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Molecular characterization of a novel role of p63 in controlling BMP signaling during embryonic skin development

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I. LIST OF ABBREVIATIONS

TA, transient amplified cells; Krt, Keratin; Lce, Cornified Envelop Gene; ORS, outher root sheath; IRS, inner root sheath; IFE, interfollicular epidermal cells; HFs, hair follicle stem cells; SGs, sebaceous glands cells; Shh; Sonic hedgehog, FGFs, fibroblast growth factors; BMP, bone morphogenetic protein; TGF-β, transforming growth factor-β; SAM, Sterile Alpha Motif domain; TID, transcriptional inhibitor domain; TA, transactivation; SUMO, small ubiquitin modifier-1; AER, apical ectodermal region; TUNEL, terminal dUTP nick-end labeling to detect apoptotic cells; BMPR, bone morphogenetic protein receptor; Smurf1, Smad ubiquitination regulatory factor-1; TAK1, TGF-beta-activated Kinase; ES, Embryonic Stem Cells; EEC; Ectrodactyly, Ectodermal Dysplasia, and Cleft Lip/Palate Syndrome; LMS; Limb Mammary Syndrome; ADULT, Acro-Dermato-Ungual-Lacrimal-Tooth; AEC, Ankyloblepharon-Ectodermal defects-Clefts Lip/palate; RHS, Rapp-Hodgkin Syndrome; DBD, Dna Binding Domain; ABBP1, Apobec-1-binding protein-1; FGFR2, fibroblast growth factors receptor 2; siRNA, small interfering RNA; RT, reverse transcriptase; ChIP, chromatin immunoprecipitation; ERK, extracellular signal-regulated kinase; FDR, false discovery rate; PPDE, posterior probability density estimate; DIG, digxigenin; TSS, transcription Start Site; BRE, Bmp-responsive-elements; CAGA-luc, TGF-beta responsive elements; 3TP-luc, TGF-beta responsive element; HDAC, Histone deacetylase ; TSA, Trichostatin A;

II. ABSTRACT

p63, a p53 family member, plays an essential role in epidermal development by regulating its transcriptional program. Here we report a previously uncovered role of p63 in controlling BMP/Smad signaling, which we find to be essential for maintaining low expression levels of several non-epidermal genes. P63 represses transcription of the inhibitory Smad7, thereby sustaining BMP signaling. In the absence of p63, compromised BMP signaling leads to inappropriate non-epidermal gene expression in postnatal mouse keratinocytes and in embryonic epidermis. Reactivation of BMP signaling by Smad7 knockdown and/or –to a lesser extent– by BMP treatment suppresses expression of non-epidermal genes in the absence of p63. Canonical BMP/Smad signaling is essential for control of non-epidermal genes as use of a specific inhibitor, or simultaneous knockdown of Smad1 and Smad5 counteract suppression of non-epidermal genes. Our data indicate that p63 prevents ectopic expression of non-epidermal genes by a conserved mechanism involving Smad7 repression and consequent enhancement of BMP/Smad signaling.

Human syndromes caused by mutations in p63 gene resemble the phenotype of p63 knock-out mice characterized by ectodermal dysplasia, split hand/foot malformation and orofacial clefting. To understand the molecular defects of AEC syndrome, in our laboratory has been generated a knock-in mouse model for AEC syndrome. We searched for the expression of non-epidermal genes and known direct targets of p63 such as Bmp7 and Smad7 in AEC mutant mice. We found that neither non-epidermal genes or Bmp7 and Smad7 change their expression in this mouse model. Interestingly, we observed that known targets of p63 did not change their expression in AEC mutant mice. Here, we found that AEC mutation affects gene expression of small group of genes. Among them we identify p53 family members, p73 that is inhibited in AEC mutant epidermis demonstrating that p73 is a direct targets of p63.

1. INTRODUCTION

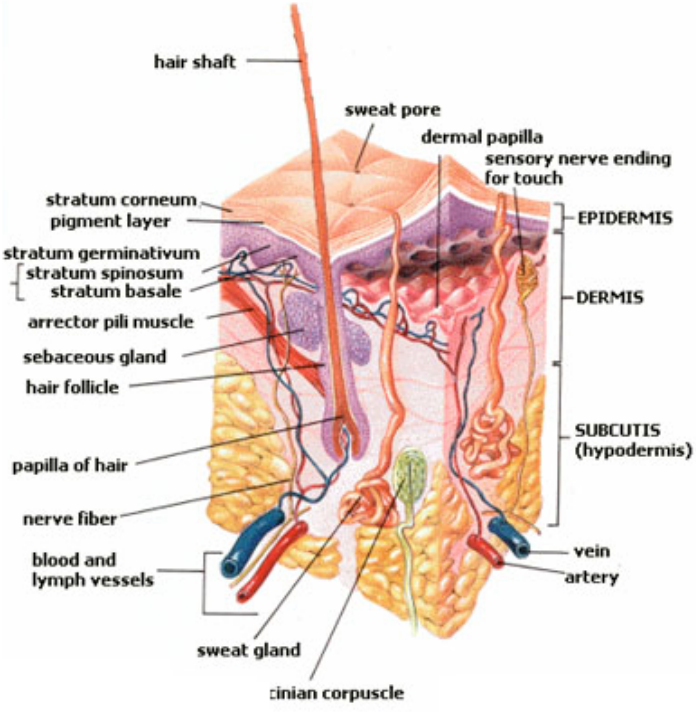
1.1 The skin and its structure

The skin is the first barrier of our body that protects us from infectious agents, hazardous substance, UV radiation, mechanical stress. Mammalian skin is a stratified epithelia and the epidermis represents the major part of epithelium covering the body. The epidermis is derived primarily from ectoderm germ layer as its appendages. The other components of the skin is the dermis that gives rise from the mesoderm germ layer and that recovers a crucial role in promoting epidermal development and hair placodes formation during embryogenesis.

The dermis is tightly connected to the epidermis by a basement membrane. Structural components of the dermis are collagen, elastic fibers, and extrafibrillar matrix. On the other side the dermis is connected with hypodermis and it preserves the body from stress and strain. The dermis structure is divided into two areas: a superficial area adjacent to the epidermis, called the *papillary region*, and a deep thicker area known as the *reticular region*. In itself there are located many mechanoreceptor/nerve endings that provide the sense of touch and heat. The dermis also contains the hair follicles, sweat glands, sebaceous glands, apocrine glands, lymphatic vessels and blood vessels. The blood vessels provide nourishment and waste removal from the dermis and epidermis.

The dermis shows a crucial role during early stage of epidermal keratinocytes development releasing inductive signals to promote ectodermal progenitors to specify epidermal commitment (1). The epidermis constantly replenishes itself by a proliferation and differentiation process. In human, it is estimated that the epidermis turns over every 40-56 days (2; 3), whereas in mice the estimated epidermal turnover time is 8-10 days (4). This constant turnover of the epidermis is mediated by epidermal stem cells which reside in the basal layer, each one generates a columns of epidermal proliferative units composed by Transient Amplified (TA) cells (5). The TA cells reside in the basal layer and they undergo a few rounds of cell division before initiate an asymmetric cells division to lead terminal differentiation (6;7). After few rounds dividing cells in the basal compartment of the epidermis continually execute a program of terminal differentiation.

As cells exit from the basal layer and begin their journey towards the skin surface, the cells switch expression of basal layer specific keratin s, Keratin5 and Keratin 14 (Krt5; Krt14) , to Keratin1 and Keratin10 (krt1; Krt10) typical of the spinous layer (8). The cells of spinous layer expressing the intermediate filament of Krt1/Krt10 are connected trough cell-cell junctions known as desmosomes. These connections provide a cohesive, integrated mechanical structure across and within stacks of epithelial sheets. Keratin6, Keratin16 and Krt17 (Krt6; Krt16 and Krt17) are also expressed suprabasally, but only in hyperproliferative condition such as wound healing.



This keratin network not only remodels the cytoskeleton organization for migration but also regulates cell growth through binding to the adaptor protein 14-3-3σ and stimulating Akt/mTOR signaling (9;10).

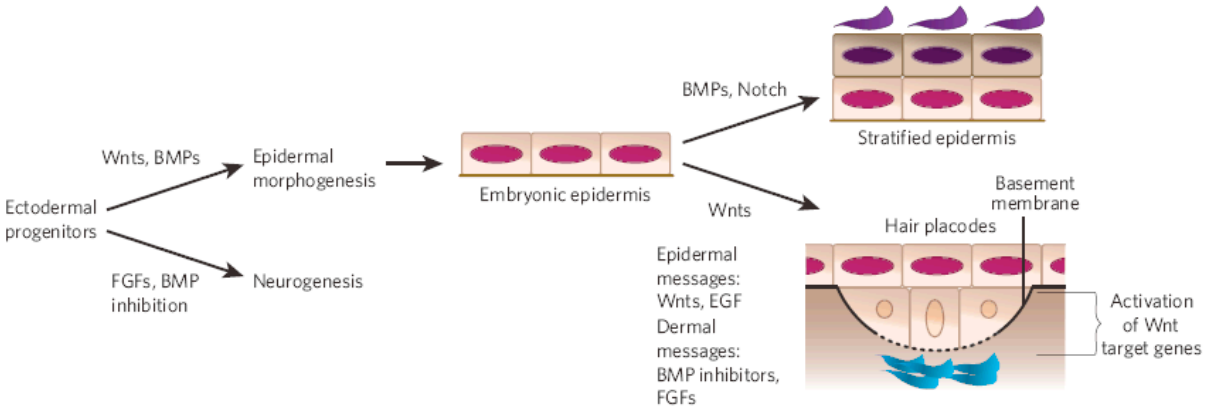
As spinous cells progress to the granular layer, they produce electrondense keratohyalin granules packed with the protein profilaggrin which, when processed, wrap up keratin intermediate filaments even more to generate large macrofibrillar structures. In addition, cornified envelope proteins (Lce), which are rich in glutamine and lysine residues, are synthesized and deposited under the plasma membrane of the granular cells. As response to the increased permeability to the calcium, the cells activate transglutaminase, generating γ-glutamyl ε-lysine crosslinks to create an indestructible barrier. The final steps of terminal differentiation involve the destruction of cellular organelles including the nucleus, and the extrusion of lipid bilayers, packaged in lamellar granules, onto the scaffold of the cornified envelope. The dead stratum corneum cells create an impenetrable layer that is continually replenished as inner layer cells move outwards and are sloughed from the skin surface (11).

The hair follicle shows a more complex structure, composed at least of seven distinct cell types organized in three compartments: an outer root sheath (ORS), an inner root sheath (IRS) and hair shaft (12). In the epidermis two different population of stem cells have been found the interfollicular stem cells and hair follicle stem cells. The hair stem cells reside in the bulge region, a morphologically distinct area of the hair follicle located in the mid portion of the follicle (13). One gene that is expressed at high levels in the bulge region is keratin15 (Krt15). Krt15 expression is also detected in the interfollicular epidermis, although at lower levels (14). During physiological growth of the skin, interfollicular and hair follicle stem cells contribute to their respective compartment of origin. However, transplantation experiments and lineage tracing experiments have demonstrated that, in response to injury, firstly bulge-derived keratinocytes participate into repair of the interfollicular epidermis (15;16). Within the wound healing, the bulge derived cells survive transiently and they are rapidly replaced by progeny of interfollicular stem cells. Consistent with these findings, mice that lack hair follicle show delay in wound healing (17). However, wound healing ultimately takes place, presumably due to the activation of interfollicular epidermal stem cells.

Upon severe injury, the interfollicular Stem Cells (IFE) can even regenerate hair follicle stem cells (HFs). Thus, epidermal cells are also multipotent (18). Recent studies with lineage tracing of single basal cells in tail skin, suggests that, while the majority of labeled cells are lost within 3 months, some survive and clonally expand in size over time. This behavior seems indicate that discrete epidermal proliferating unit composed of one stem cell surrounded by a steady-state pool of ~10 transit-amplifying progeny that subsequently exit the niche and terminally differentiate (7). The exact identity and location of interfollicular epidermal stem cells remains unknown. Recently, a population of murine HF stem cells was identified and these populations are enriched for Lrg1 and Blimp1. These cells give rise to IFE and Sebaceous Glands cells (SGs) when stimulated by retinoic acid in vivo (19). It has been suggested that these cells might be the elusive IFE stem cells. However, three different lineage tracing studies using Cre-recombinase driven by either *Shh*, *Sox9*, or *cytochrome P450* promoters each document that mouse IFE harbors its own resident progenitors, which can sustain long-term epidermal homeostasis (20; 21; 22).

1.2 Signaling pathways in epidermal development.

After gastrulation, the embryo surface emerges as a single layer of ectoderm and specific signals determinate the commitment of neuroectodermal cells to neural or epidermal fate (23). One of the most important signaling required for this process is the Wnt signaling that plays a crucial role in early step of ectodermal commitment. Wnt signal blocks the ability of ectoderm to respond to fibroblast growth factors (FGFs). In the absence of FGF signaling the cells are prone to respond to bone morphogenetic proteins (BMPs), initiating the epidermal development. Conversely, the acquisition of neural fate depend on the absence of a Wnt signal. In this conditions the ectoderm is able to receive and translate activating signal mediated by FGFs to induce neurogenesis. Loss of Wnt signaling induces the expression of specific inhibitors of BMP signaling (Follistatin, Chordin or Noggin) that induces the neural fate (24) (Figure 1).



Adapted from Fuchs E. Nature Review 2007

Figure 1. Signaling pathways in early embryogenesis. *In early stages of embryogenesis the Wnt signaling play an important role. In the absence of Wnt signal, ectodermal progenitors responds to FGFs, downregulate BMP signaling and progress towards neurogenesis. On the other hand the Wnt signaling blocks the ability of early ectodermal cells to respond to FGFs, allowing them to respond to BMP signaling to drive and epidermal fate. As development progress, a single-layer expressing Wnt signaling is formed. Some cells fail into respond to Wnts signal, and these become fated to became epidermal cells trough BMP, EGF, and Notch signaling. The cells that do respond to Wnt signaling also receive underlying FGF and BMP inhibitory signals from mesenchymal compartment and, together, these signals the cells to make an appendage (hair follicle).*

The embryonic epidermis that results from this process consists of a single layer of multipotent epithelial cells expressing the ectodermal markers Keratin 8 and keratin 18 (Krt8 and Krt18) (25; 26). When the commitment to stratification occurs, Krt8 and Krt18 expression is down-regulated with concomitant induction of Krt5 and Krt14 expression (E13.5). In the adult Krt5 and Krt14 are only expressed in stratified epithelia and never expressed in single-layered epithelia (27).

One gene that is essential for this switch and for the commitment to stratification is the transcription factor p63, a p53 family member (28). In the primordial epidermis a transient protective layer known as periderm recovers the whole embryo. This layer is characterized by Krt17 expression (29). During late stages of skin development the peridem is lost when the epidermal cells begin to stratification and differentiate becoming impermeable.

As described before, epidermal development is due to a complex signaling network in early stage of embryogenesis where the BMP signaling shows a key role as determinant of ectodermal specification in low vertebrates (30; 31). In mammals a putative BMP function in regulating epidermal fate or specific gene expression has not been demonstrated consistent with a possible redundant function among the BMP family members or with other signaling pathway. The BMP signaling during late stage of embryogenesis show a crucial role in the hair follicle morphogenesis, it is active in the interfollicular epidermis in which represents as much an epidermal promoting signal as it is a follicle inhibitory signal (32; 33; 34) (Figure 1).

1.3 The transcription factor p63.

The p63 gene is a member, together with p73, of the p53 family. All three proteins show high amino acid homology and share three functional domains commonly found in transcription factor: an N-terminal transactivation domain which shares 25% homology with N-terminal part of p53, a central DNA binding domain which shares 65% of homology with the corresponding p53 domain and C-terminal tetramerization domain, which shares 35% of homology with the oligomerization domain of p53 (Figure 2). The p63 and p73 genes are transcribed at least in six different isoforms due to two alternative promoter at the N-terminus of the gene. The two alternative promoters give rise to TA and ΔNp63 transcript. In addition

to use two different promoters, the p63 and p73 genes increase their complexity by alternative splicing at the C-terminus, which gives rise to three different carboxyl terminal isoforms named α, β, γ and seven isoforms (α, β, γ, δ, ε, ζ, η) respectively for p63 and p73 (35; 36) (Figure 2).TAp63 isoforms contain an N-terminal transactivation domain, whereas ΔNp63 isoforms lack this domain and show an alternative transactivation domain (35; 37). In addition, the p63α isoforms contain a sterile alpha motif (SAM) domain at C-terminal of the gene, which is absent in p53 (38; 39). This domain is a protein-protein interaction domain. It is an evolutionary conserved domain commonly found in proteins controlling developmental processes such as several Eph receptor tyrosine kinase (40). Although the SAM domains are known to be involved in protein-protein interactions, in vitro studies demonstrated that SAM domains of p63 can also bind to RNA or lipids (41;42; 43). At this time the protein partners of SAM domain of p63 is unknown and is one of the field unexplored into study of p63. Another unique domain of p63alpha isoforms is the post-SAM domain Transcriptional Inhibitor Domain (TID) which has been shown to have an inhibitory function (44; 45). The transactivation inhibitory domain of p63 binds to the N-terminal TA domain masking residues that are important for transactivation (45). In fact, p63 isoforms that contain the γ and β C-termini are associated with higher transactivation competency that the ones with α terminus protein (46). The lack of TA domain in ΔNp63α isoforms suggests that they are transcriptionally competent. Since ΔNp63 isoforms retain the oligomerization and DNA binding domains, it is plausible that they act as dominant negative inhibitors of p53 and TA containing p53 family members (35; 47). Indeed, numerous studies show that co-expression of ΔNp63α with either TAp63, TAp73, or p53 has inhibitory effect on TAp63-mediated transcription. A plausible mechanism is due to 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 (48; 49; 50). 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 (44).

Different studies reported that the stability as well as the transcriptional activity of p63α is regulated by sumoylation of this domain by small ubiquitin modifier-1 (SUMO-1) (51; 52). Surprisingly, overexpression of SUMO-1 caused a decrease in DNp63alpha but not in TAp63alpha protein.

