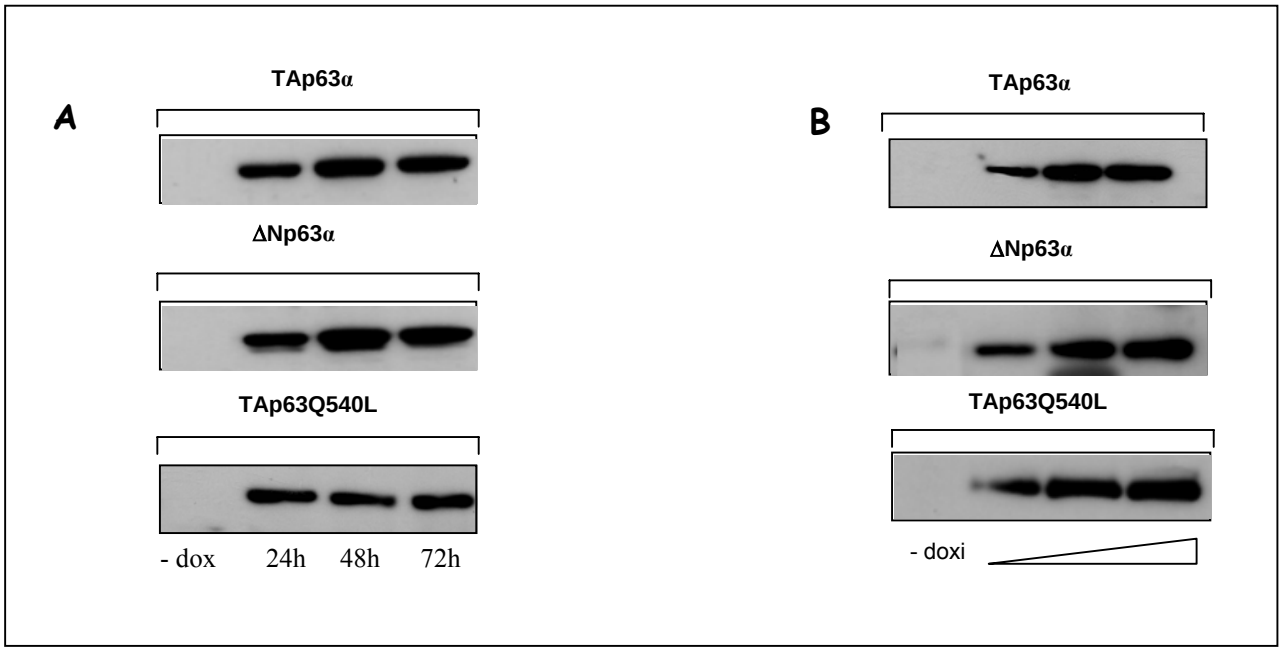


Figure 1. Expression of p63 isoforms in H1299 stable clones.

Western Blot analysis for detection of the p63 protein expression levels in Dox-inducible H1299 cells. Cells were harvested and p63 expression level was analized at the indicated time points and with different amounts (0.5, 1 and 2μg) of inductor. Equal amounts of soluble lysates (30μg) from uninduced and induced clones expressing wild-type TAp63α, ΔNp63α or the mutant TAp63Q540Lprotein were evaluated for p63 protein levels by western blotting using an anti-p63 antibody (4A4, Santa Cruz). A) The timing of expression was assessed at 48 hours after inductor, while B) expression upon addition of different amounts of doxicycline into the medium was assessed at 1μg.

The high degree of similarity between p53, and p63 DNA-binding domains, as well as transactivation and oligomerization domains, suggested that p63 could regulate p53 target genes. Actually, It has been reported that some p63 isoforms such as TAp63, are able to regulate the transcription of p53 target genes such as p21WAF and MDM2 (Yang et al; 2000). In order to verify the transcriptional activity of ΔNp63α as well as the effect of Q540L point mutation on the activity of TAp63α I tested the effect of TAp63α, ΔNp63α and the Q540L mutant expression on p21WAF and MDM2 endogenous gene expression. Stable cell lines expressing, respectively, TAp63α, ΔNp63α and TAp63Q540L were induced for 48 hours with different amounts of doxycycline. The results of Western Blot analysis showed an increase of p21WAF and MDM2 protein expression levels upon TAp63α and ΔNp63α induction, though to a different extent. In contrast, AEC mutant Q540L appeared to reduce p21 and MDM2 expression (Figure 2A). I have also analyzed the effect of Q540L point substitution on the ability of TAp63α wt protein to transactivate the expression of a CAT reporter gene under the control of the p21WAF gene promoter. A fixed amount of p21/WAF promoter-CAT construct was transiently transfected in H1299 cell lines along with increasing amounts of plasmids encoding p63α proteins. As shown in Figure 2B, the Q540L mutation strongly impairs the ability of TAp63α to induce the p21/WAF gene promoter, whereas ΔNp63α is a mild activator.

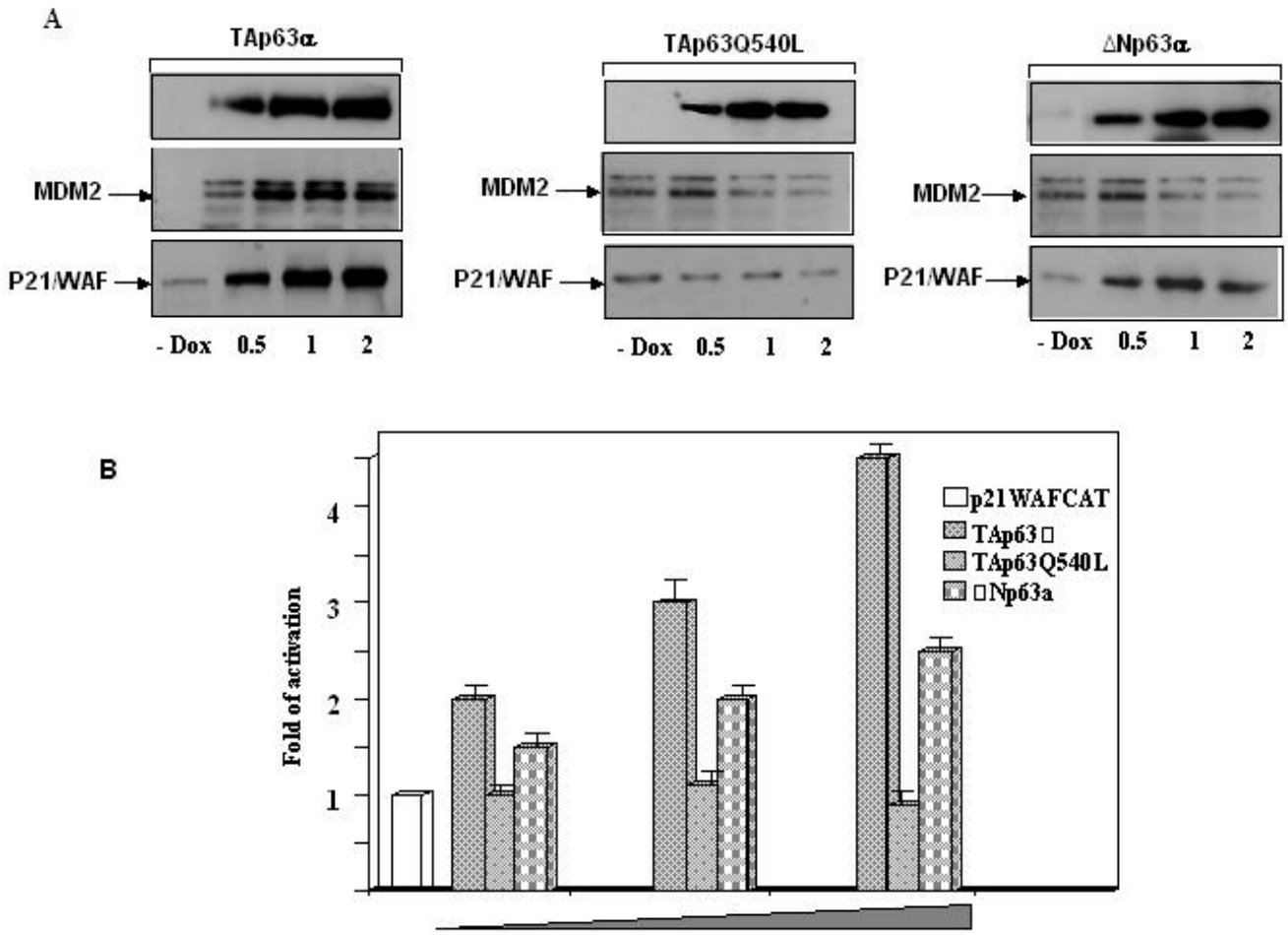


Figure 2. TAp63αQ540L has lost the ability to activate p21/WAF and MDM2 gene expression.

A) Western Blot analysis showing expression of TAp63α wt, TAp63αQ540L and ΔNp63α proteins in stable clones at 48h 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.

B)H1299 were transiently transfected with 2μg of p21/WAF-CAT reporter plasmid/dish alone or with different amounts of each p63expressing plasmid (1, 2 or 3 μg). After 48h, cells were harvested, and CAT acivity 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 represent the mean of triplicate assays from three independent experiments. Standard deviation are also indicated.

1.2) Subcellular localization of mutant TAp63αQ540L

TAp63α and ΔNp63α proteins are located in the nucleus where they act as transcriptional factors (Calabrò et al; 2004).

For this reason, we have supposed that the lack of transcriptional activity of the TAp63αQ540L protein could to be due to its inability to relocate in the nucleus.

So, I performed subcellular immunolocalization assay on induced or uninduced stable clones expressing TAp63α , ΔNp63α and mutant Q540L protein to compare TA, ΔNp63α wild type and TAp63αQ540L subcellular distribution in Tet-On stable H1299 cells. Fourty-eihgt hours after induction, cells were fixed and immunorevealed with antibody to detect p63 protein. Inspection of the subcellular localization of TAp63α, ΔNp63α and TAp63αQ540L protein expressed in our inducible clones, by immunofluorescence, showed that, both p63α wild type and mutant Q540L proteins were uniformly distributed in the nucleus with nucleolar sparing (Figure 3). Therefore, the Q540L amino acid substitution does not alter TAp63α subcellular distribution.

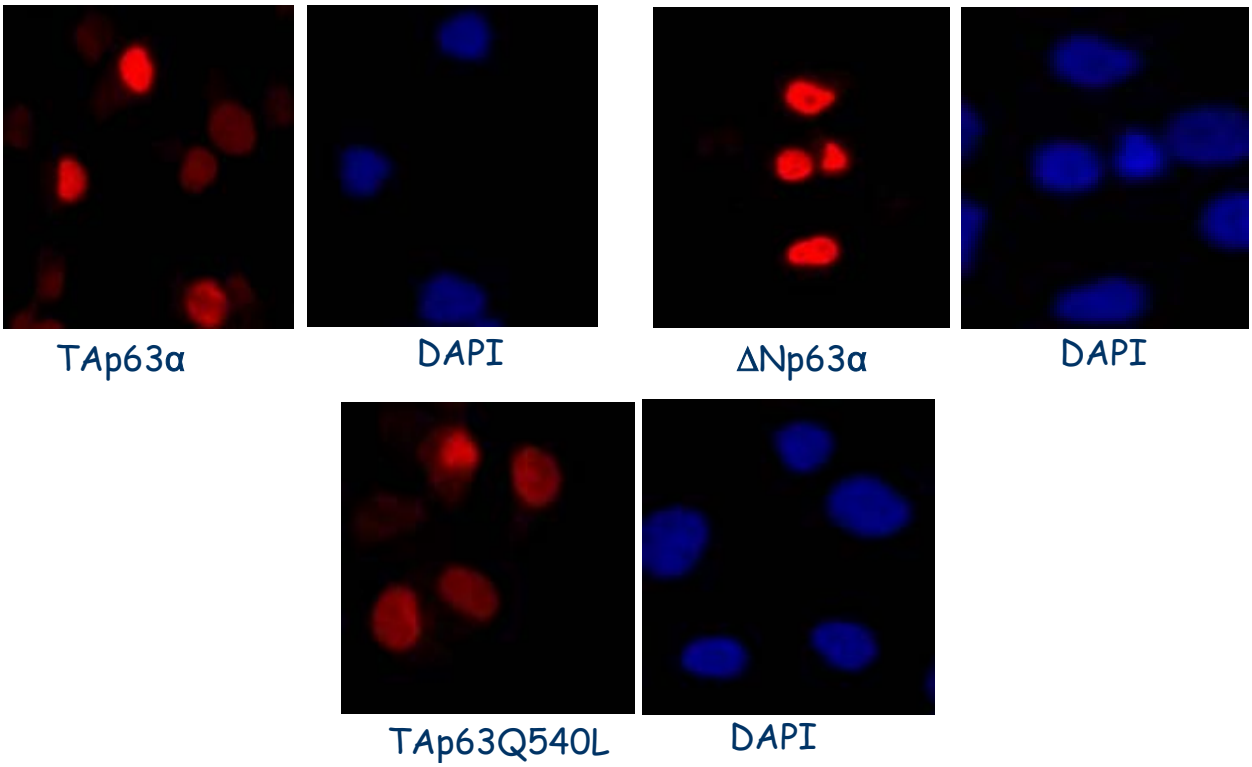
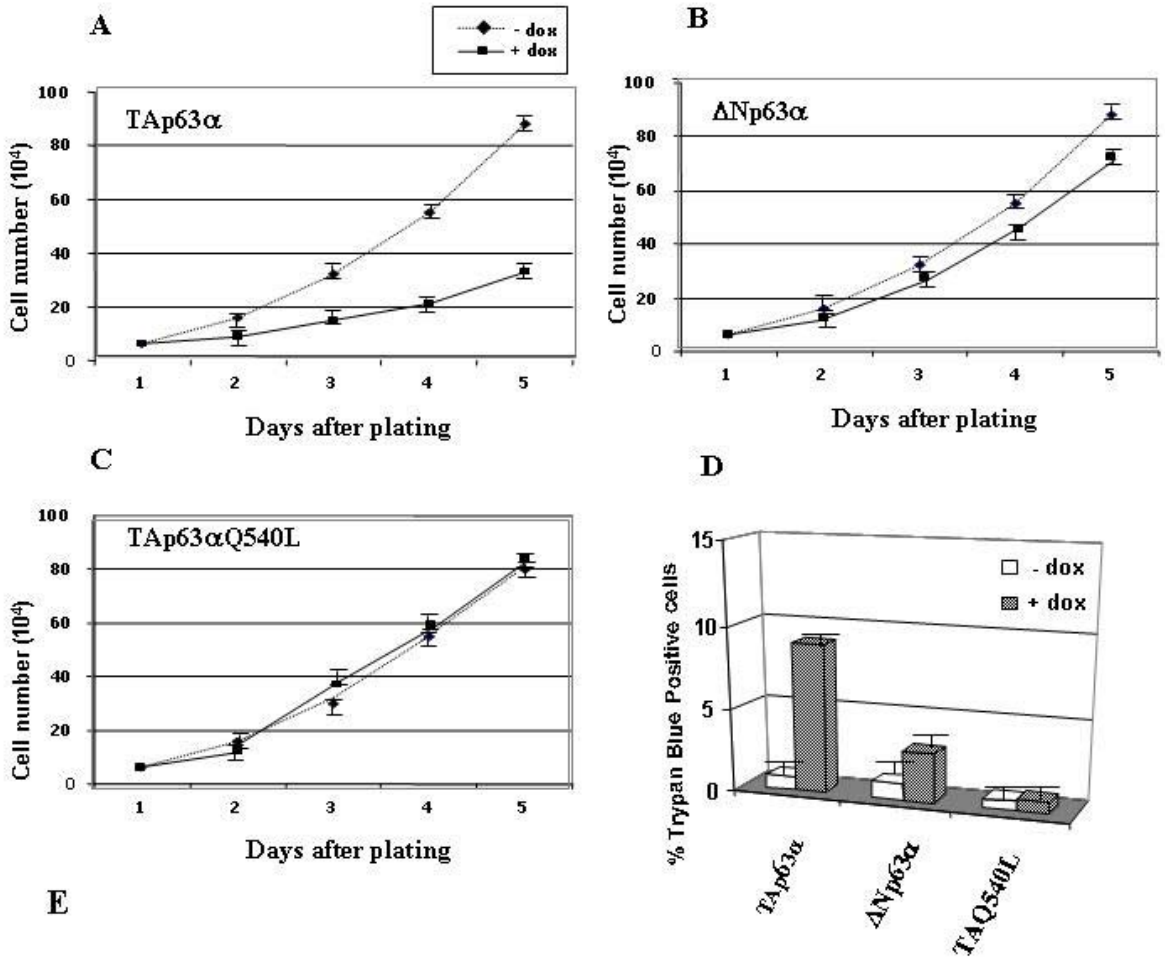


Figure 3. The Q540L AEC-mutant localize into the nucleus. Subcellular localization assay are performed in TAp63α , ΔNp63α and TAp63αQ540L stable cell line under induced (+dox) condition. The cells were examined under a fluorescence microscope. As shown, both p63α wild type and Q540L mutant protein localize into the nucleus.