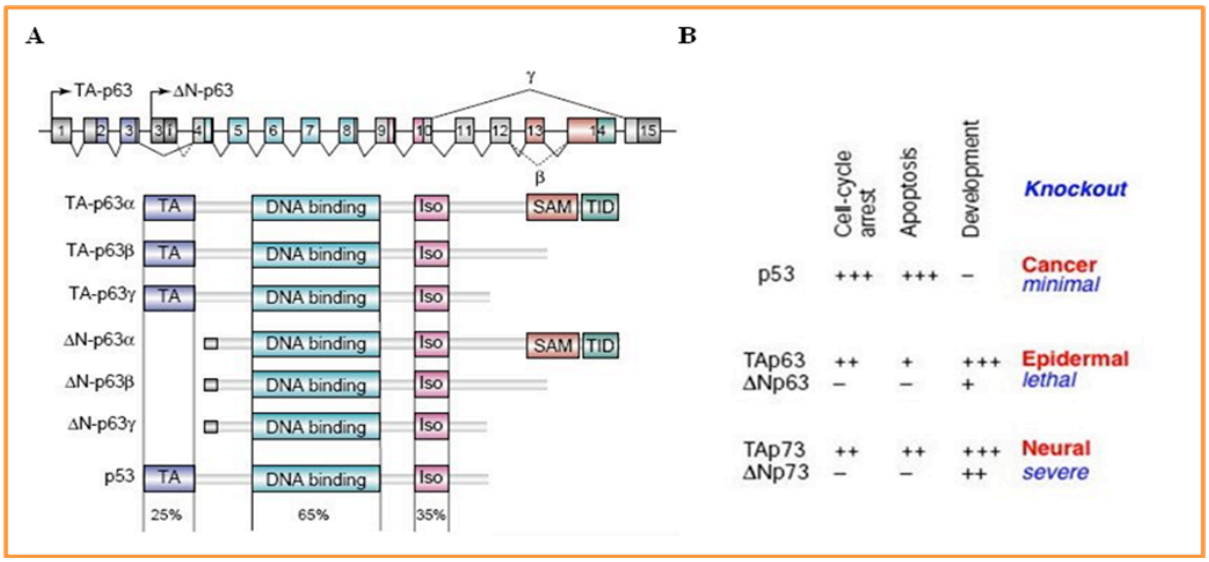


Figure 2. p53 family*. A) The p63 gene encodes a tetrameric transcription factor that can be expressed in at least six isoforms with widely different transactivation potential that share an identical DNA binding domain. Alternative transcription start sites give rise to transactivation (TA) isoforms, encoding proteins with a canonical transactivation domain similar to p53. B) A very high degree of homology is seen in the different domains of p53, p63 and p73. All three members of the family are able to regulate cell cycle arrest, apoptosis and in parallel show developmental effects. However, the knockout mice reveal striking differences, with p63 showing mainly an epidermal phenotype, and p73 a neuronal phenotype.*

This apparent difference in protein stability in response to sumoylation may be caused by the interaction between the TAp63α transactivation domain and the post-SAM TID, which may mask the sumoylation site (52).

The expression pattern of p63 in early stage of embryogenesis at E9.5 is restricted within oral ectoderm, in the ectodermal part of limb buds (Apical Ectodermal Region, AER) and tail bud region, whereas in later stages of embryogenesis the expression of p63 is restricted to interfollicular epidermis and in outer root sheath of hair follicle. The essential role of p63 in skin development was revealed by two independent studies of p63-null mice generated trough two different knockout gene strategies. These mice lack stratified epidermis, producing a

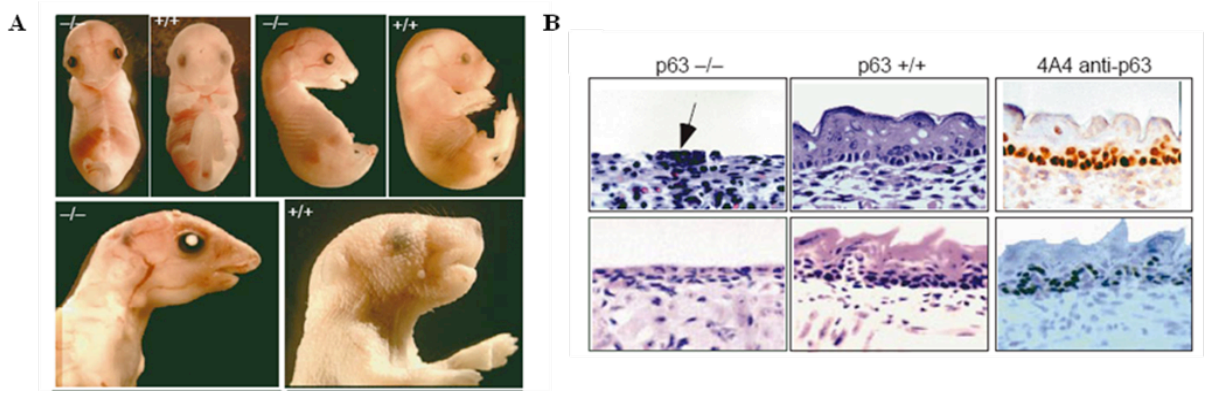
disorganized single layered surface epithelium that is negative for epidermal markers such as Krt5 and Krt14 (53; 54). Both two groups showed that the p63 null mice lack the epidermis and its appendages, associated to limb truncation, craniofacial abnormality. Although, the mutant phenotype in these two studies is similar, the interpretation of the biological function of p63 remains controversial. Nonetheless, these mouse models have been showed a unique role played by p63 during development, in contrast to the p53 null mice that it doesn't show development defects (Figure 2).

1.4 p63 knock-out: toward a comprehension of p63 physiological function.

To understand the biological functions of p63, two different mouse models have been generated. Although, the phenotype of p63 null mice reported by both group was similar, the interpretation is dramatically different. In succession, the biological interpretations are showed based on the evidence of knockout mouse model and other studies.

a) p63 is required for stem cell proliferative potential

p63 gene and in more detail the ΔNp63α isoforms is highly expressed in the basal layer of epidermis and in the highly proliferative compartment of the other stratified epithelia as the cervix, urogenital tract, prostate and breast tissues (35; 55).The knockout mouse model generated by McKeon's group is born alive but has striking developmental defects. Their limbs are absent or truncated, defects caused by a failure of the apical ectodermal ridge (AER) to differentiate. The lack of a proper AER limb buds in p63 null mice results from a failure of the ectoderm to undergo growth and differentiation that give rise to this stratified epithelium. At birth, p63-deficient mice have striking and visible skin defects; in fact, they die within few hours from the birth for 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 (Figure 3).
The skin lacks expression of the basal layer markers as Krt5 and Krt14 and also spinous layer markers Krt1 and Krt10, although the isolated patch of the epidermis showed the expression of late differentiation markers such as Loricrin, Involucrin, Filaggrin.



Adapted from Yang A. Cell 1999

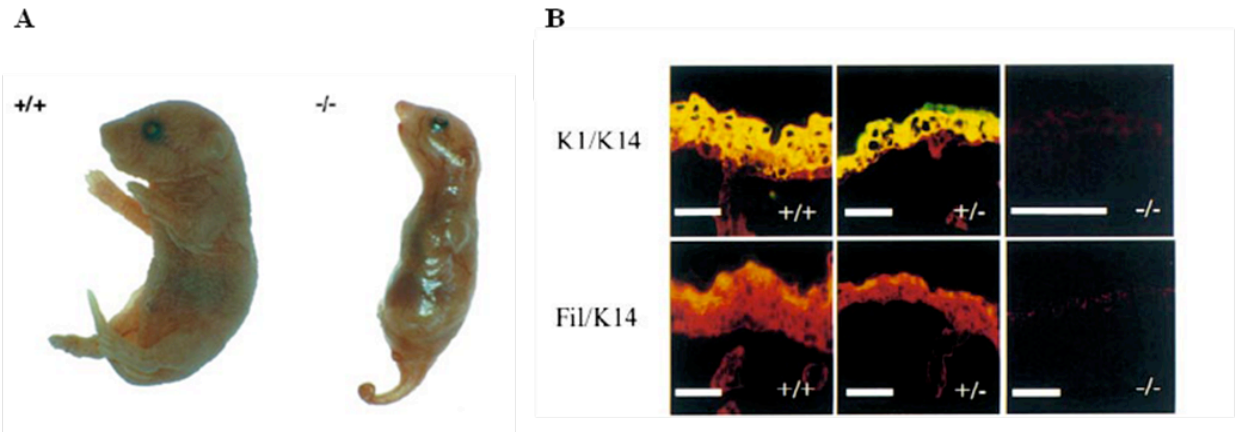
Figure 3. Phenotype of p63 knockout mice . *A) The p63 null mice at birth show defects in limb formation craniofacial defects associated with skin and appendages aberrant development for lack of stratification and differentiation. B)Defects in stratified epithelial differentiation in p63-deficient mice. H&E staining of the epidermis at E17, p63-/- mice lacking squamous stratification in the epidermis (top) and tongue epithelium (bottom). Middle, wilde-type H&E control mice showing extensive stratification. In the right the basal staining with anti-p63 antibody to show the endogenus expression of p63 in the epidermis*

The authors argue that p63 is required for the maintenance of epidermal stem cell, because the p63 null mice showed in late stage of embryogenesis and in newborn mice isolated patch of disorganized epithelial cells expressing the late differentiation markers. The epidermis appears as disorganized epithelia. The cells showed highly pyknotic nuclei which positive for TUNEL indicating apoptosis processes. This study concluded that p63 is required for the initial development and continued regeneration of the epidermis and that the loss of p63 in the tissues failed to maintain the proliferative potential of stem cells (35). In addition, to reinforce this hypothesis the same authors in another study strongly showed that p63 is dispensable to maintain the proliferative potential of epithelia stem cells of the thymus and epidermis (56). The thymus has an epithelial component derived to ectoderm germ layers. In p63 null mice this region exhibited a hypoplastic phenotype compared to wild-type. Unlike the epidermis, the p63 null thymus showed the typical markers for epithelia development and in this case was fully competent to support the maturation of developing T cells. However, the hypoplastic phenotype of the thymus is due to a reduction in proliferative potential of stem cells in thymus compartment and in epidermis. They showed by colony- forming assay that p63 is involved in proliferative potential of epidermal keratinocytes. Clones lacking p63 had a reduction in cell size for decrease of proliferative potential of stem cells (56). These data, support the

hypothesis that p63 is involved in maintenance of proliferative potential of stem cells in stratified epithelia.

b) p63 is required for cell differentiation

The knockout mice generated by Allan Bradley showed defects in limb formation due to failed to stratification of Apical Ectodermal Region (AER), craniofacial defects associated with skin and appendages aberrant development for lack of stratification and differentiation (53). The authors showed that in p63 null mice all structures that required the ectodermal-mesenchymal signal were compromised because the ectoderm failed to receive the signal. They showed that the skin of p63 null mice is covered by a single layers of ectodermal cells or flattened epidermal cells, lacking the differentiate layers (Figure 4).



Adapted from A.A Mills. Cell 1999

Figure 4. The phenotype of p63-deficent newborn mice. *A) The p63 null mice show severe limb and skin defects. B) The expression of different markers in the epidermis of p63 null mice show the staining for Krt14 in red and Krt1, filaggrin and krt6 in green. Krt14 is weakly express in p63 null mice, whereas Krt1, filaggrin and Krt6 are not detectable in the epidermis*

These results suggested that p63 is determining factor of stratification, because they did not detect the expression of any early or late differentiation markers in the epidermis of p63 null mice. The stratified epithelia showed a single disorganized layer well visible in the epidermis

(53). This model supported the hypothesis that p63 is required for simple epithelial cells to commit to a stratified epithelial lineage during development.

Complementary studies show that over-expressing ΔNp63 in keratinocytes culture blocks Ca2+-induced growth arrest and terminal differentiation suggesting a key role of ΔNp63 in controlling the differentiation process (50). In addition Roop et al. showed that ectopic expression of TAp63 in epithelial cells, but not ΔNp63, coverts a normal Krt8 positive epithelia cells into Krt5/Krt14 expressing cells, indicating that TAp63 is sufficient to drive epidermal specification and promoting differentiation (57). The same authors have demonstrated a balance from TAp63 and ΔNp63 in embryonic development, in which the TAp63 isoforms played a key role in early embryogenesis to promote the epidermal specification, whereas in late stage the TAp63 expression left place to ΔNp63 isoforms involved in maintenance of proliferative state of basal layer. They conclude that the balance of TAp63 and ΔNp63 is crucial to determinate the proliferative state of keratinocytes. The proposed model of Bradley and Roop argue that p63 is required for commitment to stratification and differentiation (57).

Other studies reported that ΔNp63α represses the expression of p21 and 14-3-3σ, two genes induced during epidermal terminal differentiation, and the expression of these genes are required for cell cycle progression including cyclin B2 and cdc2 (58). These genes are directly inhibited by p63 in the basal layer where the cells are undifferentiated. Their induction represents an important step to initiate terminal differentiation program. p21 induction is mediated by two different mechanisms: Rb-Jk that directly binds its promoter and the activation of Notch pathway. The Notch pathway is required for differentiation process whereas the ΔNp63α maintains the undifferentiated state. The balance between un-differentiation and differentiation state is regulated by cross-talk between p63 and Notch (59). In primary mouse keratinocytes, p63 expression counteracts the ability of Notch1 to restrict growth and promote differentiation in keratinocytes, effect mediated by repression of Hes1 an activator of Notch. In these way the cells in the basal layer proliferates whereas, when the cells escape to highly proliferative state show a reduction of ΔNp63α and the induction of Notch activity inducing cell differentiation. These studies showed that p63 and in more details the TAp63 isoforms in involved in early stage of embryogenesis to drive epidermal specification

whereas the ΔNp63α is important for maintenance of proliferative state of epidermal cells in the basal layer inhibiting the terminal differentiation.

c) p63 is required for cell adhesion

In the epidermis and in all epithelia, cell adhesion has a fundamental role into support epithelia formation and structure. Three different types of junction complexes mediate cell-cell adhesion in epithelia: tight junction, adherents junction and desmosomes junction. While the tight junction perform a role in establishment of barrier formation and in para-cellular transport, the adherents junction and desmosome are crucial in promoting cell adhesion. The importance of these junctions for epidermal integrity is highlighted by the autoimmune or genetic blistering diseases in humans caused by dysfunctional desmosome components (132). Similarly, mouse model lacking desmosomal components are prone to epithelial blistering (60). Cell adhesion process as attachment to the extracellular matrix via integrins is an important step not only for structural organization of tissues but also for epithelial cell proliferation, migration, differentiation and survival. Therefore, physical detachment from the extracellular matrix for defects in cells adhesion results in induction of apoptosis or anoikis in a wide range of epithelial cell types (61; 62). Loss of p63 causes the reduction in gene expression of adhesion molecule with consequent cell death. The ability of p63 to regulate key matrix adhesion proteins could play an important role in maintenance of tissue integrity since the attachment of cells to underlying basement membrane is crucial for epithelial tissue integrity and homeostasis. Carrol et al in 2007 showed the reduction of multiple adhesion molecules in p63 null mice (63;65). They found that p63 directly controls the gene expression of Perp a desmosomes junction. Perp is highly express in stratified epithelia and it is a component of desmosomes junction. Interestingly, *Perp-null* mice showed lethally associated with the presence of epithelia blistering in the skin and in oral epithelium, some phenotype observed in p63 null mice (64). Moreover, they showed also that p63 directly controls the expression of other adhesion molecules as integrin alpha 3, 4, 5 and 6 and laminin-2. Genes encoding many classes of proteins involved in multiple aspect of cell adhesion were regulated by p63, including extracellular matrix component, integrins, adherents and desmosomal junction (63;65). Accordingly with these data, the down regulation of p63 in breast cancer

cells (MCF10A) or in mouse primary epithelia cell, induced cell detachment and subsequent apoptosis. Interestingly, this affects are specific of down-regulation of ΔNp63 isoforms, whereas the reduction of TAp63 isoforms had a little phenotype. Conversely, the induction of ΔNp63α and TAp63γ isoforms protected the cells from death induced by detachment of the cells from matrix. These finding suggest that the ΔNp63 isoforms are essential for adhesion process and survival in epithelial cells in vitro (63;65).

1.5 BMP signaling in skin development.

Skin morphogenesis is a complex process resulting from a cross-signal from ectodermal and mesenchymal component of the embryo involving Wnt, FGF and BMP signals. BMP signaling together with its antagonist and BMP receptor perform many roles in skin and appendage morphogenesis and control a large number of biological processes as cell proliferation, differentiation, apoptosis and cell fate decision not only in skin but also in other tissues.

The **B**one **M**orphogenetic **P**rotein (BMPs) is secreted signaling molecules that belong to **T**ransforming **G**rowth **F**actor-**β** family and perform their biological function via interaction with specific receptor. The BMP signaling is modulated by different mechanisms: at extracellular levels thought the BMP antagonist that recovers the main role in regulating the magnitude and spatio- temporal specificity of the signal. On the other hand, the signal is also controlled at intracellular levels by the inhibitory-Smad or MAP kinase and finally, in the nucleus by controlling the transcription of BMP target genes (66). The BMP family consists of more than 20 secreted proteins that share structural homology that are able to form homo-heterodimers. The intensity of signal and the spatio-temporal specificity is due to a structurally distinct protein families in which there are Noggin, Chordin, Follistatin, Cerberus/DAN family and Ectodin. All of these proteins selectively bind distinct members of BMP family restricting the action to specific tissues compartment. The signaling is activated by binding of BMPs on transmembrane receptor complex formed by type I and type II receptor. Combinatorial interaction in the tetrameric receptor complex allow differential ligand binding or differential signaling in response to the same ligand (67). One receptor combination often binds different ligands, and pattern of ligand and receptor expression often dictate which receptor are

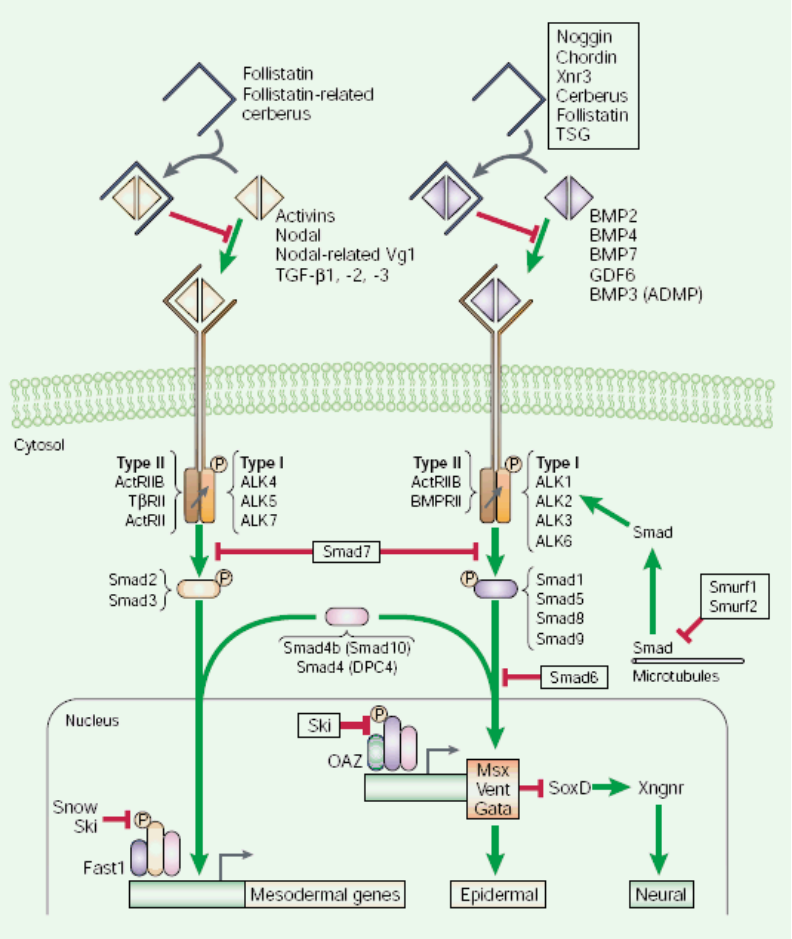
activated. The BMP signal can be transduced by combination of type II receptor (ActRII and ActRIIB) with type I receptor (BMP-RIA and BMPRIB). In addition, the BMP type II receptor (BMPRII) can combine with three type I receptor as, BMPRIA, BMPRIB, and ActRI/ALK2. The binding to specific receptor leads the activation that induces the phophorylation of intracellular domain of type I receptor by the type II receptor kinase and leads a transmission of an intercellular signal thought BMP/Smad canonical pathway or BMP/MAPK non-canonical pathway (68; 69; 70;71).

The activated type I receptor induces the posphorylation of R-Smad proteins (Smad1, Smad5, Smad8) at their C-terminal domain (72) (Figure3).Among the R-Smads family there are Smad2 and Smad3 but they are activated by ActRI and TGF-bRI kinases and are not mediators of the BMP signaling pathway (72). R-Smad phosphorylation in turn induced the recruitment of Smad4 and the formation of heteromeric complex that are able to translocate into the nucleus to control gene expression of direct targets of BMP signaling.

The second negative control of BMP signaling is performed by inhibitory Smads, Smad6 and Smad7 that act with different mechanisms to block the BMP activity. The inhibitory Smads are able to antagonize the phosphorylation of Smad1, Smad5 and Smad8 by the binding to activated BMPRI kinases. In addition, the inhibitory Smads compete with Smad4 to binding to Smad1 (73). Finally the inhibitory Smads induce the degradation of the BMP receptor and R-Smad by recruitment of Smurf1 (Smad ubiquitination regulatory factor-1), which interacts with Smad1 and Smad5 to promote their degradation by proteosome (74). More recent study, has showed a new non-canonical role of Smad7 in controlling other signaling pathway, including β-catenin (75; 76). The Smad4 and R-Smad proteins have a two highly domain conserved at N- and C-terminus of the proteins, MH1 and MH2 domain that are involved into binding to DNA and into recruitment of specific co-regulator respectively that can induce or repress the expression of downstream direct targets of BMP signaling (70) (Figure 5).

As indicated before the BMP signaling activates a Smad canonical pathway. On the other hand, BMP signaling can elicit its effects trough a non-canonical pathway. The non-canonical pathway is activated when the BMPs firstly bind the type I receptor followed by the recruitment of BMPRII (71). These interactions induces the recruitment of XIAP and/or BRAM1, an adaptor protein that link BMPRI complex to TAK1 binding protein, that in turn activates the TAK1 (77;78). TAK1 is a TGF-beta-activated kinase member of the MAPK

family. The activation of TAK1 induces the activity of p38 and JNK pathway that is known to be involved into apoptosis process.



Adapted from Ignacio Munoz-Sanjuan Nat Rev 2002

Figure 5. The BMP signaling pathway and the specification of ectodermal cell fates. *In Xenopus, activation of the bone morphogenetic protein (BMP) pathway in the ectoderm leads to the acquisition of epidermal fates, whereas inhibition of BMP signaling induces neural fates. However, signaling that is mediated by the nodal/activin branch of the transforming growth factor-β (TGF-β)pathway induces mesodermal gene expression in ectodermal cell. The ligands bind and activate a subset of type I and type II TGF-β receptors, which form heterodimers. Activation of the BMP pathway leads to phosphorylation of the signal transducers Smad1,-5, -8, which form a complex with Smad4 (DPC4) and translocate to the nucleus, where they associate with various developmentally regulated transcription factors to direct epidermal gene expression. Smad7 is thought to exert part of its inhibitory activities by preventing the activation of Smad1 and 2. In addition, the ubiquitin ligases Smurf1 target Smad1 and smad2 and the TGF-β receptors for degradation by the proteasome.*

Interestingly, the BMP signaling displays different role depending cell context and timing. Many studies demonstrated that the BMP signaling is able to be repressor or activator of

transcription in the same cells, action that depend only from recruitment of co-transcription factor are able to give different role at BMP signal in the cells (70; 79).

The BMP ligands are differential expressed in the epidermis, BMP2 and BMP4 are expressed respectively in hair follicle compartment and in the mesenchymal component of the skin during embryogenesis (80; 81). Whereas, BMP6 is restricted to differentiated layers during late stages of embryogenesis and in post natal life in mice. Finally, the Bmp7 is expressed in the basal layer of epidermis during embryogenesis and interestingly in p63 null mice has been shown that its expression is reduced at E14 (80; 82; 83).

Data in vitro in primary mouse keratinocytes showed the main role performed by BMP2 and BMP6 in controlling proliferation and differentiation of keratinocytes (84; 85; 86). In addition, has been demonstrated that BMP6 stimulates the differentiation and induces Krt1 expression in primary mouse keratinocytes (85).

Many are the function recovered by BMP signaling in vivo as well as in vitro and these effects underline the pleyotropic function of the signaling and the redundancies due to the possibility of activate the pathway in different way. In fact, mouse model deleted for several component of the pathway have been generated. Some of them have little phenotype due to the redundancies of BMPs family in the epidermis. The major of them showed defects in hair follicle morphogenesis or failed into growth and cycle progression of the hair follicle. The BMP signal represent an important signaling in hair follicle but in this work has not been addressed this issue.

1.6 p63 and BMP signaling evidences for direct functional correlation.

BMP signal is an important determinant of epidermal fate specification acting as epidermal inducers and suppressing neurogenesis. During embryogenesis, epidermal and neuronal precursors are supposed to be derived from a common neuroectodermal precursor. In *Xenopus* embryos, epidermal commitment is induced by BMP-4 in the ventral part of the egg, while its absence within the dorsal part leads to a "default neural" program (87).

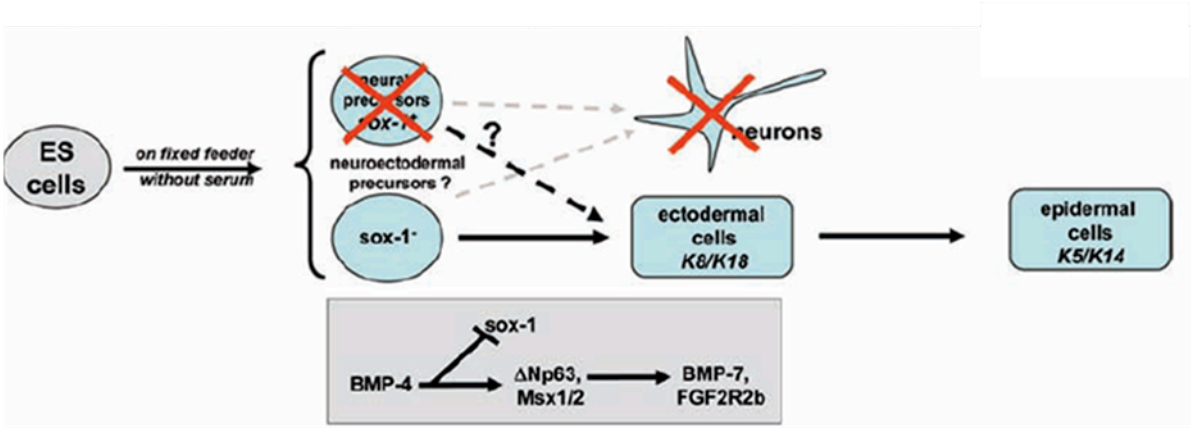
Little is known on the role of p63 and BMP signaling in mammals systems. Thus, in low vertebrate it has been demonstrated that p63 is important into promoting ectodermal proliferation and differentiation and its expression is an early events in xenopus epidermal

development (88). In addition, it has been shown that the down-regulation of ΔNp63 by morpholino injection in the early Xenopus embryo potentiates mesoderm formation whereas ectopic expression of ΔNp63 inhibits mesoderm formation inducing epidermal commitment, suggesting that ΔNp63 is a key function in defining a squamous epithelial phenotype activating or suppressing mesodermal cell fates during early development (89).

Other studies have been performed that showed a key role of p63 in controlling epidermal commitment in vivo by inhibition of ΔNp63 in zebrafish embryos. The lack of p63 disturbs skin formation and AER maintenance while overexpression of ΔNp63 is sufficient to block anterior neural specification while promoting early steps of epidermal fate, even in embryos lacking BMP-4 signaling (90; 91). These two studies into zebrafish development suggest that ΔNp63 plays a dual role in the early steps of epidermis formation; it acts as both an epidermal inducer and an inhibitor of neuroectodermal formation and later on, it is required for the epidermal cell proliferation (90; 91).

The Embryonic Stem (ES) cells are pluripoten stem cells derived from inner cell of the blastocyst. These cells can reproduce in vitro the early stage of embryogenesis. The use of cell model is able to discover new molecules or known molecule that could direct the different commitment of ES cell to different fate. This strategy, at this moment, could represent new frontiers for regenerative medicine. One of these cases is the differentiation of ES cell to produce epidermal cells trough different approach. Aberdam and co-workers showed that a synergistic effect of the mesenchymal extracellular matrix and BMP4 is able to induce mouse ES cells to differentiate into keratinocytes (92). In mammals as well as low vertebrate, BMP4 plays a similar critical role in epidermal commitment and displays an inhibitory effect on neural induction (93). Neural differentiation process of ES cells is inhibited by BMP-4 through specific apoptosis process of Sox-1+ neural precursors cells that leads the epidermal fates (94). They suggest the existence of bipotent ES derived neuroectodermal precursors able to become either neurons or epidermal cells depending on the presence or the absence of BMP-4, respectively. Finally, an important role performed by p63 is elicited in this system. Aberdam et al showed that the drastic switch to ectodermal commitment occurs through the activation of ΔNp63 in early stem of differentiation of ES cells after BMP4 addition, whereas TAp63 is never activated during this commitment process (Figure 6).

p63 loss of function drastically prevents ectodermal cells to commit to the Krt5/Krt14 positive stratified epithelial expression while gain of function experiments show that ΔNp63 allows this commitment. Interestingly, they showed that other epithelial cell fates are not affected, allowing the production of Krt5/Krt18 positive epithelial cells (95). By these evidence emerges that p63 could have an uncovered role in promoting cell fate determination in association with BMP signaling and in maintaining the cell identity of the epidermal cells, by day little is discovered.



Adapted from Matthieu Rouleau Cell Cycle 2007

Figure 6. Schematic representation of ectodermal commitment and epidermal differentiation of ES cells. *A) ES cells cultured on fixed NIH-3T3 cells under serum-free conditions efficently differentiate into neural precursor. When BMP4 is added to medium from day 3 to 5 neural commitment is prevented while ectodermal cells (Krt8 and Krt18) are produced. Same of theme became keratinocytes (Krt5/Krt14) after the addition of serum. Soon after the addition of BMP4, ΔNp63 is induced. This induction is followed by activation of ΔNp63 and its target gene Bmp-7 and FGF2R2b.*

1.7 p63 and related human autosomic dominant disorders.

The transcription factor p63 is associated to several autosomic dominant human syndromes causative of ectodermal dysplasia, orofacial clefting and limb malformation. Ectodermal dysplasia is manifested as the abnormal development or growth of tissues and structures that are developed from ectoderm. In this condition skin, hair, teeth, nails and several exocrine glands, such as sweat and sebaceous glands are usually abnormally developed.

This gene is characterized by several point mutation in all domains that causes different specific syndrome in human. The mutations identified in this gene are causative of five different syndromes in human (Figure 8). The clinical signs are matched in different way in the patients. Interestingly, human phenotypes caused by mutations in p63 gene resembles the phenotype of p63 knockout mice characterized by ectodermal dysplasia, split hand/foot malformation and orofacial clefting (Figure 7).

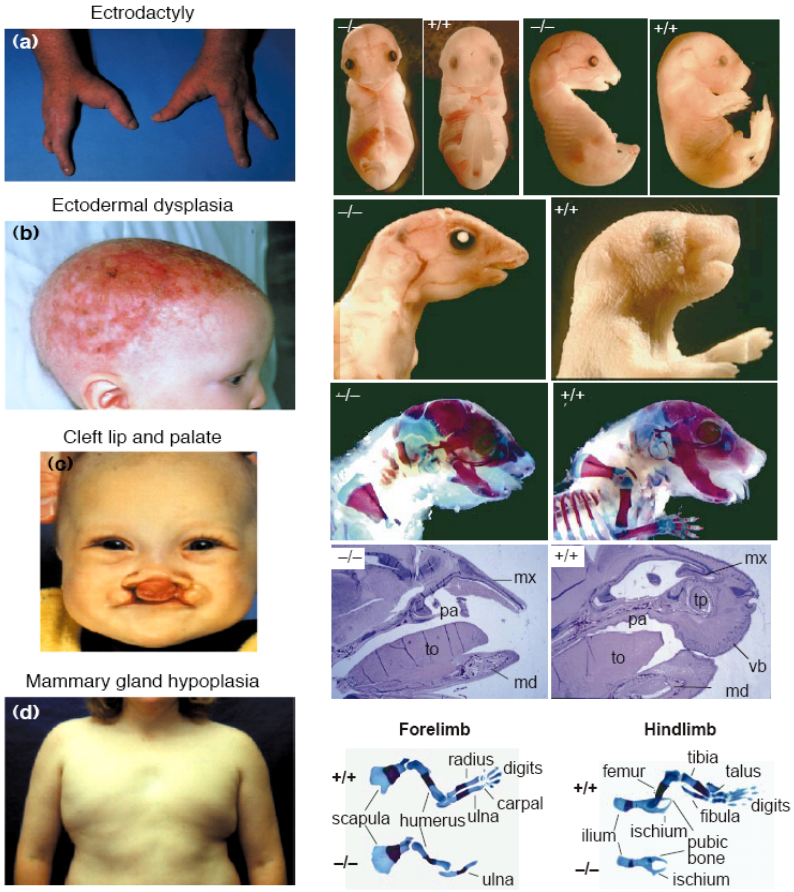


Figure 7. the phenotype of knock mouse model resembles the main signs of AEC syndrome. *p63 knockout mouse model shows the defects in limb formation due to failed to stratification of Apical Ectodermal Region (AER), craniofacial defects associated with skin and appendages aberrant development for lack of stratification and differentiation. The p63 human syndrome show ectodermal dysplasia, split hand/foot malformation and orofacial clefting.*

Adapted by Yang A Cell 1999; and McGraft JA Hum. Mol. Genet. 2001; Celli Cell 1999

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

The **E**ctrodactyly, **E**ctodermal Dysplasia, and **C**left Lip/Palate Syndrome (**EEC**) is the most common between these autosomic disorders (96). The point mutations underling this syndrome were found in the DNA binding domain of p63 and at this moment were identified

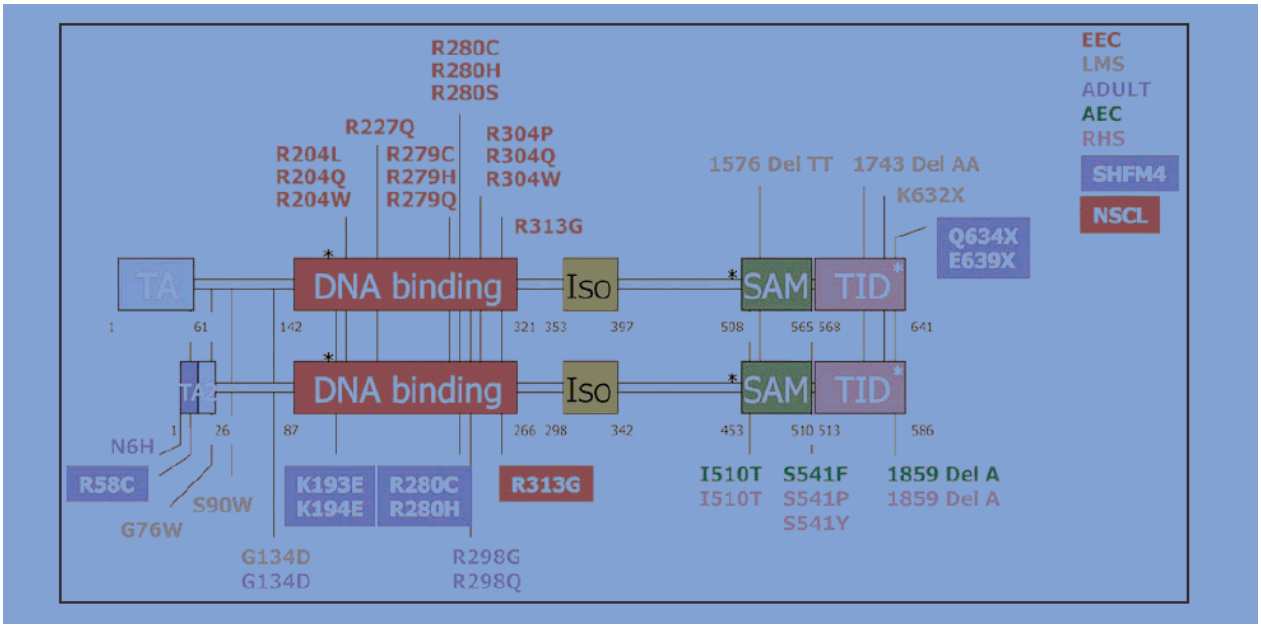
34 different mutations. The mutations are frequently located in CpG island, and some of this mutations cause the impairment of DNA binding. The autosomal dominant inheritance of EEC suggests that these mutations have a dominant negative effect on p63 function. The EEC patients are invariably characterized by one or more features of ectodermal dysplasia, which can present as defects of hair, skin, nails, teeth and glands. The genotype–phenotype is highly variable and depends to exact nature of mutation. EEC patients occasionally also have mammary gland/nipple hypoplasia (14%) and hypohidrosis (11%). About two thirds of these patients have ectrodactyly, and syndactyly is also frequent (43%). Cleft lip/palate is present in about 40% of the EEC patients, mostly as Cleft lip with or without Cleft palate (Figure 8).

Limb **M**ammary **S**yndrome (**LMS**), belongs to human syndrome associated to p63 mutation. It resembles the phenotype of EEC syndrome but shows lesser ectodermal manifestation. A consistent feature of LMS is the mammary gland and/or nipple hypoplasia (100%). Lachrymal duct obstruction and dystrophic nails are frequently observed (59 and 46% respectively), hypohydrosis and teeth defects are detected in about 30%, but other ectodermal defects such as hair and skin defects are rarely detected. 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 (96). The point mutation is found in LMS patients are located as in the N-terminus as well as in the C-terminus of the p63 gene (Figure 8).

The **ADULT, A**cro-**D**ermato-**U**ngual-**L**acrimal-**T**ooth syndrome is less characterized at molecular levels. Now, four family and three sporadic cases were identified. All three families and one sporadic cases show the point mutation in exon 8 of DBD (R298). This mutation causes the changing in an arginin located outside DNA-binding interface, so this mutation does not cause impairment to DNA binding. Two other mutations are located in the N-terminus: N6H mutation affects only the DN-isoforms and in another isolated patient a missense mutation G134D* is located just front of the DBD in exon4 (97) (Figure 8).

The **A**nkyloblepharon-**E**ctodermal defects-**C**lefts Lip/palate (**AEC**), has more dramatic phenotype than other p63 human syndromes. AEC patients show a strong skin phenotype and are characterized by eyelid fusion at birth and the absence of limb malformations. Approximately 80% of the patients have severe skin erosion at birth, which usually will recover in the first years of the life. The eyelid fusion, also called ankyloblepharon, is present in about 45% of AEC patients, but only rarely in other *p63*-associated conditions. The other

ED 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 (94%).



Adapted by Rinne T. Cell Cycle 2007

Figure8. Mutations in p63 gene. *Distribution of mutations in p63, revealing a striking genotype-phenotype correlation. The positions of mutations and aminoacid correlation are indicated.*

Lachrymal duct obstruction is seen in 50% of patients, whereas mammary gland hypoplasia and hypohydrosis occur occasionally (both 13%). Interestingly, almost 40% of patients have hearing impairment and genito-urinary defects. Cleft lip is present in 44% and cleft palate in about 80%. Limb malformations are almost absent. Ectrodactyly has never been reported, but 25% of patients have only mild syndactyly (96). The most of point mutations of AEC syndrome were located in SAM domain of p63 alpha isoforms or TI domains. The SAM domain is known to be involved in protein-protein interactions and therefore mutations in this domain are most could hamper the binding to interacting proteins. One known interactor of p63 SAM domain is the Apobec-1-binding protein-1 (ABBP1), which is a member of RNA processing machinery and known to regulate the alternative splicing of the Fibroblast-growth-factor-receptor-2 (FGFR2) towards the epithelial specific isoform. AEC mutations in the SAM domain abolish the binding to ABBP1, which most probably leads to changes in FGFR2 RNA splicing (98).