1.3) Functional analysis of stable clones expressing TAp63α, ΔNp63α and TAp63αQ540L

To test the effects of wild type TAp63α, ΔNp63α and mutant TAp63αQ540L protein on cell proliferation, I compared the cell growth profile of H1299 cells, expressing wild type TAp63α, ΔNp63α, or TAp63αQ540L. Interestingly, cells expressing wild type TA or ΔNp63α showed a reduction of cell growth rate while the cell growth profile was completely unaffected by TAp63αQ540L expression (fig. 4A, B and C). By the trypan blue dye exclusion assay, I could estimate that at 72 hrs after induction, TAp63α expression induced a 9.2% of cell death while ΔNp63α a 3.4 %. Again TAp63αQ540L had completely no effect on cell viability (fig. 4D). Then, I performed flow cytometric analysis on p63 inducible clones. In a dox free culture medium, the cell cycle profile of the three p63 stable cell lines and parental H1299 cell line were comparable (fig. 4E and data not shown). However, at 48 hrs upon addition of 1 μg/ml of dox, both wild type TA and ΔNp63α caused a G1 cell cycle arrest, with a parallel reduction in S and G2/M phases and a significant increase of sub-G1 events (fig.4E). In contrast, when the TAp63αQ540L mutant was induced, the percentage of cells in G1 phase was unaffected and a slight increase in S phase with a corresponding decrease in G2-M phase was observed (fig. 4E).



E

	TAp63α		ΔNp63α		TAp63αQ540L	
	- Dox	+ Dox	- Dox	+ Dox	- Dox	+ Dox
sub-G1	0.8[1]+/- 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 4. **The Q540L aminoacid substitution impairs the ability of wild type TAp63α to induce a G1 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. Standard deviations are also indicated.

1.4) Microarray analysis

The finding that TAp63α inhibits cell proliferation and induces cell death, whereas the TAp63αQ540L mutant lacks these capabilities prompted us to generate a comprehensive profile of differential gene expression by Microarray analysis. I extracted and purified total RNA from stable H1299 cell lines (see Matherial and Methods). Microarray analysis was performed in the lab of Prof. Calogero in Turin. Four prototypic situation were evaluated: a) TAp63α wt without induction, b) TAp63α wt with induction, c) TAp63αQ540L without induction, d) TAp63αQ540L with induction. Three biological replicas generated for all four situation were used to synthesize biotinylated cRNA for hybridization on arrays containing 54675 probes. Microarray data have shown that there is a clear up-modulation of the wild type and mutant p63 proteins upon induction with doxycycline (fig. 5). Up-modulation of p63 was also confirmed by real-time PCR (data not shown). A total of 45 probe set ids were identified as differentially expressed and associated to 36 gene IDs. Quantitative RT real-time PCR (QPCR) validation was done for 11 out of 36 genes. A total of 10 annotated genes, that were transactivated upon induction of wild type TAp63α (fig. 5D), resulted not to be responsive to the TAp63αQ540L mutant. Among this set of genes, 4 of them (GDF15, CDKNIA/p21WAF, MDM2, ARX) were selected to be also investigated by QPCR for their responsiveness to TAp63α, ΔNp63α and TAp63αQ540L (fig. 6). GDF15 and CDKNIA-p21WAF appeared to be

significantly transactivated by TAp63α only. GDF15 was already activated at 12 hrs of induction (fig. 6A) while p21WAF after 24 hrs (fig 6B). MDM2 and ARX were similarly transactivated by wild type TA and ΔNp63α (fig. 6C and D) but none of these four genes were modulated by the TAp63αQ540L mutant (fig 6A and D). According to data that I obtained from both CAT reporter assay and western blot analysis, also Microarray data have showed no increase of p21/WAF and MDM2 endogenous proteins in TAp63αQ540L stable cells upon induction.

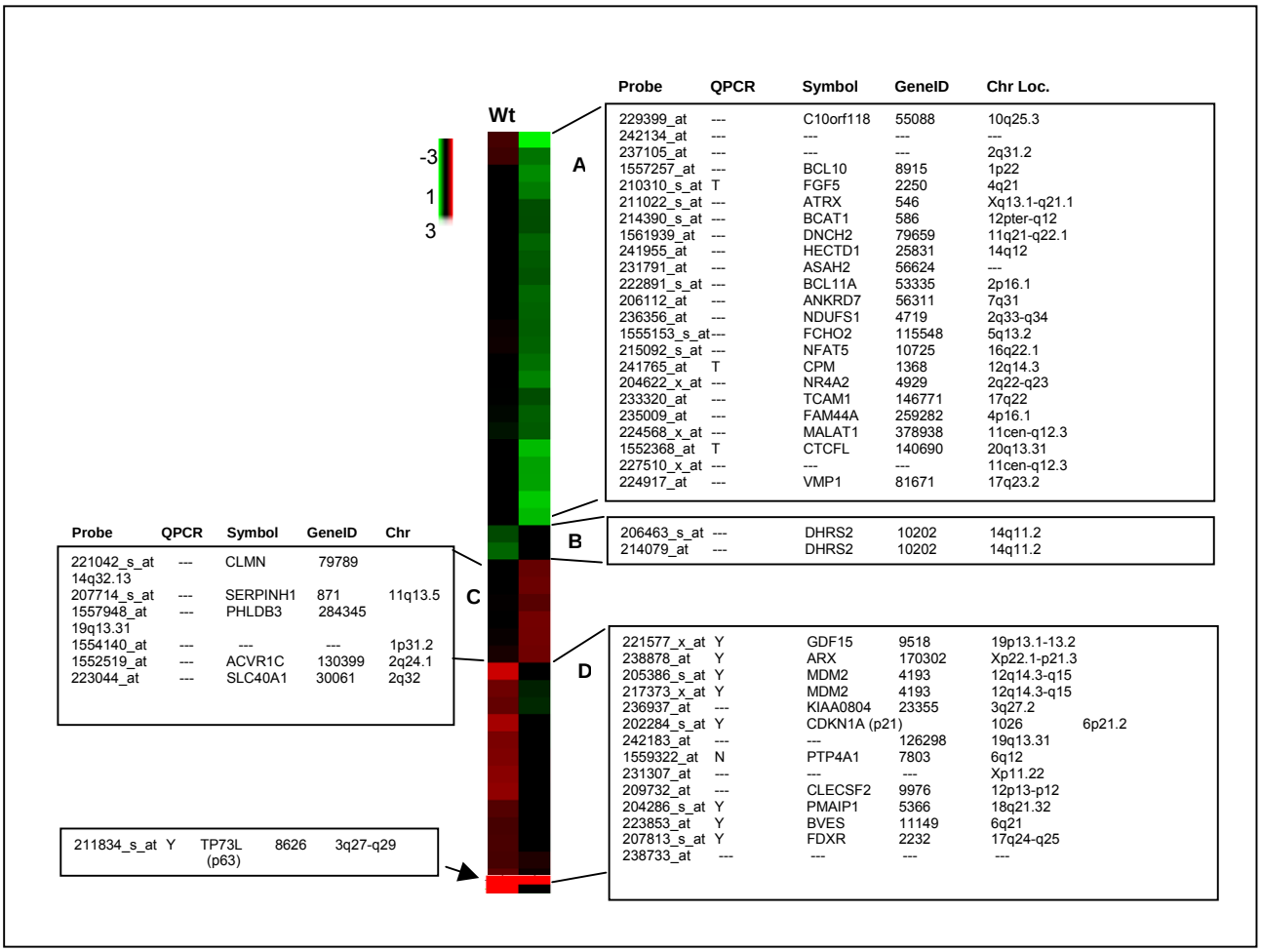


Figure 5. Clustering of probe sets differentially expressed between TAp63α wt and Q540L mutant stable cell lines. Cluster A refers to genes which are not significantly modulated upon induction of TAp63α wild type and are instead down-modulated by TAp63αQ540L expression. Cluster B includes only one gene which is down-modulated by TAp63α wild type expression and not significantly modulated by TAp63αQ540L. Cluster C encloses genes which are not significantly modulated upon induction of TAp63α wild type and are instead up-modulated by the TAp63αQ540L mutant protein. Cluster D refers to genes transactivated by the wild type TAp63α protein and characterized by a loss of regulation by the TAp63αQ540L mutant.

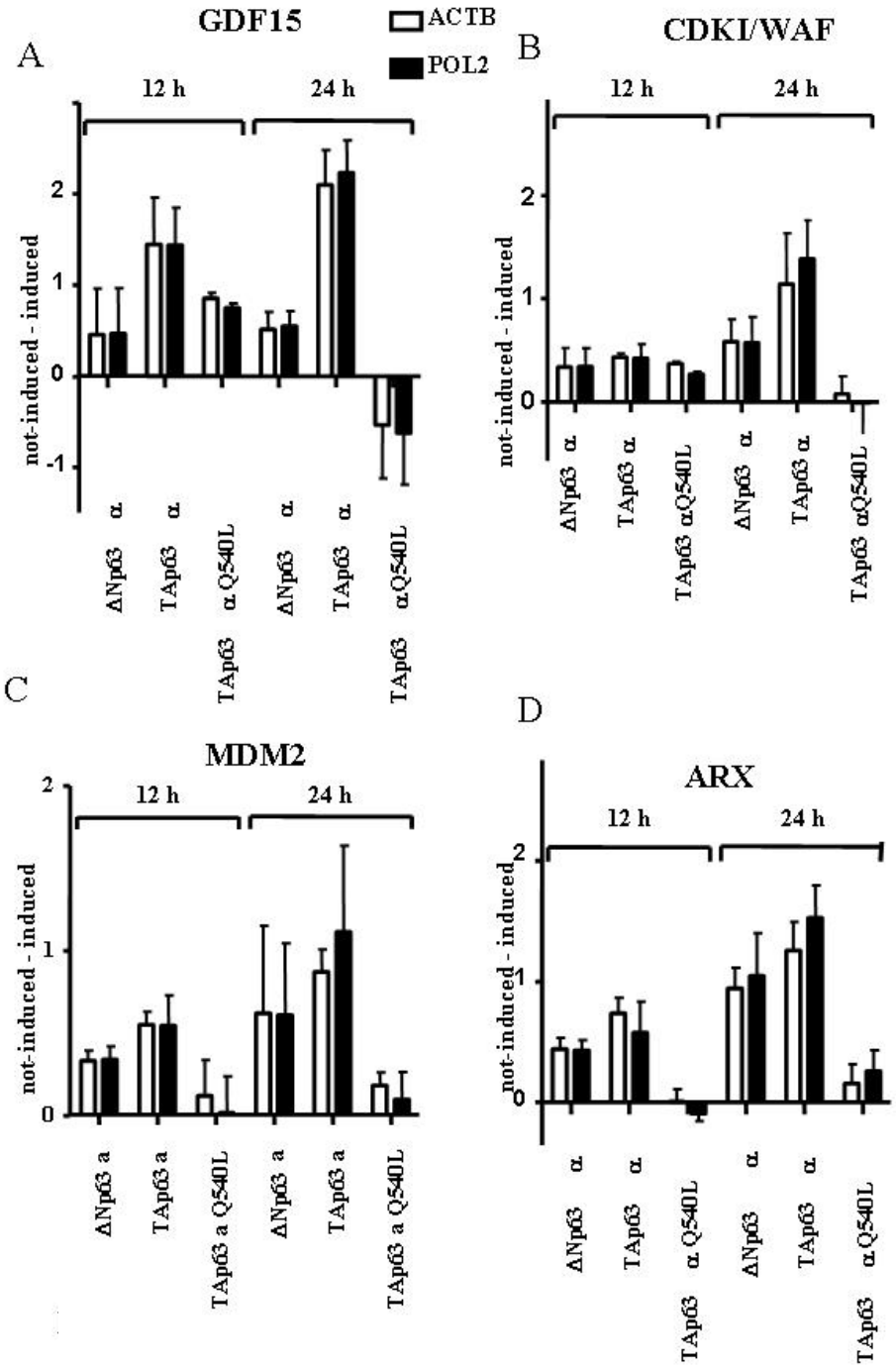


Figure 6. QPCR data related to 4 genes transactivated by TAp63α wild type and characterized by a loss of control by the Q540L mutant. Gene expression was analyzed at 12 and 24 hours upon addition of doxycycline to ΔNp63α, TAp63α and Q540LTAp63α inducible cell lines. Target gene mRNA levels were normalized for ACTB (white bars) and POL2 (black bars) and expressed as –DDCt (i.e. not-induced cell line-induced cell line Cts).

1.5) TAp63αQ540L binds to p21 promoter sequences in vitro and interacts with Sp1 in vivo.

The absolute inability of the TAp63αQ540L protein to transactivate the p21WAF promoter prompted us to verify whether the mutant protein was still capable to bind to the p53-consensus sequence of the p21WAF promoter. Thus I compared the DNA binding capacity of wild type and mutant TAp63α proteins 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 (Calabrò, Mansueto et al. 2004). Incubation of the radiolabeled oligonucleotide with *in vitro* translated TAp63α or TAp63αQ540L mutant, led to the formation of specific protein-DNA complexes (Fig.7 A, lanes 2 and 6). The specificity of the TAp63-DNA complexes was tested by a competition experiment: a 100x cold molar excess of the same oligonucleotide used as probe completely abolished the binding, while an unrelevant control oligonucleotide had no effect (Figure 7, lanes 3 and 4; 7 and 8). The identity of the TAp63α-DNA complexes was confirmed by a supershift experiment (Figure 7, panel A, lanes 5 and 9) by incubating the in vitro translated TAp63α proteins prior to the binding reaction, with an antibody recognizing the p63 DNA-binding domain. By Western blot analysis we verified that the relative abundance of the *in vitro* translated TAp63 proteins was comparable (data not shown). These observations indicate that the wild type TAp63α protein 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.

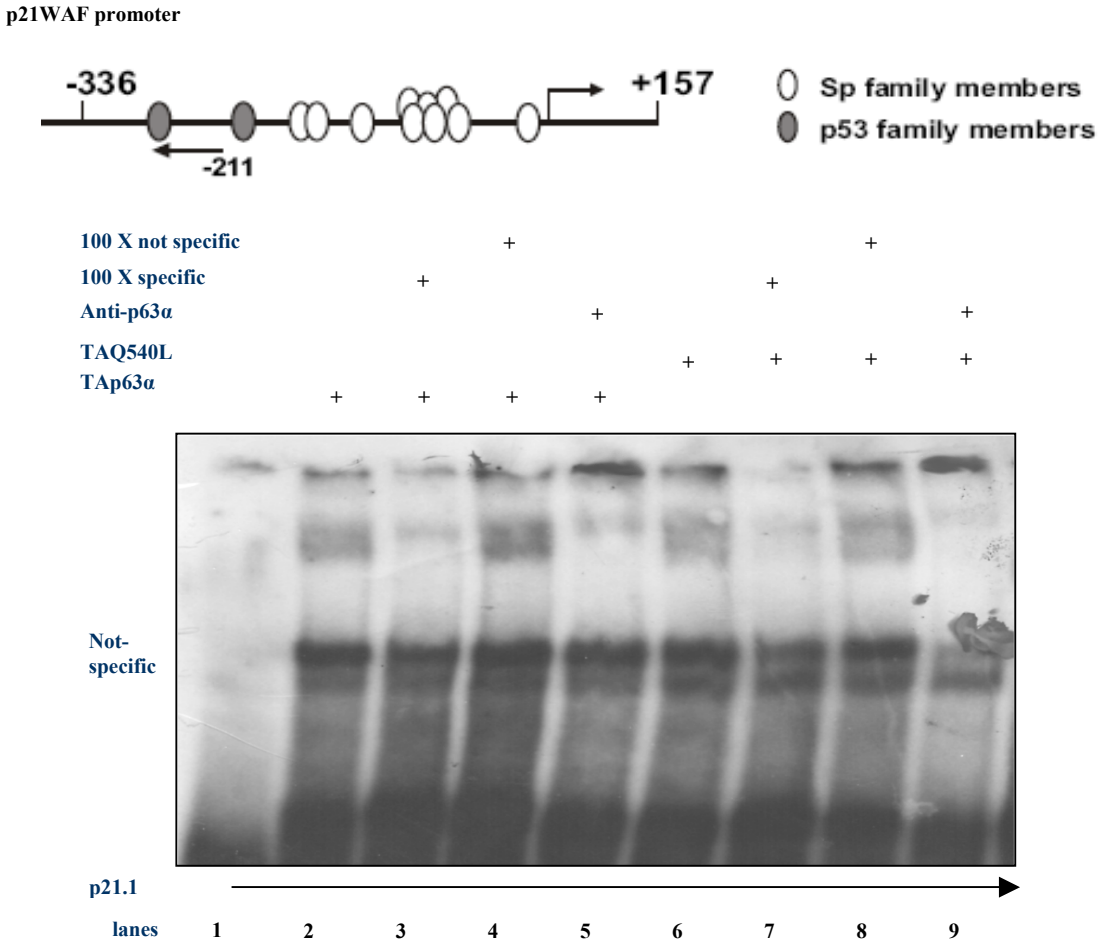


Figure 7. TAp63αQ540L binds to p53 consensus site in the p21/WAF target promoter and it is able to associate with the Sp1 transcription factor.