The **R**app-**H**odgkin **S**yndrome (**RHS**) show a phenotype very similar to AEC syndrome. The differences discussed earlier in several papers are the absence of ankyloblepharon in RHS and the more severe skin phenotype in AEC. Other ED symptoms, such as orofacial clefting and the near absence of limb malformations are similar to AEC. These two conditions could be considered as a single entity, since the ankyloblepharon is present only in about 45% of AEC syndrome patients, and therefore is not a discriminating factor. Although, the severity of the skin phenotype is obvious and 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 (99). They are either point mutations in the SAM domain or deletions in the SAM or TI domains (100; 101; 102). Frequently, in the same family affected by p63 human syndrome are found variable phenotype. It is clear that variability within the family may be reflecting the presence of modifier genes. At this moment it is unknown but in literature it is known the modifier gene for p53 in human cancer and at least one of these has also been shown to affect p63 protein levels and transcription (103; 104).

1.7 Aims of the thesis.

My research activity, during the last three years, focused on the elucidation of the molecular mechanisms through which p63 exerts its function in primary mouse keratinocytes and in embryonic epidermis. Complementary approaches were used from the identification of p63 specific transcriptional targets and to identify the signaling pathways downstream p63. The study of relationship from p63 and the BMP signaling has identified a new role of p63 in controlling gene expression of non-epidermal genes. In addition, I investigated if this specific regulation occurred also in knock-in mouse model generated in our laboratory. Part of this studies is published in Journal of Biological Chemistry in August 2009. The second part of the study is unpublished and is the objective of my recent research activity.

2. MATERIAL AND METHODS

2.1 Cell cultures, transfections of siRNA.

Mouse primary keratinocytes were isolated from 2-day-old Swiss CD1 mice and cultured under low-Ca^{2+} conditions (0.05 mM) in the presence of 4% Ca^{2+}-chelated fetal bovine serum (Invitrogen, Carlsbad, CA), and epidermal growth factor (Invitrogen). The cells are cultured in 8% CO_2 and 34 °C. A total amount of 200nM siRNA (Stealth siRNA, Invitrogen) for pan-p63, for specific p63 isoforms, and/or for Smad7, Smad1, Smad5, or control medium GC rich siRNA (Stealth siRNA, Invitrogen) were transfected by Lipofectamine 2000 for 5h. After 5h the medium were changed and replacing in medium with low-Ca^{2+} conditions (0.05 mM) in the presence of 4% Ca^{2+}-chelated. In some experiments cells were treated with BMP7 (20ng/ml) (R&D Systems) 24 hrs after transfection. The BMP type I receptor inhibitor LDN-193189 (200 nM) was given to the cells 30' before BMP7 addition.

2.2 Transfections, constructs, reporter assays.

The tansfections were performed 5 days after plating using Lipofectamine 2000 (Invitrogen). siRNA oligonucleotides specific for p63 and relative isoforms were designed by invitrogen tools. Reporter plasmids (250ng) were co-transfected with pCMV2-FLAG-ΔNp63α or pCMV2-FLAG control. A 4.3kb Smad7 promoter (3.6 kb promoter region and 0.7kb 5'UTR upstream) (105) was cloned into the XhoI-HindIII sites in the pGL3 reporter plasmid (Promega). The Smad7 fragment (-3.0/-2.6) was generated by deletion of Smad7 promoter using NheI-PstI and cloned into pGL3-TKLuc reporter plasmid. Mutations in the p63 binding sites were generated using the QuikChange Site-directed mutagenesis kit (Stratagene).
Reporter plasmids (250ng) were co-transfected with 100nM siRNA for pan-p63 (Stealth siRNA, Invitrogen). A 0.5kb p73 promoter (+ 5kb to TSS) was cloned into the KpnI-SacI sites in the pGL3 reporter plasmid (Promega). The p73 fragment (+4.25/+ 5.25) was generated by PCR amplification and cloned into pGL3-TKLuc reporter plasmid. Mutations in the p63 binding sites were generated using the QuikChange Site-directed mutagenesis kit (Stratagene). Luciferase activity was determined 48 hours (hrs) after transfection with the dual-luciferase reporter assay kit (Promega). pCMV-Renilla reporter (20ng; Promega) was used to normalize transfection efficiency.

2.3 Retroviral production.

High titer retrovirus production was obtained in HEK-293T cells by transient transfection of the pBABE-Smad7 (105) using Lipofectamine 2000 as previously described. Primary keratinocytes were infected twice with the retrovirus 24 and 48h after plating in the presence of 8μg/mL polybrene (Sigma). After 48hrs infected keratinocytes were selected with 2μg/ml puromycin for 48 hrs, and grown after selection for an additional 24 hrs in the absence of puromycin.

2.4 Analysis of gene expression, Real-time RT-PCR.

Total RNA was extracted 48 hrs after transfection from primary mouse keratinocytes using TRIzol reagent (Invitrogen), and from mouse embryonic skin (E14.5) using RNAspin Mini RNA isolation kit (GE Healthcare) according to the manufacturer's instruction. RNA samples were treated with RNase-free DNase I (Promega), and cDNA was synthesized using SuperScript Vilo (Invitrogen). Two-step real-time reverse transcription RT-PCR was performed using the SYBR Green PCR master mix in an ABI PRISM 7500 (Applied Biosystems). Levels of the target genes were quantified using specific oligonucleotide primers and normalized for Gapdh or Act-b expression.

For microarray analysis, total RNA was extracted 48h after transfection and sent to the Boston University Microarray Facility for labeling, amplification, and hybridization to the Affymetrix Mouse Genome 430A 2.0 microarrays. Raw probe intensities for each of the hybridized microarrays were normalized to gene expression levels using the dChip algorithm. We then computed the *P*-values and false discovery rate (FDR). To identify differentially regulated genes in the siRNA experiment, we used Cyber-T (Baldi and Long 2001; http://cybert.microarray.ics.uci.edu). Cyber-T estimates experiment-wide false positive and negative levels based on the modeling of *P*-value distributions by computing the posterior probability density estimate (PPDE). We selected 1920 genes with PPDE $\geq$ 0.9900 and 719 with a PPDE $\geq$ 0.9999. The use of a different threshold is suggested for a small number of replicates, typical for microarrays (106). Among the genes affected by loss of p63, 106 genes were upregulated more than 5-fold by p63 knockdown (Table 2). Tissue expression profiling of upregulated genes was obtained from a custom made mouse GNF1M (MAS5) GNF gene expression database (http://symatlas.gnf.org) (107). Among 72 genes that were upregulated

more than 5-fold by p63 knockdown (FDR<0.25), 48 (67%) were not expressed in normal epidermis, but rather in other tissues.

2.5 ChIP assay.

Approximately 3 x10^6 mouse keratinocytes were fixed with 1% formaldehyde in growth medium at 37 °C for 10 minutes. Extracts were extensively sonicated on ice to obtain DNA fragments ranging from 200 to 800 bp in length. Chromatin was immunoprecipitated following the Upstate protocol (http://www.upstate.com). Immunoprecipitation was performed using anti-p63 (H-137; Santa Cruz Biotechnology) and anti-ERK-1 (K23; Santa Cruz Biotechnology) antibodies. Real-time PCR was performed using the SYBR Green PCR master mix in an ABI PRISM 7000 (Applied Biosystems), using specific oligonucleotide sequences.

2.6 Immunostaining, immunoblotting.

Embryos were fixed in 4% paraformaldheide and either embedded in OCT (Sakura) or in paraffin. Fluorescent signals were monitored under a Zeiss confocal microscope LSM510meta using a Zeiss EC Plan-Neofluar 63X/1.3 oil immersion objective. For immunoblotting cells were lysed in sample buffer or in 1% Triton X-100 lysis buffer (10 mM Tris-HCl pH7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA) for Smad7, and protein extracts were run on SDS-PAGE gels, transfer on Immobilon-P transfer membranes (Millipore), probed with the indicated antibodies and detected by chemiluminescence (ECL, GE Healthcare Life Sciences). The following primary antibodies were used for immunofluorescence staining: p63 (4A4, Santa Cruz Biotechnology), Krt8 (Troma-1 rat monoclonal antibody developed by Rolf Kemler, obtained from the Developmental Studies Hybridoma Bank at The University of Iowa), Cldn7 (34-9100, Invitrogen), Cldn3 (34-1700, Invitrogen), Cdh1 (610181, BD Biosciences) for paraffin-embedded tissue, Cdh1 (13-1900, Invitrogen) for frozen-embedded tissue, phospho-Smad1/5/8 (9511, Cell Signaling), Smad1 (sc-7965, Santa Cruz), phospho-Smad2 (3108, Cell Signaling), Smad2 (3103, Cell Signaling). Alexa Fluor ® secondary antibodies (Invitrogen) were used for detection. The following primary antibodies were used for immunoblotting analysis in addition to the ones listed above: Smad7 (MAB2029, R&D

Systems), polyclonal anti-Bex1 (a gift from Frank Margolis), ERK-1/2 (K-23, Santa Cruz Biotechnology), ß-actin (AC-15 Sigma). The following secondary antibodies were used for immunofluorescence staining: Alexa Fluor ® 488 goat anti-mouse (Invitrogen), Alexa Fluor ® 594 goat anti-rabbit (Invitrogen), Alexa Fluor ® 594 goat anti-rat (Invitrogen).

2.7 *In situ* hybridization.

In situ hybridization was performed on frozen sections of P1 skin and E14.5 embryos as previously described skin was fixed in 4% fresh paraformaldehyde overnight at 4°C, washed in PBS, incubated in 30% sucrose/PBS overnight at 4°C and embedded in OCT compound (Sakura). Sections of 7 μm were treated with proteinase-K 20 μg/ml for 15 min and hybridized with DIG-riboprobe in 50% formamide overnight at 60°C. After extensive washings, slides were incubated with anti-DIG-alkaline phosphatase antibodies (Roche), washed and incubated in NBT/BCIP solution (Roche), 1 mM levamisole (Sigma) for 6–12 h. Digoxigenin (DIG)-labeled antisense and sense RNA probes were transcribed from the SP6 and T7 promoters using a DIG labeling kit (Roche Applied Science) according to manufacturer's instructions. The Smad7 probe corresponding to a 485bp cDNA fragment was kindly provided by Xiao-Jing Wang (Han, 2006). The Bmp6 probe was synthesized from a 893bp SacI-EcorI cDNA fragment kindly provided by Dr. M. Mikkola. The Bmp7 probe corresponds to a 440bp long cDNA fragment generated by PCR and inserted in the pCR®II vector vector (Invitrogen). For PCR oligonucleotide sequences see Table S2. DIG labeling was monitored under a Zeiss Axioskop2 plus microscope using a Zeiss Plan-Neofluar 20X/0.50 objective.

2.8 Mice and Skin Explants.

All experiments performed with mice were conducted under IACUC approval. p63-null mice (B6.129S7-Trp63^{tm1Brd}/J) were obtained from the Jackson Laboratory. For skin explants, dorsal skins from embryos at E14.5 were laid on culture plate insert (Millipore) and cultured in the presence or in the absence of BMP7 in DMEM with or without 5% FBS, overnight at 37 °C and 5% CO_2.

2.9 Oligo sequences for gene expression analysis, cloning, siRNA sequences.

Oligonucleotide Primers for Real Time RT-PCR

Gapdh
GTATGACTCCACTCACGGCAAA
TTCCCATTCTCGGCCTTG

Actinβ
CTAAGGCCAACCGTGAAAAGAT
GCCTGGATGGCTACGTACATG

p63
CATGAGCTGAGCCGTGAGTTC
GGCTGTTCCCTTCTACTCGAA

Krt14
TGACGTCTCCACCCACCTG
ACCACGAGGAGGAAATGGC

Krt8
TGCTCATGTTCTGCATCCCA
GATCACCACCTACCGCAAGC

Cldn7
ACAGGAGCAAGAGAGCAGGG
CTGCCATCTTTATCGGCTGG

Tmprss2
CCTACATACAGACTAAATGTGCAA
GAAATAACCAACCAACAGCAAAGA

Bex1
TGACCACCATGATGAGTTTTGC
TCCCCATGTCATCTTCAGAGAA

Smad6
GGCTGTCTCCTCCTGACCAGTA
CAATGTAGAATCGGACAGATCCAG

Smad7
GAAGGTGGTGCCCACTTTCA
AACGAGAGTCAGCACTGCCA

Bmp7
CATCGTCCAGACACTGGTTCA
AGCAGGGCTTGGGTACTGTG

Bmp2
GGCCGTTTTCCCACTCATCT
CCATCACGAAGAAGCCGTG

Bmp4
TGAGGAGTTTCCATCACGAAGAA
CACTGGTCCCTGGGATGTTC

Bmp6
TGTCCAACAAAAATAGGTCAGAGT
AAGTCTTGCAGGAGCATCAGC

Lce1g
TGGTACAGGAGGAGAACACGC
GAGGGAAGCAGGAGGAGAGG

Lce1d
TCGTCTTGCTCCAGAGCACTCACC
GGAGGCTGGCACTGCTGTTGG

Lce1a2
GTTCTGCTGGCAGGACATCT
GCCCAAGGATCTTGTACTGC

Id1
GAGCAGCAGGTGAACGTCCT
TCCTTGAGGCGTGAGTAGCA

Id2
TGATGCAGGCTGACGATAGTG
TCTTGGACCTGCAGATCGC

Smad1
ATTGAAAACACCAGGCGACATA
CCAACGTAATAAAGGTGGACTCCT

Smad5
ACTATTGAAAACACTAGGCGGCATA
CACCTCCCCACCAACGTAGTA

Oligonucleotide Primers for ChIP

Smad7 -0.2Kb
GCGAAACACAATCGCTTTTTT
CGTCACGTGGCCGTCTAGA

Smad7 -2.7Kb
ATCTGTTTTTACCCGGGCCT
CGTGAGTGGTGCTAATCCCCT

Smad7 -3.1Kb
GTGAGGCGAAAGAAGAGCCC
GCTCTGACTGGCTTGTATGCC

siRNA oligonucleotides

Smad7: AGTCAAGAGGCTGTGTTGCTGTGAA
Smad7_2: CCCATCACCTTAGTCGACTCTGTGA
Smad1_a: CATATTGGGAAAGGAGTCCACCTTT
Smad1_b:CAATCCTATTTCATCCGTGTCTTAA
Smad5_a: CAGAGATGTTCAGCCTGTCGCCTAT
Smad5_b: ATTCATAGTAGACAATCGAACACCA

Cloning of Bmp7 probe for in situ hybridization:
TCTTCCACCCTCGATACCAC
CCGGATACTACGGAGATGGA

Cloning of p73 promoter region +4.75/+5.25
AGCTTGGTACCTTGCCTGGGGTTAGTGACTG
AGCTTGAGCTCAGACAACTCGCCTTTGCTGT

3. RESULTS

3.1 p63: crucial regulator of gene expression in epidermal cells.

To shed light on the role of p63 in controlling gene expression in epidermal cells, our laboratory previously performed a global gene expression profile analysis identifying new putative targets of p63 (108). As a model system, we used primary mouse keratinocytes isolated from 2-day-old Swiss CD1 mice where p63 expression was suppressed by specific double strand small interfering RNA (siRNA) oligonucleotide targeting p63. Using this approach, we identified members of the TGF-β family and their regulators that were affected by p63 knockdown (Table I). Some members of the TGF-β were varied in p63 knockdown cells but we focalized our attention on Bmp7 a member of BMP signaling and its negative regulator Smad7. These two genes were inhibited and induced respectively by p63 knockdown. So, we selected them for further investigation, as potential p63 targets genes. Other members of TGF-β family changed their expression in primary mouse keratinocytes, however these variations did not reflect difference in TGF-β signaling activity as described below.

3.2 p63 directly controls Bmp7 expression in epidermal cells.

We used siRNA oligonucleotides targeting the DNA-binding domain of p63 to knockdown p63 expression. The DNA-binding domain is conserved in all p63 isoforms and thus represented an ideal region for knockdown siRNA strategy (Figure 2A). Loss of p63 affected gene expression of TGF-β family members in epidermal cells. We focalized our attention on Bmp7 a members of BMP signaling and its negative regulator Smad7. These two genes were inhibited and induced respectively by p63 knockdown from gene expression profiling in p63 knockdown keratinocytes. Forty-eight hours after siRNA transfection we analyzed Bmp7 expression in p63 knockdown cells by real time RT-PCR. As showed in Figure 9A, Bmp7 was the most highly expressed ligand of the BMP family in keratinocytes and was inhibited by p63 knockdown. Specific knockdown of TAp63 and p63γ isoforms had no effect on Bmp7 expression whereas specific knockdown of ΔNp63 and p63α isoforms inhibited Bmp7 expression, demonstrating a specific regulation operating by the ΔNp63α isoform in epidermal cells (Figure 9B). To confirm this data *in vivo,* we isolated *p63 null*

skin at E14.5 and measured by real time RT-PCR the expression levels of Bmp family members. *p63 null* skin displayed lower levels of Bmp7 than wild-type controls (Figure 9C) accordingly with *in vitro* data. In contrast, loss of p63 had little or no effect on Bmp2 and Bmp6 either in isolated keratinocytes or in embryonic skin (Figure 9A and 9C). Bmp6 was modestly induced by p63 knockdown. However, Bmp6 expression was very low under non-differentiating conditions, consistent with its suprabasal expression in newborn skin (Figure 10A) and in the developing murine epidermis (Lyons. K.M. et al Genes & development 1989, Wall, N. A., JCB 1993). Bmp4, which is mainly expressed in the mesenchimal component of the skin, was unaffected by p63 knockdown in cultured keratinocytes, whereas it was reduced in *p63 null* skin, suggesting that p63 may also indirectly control Bmp4 expression in the adjacent dermis.

By *in situ* hybridization, we detected Bmp7 mRNA levels in the basal layers of newborn epidermis and in embryonic epidermis of *p63 null* skin and control littermate at E14.5 (Figure 10A and 10B). Bmp7 partially co-localized with p63 mRNA in newborn epidermis while their expression overlapped during embryogenesis suggesting a putative common function of p63 and Bmp7 in skin development.

To determine if p63 is involved in control of Bmp7 we analyzed the Bmp7 gene expression at early times point after p63 knockdown. Upon siRNA transfection, p63 protein levels decreased quickly with a complete suppression at 8hrs. In parallel, strong reduction of Bmp7 occurred at 8 hrs, suggesting a putative direct regulation of Bmp7 by p63 (Figure 11A and 11B). To explore the possibility that Bmp7 may be a transcriptional target of p63, we performed a ChIP assay in primary mouse keratinocytes. We identified three phylogenetically conserved p63-binding hemi-sites located in the Bmp7 first intron at +2.4kb from the TSS (Figure 11C , right panel), in a region corresponding to a recently identified human genomic region bound by all p53 family members in a breast cancer cell line (109) (Figure 11C, left panel). Taken together these data showed that p63 directly controls Bmp7 in epidermal cells by direct binding to a specific consensus sequence located in a genomic region downstream transcription start site TSS.