(A) The indicated p63 proteins were *in vitro* translated by using TnT reticulocytes from Promega and 0.5 µg of each p63 plasmid DNA. Equal amounts of the individual reactions were subjected to EMSA using a ^{32}P-labelled oligo containing a p53-binding site present in the p21 promoter (p21.1 probe). Cold competition was performed using 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 used, adding them to the sample prior to the binding reaction (lanes 5 and 10).

The promoter of the human p21/WAF gene is characterized by a set of six Sp1 binding sites located in the proximal region (nucleotides –120 to –40) and two distal p53 binding sites. These proximal Sp1 binding sites were shown to be essential for the activation of p21/WAF promoter by p53 (Koutsodontis et al; 2005). Since it has previously been reported that the γ isoform of TAp63 directly interacts with Sp1, we decided to investigate on whether TAp63α was also able to interact with Sp1 and, in this case, which was the effect of the Q540L substitution on this interaction. I performed co-immunoprecipitation experiments in TAp63α and TAp63αQ540L expressing cell lines induced or not with doxycycline. As shown in fig. 8, both wild type and mutant TAp63α proteins were immunoprecipitated using a Sp1 polyclonal antibody. The reciprocal immunoprecipitation, detecting Sp1 protein using the p63 monoclonal antibody did result in Sp1 being immunoprecipitated (data not shown).

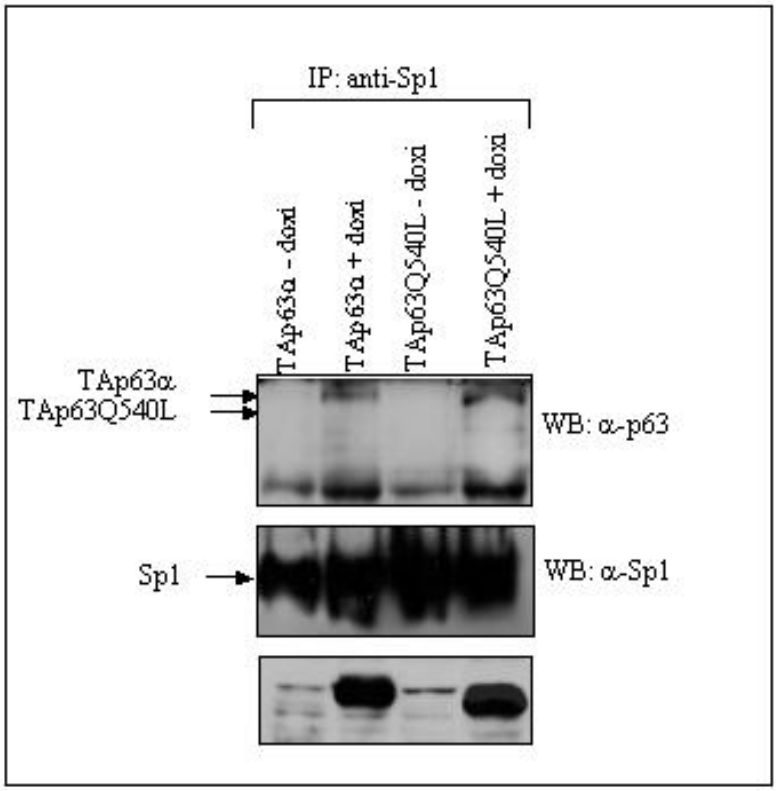


Figure 8. TAp63αQ540L associates with Sp1 transcription factor.

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.

1.6) TAp63αQ540L does not bind to p21 promoter sequences in vivo

In vitro DNA-binding assay suggested that the Q540L mutation does not affect the ability of the p63 protein to bind DNA. However, given the important role of chromatin structure in the regulation of gene expression by transcription factors and cofactors I would verify if the Q540L mutant protein was still able to bind p53-consensus sites of the p21/WAF promoter, also an *in vivo* context. Thus, I performed Chromatin Immunoprecipitation Assay in H1299 cells transfected with TAp63α wild type or mutant Q540L encoding plasmids. Cross-linked chromatin was extracted and immunoprecipitated with anti-p63 antibody. DNA was purified and appropriately primers designed to amplify the p53-response element in p21/WAF, Dlx3, Jagged 2 and IKK promoters. In contrast with the previous reported data, obtained with an in vitro assay, in vivo cromatin Ip revealed that the AEC mutation impairs the ability of p63 to bind DNA. As shown in figure 9, wild type TAp63α protein specifically binds to promoter region of assayed genes, while the Q540L mutant shown significantly reduced ability to bind DNA. A Western Blot analysis of the transfected proteins have showed no significant differences in the relative abundance of p63α wild type and mutant protein (data not shown).

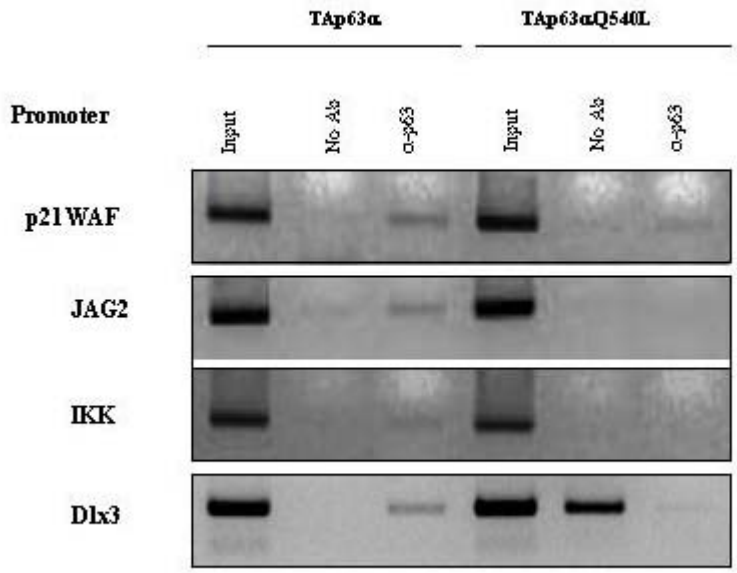


Figure 9. The Q540L substitution impairs DNA binding ability of p63. H1299 cells were transfected with TAp63α wild type or mutant Q540L encoding plasmids. Formaldeyde cross-linked chromatin was immunoprecipitated with anti-p63 4A4 antibody. Eluted DNA was PCR amplified with primers specific for different p63 (JAG 2, DLX 3) and p53 (p21WAF, IKK) target promoters genes.

2) INVESTIGATION ON P63 PROTEIN DEGRADATION Dlx3-MEDIATED.

2.1) Dlx3 downregulates p63 protein level.

Our group has also demonstrated that AEC-derived TAp63 proteins exhibit an impaired ability to transactivate Dlx3, thus suggesting that the misregulation of the Dlx3 gene is involved in the pathogenesis of AEC. It is interesting to note that p63 and Dlx3 mutations resulted in partial-overlapping phenotypes characterized by defects in the same structure, such as hair, teeth and bone, and by absence of limb malformation (Price et al; 1998) suggesting that these genes belong to a common signaling cascades regulating epidermal development.

A recent work published by our group in collaboration with Dr. Morasso, has previously reported that calcium-induced differentiation of primary keratinocytes resulted in induction of Dlx3 gene expression that parallels a decrease of ΔNp63α specific transcripts (Radoja N. et al. 2007). Furthermore, transient expression of Dlx3 induced a remarkable decrease of endogenous ΔNp63α protein expression both in primary Human Embryonic keratinocytes (HEK) (data obtained in lab of Dr. Morasso from NIH of Bethesda) and immortalized HaCaT keratinocytes (Fig.10, panels A and B). As we suspected that Dlx3 was down-regulating ΔNp63α at the protein level, I transfected ΔNp63α or TAp63α expression vector alone, or in combination with increasing amount of Dlx3, in Saos2 cells, a human osteosarcoma-derived cell line where p63 is not normally expressed. I transfected Saos2 cells with the indicated amount of expression vector by LipofectAMINE 2000 reagent and, after

24 hours, cells were lysated and protein concentrations determined using the Bio-Rad protein assay. Western blot of whole extracts and specific immunodetection with anti-p63 (4A4) antibodies and anti-Flag antibodies showed that Dlx3 caused a remarkable decrease of ΔNp63α protein (Fig. 10 C). This effect was at protein level, since no difference in ΔNp63α specific transcript was detected when I performed semiquantitative RT-PCR on total RNA from Saos2 cells transfected with ΔNp63α alone or with Dlx3 (Fig.10 C). Compared to ΔNp63α, the abundance of TAp63α protein was less affected by Dlx3 expression (Fig.10 C). I obtained similar results in different cellular context such as U2OS, HeLa, and H1299 cells. It has been previously demonstrated that p53 itself was able to associate with ΔNp63α inducing its degradation by a caspase-dependent mechanism (Ratoviski et al; 2001), so, in order to investigate on whether Dlx3-mediated p63 degradation was involving p53 activity, I performed experiments in (p53-/-mdm2-/-) Mouse Embryo Fibroblasts, MEFs (Fig.10 D). Thus I transfected MEFs with p63 alone or along with increasing amount of Dlx3 expression vector. Even in MEFs, I observed a decrease of p63 protein, in response to Dlx3, indicating that the mechanism responsible for the reduction of ΔNp63α protein, was independent from both p53 and MDM2.

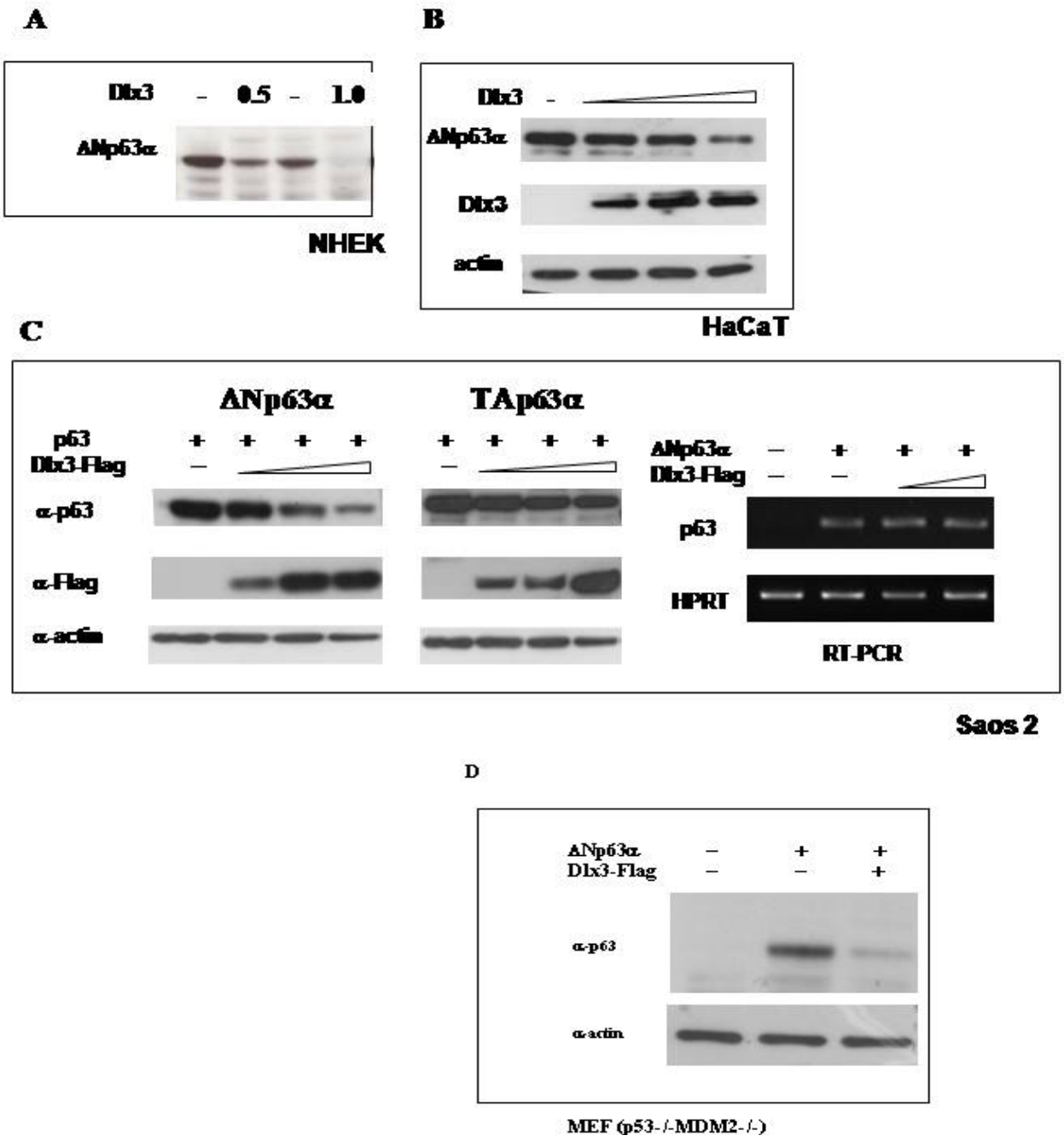


Figure 10. Dlx3 reduces p63 protein level.

(A) NHEK stable cell lines were induced for Dlx3 expression and cellular lysate were analyzed for ΔNp63α expression. (B) Western Blot analysis of HaCaT cell extracts 48 hrs upon transfection with increasing amounts (0.5; 1.0 and 1.5 µg) of Flag tagged Dlx3 expression vector. Endogenous ΔNp63a was revealed with 4A4 antibodies, Dlx3 with anti-Flag antibodies. Actin was checked as loading control. (C) Saos2 cells were cotransfected with 0.2 µg of ΔN or TAp63α plasmid DNA and increasing amounts (0.5; 1.0 and 1.5 µg) of Flag-Dlx3 expression vector. 48 hrs after transfection cell extracts were analyzed by Western blot and specific immunodetection. RT-PCR. Level of exogenous ΔNp63α mRNA upon cotransfection with increasing amount (1.0 and 1.5µg) of Dlx3-Flag expression plasmid in Saos2 cells. (D) Mouse Embryo Fibroblasts (p53-/- MDM2-/-) were transfected with 0.2 µg of ΔNp63α alone or together with 1.0 µg of Flag-Dlx3 expression vector. At 48 hrs after transfection cell extracts were analyzed by Western blot and specific immunodetection.

the protein synthesis inhibitor cycloheximide. I transfected Saos2 cells with p63 encoding vector alone or with Dlx3 expression plasmid, and 16 hours after transfection, cells were treated with cycloheximide to block protein synthesis. At the indicated times after exposure to the drug, cells were harvested and the extracts analyzed by Western Blot and probed with an anti-p63 and anti-flag antibodies. Transfected ΔNp63α protein was very stable with a half-life being greater than 9 hours; expression of Dlx3 reduced ΔNp63α protein level and the decay was faster, with a half-life between 5 to 7 hours (fig. 11).

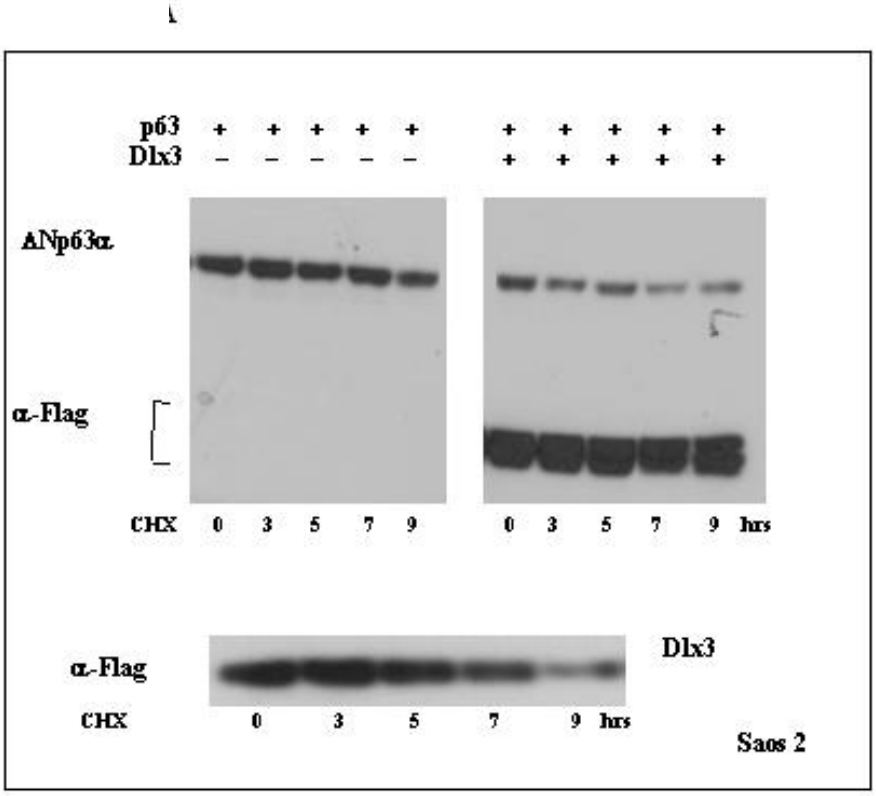


Figure 11. Dlx3 affects ΔNp63α protein half-life.

Saos2 cells were transfected with 0.2 μg of ΔNp63α, expression plasmid alone or along with a fixed amount (1μg) of Dlx3-Flag encoding plasmid. 16 hours after transfection cells were treated with 40μg/ml of cycloheximide. At the indicated times, cells were lysated and cellular extract were analyzed by Western Blot and specific immunodetection. Saos 2 cells were also transfected with Dlx3-Flag expression plasmid alone to determine Dlx3 protein half-life.

As it has been reported that both proteasome and lysosome are involved in p63 protein degradation (Ghioni et al. 2005, Rossi et al. 2006), I decided to investigate whether Dlx3-driven p63 degradation was proteasome or lysosome-dependent. Reduction of p63 by Dlx3 was efficiently reversed by the proteasome inhibitors MG132 and ALLnL while no effect was observed treating cells with the lysosome inhibitor chloroquine or ammonium chloride (Figure 12 A and data not shown). Finally, to determine if a physical association between p63 and Dlx3 protein was required to induce p63 protein degradation, I immunoprecipitated ΔNp63α from lysates of Saos2 cells expressing p63, Dlx3 or both. Whole extracts were subjected to Western blot and specific immunodetection with anti-p63 (4A4) antibodies. Dlx3 was revealed with anti-Flag antibodies. I also performed the reciprocal experiment and, in both cases, I failed to see interaction between p63 and Dlx3 (Figure 12 B and data not shown). These data suggest that Dlx3 is activating a protein degradation pathway that leads to the reduction of p63 level and that this molecular mechanism does not require a direct interaction between the two proteins.

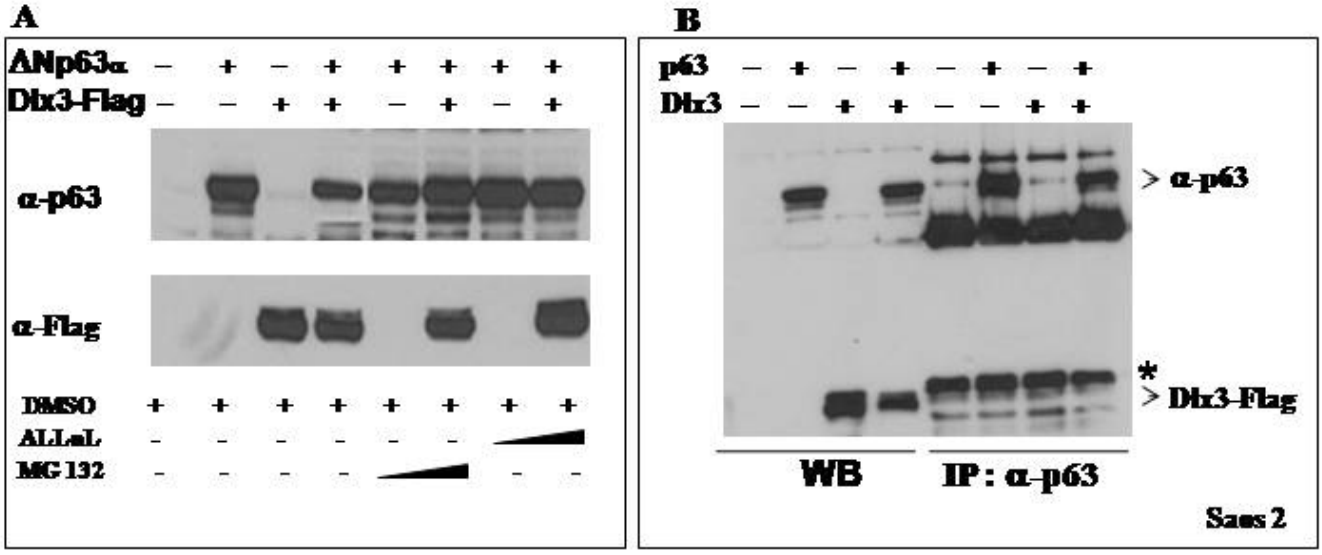


Figure 12. Dlx3 mediates proteasome-dependent degradation of p63 and its function does not require p63-Dlx3 physical association.

(A) Saos 2 cells were transfected with the expression vector for ΔNp63α (0.2μg) with or without a fixed amount (1μg) of Dlx3 expression vector in the presence or absence of 10μM of MG132 or ALLnL. Total lysates were separated by SDS-PAGE and blotted with antibodies against p63 (4A4) or Flag to detect Dlx3 protein. (B) Coimmunoprecipitation of ΔNp63α with Dlx3. Saos2 cells were transfected with 0.2μg of ΔNp63α plasmid alone or togheter with Dlx3-Flag expressing plasmid. Cellular extract were immunoprecipitated with anti p63 4A4 antibody and immunocomplex were analyzed with anti-p63 and anti-Flag antibodies.

2.2) Dlx3-induced ΔNp63 degradation requires specific Serine and Threonine residues located in the p63 α and β carboxyterminal tails.

Although ΔNp63α is the most abundantly expressed p63 isoform in adult skin, I wished to investigate whether Dlx3 could also reduce the level of the remaining ΔNp63 isoforms. Thus, I overexpressed ΔNp63β or γ, in Saos2 cells, along with increasing amount of Dlx3. Interestingly, the levels of ΔNp63β was significantly reduced by Dlx3 while ΔNp63γ was almost completely unaffected (Fig. 13 A). These results suggested that aminoacid residues located in the α and β tail of p63, could confer the sensitivity to the degradation mechanism triggered by Dlx3 expression. To identify these residues , I tested two constructs expressing carboxyterminal truncated ΔNp63 proteins, named ΔNp63Δ408 and Δ373 (a schematic representation is shown in Fig. 12 B), kindly provided by Dr. Guerrini (University of Milan). I transfected each of them into Saos2 cells, with or without Dlx3 and compared with wild type ΔNp63α for the sensitivity to Dlx3-mediated degradation. Interestingly, the ΔNp63αΔ373 protein was almost unaffected by Dlx3, while ΔNp63αΔ408 was efficiently degraded (Fig. 13 B).

Thus, I concentrated my attention on the region encompassing aminoacid 373 to 408 of ΔNp63; by the NetPhos 2.0 bioinformatic analysis I identified two potential phosphoacceptor sites, located in this region, a Serine at position 383 (score 0.866) and a Threonine at position 397 (score 0.6). Furthermore, using the ELM

bioinformatic resource (http://www.elm.eu.org), we found that Serine383 was included in a PIKK (PI3K related kinases) phosphorylation motif, while Threonine 397 was a potential MAPK phosphorylation site. Interestingly, Serine383 and Threonine397 also belong to the Phospho Cluster IV, as described by Finlan and Hupp (Lee E. Finlan and Ted R. Hupp; 2007). In the laboratory of Dr. Guerrini at the University of Milan it was generated, by site-directed mutagenesis, the ΔNp63αS383A construct bearing a Serine to Alanine substitution at position 383 and the ΔNp63αT397A expression plasmid, bearing a Threonine to Alanine substitution at position 397. Strikingly, substitution of either Ser383 or Thr397 caused a remarkable decrease of ΔNp63α sensitivity to Dlx3-driven degradation (Figure 13 B). In addition, it was generated the ΔNp63αS383AT397A protein bearing both aminoacid substitutions. As expected, the S383T397 double mutant was almost completely resistant to Dlx3-mediated degradation (Figure 13 B). These results indicate that the mechanism through which Dlx3 causes p63 protein degradation is dependent on specific serine/threonine residues located in the p63 carboxyterminal α and β tails. Consistently, ΔNp63γ lacking these residues, was almost completely refractory to Dlx3-induced degradation (Figure 13 B).

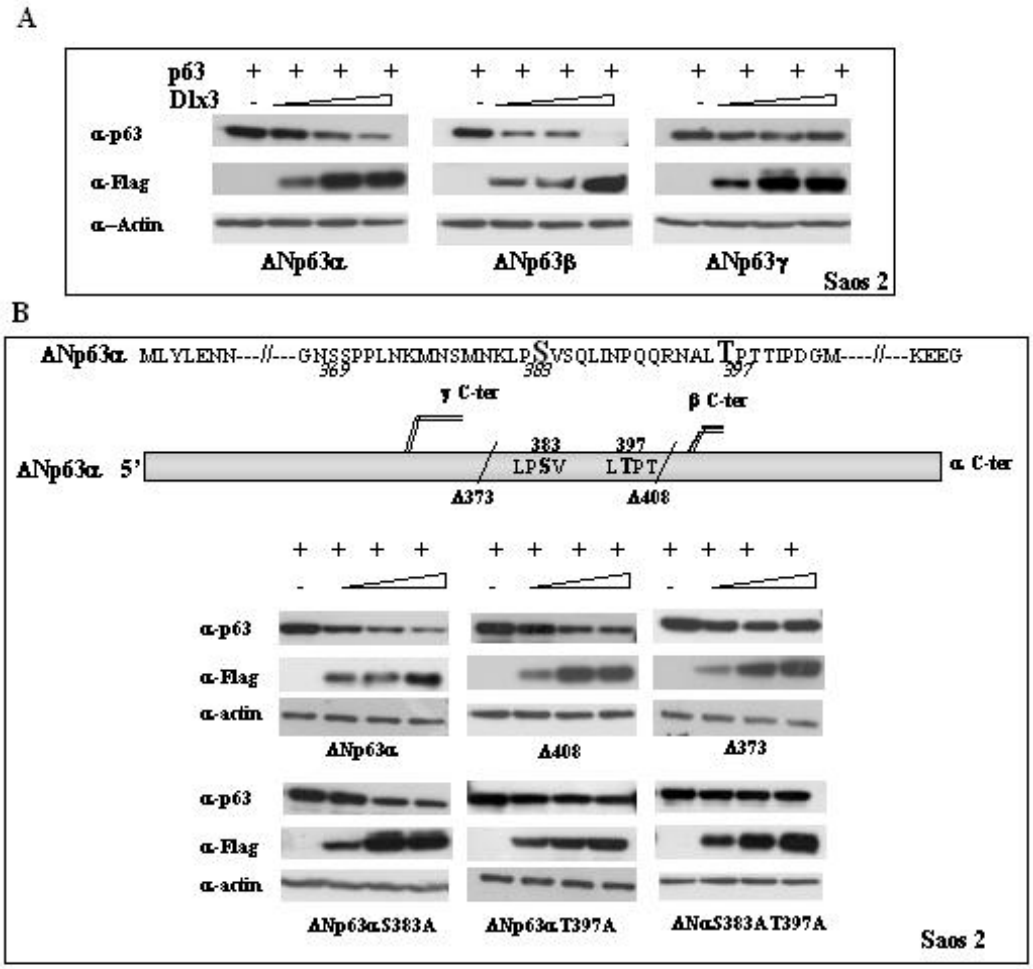


Figure 13. Carboxyterminal α and β tails of p63 confer to ΔNp63 sensitivity to Dlx3 elicited degradation.

(A) ΔNp63α, β and γwere compared for their sensitivity to Dlx3-mediated degradation by cotransfection and immunoblot experiments in Saos2 cells. for their sensitivity to dlx3-mediated degradation by cotransfection and immunoblot experiments in Saos2 cells. 0.2 µg of p63 expression vectors were transfected in Saos2 cells along with increasing amount of Dlx3 plasmid (0.5, 1.0 and 1.5 µg). P63 and dlx3 were revealed with 4A4 and anti-Flag antibodies, respectively. (B) Schematic representation indicating the positions where stop codons were inserted to generate the Δ373, Δ390 and Δ408 truncated version of ΔNp63α , the Ser383 and Thr397 that have been replaced with Alanine have also been indicated. ΔNp63α was compared with deletion (Δ373, Δ390 and Δ408) and point mutants (ΔNaS383A, ΔNp63αT397A, and ΔNαS383T397A) for their sensitivity to Dlx3-mediated degradation. 0.2 µg of each ΔNp63α construct was transfected in Saos 2 cells along with increasing amount of Dlx3 plasmid (0.5, 1.0 and 1.5 µg). P63 and dlx3 were revealed with 4A4 and anti-Flag antibodies, respectively. Actin was used as a loading control.

To confirm that S383 and T397 were involved in Dlx3-mediated p63 degradation I expressed the ΔNp63αS383AT397A double mutant in Saos2 cells, with or without Dlx3, and measured p63 protein half-life in cycloheximide-treated cells. The half-life of ΔNp63αS383AT397A mutant, in presence of Dlx3, was greater than 9 hours (Fig.14) while that of the wild type protein was among 5 and 6 hours (Figure 11) implying that Dlx3-induced p63 protein degradation was impaired preventing phosphorylation of Serine383 and Threonine397. On the other hand, ΔNp63αS383A and ΔNp63αT397A proteins also exhibited increased half-life in presence of Dlx3, confirming that both residues were involved in the mechanism of Dlx3-mediated p63 degradation (data not shown). Next, I tested if replacement of Serine383 and Threonine397 with Alanine, was dramatically changing their subcellular localization. To answer this question I performed immunofluorescence microscopy and I could verify that the ΔNp63αS383AT397A protein was normally distributed into the nuclear compartment, both in absence and presence of Dlx3 exogenous expression (data not shown).

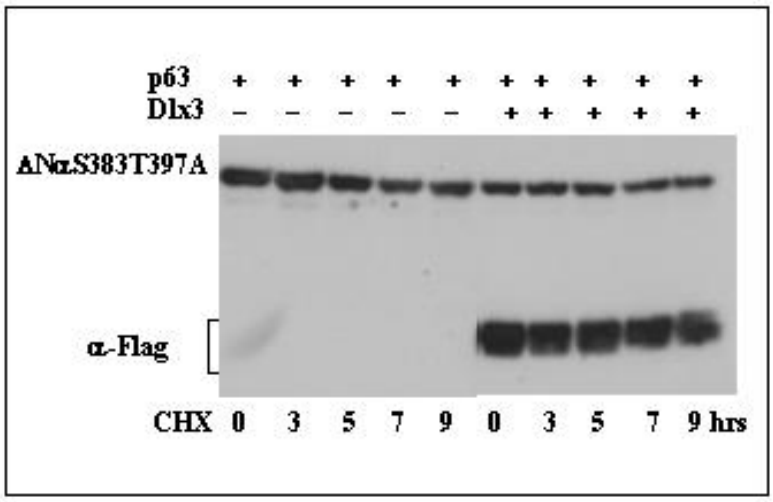


Figure 14. The ΔNp63αS383AT397A mutant increase half-life in presence of Dlx3.
Saos2 cells were transfected with the ΔNp63αS383AT397A double mutant with or without Dlx3, and p63 protein half-life was measured in cycloheximide-treated cells (40μg/ml). At the indicated times, cells were lysated and cellular extract were analyzed by Western Blot and specific immunodetection.

2.3) Dlx3-mediated p63 degradation is impaired by inhibiting Raf signaling pathway.

Dlx3 is induced in primary mouse keratinocyte by increasing the level of extracellular calcium and in parallels a reduction of ΔNp63α protein is observed (Radoja N. et al. 2007). As substitution of Threonine397 and Serine383 with Alanine prevented the reduction of ΔNp63α protein level upon Dlx3 transfection, we hypothesized that phosphorylation of these specific residues was a prerequisite for Dlx3-induced ΔNp63α protein degradation. Park and Morasso reported that Dlx3 is activated through a protein kinase C-dependent (PKC) pathway (Park GT and Morasso MI; 2001). As PKC can signal to MAP kinases through Raf1 (Seo HR et al; 2004) I examined the status of the Raf signalling major components upon transfection of increasing amount of Dlx3 in HaCaT keratinocytes. By using antibodies that specifically recognize the phosphorylated active forms of Raf1 and MAPKs (ERKs and p38MAPK) I found that phosphorylated Raf1 and ERKs increased markedly in response to Dlx3 expression (Figure 15). By contrast, phosphorylated p38MAPK was reduced (Figure 15). Similar results were obtained in Saos2 cells (data not shown). I examined the possible contribution of Raf and the other components of its pathway on Dlx3-mediated p63 degradation by using specific pharmacological inhibitors (figure 16). I transfected Saos2 cells with a fixed amount of p63 and Dlx3 expression vectors exposed to 10μM GW5074 for 3, 6 and 8 hours. As shown in Figure 16 B, incubation of cells with GW5074, a specific Raf inhibitor, resulted in a substantial recover of p63 protein levels and a clear decrease of Dlx3

protein level suggesting that p63 degradation, induced by Dlx3, was mediated by Raf activity. It has to be noted that incubation of cells with GW5074 did not result in change of ΔNp63α level if ΔNp63α was transfected without Dlx3. However, as shown in figure 16 D, treatment of p63 and Dlx3 transfected Saos2 cells with PD98059, the specific ERK inhibitor, appears to reduce exogenous p63 protein level when ΔNp63α was transfected both alone and with Dlx3. This observation suggests that ERKs are involved in a pathway of p63 protection independently from Dlx3. Conversely, specific inhibition of p38MAPK with SB203580 had absolutely no effect on dlx3-mediated p63 degradation (Figure 16 C).