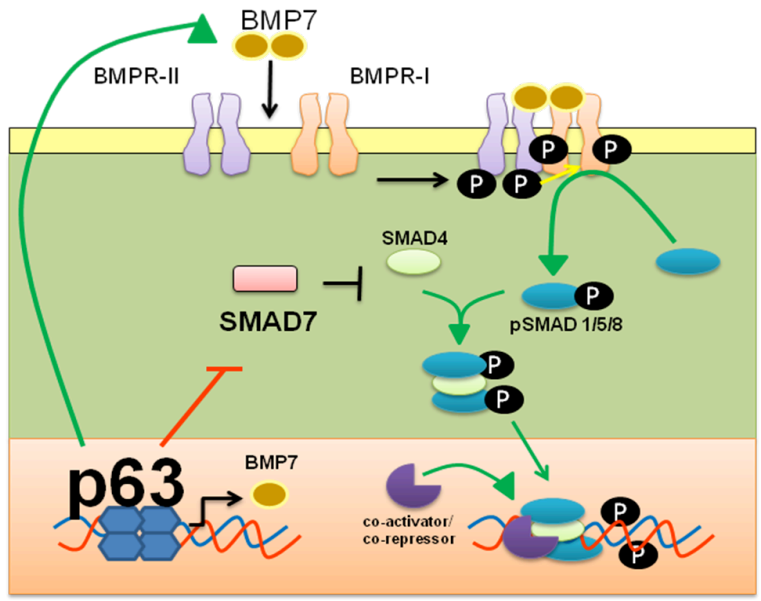
3.3 p63 directly controls Smad7 expression in epidermal cells.

p63 knockdown keratinocytes showed the increase of Smad7 mRNA levels 48hrs after transfection as well as at protein levels, without affecting the related inhibitory Smad6 (Figure 12A and 12C). Specific knockdown of ΔNp63 and p63α isoforms induced Smad7 expression, whereas knockdown of TAp63 and p63γ isoforms had no effect on Smad7 expression (Figure 12B), suggesting that the ΔNp63α isoform is required for Smad7 repression. As demonstrated by real time RT-PCR Smad7 expression was higher in p63-null skin at E14.5 than in wild-type controls, confirming that p63 exerted its effect also *in vivo* (Figure 12D). In line with these data, *in situ* hybridization analysis demonstrated that higher Smad7 mRNA levels are found in *p63 null* skin than in wild-type skin (Figure 12E).

To determine if p63 is involved in control of Smad7, we analyzed the Smad7 gene expression at early times point after p63 knockdown. Smad7 was strongly reduced at 8h after p63 siRNA transfection, suggesting that Smad7 could be a direct target gene of p63 (Figure 11A and 13A). To test this hypothesis, we searched for p63-binding site in a 48.5kb genomic sequence covering the Smad7 locus. Using chromatin immunoprecipitation- (ChIP-on-chip), we identified a single putative p63-binding region located at -2.7kb from the TSS. The p63-binding region was found in an evolutionary conserved genomic sequence containing four canonical p63 binding hemi-sites (Figure 13B). ChIP followed by real time PCR with two independent sets of oligonucleotides confirmed that p63 specifically bound this genomic region (Figure 13C). In addition, p63 overexpression significantly inhibited the activity of a 3.6kb *Smad7* promoter, as well as the activity of a 0.4kb fragment containing the p63 binding sites (Fig. 13D, left panel). Conversely, p63 knockdown resulted in enhancement of the activity both of the Smad7 promoter and of the portion containing the p63 binding sites (Fig. 13D, right panel). Mutations in three canonical p63 binding hemi-sites in the promoter fragment failed the ability of p63 knockdown to enhance promoter activity. Thus we demonstrated the direct regulation of p63 on Bmp7 and Smad7 two signaling molecules belonging to the TGF-β/BMP pathway.

3.4 p63 positively controls BMP signaling.

As described before p63 directly regulates Bmp7 and Smad7 expression in epidermal cells. Bmp7 is the most highly expressed member of the BMP family in keratinocytes. On the other hand, inhibitory Smad7 is expressed at low levels. In the absence of p63 we observed a reduction of Bmp7 and an increase of Smad7 expression (Schema I).



Schema I: p63 directly regulates Bmp7 and Smad7 expression. This regulation determinates the activation of the pathway.

As Smad7 is a common regulator of BMP signaling as well as TGF-ß signaling, we investigated if p63 displayed a specific effects on BMP signaling pathway. To this aim we co-transfected different amounts of a ΔNp63α expressing vector with BMP (BRE) or TGF-ß (CAGA and 3TP) responsive reporters in primary mouse keratinocytes. As shown in Figure 14A, p63 resulted in a dose-dependent induction of the BMP responsive element, without affecting the TGF-ß one. Conversely, p63 knockdown resulted in specific inhibition of the BMP responsive element without affecting the TGB-ß responsive elements (Figure 14B). Expression of two well-characterized BMP targets genes, Id1 and Id2, were strongly inhibited by p63 knockdown (Figure 14C). In parallel, p63 knockdown resulted in a significant reduction in Smad1/5/8 phosphorylation under basal conditions and upon BMP7 treatment (Figure 14D). In contrast, p63 knockdown had no effect on Smad2 phosphorylation, either under basal conditions or upon TGF-ß1 stimulation (Figure 14D and data

not shown). To assess whether p63 regulates BMP signaling during embryonic skin development, we measured Smad1/5/8 phosphorylation in *p63 null* and in wild-type counterpart. Immunoblotting analysis and immunofluorescence staining using anti phospho-Smad1/5/8 revealed strong activation of BMP signaling in embryonic epidermis of wild-type epidermis, whereas decrease signal was showed in p63-null epidermis (Figure 14E and 14F). In contrast, Smad2 phosphorylation was similar in wild-type and in *p63 null* skin at this embryonic stage (Fig. 14D). Taken together, these data indicate that p63 positively regulates the activity of BMP signaling in primary mouse keratinocytes and in embryonic epidermis, without significantly affecting TGF-ß signaling.

3.5 Loss of p63 induces the expression of non-epidermal genes in primary keratinocytes and in embryonic epidermis.

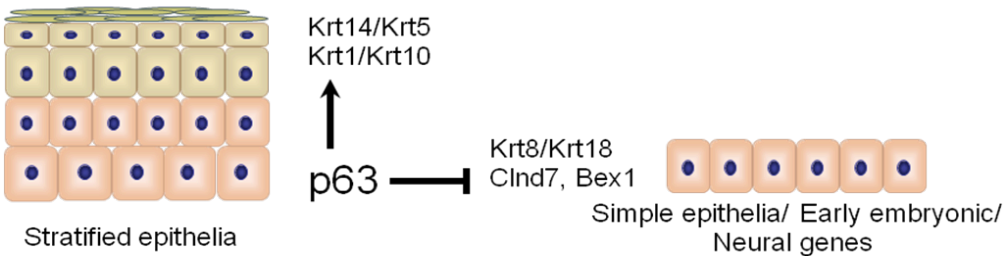
In parallel with a regulation operating by p63 on BMP signaling, gene expression profiling reveled that large set of genes are induced by loss of p63. These genes are preferentially expressed in other tissues (non-epidermal genes), including the previously reported Krt8 and Krt18 (110; 111), and were highly expressed in early development, simple epithelia, in neural tissues (Table II) (Schema II).

Epidermal genes (stratified epithelial keratins)

Gene Symbol	p63KD/ctr
Krt1	0.3
Krt10	0.3
Krt6b	0.3
Krt14	0.5

Non-epidermal (simple epithelial keratins)

Gene Symbol	p63KD/ctr
Krt8	51.1
Krt18	17.0
Cldn3	17.5
Cldn7	7.4



Schema II. Non–epidermal genes are induced in p63 knockdown epidermal cells, whereas in physiological condition p63 represses the expression of these genes.

Aberrant expression of non-epidermal genes were confirmed in several independent sets of primary mouse keratinocytes in the absence of p63 at the RNA and protein levels (Figure 15A and 15B). Specific knockdown of the ΔNp63 and the p63α isoforms strongly induced the expression of these proteins, indicating the main role exerted by ΔNp63α in controlling non-epidermal genes expression in keratinocytes. Interestingly, we found that the induction of non-epidermal genes were related with the reduction of BMP activity, measured by the amount of p-Smad1,5,8 phosphorylation, in p63 knockdown cells (Figure 15B). Given that, we analyzed the expression of non-epidermal genes in total embryonic skin of p63-null embryos and their wild-type counterparts. Non-epidermal genes were strongly induced in embryonic epidermis of p63-null mice both at the RNA and protein levels at E13.5 and E14.5 suggesting that their aberrant expression is an early event in embryonic skin lacking p63 (Figure 15C and 15D). Krt14 and Krt1, specific keratin-markers expressed respectively in the basal layer and in spinous layer of the epidermis, were down-regulated in p63 null skin consistent with previous reports (Figure 15E and data not shown) (53; 54). A shown in Figure 15E, aberrant expression of non-epidermal proteins in p63-null mice occurred also in snout skin and in of E15.5 mice. The *p63 null* epidermis appeared as a multilayer tissue defective of proper differentiation markers. Non-epidermal genes expression after E16.5 is extremely difficult due to the detachment of epidermis from dermis in p63 null skin. Taken together these data indicated that p63 is required to suppress several non-epidermal genes during embryonic skin development and in postnatal keratinocytes. The aberrant expression of non-epidermal genes was related with decrease of BMP activity.

3.6 p63 controls non-epidermal genes through BMP signaling activation.

To test whether p63 directly controlled non-epidermal genes expression in epidermal cells, we analyzed the effects of p63 knockdown at early times after siRNA transfection. We measured the expression of several non-epidermal genes in p63 knockdown versus control cells. The p63 expression is already downregulated at 8h after siRNA transfection (Figure 11A), as its direct targets, Bmp7 and Smad7 (Figure 11B and 11B and 12A).On the other hand, the non-epidermal genes showed a different gene expression profile. They were first induced at 24hrs and strong accumulation was observed at 48hrs (Figure 16A). Thus, p63 could regulate non-epidermal gens through indirect mechanisms that might require the activation or repression of other pathways. As

shown in Figure 15B, non-epidermal gene induction in p63 knockdown cells were related with a decrease of BMP signaling, therefore we hypothesized that this event was a functional linked in epidermal cells.

BMP signaling plays crucial roles in cell fate determination of ectodermal cells during early development in lower vertebrate by suppressing alternative fate (23). Moreover, the main role of BMP signaling in promoting epidermal and suppressing neural fate has been shown also in ES cells (94;95). For these reasons we hypothesized that p63 could maintain low levels of non-epidermal genes via a BMP-dependent mechanism.

To investigate whether p63 regulated non-epidermal gene expression through BMP signaling dependent mechanism, we re-activated the BMP signaling in p63 knockdown keratinocytes by either Smad7 knockdown and/or by BMP7 treatment. In this way, we could restore physiological levels of non-epidermal genes in p63 knockdown cells.

Smad7 knockdown resulted in a significant re-activation of Smad1/5/8 phosphorylation, indicating the reactivation of BMP signaling in the absence of p63 (Figure 16C). However, no effects was observed on Smad2 phosphorylation accordingly with our previous data (Figure 16C). Interestingly, siRNA targeting Smad7 resulted in strong down-regulation of non-epidermal gene expression in p63 knockdown keratinocytes both at the RNA and protein levels (Fig. 16B and 16C), suggesting that Smad7 depletion counteracted the effect of p63 knockdown on BMP signaling. The same effect was obtained by treating p63 knockdown cells with recombinant BMP7 (Figure 16B and 16C). Concomitant Smad7 knockdown and BMP7 treatment had little additional effect on the expression of non-epidermal genes as compared to each treatment alone, suggesting that these treatments repressed non-epidermal genes through overlapping mechanisms. Neither Smad7 knockdown nor BMP7 treatment affected p63 expression, excluding a feedback loop mechanism.

Intriguingly, we showed a reduction of non-epidermal gene expression by treatment of epidermal cells with SB431542 a selective inhibitor of TGF-ß type I receptors. As showed in Figure 17A, p63 knockdown cells selectively treated with SB431542 displayed reduction of Krt8 expression and concomitant induction of Smad1,5,8.

A concomitant treatment of the cells with SB431542 or BMP7 did not show additional effects, thereby a possible explanation is that the inhibition of TGF-β signaling may result in a release amount of Smad4, a crucial mediator of both BMP and TGF-ß signaling (Figure17A).

To demonstrate that loss of BMP signaling contributes to the expression of non-epidermal genes in p63-null epidermis, skin explants were isolated at E14.5 from *p63*-null and wild-type mice and cultured with or without BMP7. BMP7 treatment significantly down-regulated Krt8 expression in p63-null skin (Figure 15D), consistent with a role of BMP signaling in repressing non-epidermal genes in the embryonic epidermis. To show that the BMP signaling specifically regulates non-epidermal genes in epidermis, we measured the expression of Krt14 in p63-null skin under treatment with or without BMP7. As previously reported, Krt14 is a marker of basal layers of epidermis was down-regulated in p63 null skin. Interestingly, BMP7 treatment was insufficient to rescue loss of Krt14 expression in p63-null skin explants, indicating that BMP7 elicited a selective effect on non-epidermal genes in embryonic epidermis (Figure 15D).

Thus, Using these keratinocytes as a model system and skin explants isolated from p63 null skin, we provide functional evidence for the involvement of BMP signaling as negative control on non-epidermal genes expression downstream of p63.

3.7 Loss of p63 induces late differentiation markers independently from BMP signaling.

As described before, we showed that loss of p63 induced non-epidermal genes in isolated epidermal cells and in embryonic epidermis. p63 knockdown caused also increase levels of several late differentiation markers such as members of the cornified envelope genes family (Lce) (Table II). To address if late differentiation markers were under control of BMP signaling we performed the same experiments made for non-epidermal genes. In p63 knockdown cells we showed that neither Bmp7 treatment nor Smad7 knockdown rescued the effect of p63 knockdown on the differentiation genes (Figure 18), indicating that BMP signaling selectively restored low levels of non-epidermal genes without affecting late differentiation genes. Taken together these and previously results showed that BMP signaling specifically regulates non-epidermal genes expression in keratinocytes, indicating a specific role of BMP signaling in epidermal cells.

3.8 Canonical BMP/Smad signaling regulates the expression of non-epidermal genes.

Our results indicated that p63 positively controls BMP signaling by induction of Bmp7 expression and repression of the inhibitor Smad7. BMP proteins binds their specific receptor and leads to induction of canonical signaling pathway mediated by Smad1/5/8 protein. On the other hand, it has been recently demonstrated that the binding of BMP on its specific receptor leads to activate of non-canonical pathway mediated by MAPK including p38 (112). In addition, recent work has shown that the inhibitory Smad7 plays crucial roles not only by regulating R-Smad but also to regulate independent pathways (75;76).

To further explore if p63 controlled non-epidermal gens by BMP/Smad canonical pathway we firstly measured the expression of Smads in primary mouse keratinocytes. Smad1 and Smad5 were highly expressed in primary mouse keratinocytes whereas Smad8 was absent (data not shown). To investigate the contribution of Smad1 and Smad5 in BMP/Smad canonical pathway we used two different approaches. Firstly, we used a specific small-molecule named LDN- 193189 that is able to block BMP type I receptor kinases selectively inhibiting Smad1 and Smad5 phosphorylation and BMP signaling activity (113). As shown before, the induction of non-epidermal genes due to p63 knockdown was rescued by the addition of BMP7. Co-treatment with BMP7 and LDN-193189 in p63 knockdown keratinocytes could not rescue the expression of non-epidermal genes suggesting that p63 controlled BMP/Smad canonical pathway (Figure 19A). As control of proper LDN-19318 function, we measured the expression levels of BMP downstream targets such as Id1 and Id2 (data not shown). To further demonstrate that the rescue occurred trough the canonical BMP/Smad pathway we designed specific siRNA oligonucleotide targeting Smad1 and Smad5. Specific knockdown of Smad1 or Smad5 alone had no effect on BMP direct targets Id1 and Id2 indicating the redundant role playing by BMP-responsive Smads in epidermal cells, while concomitant Smad1/5 knockdown inhibited the expression of direct targets of BMP signaling (figure 19B and 19D). We then tested if the activation of Smad1/5 by phosphorylation dependent mechanism is required for suppression of non-epidermal genes. Co-silencing of p63 with double knockdown of Smad1/5 in the presence of BMP7 treatment could not rescue the expression of Krt8 and Bex1, two non-epidermal genes (Figure 19C).

Finally, we decided to investigate the contribution of Smad7 on BMP/Smad canonical pathway. We performed double knockdown of Smad1/5 in presence of co-silencing of p63 and Smad7 showing that in this condition the silencing of Smad7 could not rescue the expression of Krt8 and

Bex1(Figure 19D). These data were confirmed also at protein levels as showed in Figure 19E.
Finally, we investigated the BMP/non-canonical pathway and in more details the p38 MAPK. We measured by western blot analysis the phosphorylation of p38 and we observed no effect on p38 phosphorylation in p63 knockdown cells versus wild-type cells (data not shown).
Taken together, these results strongly demonstrate that BMP/Smad canonical pathway is required for suppression of non-epidermal genes downstream of p63.

3.9 Loss of BMP signaling induces the expression of non-epidermal genes in keratinocytes.

Our results showed a crucial role played by p63 in controlling BMP signaling to suppress non-epidermal markers in epidermal cells. As showed BMP signaling plays a key role in our system, so we asked if the reduction by itself of BMP signaling in epidermal cells is necessary and sufficient to induce aberrant expression of non-epidermal genes.
To address this issue, we used two different approaches. First of all, we over-expressed Smad7 via retroviral vector in keratinocytes than we treated the cells with specific inhibitor of BMP signaling LDN-193189. Smad7 plays an inhibitory effect on BMP and on TGF-β signaling. Thus, to investigate the specific effect played by Smad7 in controlling BMP and not TGFβ signaling, we over-expressed Smad7 by retroviral infection in primary mouse keratinocytes. Smad7 overexpression inhibited BRE-Luc activity without affecting CAGA-Luc (Figure 20A), indicating a selective role in repressing BMP signaling in epidermal cells. Interestingly, we showed that overexpression of Smad7 by itself in keratinocytes is sufficient to induce the expression of non epidermal genes although more modestly than p63 knockdown (Figure 20B). These data are in agreement with previous results shown in Figure 19D in which we concluded that Smad7 played a crucial role in controlling BMP/Smad canonical pathway.
As a second approach, we used a selective small-molecule LDN-193189 that blocks BMP type I receptor kinases. As control, we measured the BMP-Luc by luciferase assay in primary mouse keratinocytes transfected with ΔNp63α and treated with LDN-193189 for 24h. The BMP responsive elements was induced by ΔNp63α whereas resulted in a drastic inhibition by LDN-193189 treatment (Figure 20C). On the other hand, keratinocytes treated for 24h with LDN showed the induction of non-epidermal genes although more modestly than p63 knockdown (Figure 20D).

By this approach we concluded that BMP signaling is a crucial negative regulator of non-epidermal genes in keratinocytes. However, in embryonic epidermis Smad7 overexpression (76) or homozygous deletion of *Bmp7* (114), or double homozygous deletion of BMP type II receptor *Bmpr2a*/*Acvr2a* (115) were insufficient to induce non-epidermal genes (data not shown) suggesting that inactivation of BMP signaling is by itself in vivo to inhibit non-epidermal genes.

3.10 Transcriptional targets of AEC mutant p63.

Missense mutation in the SAM domain of p63α isoforms is the main cause AEC syndrome characterized by skin fragility and erosion at birth (96). The induction of non-epidermal markers observed in the absence of p63 could be a cause of skin fragility. To test this hypothesis, we measured gene expression profiling in the epidermis derived from wild-type mice versus AEC mutant mice generated in our laboratory by Dr. Ferone Giustina. Now, she are investigating on the molecular basis of AEC syndrome and she characterized the phenotype of AEC mutant mice.
Surprisingly, we observed that non-epidermal genes were not affected. In addition, most of known direct p63 target genes did not change in AEC mutant epidermis such as Bmp7 and Smad7. Interestingly, in AEC mutant epidermis we identified a small group of genes affected by AEC mutation. Among them we identified the third member of p53 family p73. We confirmed p73 expression in different sets of epidermis isolated from AEC mutant mice and wild-type littermates (Figure 21A). We analyzed the possibility that p73 was directly regulated by p63, by searching p63-binding site in p73 gene. To facilitate this work, we analyzed p63 ChIP-on ChIP data performed in human cells published by Yang A. et al in 2006 to identify putative binding sites (116). Using Chromatin Immunoprecipitation assay (ChIP), we demonstrated that putative p63-binding region is located in intron 1 at +5Kb from TSS of p73 and that effectively p63 bound this region in epidermal cells isolated from AEC mutant mice and control mice (Figure 21B). The p63-binding region was centered on an evolutionary conserved genomic sequence. ChIP followed by real time PCR confirmed that p63 specifically bounds this genomic region (Figure 21C). We cloned the region from +4.75 to +5.25 to TSS of p73 in a luciferase vector (pGL3TK-Luc) and analyzed the activity in vitro. As shown in (Figure 21D), p63 knockdown cells showed a reduction of promoter activity of p73. We mutated two canonical p63 binding sites in the first intron of p73 and we demonstrated that

one of them abolished the ability of p63 knockdown to inhibits promoter activity (Figure 21D). This results will be confirmed in epidermal cell isolated from AEC mutant and control mice.

Taken together this results showed a novel target of p63 that may have a key role in pathogenesis of AEC syndrome. p73 is a member of p53 family and recent work has shown a new role of p63 with p73 in promoting cells survival of embryonic neural precursor cells antagonized p53 effects (117). So we are investigating if p63 together with p73 control a specific gene expression program in epidermal cell to promote cell survival.

4. Discussion

p63 gene, a homologue of the tumor suppressor p53, is crucial for the development and maintenance of squamous epithelia. It is specifically expressed in the basal layers of stratified epithelial tissues and is considered a specific marker for these cell type. p63 played a crucial role in controlling gene expression in epidermal cells. To date, the interaction with other signaling during embryonic skin development is still poorly understood.

Here, for the first time we shed light on a new role of BMP/Smad signaling downstream of p63 in suppressing non-epidermal lineage markers in isolated epidermal cell and in skin embryonic development. We show that lack of p63 in epidermal cells induce the expression of non-epidermal lineage markers as the previously reported Krt8 and Krt18 (57), as well as genes expressed in early stages of embryogenesis, or in neural and simple epithelial tissues. The induction of non-epidermal gene occurred also in vivo in p63 null mice at until E15.5. Non-epidermal gene expression after E16.5 is extremely difficult to assess due to the detachment of the epidermis from the dermis in p63 null skin. In parallel, loss of p63 in squamous cell lines elicits the induction of non-epidermal genes (118), indicating that p63 may be involved in controlling a proper gene expression program in stratified epithelia both in human and in mouse.

In this work, we show that p63 directly controls the expression of Bmp7, a member of TGF-β family in epidermal cells, and we demonstrate that p63 binds a highly genomic conserved region in the Bmp7 first intron. In situ hybridization shows reduced levels of Bmp7 expression in p63 null mice although the mRNA levels of Bmp7 is still detectable, suggesting that Bmp7 is positively controlled in this context also by other transcription factors. On the other hand, we show the inhibitory effect of p63 on Smad7 expression. Smad7 is a negative regulator of TGF-β family and acts on BMP and on TGF-β signaling. Smad7 is a direct target of p63 which bins to a conserved genomic region on Smad7 promoter. Loss of p63 in primary mouse keratinocytes increases the levels of Smad7, therefore the BMP signaling activity is lost in p63 knockdown cells. To reinforce these data, in situ hybridization shows increased mRNA levels of Smad7 in p63 null mice indicating that this misregulation occurred also in vivo.

It has been known that p63 can work as an activator or repressor of transcription depending on the promoter context but the co-activator or/and co-repressor that works with p63 are still unknown. Our work identify two direct targets of p63 Bmp7 and Smad7 and it could be interesting to identify

the protein partners that differentially control Bmp7 and Smad7 expression in epidermal cells in a future studies.

The decrease expression of Bmp7 and the increased levels of Smad7 cause the inhibition of BMP signaling in p63 knockdown cells and in the epidermis of p63 null mice. We show that BMP signaling is reduced in p63 knockdown cells whereas, no effect is observed in TGF-β signaling. In addition, this work strongly demonstrates that Smad7, an inhibitor protein of the TGF-β family, preferentially acts on BMP signaling rather than on TGF-β in epidermal cells.

We identify that the increased levels of non-epidermal genes in primary mouse keratinocytes and in p63 null mice is related to a decreased activity of BMP signaling, thus suggesting a functional association between these phenomena. We confirmed this hypothesis restoring proper expression of non-epidermal genes in p63 knockdown cells cultured in the presence of BMP7. Smad7 knockdown elicits the same effect, indicating that also Smad7 acts on BMP signaling.

BMP signaling plays different function in controlling cell proliferation, differentiation, apoptosis and cell fate decision (66). These functions are exerted by Smad-canonical and/or non-canonical pathways such as trough MAP-kinase activation (71; 77;78). Here, we demonstrate that p63 induces the BMP/Smad canonical pathway in epidermal cells to repress the non-epidermal genes expression. In addition, we demonstrate that Smad7 in primary mouse keratinocytes acts also on BMP/Smad canonical pathway. It has been reported that induction of Smad7 in primary mouse keratinocytes induced the expression of Krt8. This induction is dependent to increased levels of Cripto in cooperation with Smad7 (105). In our system, we do not observe the increase levels of Cripto in p63 knockdown cells, suggesting that Smad7 works independently to induce non-epidermal gene expression. In conclusion , we show that the overexpression of Smad7 or blockage of the BMP signaling can induce the expression of non-epidermal genes in primary mouse keratinocytes, although more modestly than p63 knockdown, suggesting that the non-epidermal genes are under the control of BMP signaling downstream p63.

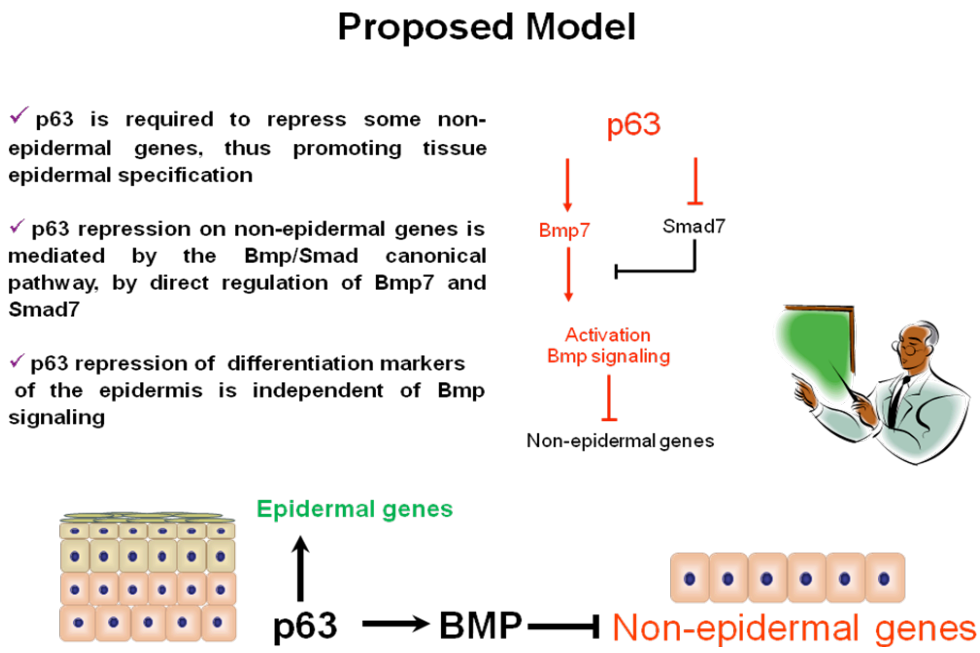
However transgenic mice over-expressing Smad7 (76) or double homozygous knockout of BMP type II receptor Bmpr2a/Acvr2a (115) are insufficient to induce non-epidermal genes, suggesting that lack of an epidermal phenotype in mice carrying a deletion of single components of the pathway may be due to expression least in part to functional redundancy among the components of the signaling. Alternatively, other pathways in vivo may be required for non-epidermal gene expression. Among the BMP receptors, Bmpr1A/ALK3 plays a crucial role in hair follicle

development with little phenotype in the epidermis (119; 120; 121). However, Bmpr1B/ALK6 and Acvr1/ALK2 are also expressed in the embryonic epidermis (122; 123; 124), and Acvr1/ALK2 is the main receptor for BMP7 (125; 126; 127), suggesting that depletion of multiple receptors may be required to observe an epidermal phenotype. Interestingly, it has been reported that Bmp7 null mice showed defects in urethra epithelium and lack of Bmp7 in urethra epithelium is by itself sufficient to induce Krt8 expression (128), suggesting that in the epidermis the expression of non-epidermal genes are controlled by multiple BMP family members or by multiple mechanisms underlying a major complexity of the physiological context of the epidermis.

This work describes a uncovered role of p63 in controlling BMP signaling and for the first time demonstrates a direct correlation with p63 and BMP signaling in epidermal cells. Here, we demonstrate that p63 is crucial transcription factor for specification of epidermal gene expression program. Its absence determinates the aberrant expression of non-epidermal markers that could partially explain the phenotype observed in p63 null mice. The epidermis results as a single layers lacking the expression of stratified epithelia markers and cell-adhesion molecules crucial for the integrity of the tissue (63; 65) and acquired the expression of gene involved in early embryogenesis as Krt8 and Kr18 or genes express in neural or simple epithelial tissues. These aberrant expression could modify the mechanical strength of the epidermis leading skin fragility or to lead an primordial epidermal layer is unable to precede in proper differentiation program activating an incorrect expression program . Loss of p63 causes not only the reduction of Bmp7,but also reduction of Bmp4 (our results). Bmp4 is preferentially expressed in mesenchymal compartment of the skin and has been reported as an important positive regulator of epidermal fate (94; 95). Reduced Bmp4 expression in the absence of p63 could also play a role in the observed induction of in non-epidermal genes. The reduction of BMP4 and BMP7 in the absence of could explain the strong reduction observed in BMP activity during embryogenesis. This work elucidates a new functional correlation between p63 and BMP signaling that could represent an important step during early embryogenesis for appropriate gene expression program.

In addition to BMP signaling control, there might be other mechanisms involved in regulation of non-epidermal genes in epidermis. Recent studies have reported that chromatin remodeling is an additional control of gene expression in epidermal cells . This control represses genes involved in terminal differentiation in the basal layer of the epidermis (129; 130; 131). In our system, we observed the induction of late differentiation markers in the absence of p63. Thus, we hypothesize

that non-epidermal genes as for late differentiation gene may be regulated by different mechanisms due to a double control by BMP signaling and chromatin remodeling downstream of p63. This mechanism is currents unknown and will be a key subject for subsequent investigation. Moreover, our preliminary data show that the inhibition of HDAC with *Trichostatin A* (TSA) induces the expression of non-epidermal genes although more modestly than p63 knockdown (data not shown), suggesting a putative role of chromatin remodeling in controlling non-epidermal genes.



Proposed Model: *p63 Suppresses Non-epidermal Lineage Markers in a Bone Morphogenetic Protein-dependent Manner via Repression of Smad7*

Finally, in our laboratory has been generated a knock-in mouse model for AEC syndrome. This is the first mouse model for AEC syndrome and closely resembles the phenotype of AEC patients. We found that neither non-epidermal genes or Bmp7 and Smad7 changes their expression in AEC mutant mice. Thus, we conclude that mutations in SAM domain affects different functions of p63. Interestingly, we observed that the known targets did not change their expression in AEC mutant mice reinforcing our hypothesis that p63 through SAM domain exerts a selectively function in the epidermis. Here, we found that AEC mutation affects gene expression of a small group of genes. Among them, we identify the p53 family members, p73 that is inhibited in AEC mutant epidermis.

We demonstrate that p73 is a direct targets of p63 and that the L514F mutation of AEC syndrome did not alter the binding to DNA of p63. The ChIP assay demonstrates that p63 efficiently bound the DNA compared to wild-type suggesting that the mutation in SAM domain alters the recruitment of co-activator. A recent work showed a new role of p63 together with p73 in promoting cells survival of embryonic neural precursor cells antagonized p53 effects (117). So we are investigating if p63 and p73 control a specific gene expression program in epidermal cell to promote cell survival and to control specific gene sets in epidermal progenitor.

The AEC mutant mice shows a hypoplastic epidermis. Recent data obtained in our laboratory by Dr. Ferone Giustina demonstrates that hypoplastic phenotype is due to a reduction of epidermal progenitor cells. Thus we hypothesize that p63 together with p73 could regulate the survival of stem cells. This regulation could also involve p73 that with p63 could activate a specific expression program to promote cell survival. The mutations in the SAM domain could create a dominant negative mechanism altering the formation of specific tetramers formed by p63 and p73 and determinates the loss of function.

Taken together these data show an uncovered role of p63 in controlling of non-epidermal genes through BMP signaling and in controlling p73 gene expression in AEC mutant mice. This work elucidates the multiple role of p63 in the epidermis, and reflects the highly complexity of this gene due to different isoforms and to different functions that may be carried out synergistically with others co-activators or co-repressors.

Figure 9

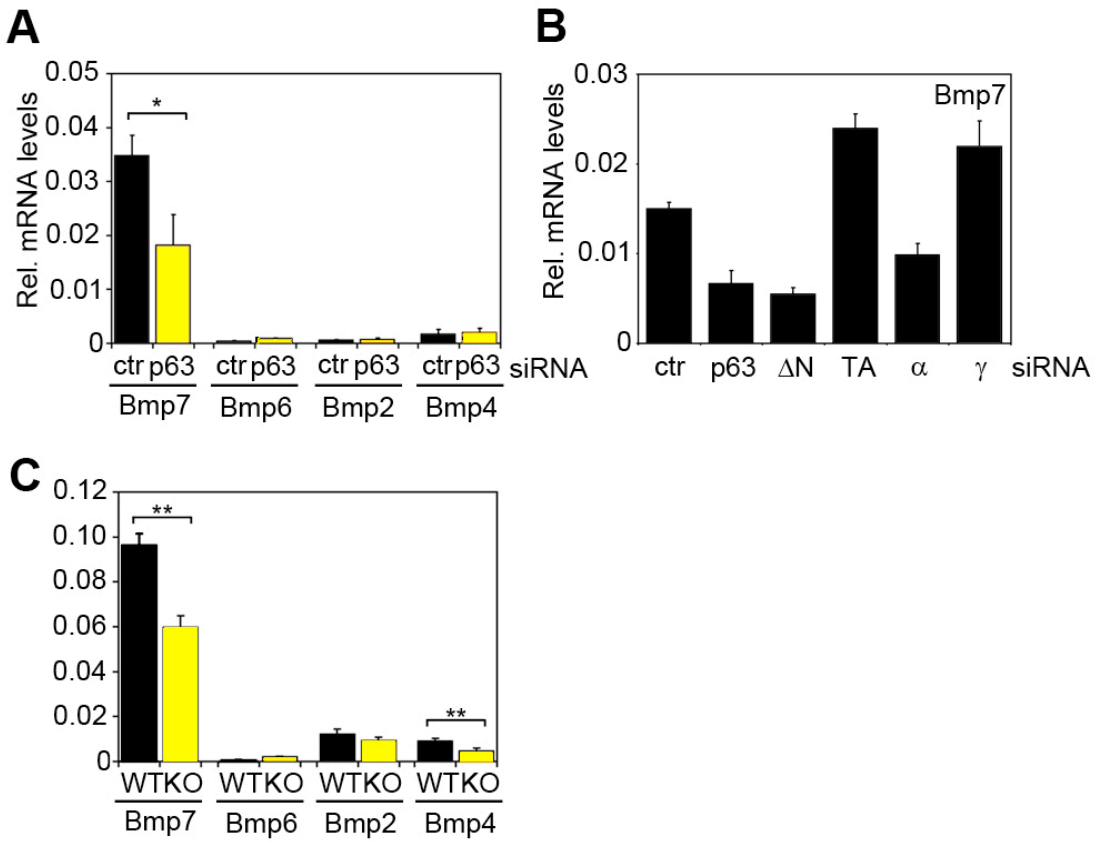


Figure 9. p63 positively regulates Bmp7expression *(A) BMPs expression was measured by Real Time RT-PCR in p63 knockdown keratinocytes transfected with 200nM of siRNA for pan-p63 and control siRNA. Values represent means of independent experiments ± SE (*P<0.05, n=4) and RNA levels were normalized versus Gapdh mRNA levels. Bmp7 is the most abundant BMP family member expressed in keratinocytes whereas low expression is found for BMP6 and BMP2. Bmp4 is mainly expressed in the mesenchimal component of the skin and was unaffected by p63 knockdown. (B) BMP7 expression was measured in primary mouse keratinocytes transfected with siRNA specific for pan-p63, ΔNp63, TAp63, p63α, and p63γ isoforms, or ctr siRNA. RNA levels were normalizedas in (A). Specific knockdown of ΔNp63 or p63α isoform elicited a strong effects on Bmp7 expression whereas no effect was observed with knockdown of TA and gamma p63 isoforms (C) BMPs mRNA levels were measured in p63 null skin and wild-type at E14.5 by Real Time RT-PCR. Bmp7 and Bmp4 are significantly down-regulated in p63 null skin (**P<0.005, n=4 embryos).*

Figure 10

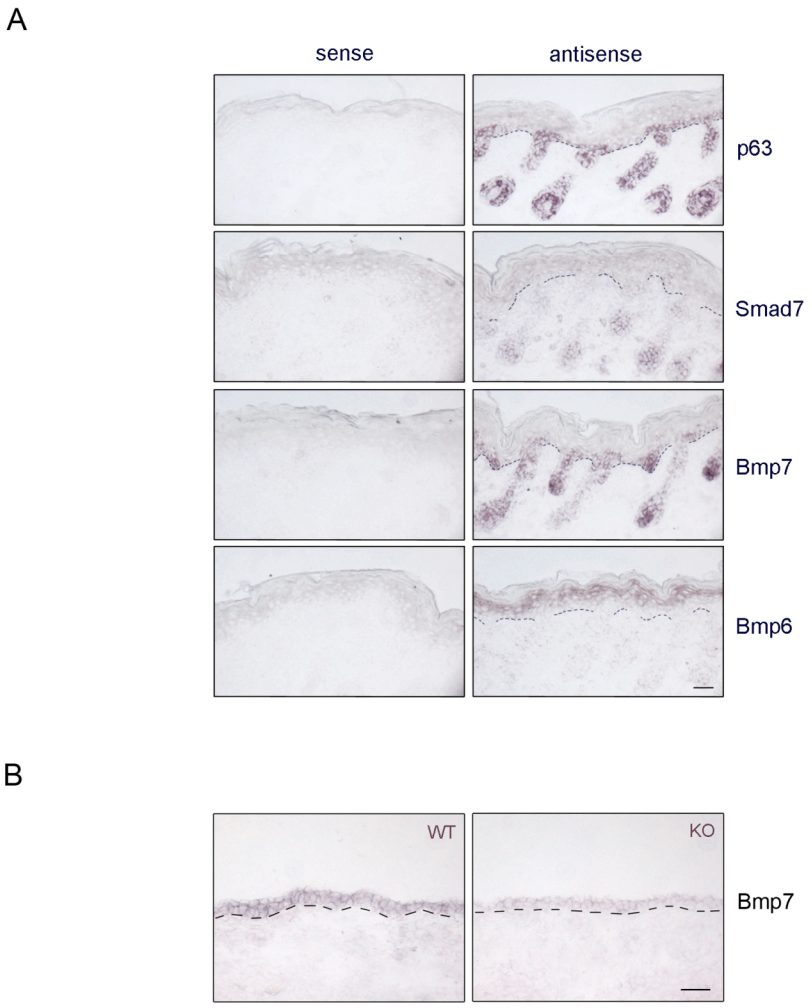


Figure 10. Expression of p63, TGF-β family and their regulator Smad7 in skin *(A) p63, Smad7, Bmp7 and Bmp6 mRNA levels was determined by in situ hybridization in newborn mouse skin at P1. Frozen sections were hybridized with digoxygenin-labeled antisense probes. Corresponding sense RNAs were used as control. The dashed lines indicate the dermal–epidermal junction. Scale bar 60µm. (B) In situ hybridization of Bmp7 mRNA was performed on frozen sections of p63 null (KO) and wild-type (WT) embryos at E14.5. Scale bar 50µM.*