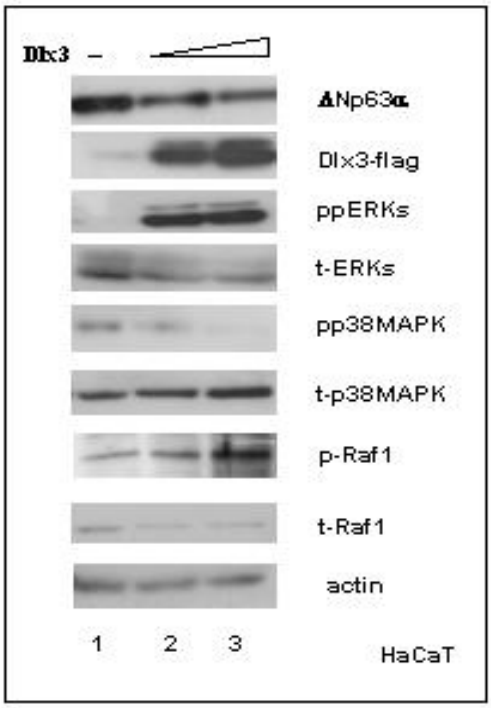


Figure 15. **Raf1 and ERKs increased markedly in response to Dlx3 expression**.

HaCat cells were transfected with an increasing amount (1 and 1.5μg) of Dlx3-Flag expression plasmid. 24 hours after transfection, cells were lysate and extract was analyzed by Western Blot analysis and immunodetection with specific antibodies.

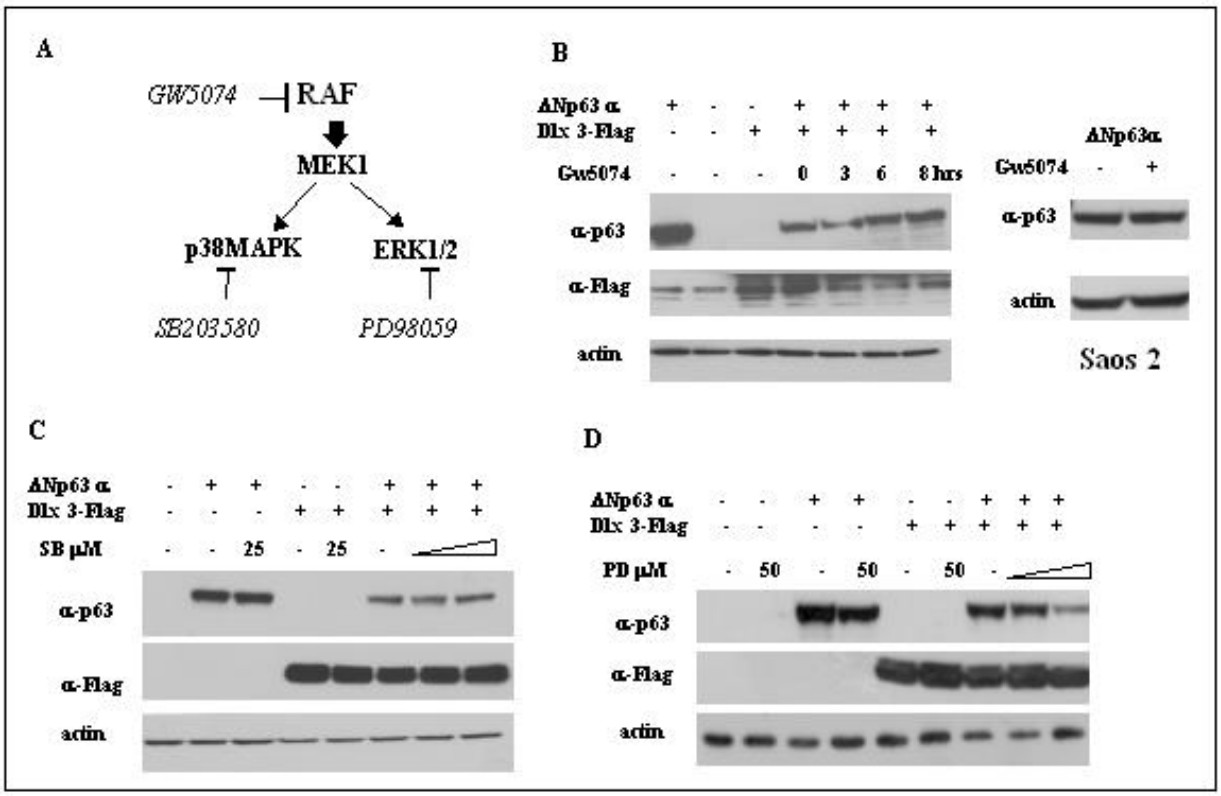


Figure16. Dlx3-mediated p63 degradation is impaired by inhibiting Raf signaling pathway. (A) Schematic representation of specific pharmacological inhibitors of the Raf signalling pathway and their target . (B) Saos2 cells were transfected with p63 encoding plasmid (0.2µg) alone or togheter with a fixed amount (1µg) of Dlx3-Flag expression plasmid. 16 hours after transfection cells were treated with 10µM of GW5074. At the indicated the indicated times, cells were lysated and cellular extract were analyzed by Western Blot and specific immunodetection.

(C) Saos2 cells transfected with p63alone or along with Dlx3 were treated for 15 hours with two different amount (25 and 50µM) of SB203580, a specific p38MAPK inhibitor and (D) with a fixed amount (50µM) of PD98059, a specific ERKs inhibitors. Cells were lysated and extract was analyzed by Western Blot and specific immunodetection.

To further explore the role of Raf kinase activity in p63 degradation induced by Dlx3, I transfected, in Saos2 cells, increasing amount of a construct expressing the kinase active domain of Raf1, Raf(BxB) (Pearson et al. JBC 2000), with a fixed amount of Dlx3. As shown in Figure 17 B, coexpression of Dlx3 with activated Raf resulted in a dose-dependent increase of Dlx3 protein level and, as expected, in a significant increase of phosphorilated ERKs. Moreover, when we transfected Saos2 cells with Raf BxB and ΔNp63α along with a fixed amount of Dlx3 I observed again an increase of Dlx3 protein and a more pronounced ΔNp63α protein degradation (fig. 17 A). Interestingly, in absence of Dlx3, Raf BxB seems to cause an increase of the ΔNp63α (fig. 17 A) . This effect might, presumably, be the conseguence of ERKs activation upon transfection of constitutively activated Raf BxB. Altogheter, these observations suggested that the effect of activated Raf on p63 protein levels was actually dependent on Dlx3. To confirm the relevance of Serine 383 and Threonine 397 of ΔNp63α in Dlx3-induced p63 degradation, I transfected the ΔNp63αS383AT397A mutant protein with a fixed amount of Dlx3 and increasing amounts of Raf(BxB). As shown in Figure 17 C, despite the remarkable increase of Dlx3 level caused by Raf(BxB) coexpression, the p63 double mutant protein remained unaffected.

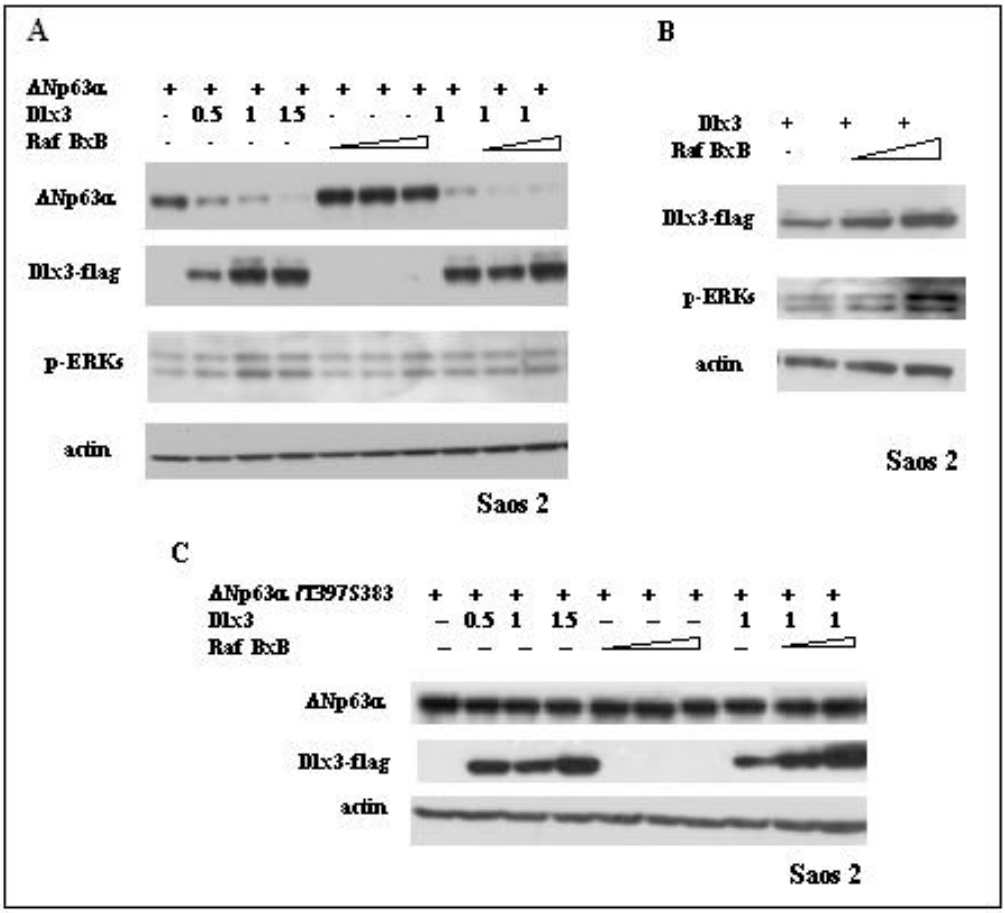


Figure 17. Involvment of Raf kinase activity in Dlx3-induced p63 degradation.

(A) Raf BxB increase Dlx3-dependent p63 degradation. Saos 2 cells were transfected with a fixed amount (0.2µg) of p63 expression plasmid alone (lane 1), p63 and increasing amount (0.5, 1, 1.5µg) of Dlx3-Flag expression plasmid (lanes 2-4), p63 and increasing amount (0.5,1,1.5µg) of Raf BxB (lanes 5-7), p63 with a fixed amount (1µg) of Dlx3(lane 8) and increasing amount (1and1.5µg) of RafBxB (lanes 9and 10). 24 hours after transfection cells were lysated and cellular extract was analyzed by Western Blot and specific immunodetection. (B) Effect of Raf BxB on Dlx3 expression levels. Saos2 cells were transfected with a fixed amount (1µg) of Dlx3-Flag encoding plasmid alone and along with increasing amount (1 and 1.5µg) of RafBxB expression plasmid. 24 hours after transfection cells were lysated and extract aanalyzed by Western Blot. (C) The ΔNp63αS383AT397A mutant protein is unaffected by coexpression of Dlx3 and Raf BxB. Saos 2 cells were transfected with a fixed amount (0.2µg) of ΔNp63αS383AT397A expression plasmid alone (lane 1), mutant p63 and increasing amount (0.5, 1, 1.5µg) of Dlx3-Flag expression plasmid (lanes 2-4), mutant p63 and increasing amount (0.5,1,1.5µg) of Raf BxB (lanes 5-7), mutant p63 with a fixed amount (1µg) of Dlx3(lane 8) and increasing amount (1and1.5µg) of RafBxB (lanes 9and 10). 24 hours after transfection cells were lysated and cellular extract was analyzed by Western Blot and specific immunodetection

Finally I sought to determine whether p63 natural mutants associated to distinct human hereditary syndromes, exhibited different sensitivity to dlx3-mediated degradation. I tested the ΔNp63αK194E and ΔNp63αQ639X mutants both derived from Split Hand and Foot Malformation 4 (SHFM4), as well as the the Hay-Wells syndrome derived ΔNp63αQ540L and the EEC-derived ΔNp63αC306R. Actually, all of the mentioned mutants were efficiently degraded by Dlx3 (fig. 18).

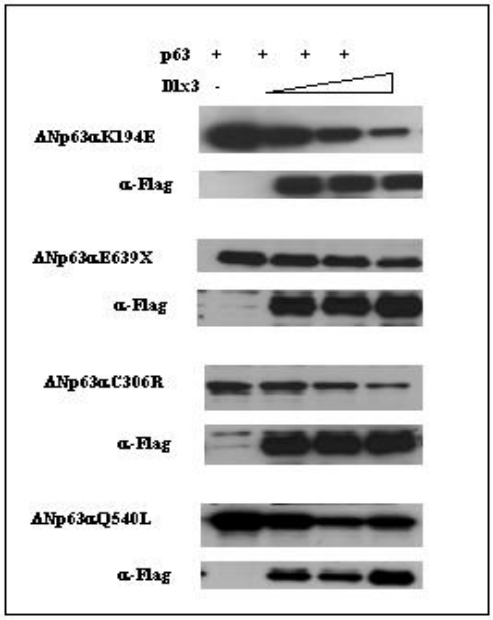


Figure 18. p63 natural mutants associated to distinct sindrome were efficiently degraded by Dlx3. Saos2 were transfected with different p63 mutant constructs alone or with increasing amount (0.5,1 and 1.5μg) of Dlx3-Flag expression plasmid. 24 hours after transfection cells were lysate and extract analyzed by Western Blot and specific immunodetection.

3) Investigation on mechanisms throught which p14ARF regulates p63 transcriptional activity.

Recently, in the lab where I worked it has been demonstrated that there is a physical and functional relationship between p63 and p14ARF. First, already pubblished data (Calabrò et al; 2004) indicate that, unlike p53, p63 is able to associate with p14ARF, both in TA and ΔN version, in different mammalian cell lines. Remarkably, overexpression of TAp63, but not ΔN, promotes the exclusion of p14ARF from the nucleus. Finally, p14ARF inhibits both transactivation and transrepression activity. To better investigate the mechanism throught which ARF inhibits p63-driven-transcription, I decided to examine whether p14ARF was able to impair the binding of p63 to a canonical p53 consensus sequence. EMSA experiments gave preliminary indications that p14ARF was able to decrease the binding of p63 to p53 consensus sequence. To gain further insights in this mechanism I performed Chromatin Ip assays to test the effect of ARF on the p63 DNA binding activity on different p53 and p63 target gene promoters. For ChIP analysis, I transfected H1299 cells with p63 alone or along p14ARF expression plasmid. Formaldeyde cross-linked chromatin was immunoprecipitated with anti-p63 4A4 antibody. Eluted DNA was PCR amplified with specific primers for different p63 (JAG 2, DLX 3) and p53 (p21WAF, 14-3-3σ) target promoters. As shown in figure 17, when cotransfected with p14ARF, TAp63α appeared to bind to the tested promoters with a lower affinity. This effect was less evident with ΔNp63α isoform. To confirm the specificity of this effect, I transfected H1299 cells with p53 and p14ARF expression plasmids and I

immunoprecipitated the chromatin with anti p53 antibody. PCR analysis of p21WAF promoter showed that ARF does not affect p53 DNA binding activity in this experimental condition.

My results suggest that when coexpressed, ARF is able to modify the DNA binding activity of p63 on different promoters thereby it might potentially alter the pattern of expression of genes regulated by p63.