Figure 11

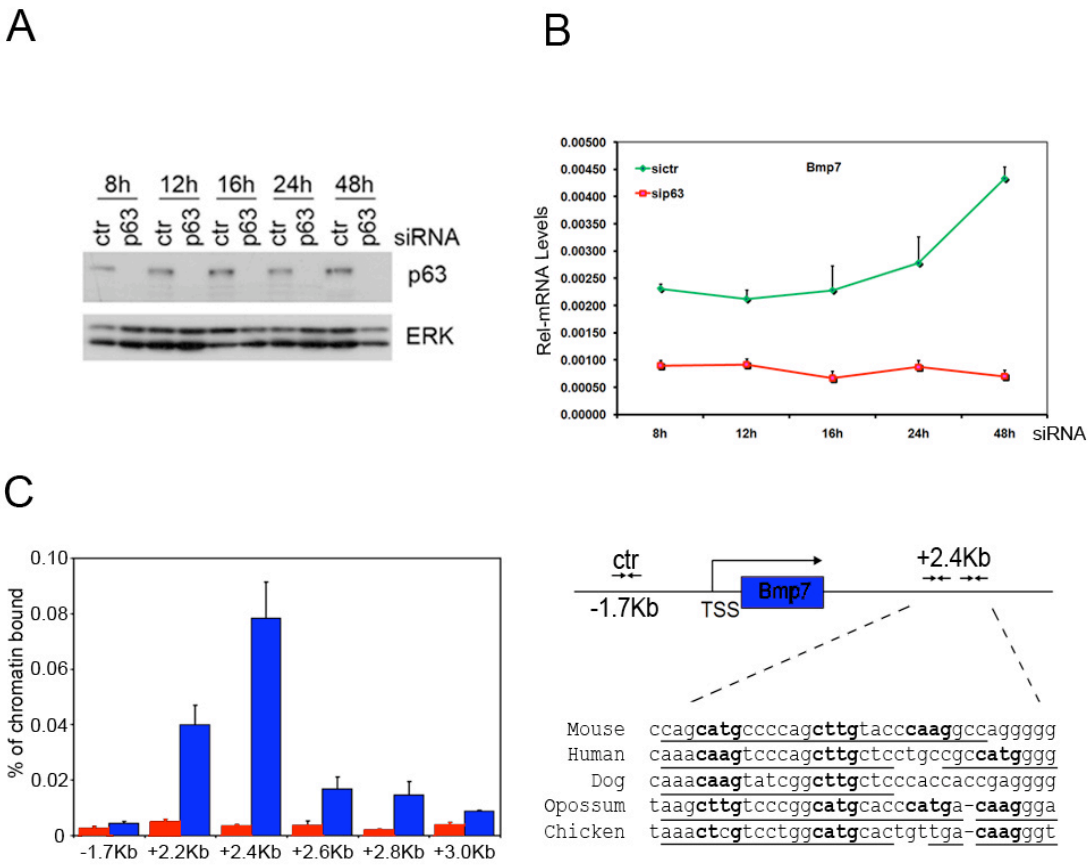


Figure 11. Bmp7 is a direct target of p63 *(A) Knockdown of p63 was performed at early time point using specific siRNA for pan p63 and control siRNA. Protein extracts were collected from 8h to 48h after transfection and were normalized using anti-ERK (ERK) antibodies. (B) Expression of Bmp7 was measured by real time RT-PCR at early time point after siRNA transfection. Red line represented siRNA for p63, green line control siRNA. RNA levels were normalized versus Actin-b mRNA levels.(C) In the right panel, the predicted p63-binding hemi-sites located at +2.4Kb from the Bmp7 TSS are indicated with their nucleotide sequence and phylogenetic conservation in multiple species. Bold nucleotides correspond to the core nucleotide sequence required for p63-binding, while underlined nucleotides are matches in the consensus sequence. ChIP followed by real time PCR was performed on mouse Bmp7 promoter. Primary keratinocytes were processed for ChIP with specific antibodies for p63 (blue bars), or unrelated anti-ERK antibodies as control (red bars), followed by real-time PCR amplification using oligonucleotide primers designed at the indicated position from the TSS. The amount of precipitated DNA was calculated relative to the total input chromatin, and expressed as the percentage of the total DNA.*

Figure 12

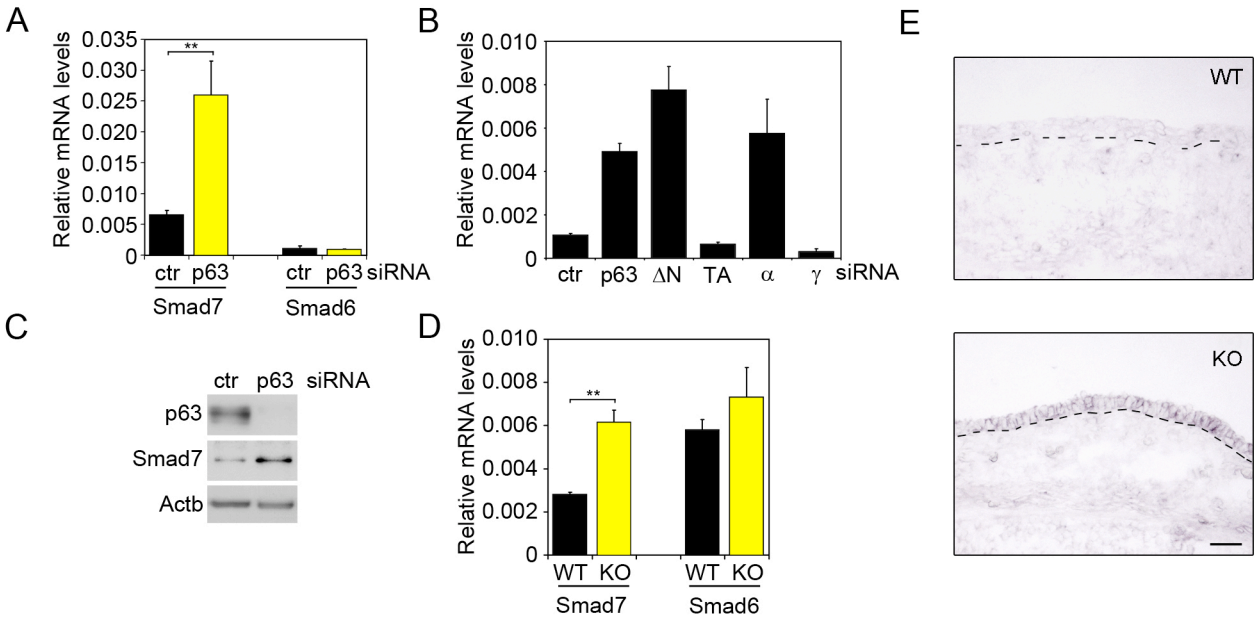


Figure 12.Smad7 is negatively regulates by p63 *(A) Smad7 and Samd6 expression was measured by Real Time RT-PCR in p63 knockdown keratinocytes using 200nM of siRNA for pan-p63 and control siRNA. Values represent means of independent experiments ± SE (*P<0.05, n=4) and RNA levels were normalized versus Gapdh mRNA. Smad7 was strongly induced by lack of p63 in keratinocytes whereas no effects was observed on Smad6 expression. (B) Smad7 expression was measured in primary mouse keratinocytes transfected with siRNA specific for pan-p63, ΔNp63, TAp63, p63α, and p63γ isoforms, or ctr siRNA. RNA levels were normalized as in (A). Specific knockdown of ΔNp63 or p63α isoforms elicited a strong effects on Smad7 expression whereas no effect was observed with knockdown of TA and gamma p63 isoforms (C) Protein extract was collected from p63 knockdown cells to perform western blot analysis. P63 knockdown cells showed the induction of Smad7 at protein levels using specific antibody recognizing Smad7 protein. Protein extracts was normalized using anti-Actinb (Actb) antibody. (D) Smad7 and Sma6 mRNA levels were measured in p63 null skin and wild-type at E14.5 by Real Time RT-PCR. Smad7 was significantly up-regulated in p63 null skin whereas no effects was observed on Smad6 expression (**P<0.005, n=4 embryos).(E) In situ hybridization of Smad7 mRNA was performed on frozen sections of p63 null (KO) and wild-type (WT) embryos at E14.5. P63 null skin showed increased Smad7 mRNA levels than control skin. The dashed lines indicate the dermal–epidermal junction. Scale bar 50μm.*

Figure 13

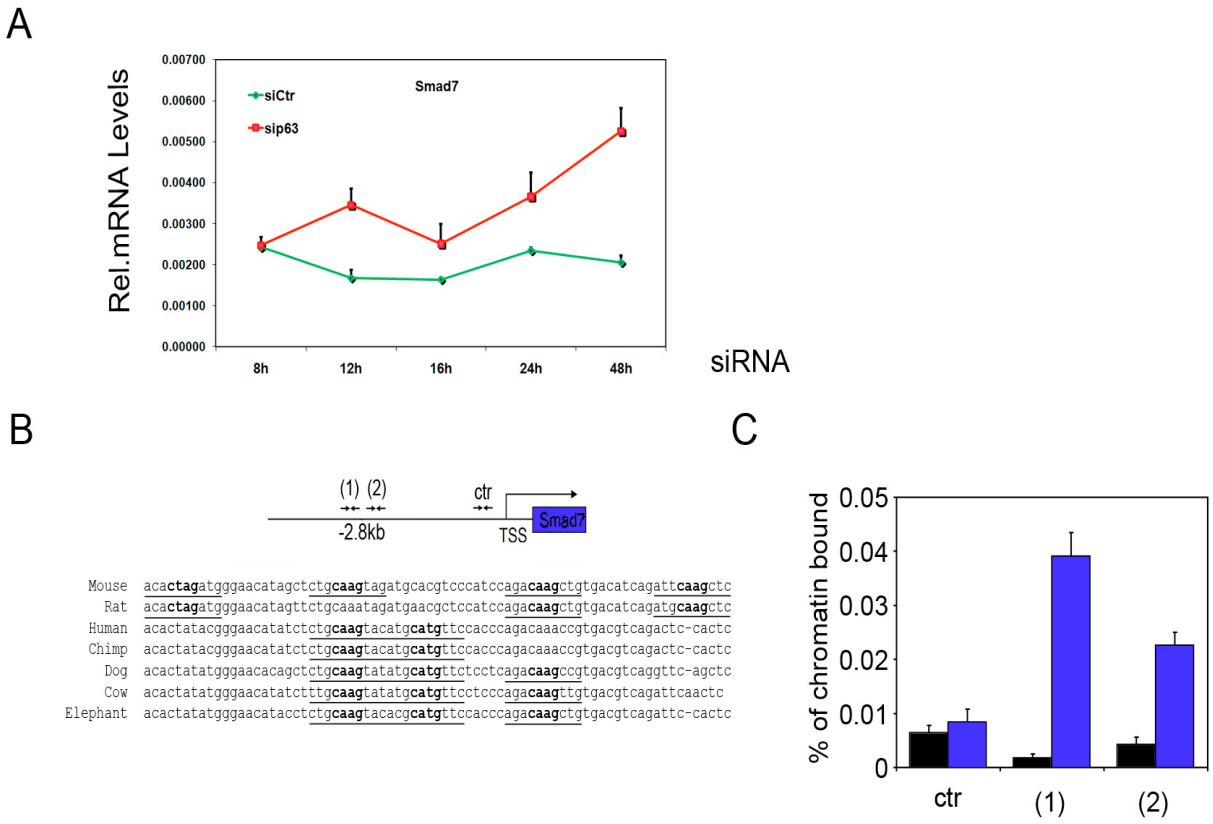


Figure 13. Smad7 is a new target of p63 *(A) The Expression of Smad7 was measured by real time RT-PCR at early time point after siRNA transfection. Red line represented siRNA for p63, green line control siRNA. Values were normalized versus Actin-b mRNA levels.(B) Predicted p63-binding hemi-sites located at -2.8Kb from the Smad7 TSS are indicated with their nucleotide sequence and phylogenetic conservation in multiple species. Bold nucleotides correspond to the core nucleotide sequence required for p63-binding, while underlined nucleotides are matches in the consensus sequence. (C) ChIP followed by real time PCR was performed on mouse Smad7 promoter. Primary keratinocytes were processed for ChIP with specific antibodies for p63 (blue bars), or unrelated anti-ERK antibodies as control (black bars), followed by real-time PCR amplification using two different oligonucleotide primers. The amount of precipitated DNA was calculated as in 10C*

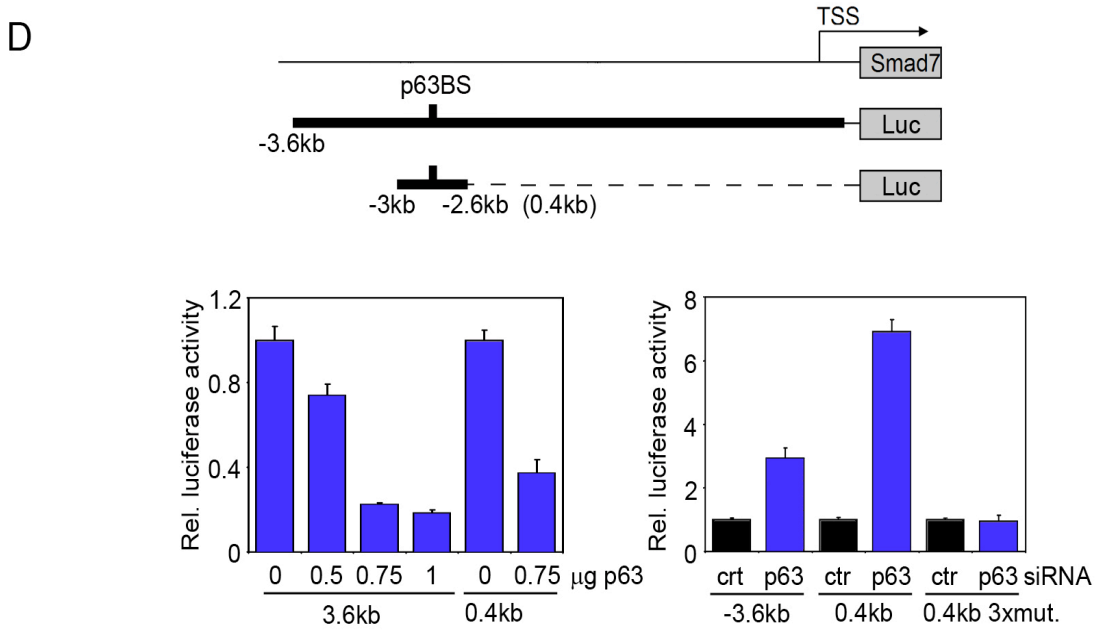


Figure 13. Smad7 is a new target of p63. *(Following up the figure 13)(D)In the upper panel, schema of Smad7 promoter cloned in pGL3TKluc with its deletions indicating the position of p63 binding site. Left panel, the activity of 3.6kb Smad7 promoter was inhibited by p63 overexpression, as well as the activity of a 0.4kb fragment containing the p63 binding sites. Right panel, on the other hand p63 knockdown resulted in enhancement of the activity both of the Smad7 promoter and of the portion containing the p63 binding sites. Mutations in three canonical p63 binding hemi-sites lose the ability of p63 knockdown to enhance promoter activity. Values of luciferase assay are normalized to Renilla luciferase activity and are expressed as fold-changes over the promoter activity in the presence or absence of p63. Values represent mean +/- S.E. of three independent experiment.*

Figure 14

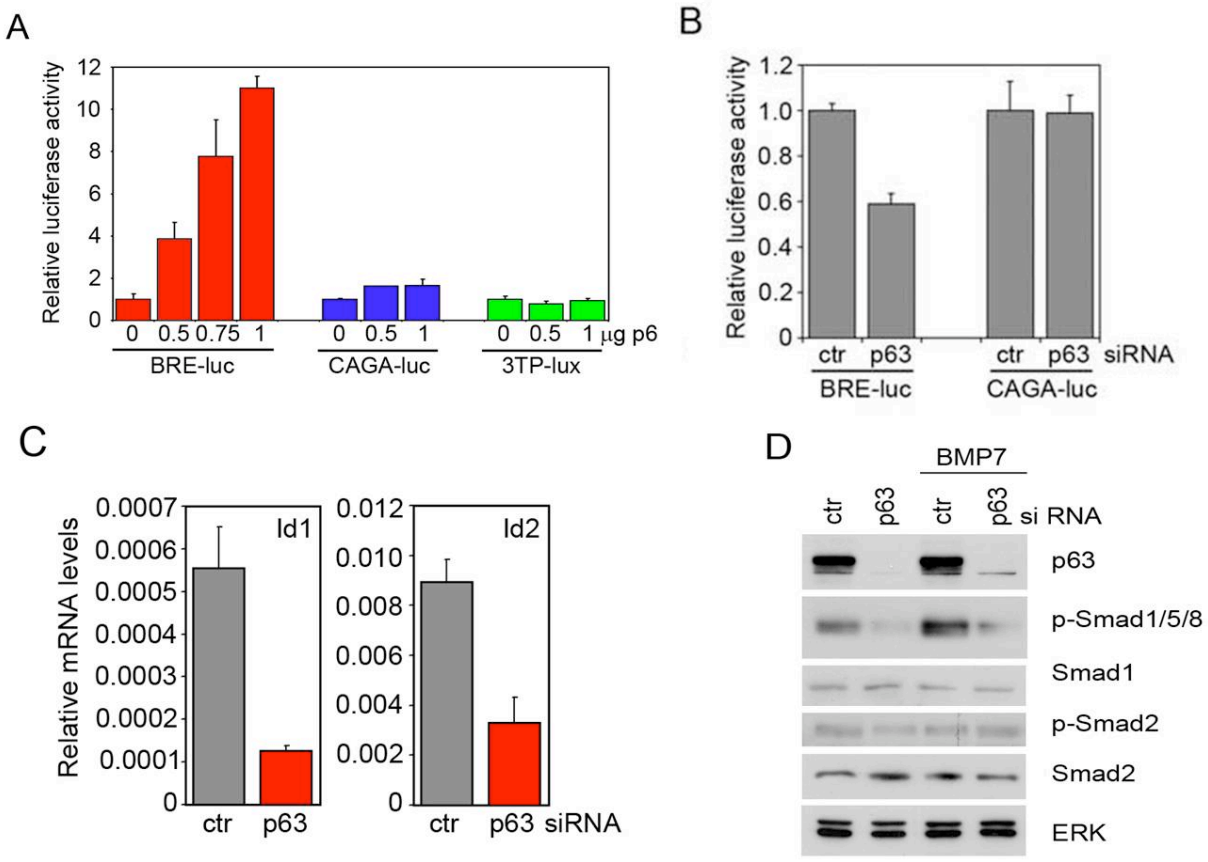


Figure 14. p63 positively regulates the BMP signaling *(A)Primary mouse keratinocytes were co-transfected with BMP reporter vector (BRE-luc) or two different reporters for TGF-ß (CAGA-luc and 3TP-Luc) and increased amount of ΔNp63α and examined 48h after transfection. Values are normalized to Renilla luciferase activity and are expressed as fold-changes over the promoter activity in the presence or absence of p63. Values represent mean +/- S.E. of three independent experiment. The increase amount of p63 displayed a strong induction of BMP reporter vector, whereas no effects was showed in TGF-ß one.(B) BMP and TGF-ß responsive elements were co-transfected with siRNA targeting p63. P63 knockdown keratinocytes displayed decrease of BMP reporter activity, no effect was showed in TGF-ß activity.(C) Id1 and Id2 were a direct targets of BMP signaling. Their expression were measured by real time RT-PCR in primary mouse keratinocytes silenced with specific siRNA targeting p63. Values were normalized versus Actinb mRNA. (D) Immunoblotting of total lysates collected from p63 knockdown keratinocytes transfected with siRNA targeting p63.The specific antibody was used to detect the activity of BMP (pSmad1,5,8) and related total protein (Smad1) and TGF-ß signaling (pSmad2) and total protein extract (Smad2). Cells lysates were normalized using anti-ERK antibody (ERK).*