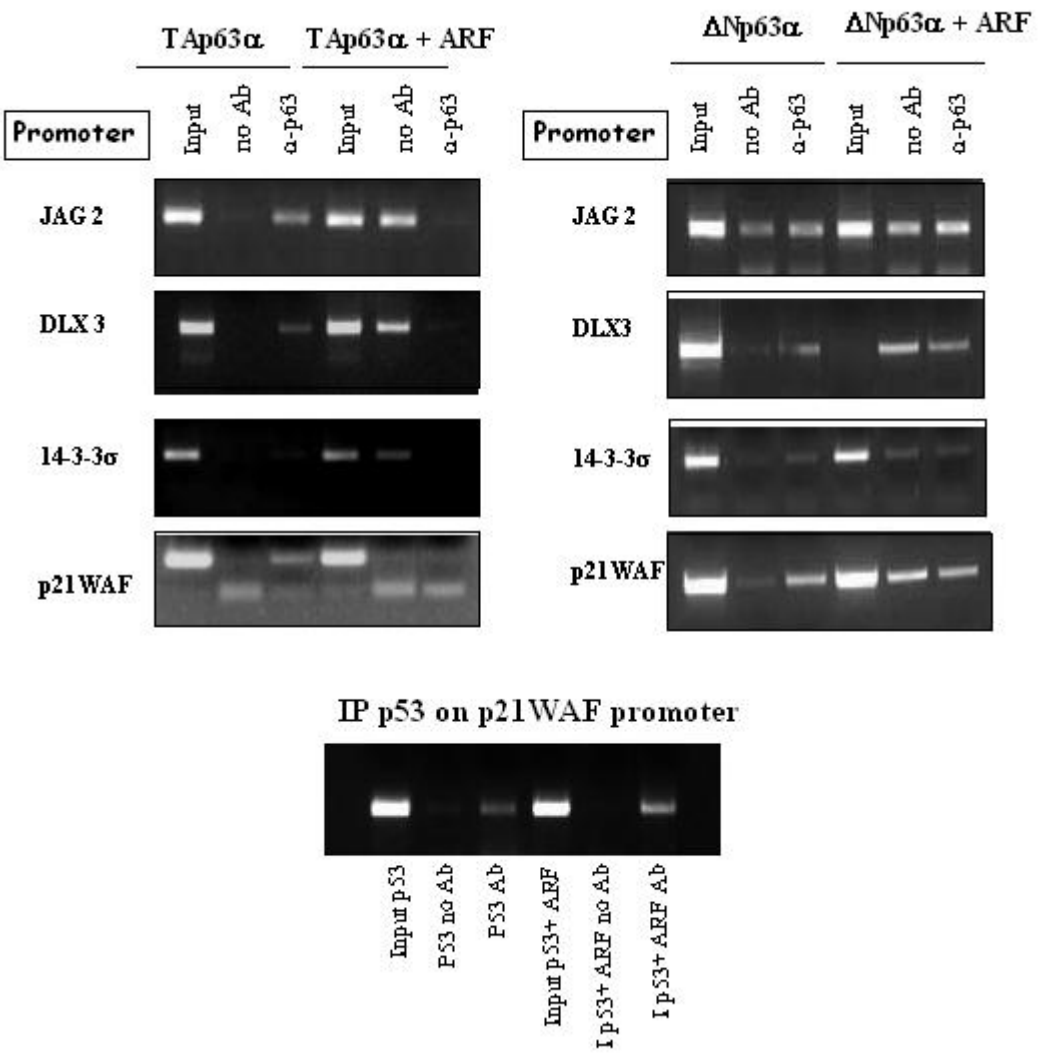


Figure 17. **p14ARF impairs DNA binding ability of p63**. H1299 cells were transfected with p63 alone or with p14ARF expression plasmids. Formaldeyde cross-linked chromatin was immunoprecipitated with anti-p63 4A4 antibody. Eluted DNA was PCR amplified with primers specific for different p63 (JAG 2, DLX 3) and p53 (p21WAF, 14-3-3σ) target promoters genes. H1299 cells were also transfected with p53 alone or with p14ARF encoding plasmids and chromatin immunoprecipitated with anti p53 antibody. Eluted DNA was PCR amplified with primers specific for p21WAF promoter.

I have also performed experiments to better define the region of p63 required for the physical association with ARF. Previously, it has been demonstrated that p63-p14ARF interaction requires the first 38 N-terminal aminoacids of p14ARF and the first 26 aminoacids of ΔNp63α. Twelve aminoacids (encompassing 15 to 26 of ΔNp63 and 109 to 120 of TAp63) among the first 26 aminoterminal residues of ΔNp63α (TA2 domain), are in common between TA and ΔN p63 isoforms. We have supposed that this stretch of 12 aminoacids contains residues that might be crucial for p63-p14ARF association. To better identify the region that mediates the interaction I took advantage of some natural occurring p63 mutations. In particular, I used the G76W point mutation that is found in Limb Mammary Sindrome (LMS) affected patients, that falls in the region under study and, precisely, in the TA2 domain of p63 (fig. 18A). The mutation was inserted both in the TA and ΔNα isoforms and tested for the interaction with ARF. Both TA and ΔNα isoform carrying the G76W point mutation were a kind gift of Dr. Hans vonBokhoven. I transfected NIH3T3, a murine ARF-/- cell line, with plasmid encoding the different p63 constructs alone or in combination with a plasmid encoding p14ARF. Cellular extracts were immunoprecipitated with an antibody against p14ARF, blotted and incubated with anti p63 and anti ARF antibody. As shown in figure 18 B, while the G76W substitution completely impairs the ability of ΔNp63 to bind ARF protein, the TA isoform retains the ability to interact with ARF suggesting that TA1 and TA2 domains could contain regions involved in p63-p14ARF association.

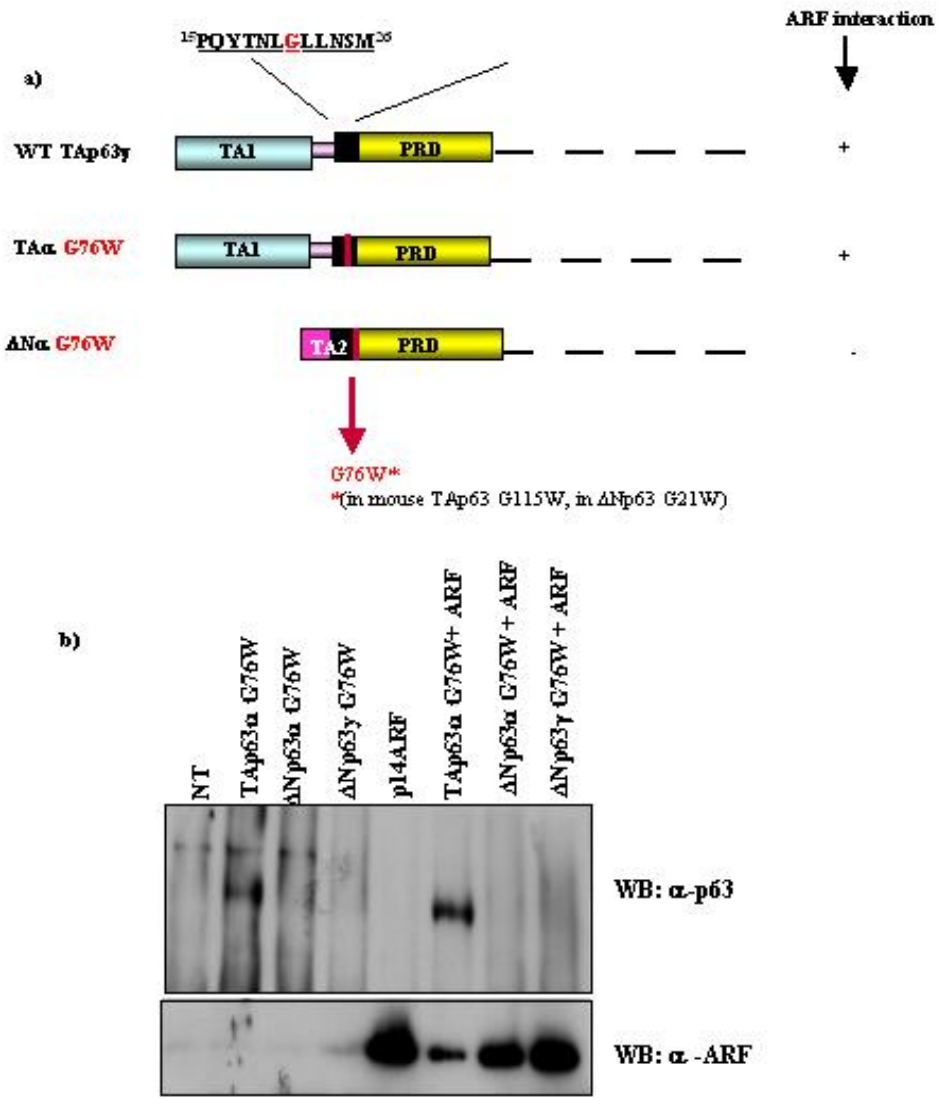


Figure 18. Identification of p63 domains involved in p14ARF interaction. **a**) Schematic representation of the p63 G76Wmutant construct. **b**) NIH3T3 cells were transfected with 1μg of expression plasmids encoding wild-type TAp63α, ΔNp63α or the indicated p63 mutant isoforms alone or togheter with 2μg of p14ARF expressing plasmid. Cellular extracts were immunoprecipitated with anti-p14ARF antibody and immunorevealed with anti-p63.

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 is believed to act as a molecular switch required for commitment to epithelial stratification.The existence of malformations due to p63 gene mutations suggests that this gene might be component of signaling cascades regulating epidermal and ectodermal appendage development. The severity of the phenotype in p63-null mice suggests that it is an upstream crucial regulator of these signaling pathway. A broad spectrum of p63 mutations are responsible for several human ectodermal, craniofacial and limb malformation. EEC and ADULT mutations are located in the DB domain of p63. They abolish p63 DNA-binding and produce higly stable, but transactivation-inert TAp63 proteins (Ying et al; 2005). AEC mutations are confined to the SAM domain. Their effect on p63 transcriptional functions are less predictable and they only affect the α isoforms.

Very few genes that are directly regulated by p63 to modulate differentiation and very few p63 protein partners that could modulate its transcriptional activity are currently known. Therefore, during my doctoral studies I have explored some of the molecular pathways that regulate p63 protein function. First, I decided to identify p63 specific transcriptional targets that were relevant for the pathogenesis of human AEC syndromes.

The Q540L mutation impairs p63 transcriptional ability.

My studies provides evidence that the Q540L amino acid substitution strongly impairs the transcriptional activity of TAp63α.

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. As indicate by my reported experiments, 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, I tested whether the mutant protein was still capable of interacting with a p53-binding motif of the p21/WAF promoter. My results indicate that both wt TAp63α and its Q540L mutant are equally active in binding to this sequence, in our in vitro system. However, my data obtained through ChIP assays indicate that the Q540L mutation affects the ability of p63 to bind its target promoters in a chromatin context. A possible explanation is that such mutation might affect the binding of p63 to a particular coactivator that could increase the affinity of the protein for its target promoters. The observation that the binding to p21/WAF, IKK, JAG and Dlx3 promoters is affected by the AEC-derived

mutation suggest that a common factor is required for the control of the expression of these genes by p63. For instance, it has been reported that Sp1 cooperates with p53, p63 and p73 in synergistic transactivation of the p21/WAF promoter.36 and my 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.

Finally, microarray analysis has revealed that specific probe sets are up or down-regulated by the mutant protein alone. These seemingly conflictual results might again be explained as a consequence of a loss of transcriptional function assuming that the above mentioned genes are p63 secondary targets, which are repressed or activated by p63 primary targets. Alternatively, we cannot exclude that the Q540L aminoacid substitution confers new transcriptional regulative properties to the TAp63α protein altering its affinity to interact with particular coactivators or corepressors.

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 littlewas 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 (Clase KL et al;

2000; Collombat et al; 2003; Yoshihara et al; 2005). 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 (Uchida et al; 2003). 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 TAp63. This regulation is completely abolished by the Q540L amino acid substitution. 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 (Collombat et al; 2003). 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(Collombat et al; 2003). Interestingly, both TAp63α and ARX transcripts decrease at E 13.5, which corresponds to the switch from TAp63α to ΔNp63α expression (Koster et al; 2004). 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 (Ishisaki et al; 2001). 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 theskin 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. Since induction of CLMN expression fits in nicely with the timing of the switch from TAp63α to ΔNp63α expression in the epithelial stratification program (Koster et al; 2004), 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 (Ripley et al; 2004), during blood vessel development, in the gut endoderm and the epicardium and in all three germ layers during avian organogenesis (Osler et al; 2004). 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 (Wang et al; 2002). p21/WAF has long been known to arrest the cell cycle. In the epithelium it is involved in maintenance of the stem cell compartment (Okuyama et al; 2004).

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 and its promoter is not or only mildly responsive to the ΔNα isoform (Westfall et al; 2003). 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 (Westfall et al; 2003). Our qPCR data and expression studies 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, they both induce a similar G1 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 transcription40 and this ability is rather dependent on the specific gene promoter. In conclusion, the difference in the growth rate profiles 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.

The growth rate profiles and cell cycle distribution of cells expressing the AEC-derived TAp63α protein are undistinguishable from those of uninduced cells, 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.

Dlx3 regulates p63 protein levels.

AEC-derived TAp63 proteins and in particular the Q540L mutant exhibits an impaired ability to transactivate Dlx3, thus suggesting that the misregulation of the Dlx3 gene is involved in the pathogenesis of AEC.

The Dlx3 gene, a target of p63, is expressed in the suprabasal layer of skin. Expression of Dlx3 in keratinocyte is activated, through a PKC-dependent pathway, by increasing the level of extracellular Ca^{2+}. The increase of extracellular Ca^{2+} is an important trigger of epidermal terminal differentiation that contributes to the decrease of ΔNp63α expression by reducing ΔNp63α gene transcription (Radoja et al; 2007). My reported data indicate that Dlx3 reduces also protein half-life and steady-state levels. I have observed this phenomenon in several cell contexts, including p53-/- and MDM2-/- mouse fibroblasts indicating that neither p53 nor MDM2 were involved in this mechanism. Interestingly, Dlx3 overexpression resulted in efficient ΔNp63α/β, but not γ, protein degradation. The evidence that ΔNp63γ is completely resistant to Dlx3-mediated degradation means that residues located exclusively in the p63 α and β, but not in the γ tail, are absolutely required for the mechanism of Dlx3-mediated p63 degradation. Although I have not provided direct evidence that Ser383 and Thr397 are phosphorylated upon Dlx3 expression, I have shown that their substitution with alanine impairs Dlx3-mediated ΔNp63α degradation, thus demonstrating that these residues are relevant for this phnomenon. However, I cannot rule out the hypothesis that additional serine/threonine residues may also have a minor contribution to this mechanism. Furthermore, I have observed that TAp63α

appears to be more resistant than the corresponding ΔN isoform, thus suggesting that the N-terminal region of p63 in some way interferes either with the phosphorylation or degradation mechanism of p63, possibly due to the minor accessibility of the TAp63α three-dimensional conformation. TAp63α expression is completely absent in the basal layer of epidermis while it becomes detectable in the soprabasal layers of epidermis where it contributes to activate Dlx3 gene expression. Interestingly, my observation that TAp63α shows a lower sensitivity to Dlx3-mediated degradation compared to ΔNp63α, provides a possible explanation to its persistence in the granular layer of epidermis where Dlx3 is actually expressed. Moreover, since terminally differentiated keratinocytes lack completely p63 expression I can speculate that Dlx3 might also contribute to TAp63α protein degradation.

Multisite phosphorylation is a common feature of many protein kinase substrates, which may enable docking interactions, integration of different kinase pathway signals or changes in the subcellular localization. Concerning this aspect, we have been able to exclude that substitution of serine 383 and treonine 397 with alanine could cause differences in the subcellular localization of p63, either in absence or presence of Dlx3 expression.

Interestingly, I have shown a direct involvement of Raf1 in Dlx3-mediated p63 degradation. Pharmacological inhibition of Raf activity was able to partially restore the basal level of p63 that was reduced upon Dlx3 expression. Moreover, the constitutively activated Raf was able to increase p63 degradation induced by Dlx3. On the basis of my data, I cannot conclude that Raf1 is the kinase responsible for p63

phosphorylation, however I have provided evidences that constitutively activated Raf enhances the levels of Dlx3 protein indicating at least one mechanism, strictly depending on Raf activity, that might significantly contribute to p63 protein degradation. In addition to Raf1 phosphorylation I have observed considerable increase of phosphorylated ERKs upon Dlx3 transfection. Activation of Raf1 by Dlx3 can signal to ERKs through MEK kinases. Normal human epidermal keratinocytes (NHEK) respond to the autocrine activated ERK signaling pathway, which contributes to the survival of keratinocytes. According to the proposed pro-survival role for ERKs, it has been demonstrated that autocrine ERK activation, at early stage of differentiation, plays a role in the regulation of cell cycle rather than the expression of the keratinocyte differentiation markers (Park et al; 2006). Autocrine activation of ERKs is not interrupted by calcium-induced differentiation but it rapidly declines in absence of EGFR ligands (Park et al; 2006). We speculate that Dlx3 while inducing p63 degradation might substain ERKs activation thereby delaying keratinocyte terminal differentiation. Alternatively, activation of ERKs might counteract apoptotic stimuli triggered by the detachment of keratinocytes from the epidermal basal layer as long as they move in the suprabasal layer of epidermis ( ). On the other side, we have found that ERKs inhibition enhances p63 protein degradation, confirming that ERKs activation protects ΔNp63α thus playing a positive role on keratinocyte survival during differentiation (Park et al; 2007).

The Raf kinase activity appears to play a crucial role in epidermal differentiation being at the cross road between signals driving cell proliferation and differentiation.

The mammalian Raf family of serine/threonine kinases consists of three highly conserved members, i.e., A-Raf, B-Raf and Raf1 (Hagemann et al; 1999). Whereas Raf1 is ubiquitously expressed, A-Raf and B-Raf display a more tissue-specific expression. The role of Raf1 in the epidermal differentiation is demonstrated by the phenotype of Raf1 targeted knock-out mice. Mice homozygous for a hypomorphic Raf1 allele ($Raf1^{tm1Zim}$) die during organogenesis. Only 5% of homozygotes are viable and display underdeveloped hair follicles, thin epidermis and abnormal epidermal layer morphology (Mikula et al; 2001). Interestingly, they also show abnormal placental labyrinth morphology strongly reminding the phenotype of Dlx3 null mice (Morasso et al; 1999). Our data indicate that Dlx3 induces Raf1 phosphorylation and moreover that inhibition of Raf kinase activity impairs p63 degradation. Although we cannot exclude the role of other member of the Raf family, Raf1 appears to be clearly involved. Further studies are currently in progress in our laboratory to understand the molecular mechanism through which Dlx3 activates Raf1 and to identify whether Raf1 or a different serine/threonine kinase is directly responsible for p63 phosphorylation.

It has been already reported that K194E and E639X mutations associated to Split Hand and Foot Malformation syndrome do not affect the ability of TAp63 to transactivate Dlx3 (Radoja et al; 2007). I have here reported that these substitutions do not alter the sensitivity of ΔNp63α to the Dlx3-dependent degradation. This is in agreement with the lack of skin phenotype of patients affected by SHFM syndrome. On the other side, the EEC-derived C306R mutation, located in the p63 DNA binding

domain, and the AEC-derived Q540L substitution, located in the p63 SAM domain, both affect the ability of p63 to transactivate a set of target genes, including Dlx3 (Radoja et al; 2007). Thus, since p63 lies upstream to Dlx3 in the molecular pathway leading to skin differentiation patients affected by EEC and AEC exhibit a severe skin phenotype.

In conclusion, my data revealed the existence of a regulatory network between p63 and Dlx3 providing new clues for thee understanding of the molecular mechanism underlying skin differentiation.

Lastly, I have also investigated on the mechanism responsible for the p14ARF-dependent counteraction of p63 transcriptional activity. My data suggest that overexpression of p14ARF is able to inhibit the ability of p63 to bind to several promoters. Respect to previously reported data (Calabrò et al; 2004) my ChIP assays indicate that p14ARF contribute to the regulation of transcription of p63/p53 target genes in their natural setting, given the important role of chromatin structure in the regulation of gene expression. Moreover, my data clearly show that ARF impairs the binding of TA and ΔNp63α also to promoters specifically regulated by p63 and not by p53 (such as Dlx3).

Hence, we could speculate that under mitogenic stimuli p14ARF physically associates with TA and ΔNp63 isoforms removing them from p63/p53-responsive promoters kepting p63 proteins inactive in a p63-p14ARF complex . This process might turn on p53 transcriptional function activating the p53-dependent checkpoint control. The physiological relevance of my observation need to be further

investigated. In particular, it will be necessary to verify my results in keratinocyte cell culture.

I have also reported that the G76W substitution associated to LMS syndrome abolish the ability of ΔNp63α, but not of TAp63α, to interact with p14ARF, confirming that residues located in the TA domain might increase the binding affinity between p14ARF and p63. Further work is necessary in order to estabilish the relevance of this finding for the comprehension of molecular mechanism responsible for the LMS syndrome,

Matherials and Methods

Plasmids

All of the p63 wild type cDNAs in the pcDNA3 vector were kindly provided by Dr. Hans van Bokhoven. The mutation Q540L was created in the lab of prof. Calogero in Turin 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 wild type sequence, digested with NdeI/NcoI to generated 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 p21WAF/CAT reporter plasmids have already been described (Calabrò et al. 2002; Ghioni et al. 2002)**.**

Plasmids. Raf1(BxB) encoding plasmid was kindly provided by Dr. A. Costanzo. Plasmid encoding carboxyterminal truncated p63 proteins, the ΔNp63αS383A and Thr397A point mutants and the ΔNp63αS383T397A double mutant were produced using the GeneEditor *in vitro* Site-Directed Mutagenesis System (Promega) following the manufacturer's instruction in the lab of Dr. Guerrini in Milan.

Cells culture, transfection and reporter assays.

Human lung carcinoma H1299 cells (p53 null, no p63 expression) were obtained from American Tissue Culture Collection and grown at 37°C in humidified 5% CO_2 in DMEM medium supplemented with 10% fetal calf serum. H1299 cells ($5x10^4$) were plated and transfected by calcium-phosphate precipitation with 20 μg of pTet-On plasmid (Clontech). 48 hrs after transfection, cells were selected adding G418 (100 mg/ml) to the colture media. After 4 weeks, single G418-resistant clones (H1299-rtTA) were picked up and expanded. The presence of the rtTA regulator in G418-resistant clones was checked performing a β-galactosidase assay on H1299-rtTA cell extracts after transient transfection with the pBGI-βgal empty construct with or without doxycyline (Sigma-aldrich) addition (1 μg/ml).

Tet-On/TAp63α, Tet-On/ΔNp63α, Tet-On/TAp63Q540L cell lines were produced as follows: 7×10^4 H1299-rtTA cells were co-transfected with each of the pBIG-p63 constructs and the pTK-Hygro vector (20 to 1 rate) by calcium-phosphate precipitation. 24 hrs after transfection each

100mm plate was splitted (1:2) and cells were selected adding 800 μg/ml of hygromycin (Sigma-Aldrich). After 4 weeks, single hygro-resistant clones were picked up, expanded and subjected to the analysis of p63 inducible gene expression by Western blot and specific immunodetection. p63 stable clones were maintained in DMEM medium supplemented with 10% Tet-Approved serum (Hyclone). CAT assays with the WAFCAT or BP100CAT reporter plasmids were performed as previously described (Calabrò et al. 2004).

The human osteosarcoma-derived Saos2 cells were maintained in RPMI 1640 medium and 10% fetal calf serum. HaCaT, Hela, and U20S cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (Euroclone) at 37°C in a humidified atmosphere of 5% (v/v) CO2 in air. Cells seeded at a density of about 70% confluence were transfected with the indicated amount of expression vector with LipofectAMINE reagent (Life Technologies. Inc.) for H1299 or LipofectAMINE 2000 for Saos2, HeLa, HaCaT and U2OS. The total amount of transfected DNA was kept constant by using the "empty" expression vector when necessary. P63 half-life was determined by addition of 40 μg/ml cycloheximide (Sigma), 16 hours upon transfection. MG132, ALLNL, Bafilomycin, NH4Cl and Chloroquine treatments were performed the day after transfection with 10 and 20 μM MG132, 10 and 20 μM ALLN, 100 nM bafilomycin A, 20 mM NH4Cl, 100 μM chloroquine or solvent alone for 6 hours. Pharmacological inhibition of serine/threonine kinases was obtained by adding 25 and 50μM PD98059 (Calbiochem), 25 and 50μM SB203580 (Calbiochem), 5 and 10 μM GW5074 (Calbiochem).

CAT assay

H1299 were transiently transfected with 2μg of p21/WAF-CAT reporter plasmid/dish alone or with different amounts of each p63 expressing plasmid (1, 2 or 3 μg). Cells were collected 48h after transfection; equal amounts of cell extracts (50μg), determined by the Bradford method (BioRad), were assayed for CAT activity using 0,1mCi of [^{14}C] chloramphenicol and 4mM acetyl-CoA. Separeted products were detected and quantified by Phosphorimager and Quantity One software.

Growth rate analysis

To determine the rate of cell growth, approximately 6 x 10^4 cells were seeded in 60 mm-diameter plates in presence or absence of doxycycline (1μg/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 using a Burker chamber. The average number of cells from two plates was used for determination of growth rate.

DNA histogram analysis

Cells were counted and seeded at 2 x 10^5/100mm plate with or without doxycycline (1μg/ml). At the indicated time points, live cells on the plates were trypsinized and both floating dead cells in the medium and trypsinized live were centrifuged and washed twice with PBS. Approximately 10^6 cells were incubated in 1 ml of 0.1% NaCitrato, 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 using the CellQuest program (Beckton-Dickinson) on 20000 total events at a rate of 150+/-50 events/second and the percentages of cells in the SubG1, G0-G1, S and G2-M phases were quantified with the ModFit software (Beckton-Dickinson**).** The percentage of dead cells/total cells was determined by the 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 using 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 in Tet-On stable cell lines was performed on doxycycline (1μg/ml) induced or uninduced condition, 10^5cells/35mm plate were grown on micro cover glasses (BDH). 48 hrs after, cells were washed with cold phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (Sigma-Aldrich), for 15' at 4°C. For immunolocalization in Saos-2 cells, 5.0 x10^5 were plated in 35

mm dish and grown on micro cover glasses (BDH). At 24 hrs after induction or transfection with the indicated vectors, cells were washed with cold phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (Sigma) for 15 min at 4°C. Cells were permeabilized with ice-cold 0.1% Triton X-100 for 10 min and then washed with PBS. P63 or Dlx3 subcellular localization were determined by using a 1:200 dilution of a monoclonal antibody against p63 (4A4) or an anti-FLAG monoclonal antibody against Dlx3-Flag (Sigma) diluted 1:2000. After extensive washing in PBS, the samples were incubated with a Cy3-conjugated anti-mouse immunoglobulin G (ImmunoResearch Laboratory) at room temperature for 30 min. After PBS washing, the cells were incubated with DAPI (4',6'-diamidino-2-phenylindole; 10 mg/ml [Sigma-Aldrich]) for 3 min. After PBS washing, the glasses were mounted with Mowiol (Sigma-Aldrich) and examined under a fluorescence microscope (Nikon). Images were digitally processed by Adobe Photoshop software.
After a rinse with PBS, fixed cells were permeabilized with ice-cold 0.1% Triton X-100 for 10 min and rinsed again with PBS. Cells were than incubated with DAPI (4,6-diamidino-2-phenylindole; 10mg/ml Sigma-Aldrich) for 3 min, and washed again with PBS. Finally, the glasses were mounted with Moviol (Sigma-Aldrich) and cells examined under a fluorescence microscope (Nikon). To detect p63 protein the H137 (Santa Cruz) and the Cy™ 3-conjugated anti-rabbit IgG (ImmunoResearch Laboratories) antibodies were used, at room temperature for 30 min. Images were digitally processed using Adobe Photoshop software.

Western immunoblot analysis

At the indicated time after transfection cells were lysed in 10 mMTris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl, 0,5% NP-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.5% sodium deoxycholate, and protease inhibitors. Cell lysates were incubated on ice for 30 min, and the extracts were centrifuged at 13,000 rpm for 10 min to remove cell debris. Protein concentrations were determined by the Bio-Rad protein assay. After the addition of 2x loading buffer (2% sodium dodecyl sulfate [SDS], 30% glycerol, 300 mM 96-mercaptoethanol, 100 mM Tris-HCl [pH 6.8]), the samples were incubated at 95°C for 5 min and resolved by SDS-polyacrylamide gel electrophoresis. Proteins were transferred to a polyvinylidene difluoride membrane (Millipore) and probed 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), anti-FLAG M2 (Sigma), anti-p-ERK (Cell Signaling), anti-p38 (Cell Signaling), anti-pp38 (Cell Signaling), anti-pRaf1 (Cell Signaling),

anti-ppRaf1(Cell Signaling). Proteins were visualized by an enhanced chemiluminescence method (Amersham).

Coimmunoprecipitations were carried out in Saos2 cells and in 5.0 x10^5 cells were plated in 60 mm dishes and transfected with the indicated vectors. Cells were harvested 24 hrs post-transfection, and cell lysates were prepared as described above. One mg of whole cell extract was precleared with 30 µl of protein A-agarose (50% slurry; Roche) and then incubated overnight at 4°C with 2µg of anti-p63 (4A4; Santa Cruz) at 4°C o.n.. Immunocomplexes were collected by incubation with protein A-agarose (Roche) at 4 °C for 4 hrs. The beads were washed with Co-IP buffer (50 mM Tris-HCl pH 7.5; 150 mM NaCl; 5 mM EDTA; 0.5% NP40; 10% glycerol), resuspended in 2X loading buffer (Sigma) and loaded in a SDS-10% polyacrylamide gel. Proteins were then transferred onto a PVDF membrane (Millipore) and probed with the indicated primary antibodies. Proteins were visualized with an enhanced chemiluminescence detection system (Amersham).

EMSA

EMSA experiments were performed as already described (Parisi, Pollice et al., 2002). P63 proteins were *in vitro* translated by using TnT reticulocytes from Promega with 0.5 µg of p63 plasmid DNA. Next, 10 µl of the individual reactions was used either for the binding reaction or 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 Westfall, Mays et al., 2003). A 100-fold molar excess of the same cold oligonucleotide or an oligonucleotide containing a consensus binding site for E2F1 was used for competition experiments. For the supershift anti-p63 antibodies (4A4; SantaCruz) or unrelated polyclonal anti-p21 antibodies (C-19; SantaCruz) were used, adding them to the sample prior to the binding reaction (30 min in ice).

Microarray sample preparation.

Total RNA (ttlRNA) was extracted and purified from stably transfected H1299 cell lines using the Concert Cytoplasmic RNA Purification Reagent (Invitrogen, Carlsbad, CA) as suggested by the manufacturer. ttlRNAs were then quantified and inspected by Bioanalyzer (Agilent Technologies) analysis. cRNAs were generated and hybridized on 12 HGU133plus2 Affymetrix DNA chips according to the Affymetrix protocol, ttlRNA (8 µg) was used for the preparation of double-stranded cDNA using the one cycle cDNA sysnthesis kit (Affymetrix, USA). The cDNA was then used as a template to synthesize a biotinylated cRNA (16 hrs, 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 min at 94°C). Affymetrix 12 HGU133plus2 array chips were hybridized with biotinylated cRNA (20 μg/chip, 16 hrs, 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 under analysis (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 artefacts was evaluated with the fitPLM function (Bioconductor package affyPLM). The probes (PM) intensity distribution was evaluated using hist function (Bioconductor package affy). Only an array from the TAp63αQ540L with induction group was found characterized by a narrow distribution of the probe (PM) intensities and was discarded.

Probe set intensities were obtained by means of GCRMA, robust multiarray analysis method (Wu, Z and Irizarry, RA, Stochastic Models Inspired by Hybridization Theory for Short Oligonucleotide Arrays Proceedings of RECOMB 2004 http://www.biostat.jhsph.edu/~ririzarr/papers/p177-irizarry.pdf). The full data set was normalized according to quantiles method (Bolstad, Irizarry et al. 2003). The hug133plus2 54675 probe sets were filtered to have an interquantile range (IQR) for each probe set greater then 0.25 (A. von Heydebreck, W. Huber, and R. Gentleman Differential Expression with the Bioconductor Project, In Bioconductor projects working papers June 18, 2004). This filtering yielded 11857. Subsequently, “Significant analysis of microarrays” (SAM) software (Tusher, Tibshirani et al. 2001) was used to identify probe sets differentially expressed between wt and mutant p63 isoforms. The identification of differentially expressed probe sets was initially done using 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 a total of 4000 differentially expressed probe sets. Subsequently, probe sets were filtered in order

to select only those characterized by a fold change≥|2| between not induced and induced cell lines in wt or mutant experiments. This filtering yielded a total of 100 probe set ids (Additional information table A). 87 out of 100 probe sets were associated to 81 Entrez Gene identifiers (gene ID; Maglott D, Ostell J, Pruitt KD, Tatusova T. Entrez Gene: gene-centered information at NCBI. Nucleic Acids Res. 2005, 33 Database Issue:D54-8.) and the remaining 13 Affymetrix ids were not assigned to any gene ID. The IQR filtered data set was also analysed using the SAM two class unpaired method (Tusher, Tibshirani et al. 2001), to highlight probe set 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 about 1. This analysis produced a total of 18 differentially expressed probe sets for TAp63α wt (16 up-modulated and 2 down-modulated) and a total of 7 probe sets for the Q540L mutant isoform (1 up-modulated and 6 down-modulated) (Fig. 1, additional information table B). Probe sets found differentially expressed using the two class unpaired method were all included in set identified as differentially expressed with the multi class method. Since a certain amount of leaking of the tet-ON system was observed in our experiments (data not shown), it is possible that differential expression between not induced and induced cell lines might be under-estimated. 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 set were 441 (Additional information table C).

To generate a robust set of differentially expressed genes to be further investigated the intersection between the 100 probe sets derived from the multi class analysis and the 441 probe sets derived by the two class analysis was selected. This intersection contains 45 probe sets linked to 36 annotated genes and 7 unmapped est (fig. 3).

The search of over expressed geneontology Biological Process themes was performed using Bioconductor GOstats package (Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang JY, Zhang J. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. 2004;5:R80.). Gene annotation was performed using Bioconductor annaffy library and hgu133plus2 annotation package (version 1.6.8).

Real-time RT quantitative PCR 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 II. Primers were designed 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 ul contained 2 ul of the cDNA, 1X SYBR GREEN PCR Master Mix (PE Applied Biosystems, Foster City, CA, USA) and 150 mM of each primers were performed with an ABI PRISM 7900HT Sequence Detection System using 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 by the SDS 2.1 software (Applied Biosystems, Foster City, CA, USA) and expressing as Ct, the number of cycles needed to generate a fluorescent signal above a predefined threshold. Target gene mRNA levels were expressed as 2^{-DCt}, normalized for ACTB and POL2B, and fold changes were evaluate as 2^{-DDCt} using as calibrator the non induced corresponding cell line, according to Livak and Schmittgen. (Livak and Schmittgen 2001).

Formaldehyde Cross-linking and Chromatin Immunoprecipitation.

H1299 ($1x10^6$ in 100mm) cell line were transfected with 5µg of TAp63α encoding plasmid or 5µg of pcDNA3-p53, alone or togheter with 5µg HA-p14ARF expression plasmid. For ChIP assay with p63α wild type and mutant Q540L, H1299 cell line ($1x10^6$ in 100mm) were transfected with 5µg TAp63α encoding plasmid or 5µg of TAp63αQ540Lexpression plasmid. 24 hours after transfection DNA and proteins were cross-linked by the addition of formaldehyde (1% final concentration) 10 min at RT before harvesting, and cross-linking was stopped by the addition of glycine pH 2.5 (125µM final concentration) for 5 min at RT. Cells were scraped off the plates, resuspended in hypotonic buffer for 5min in ice. Nuclei were spun down, resuspended in 400µl of SDS lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl pH8, and a protease-inhibitor mixture), ans sonicated to generate 500-2000 bp fragments. After centrifugation, the cleared supernatant was diluited 10-fold with immunoprecipitation buffer (50mM Tris-HCl pH8, 150mM NaCl, 5mM EDTA, 0,5% Nonidet P-40). The cell lysate was precleared by incubation at 4 °C with 20µl of protein-A beads preadsorbed with Salmon Sperma-DNA. The cleared lysate were incubated overnightwith anti-p63 4A4 antibody (Santa Cruz) anti-p53 DO1 or without any antibody. Immunocomplex were precipitated with 30µl of

protein A beads preadsorbed with salmon sperma sonicated DNA. After centrifugation the beads were washed and the antigen was elueted with 2% SDS in Tris EDTA. DNA-protein cross-links were reversed by heating at 65 °C ON, and DNA was phenol-extracted and ethanol-precipitated. Fragmented DNA was analyzed with the following primers :

p21/WAF: (F) 5' CGTGGTGGTGGTGAGCTAGA (R) 5' CTGTCTGCACCTTCGCTCCT ,

JAG 2 : (F) 5' CAAGTGGTGAACAAGGGAGACT (R) 5' ACTGCTGCCTTCTGGAAACTC

Dlx3 : (F) 5' AGAGAGGCGGAAGAGACGAG (R) 5' GAGGAGGGAGGAGAGAAGGA

IKK :(F) 5' GCAGGAGTCATGGGAGAAAA (R) 5' TTTATTTGAAGCAAAGGGAGA

Decay Rate Analysis

Saos2 cells (2.5 x10^5 in 35 mm) were transfected with ΔNp63α expression vector (0,2 μg) alone or along with pcDNA Dlx3-Flag (1μg). 18 hrs after transfection, cycloheximide (Sigma) was added to the medium at a final concentration of 40 μg/ml. Cells were harvested at the indicated time points. Total cell extracts were prepared as described above. 10 μg of cell extract was subjected to Western Blot and probed with anti-p63 and anti-FLAG antibodies and, as control, with anti-actin as a control.

RT-PCR

Saos-2 cells were transfected with a fixed amount of ΔNp63α expression vector 0.2 μg alone or with increasing amount of pcDNA Dlx3-Flag 1 and 1.5 μg. Total RNA was isolated using the RNA Extraction Kit (Quiagen). 1μg of total RNA was used to generate cDNA from each sample using one-step RT-PCR Kit (InVitrogen). Reverse-transcripts were amplified with the following p63 specifi-primers primers:

(F) 5'CCACAGTACACGAACCTGGGG (R) 5' CCGGGTAATCTGTGTTGGAG

As an internal loading control we amplified a region from HPRT gene using the following primers:

(F) 5' CCTGCTGGATTACATTAAAGC (R) 5' CTTCGTGGGGTCCTTTTC

PCR products were resolved by 2% agarose electrophoresis. RT-PCR amplification results were analyzed by Quantity One software (Biorad).

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Report

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

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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%

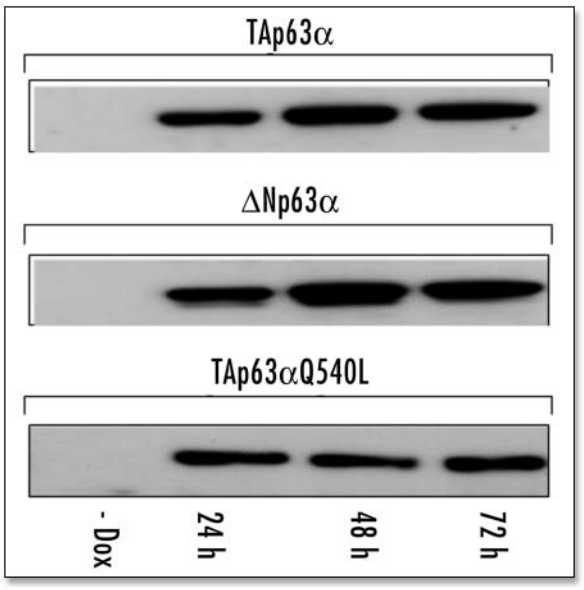


Figure 1. Expression of p63 isoforms in H1299 stable clones. Western blot analysis for detection of the p63 protein expression levels in Dox-inducible H1299 cells. Cells were harvested at the indicated time points after induction with 1 μg/ml doxycycline. Equal amounts of soluble lysates (30 μg) from 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.

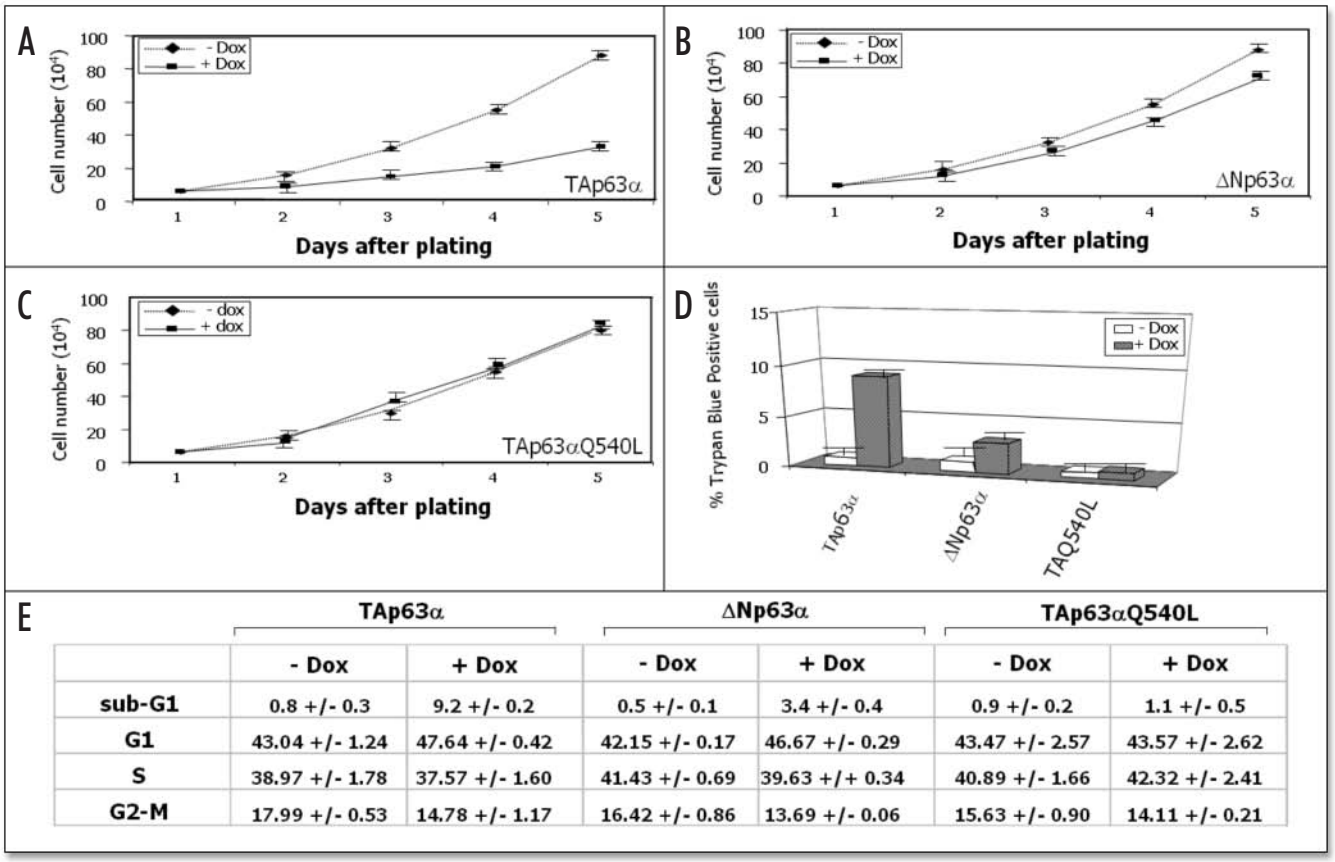
Microarray data analysis. DAT files generated for the four prototypic situations (TAp63α wt without induction, TAp63α wt with induction, TAp63αQ540L without induction, TAp63αQ540L with induction) were analyzed by GCOS (Affymetrix, USA) to generate background-normalized image data (CEL files). The presence 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).

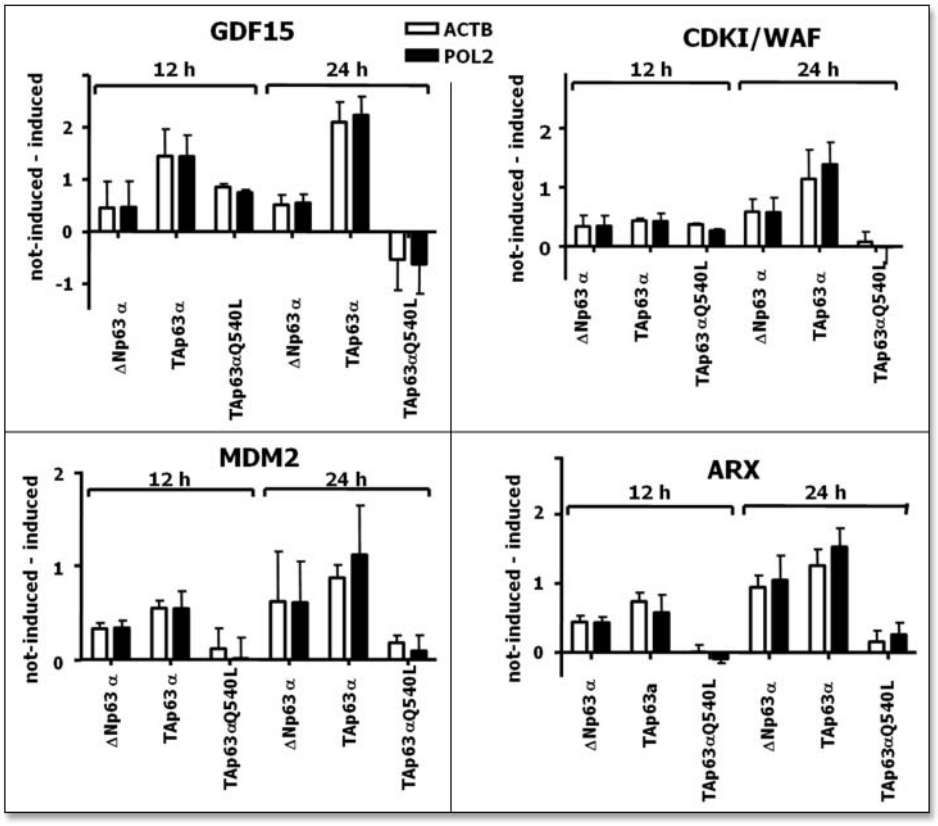

Figure 4. QPCR data related to four genes transactivated by TAp63α wt and characterized by a loss of control by the Q540L mutant. Gene expression was analyzed at 12 and 24 hours upon addition of doxycycline to ΔNp63α, TAp63α and TAp63αQ540L inducible cell lines. Target gene mRNA levels were normalized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

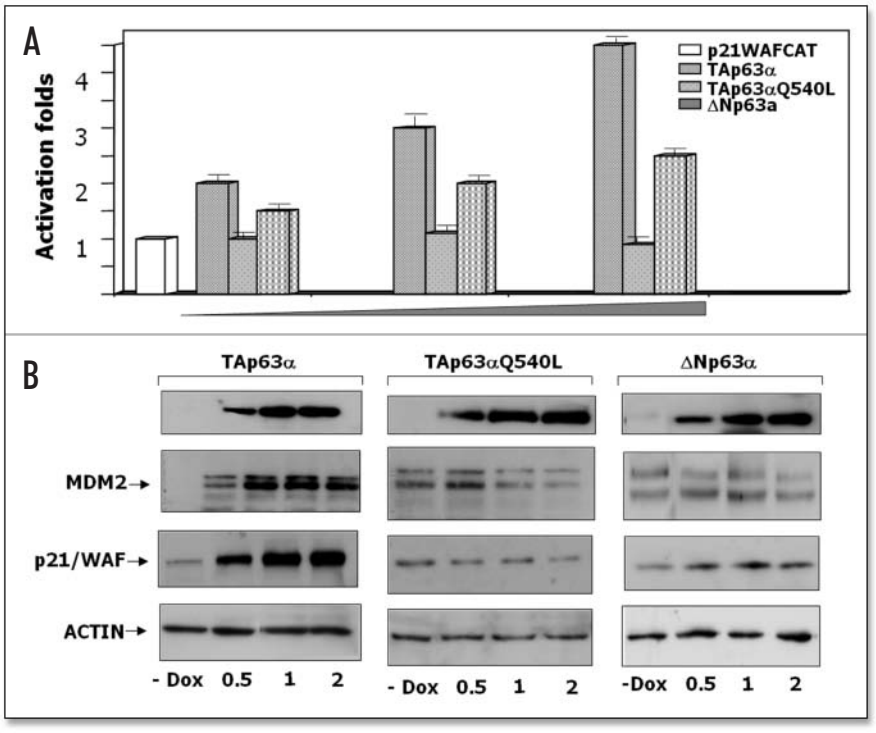

Figure 5. TAp63αQ540L has lost the ability to activate p21/WAF and MDM2 gene expression. (A) H1299 were transiently transfected with 2 μg of p21/WAF-CAT reporter plasmid/dish alone or with different amounts of each p63-expressing plasmid (1, 2 or 3 μg). After 48 h, cells were harvested, and CAT activity 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.

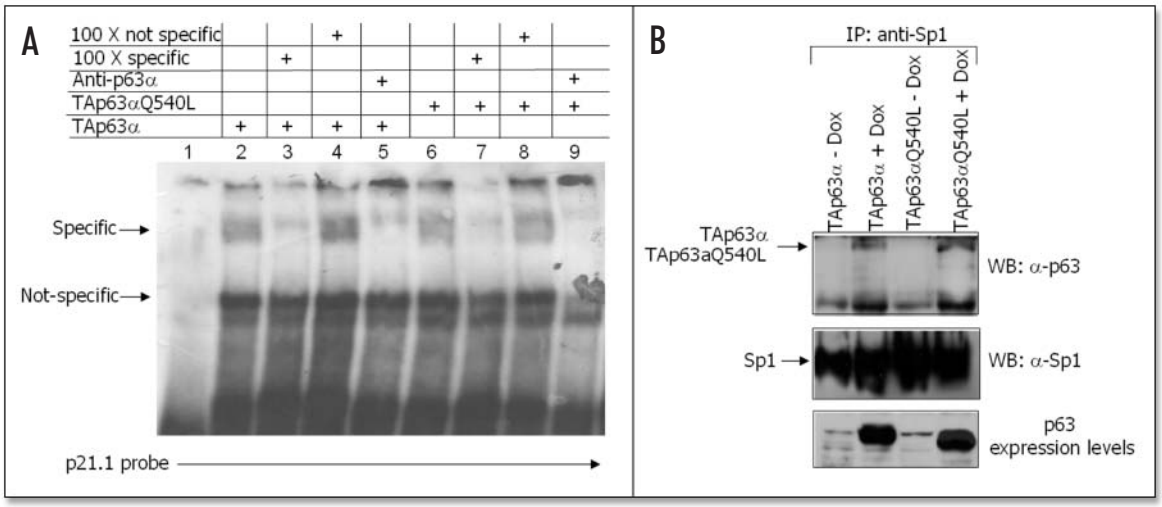

Figure 6. TAp63αQ540L binds to p53 consensus site in the p21/WAF target promoter and associates with the Sp1 transcription factor. (A) The indicated p63 proteins were in vitro translated by using TnT reticulocytes from Promega and 0.5 μ of each p63 plasmid DNA. Equal amounts of the individual reactions 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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