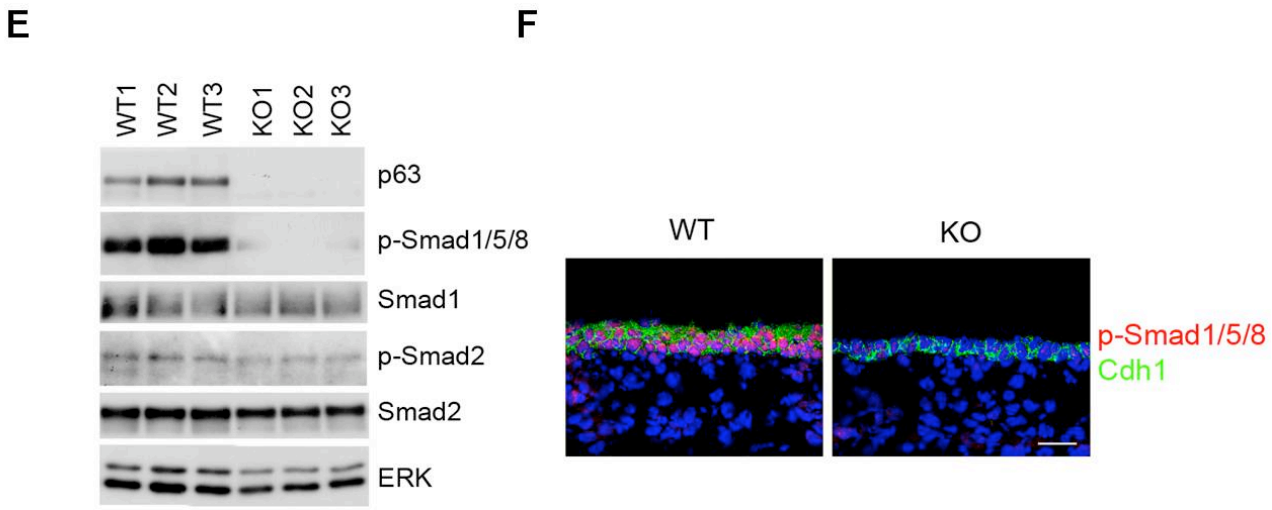


Figure 14. p63 positively regulates the BMP signaling. *(Following up the figure 14) (*E) *Immunoblotting of skin total lysates collected from p63 null mice isolated from E14.5.The specific antibody was used to detect the activity of BMP (pSmad1,5,8) and related total protein (Smad1) and TGF-ß signaling (pSmad2) and total protein extract (Smad2). Cells lysates were normalized using anti-ERK antibody (ERK)*. (F) *Immunofluorescence was performed in p63-null skin at E15.5 with specific antibody recognizing the phosphorilation on Smad1,5,8 in red and E-Chaderin specific marker of epithelia cells in green. The images was captured with confocal microscopy. Nuclei were stained with DAPI (Blue).*

Figure 15

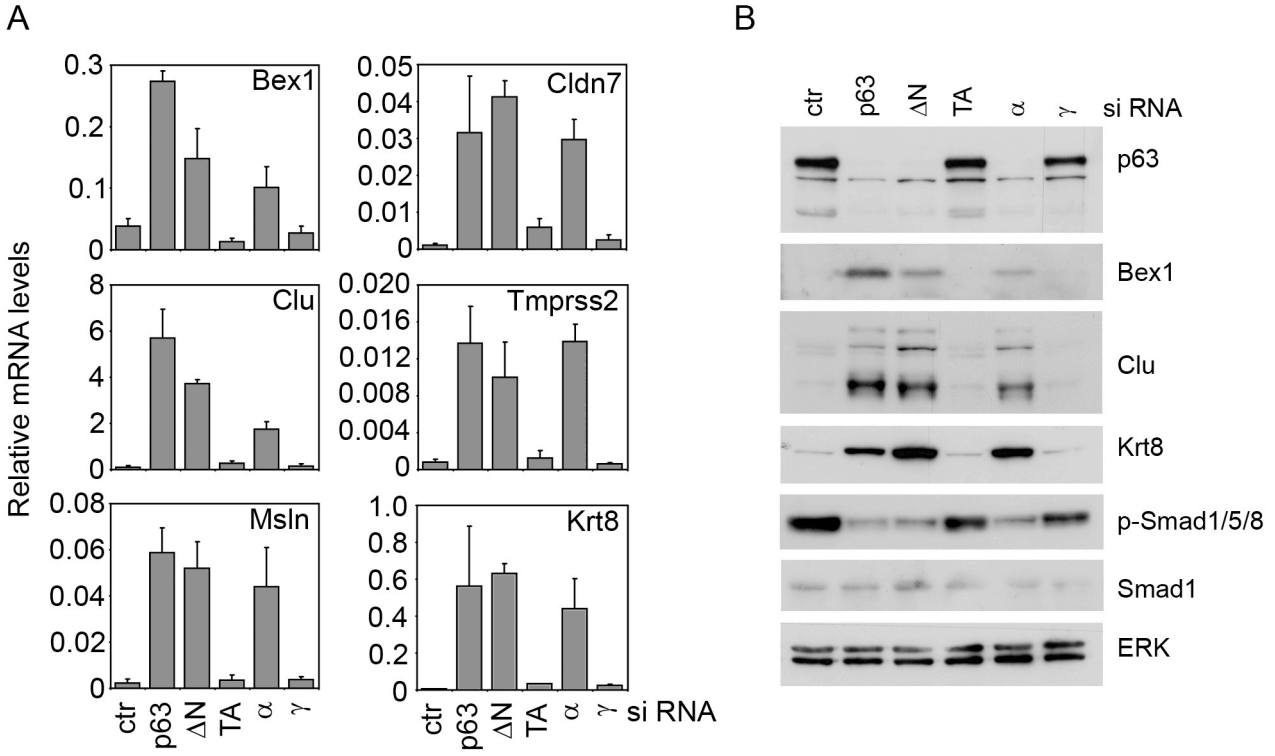


Figure 15. Aberrant expression of non-epidermal genes in the absence of p63 *(A) Non-epidermal gene expression were measured by real time RT-PCR in primary mouse keratinocytes silenced with specific siRNA for pan-p63, ΔNp63, TAp63, p63α, and p63γ isoforms, or ctr siRNA. RNA levels were normalized versus Gapdh mRNA, and represent the mean of independent experiments. Specific knockdown of ΔNp63 or p63α isoforms elicited a strong effects on non-epidermal genes expression whereas no effect was observed with knockdown of TA and gamma p63 isoforms.(B) Immunoblotting analysis of protein extract collected from p63 knockdown keratinocytes 48h after transfection. The protein extracts were normalized with anti-ERK antibody(ERK).*

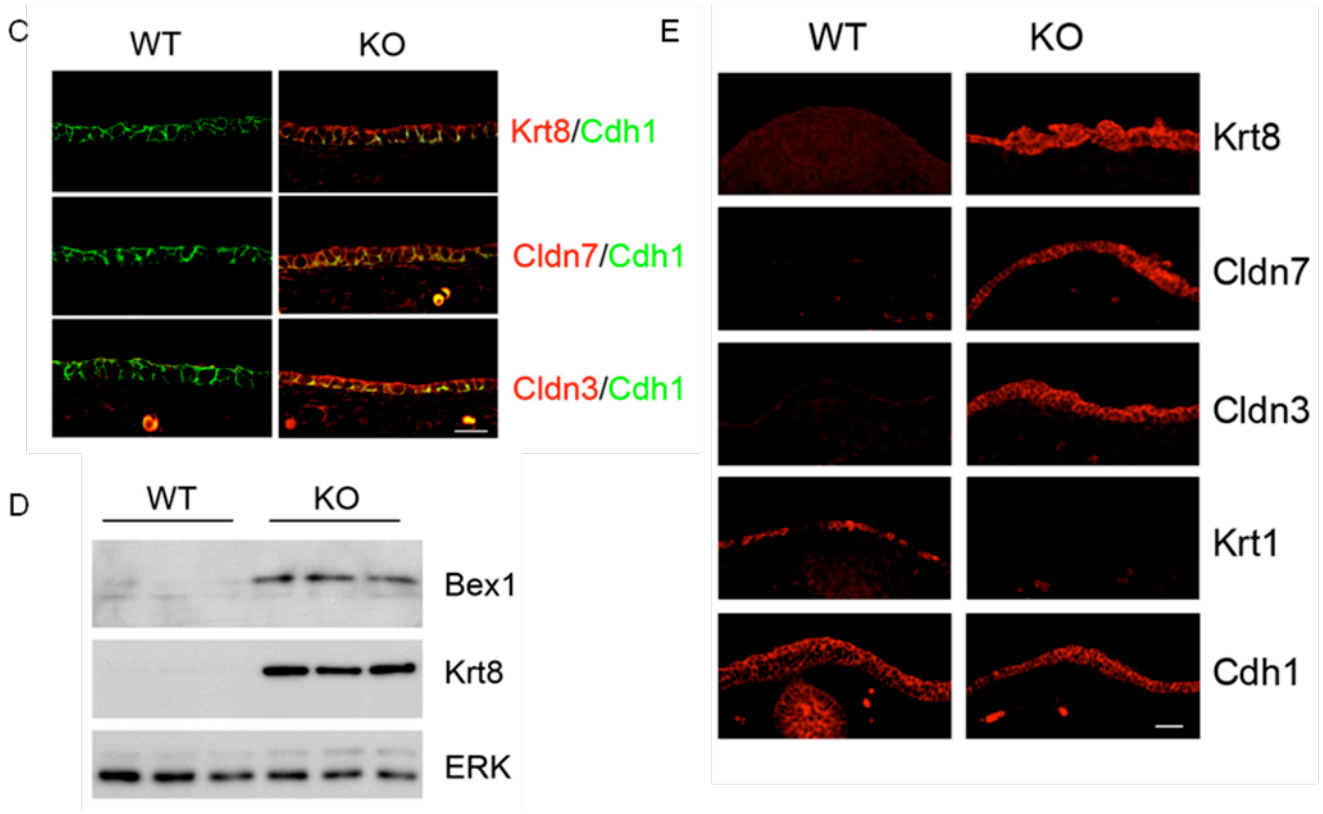


Figure15. Aberrant expression of non-epidermal genes in the absence of p63. *(Following up the figure 15). (C) Immunofluorescence analysis using antibody for Krt8, Cldn7 and Clnd3 (non-epidermal genes) in dorsal skin of p63 Knock-out mice and wild-type embryos. Non-epidermal genes were expressed in Knock-out embryos and not in wild-type epidermis, scale bar 30µm (D) Immunoblotting analysis of protein extract collected from E14.5 probed with indicated antibody. Protein extracts were normalized as in B. (E) Immunofluorescence analysis was performed with indicated antibody in embryonic epidermis isolated from E15.5 mice. Krt1 are specific markers expressed in wild type epidermis. Its expression was absent in p63 null-mice, on the other hand the non-epidermal genes were induced.*

Figure 16

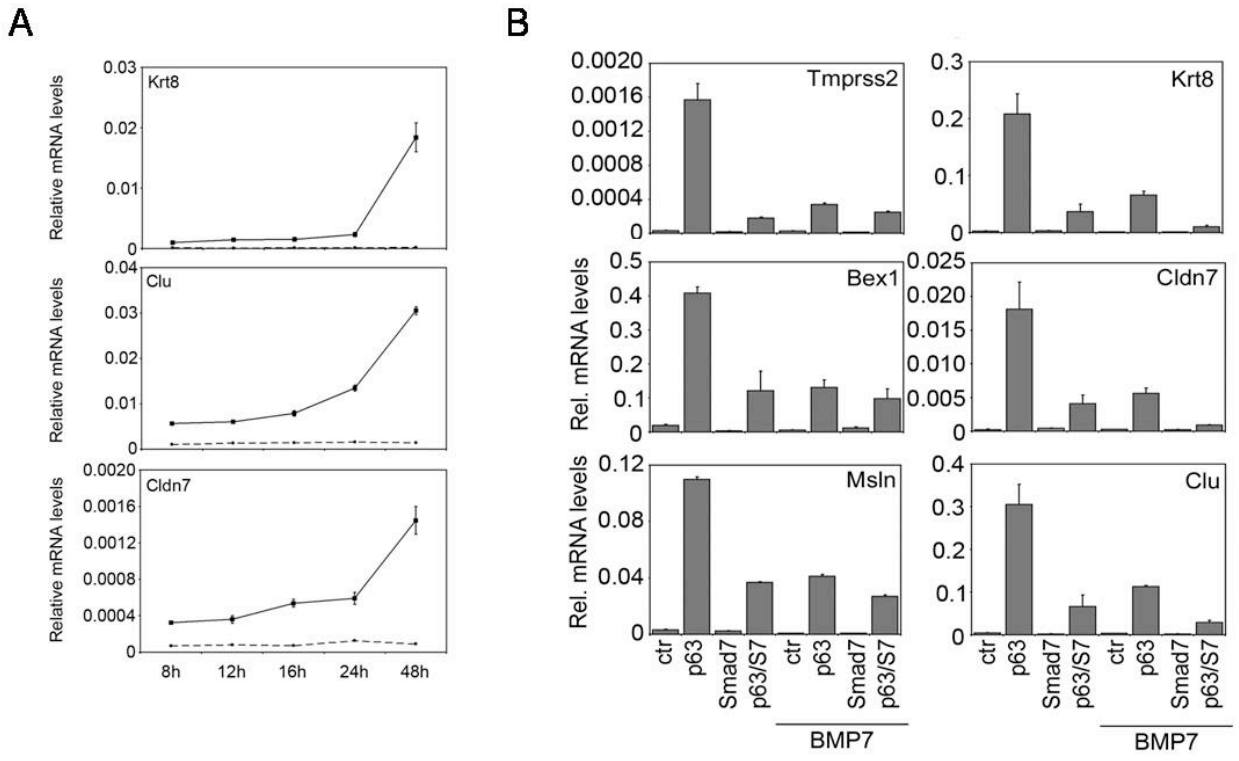


Figure 16. Re-activation of BMP signaling restores low levels of non-epidermal genes in p63 knockdown keratinocytes and p63 knock-out skin explants *(A) Non-epidermal genes were measured by real time RT-PCR at early time point after p63 knockdown. Solid line represented siRNA for p63, versus control keratinocytes dot-line. mRNA levels of Krt8, Clu, Cldn7 were showed induction after 24h after transfection. Values were normalized versus Actin-b mRNA levels.(B) mRNA expression levels of several non-epidermal genes were measured by real time RT-PCR in primary mouse keratinocytes transfected with p63, Smad7, p63 and Smad7 p63/S7 or control siRNA. Cells were treated with BMP7 for at least 24h or left untreated and collected at 48h after transfection. Values were normalized versus Actin-b mRNA levels and represented the means of independent experiments.*

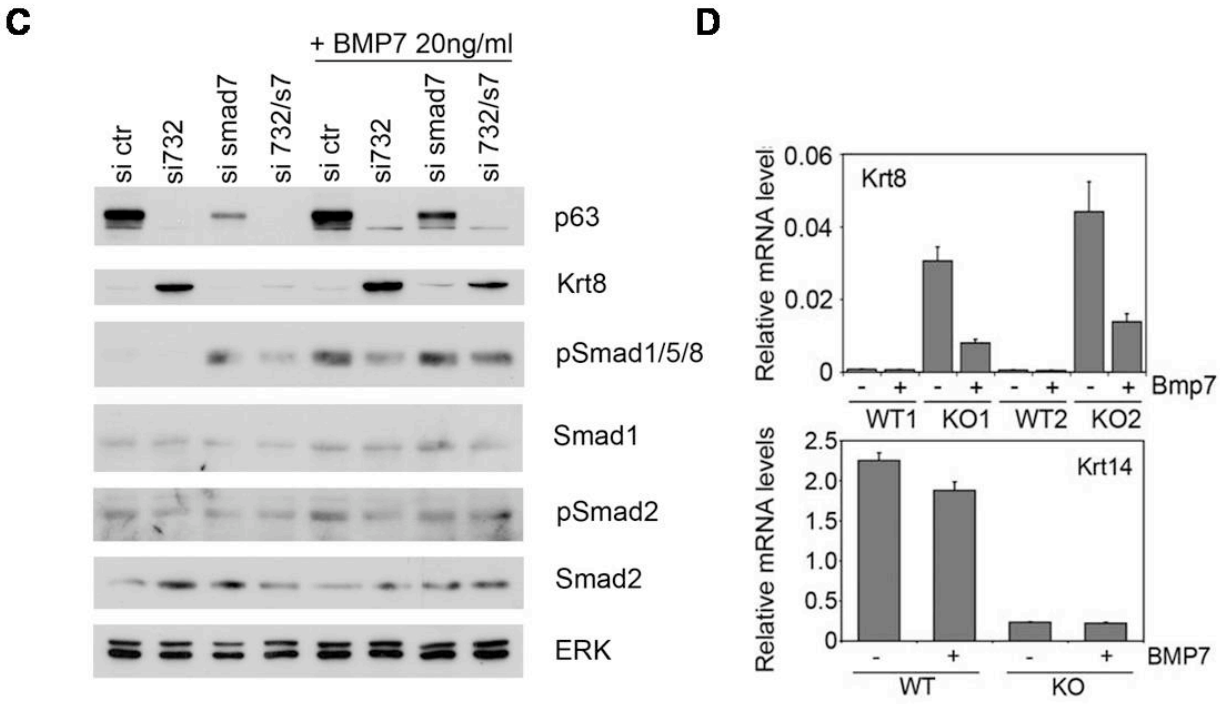


Figure 16. Re-activation of BMP signaling restores low levels of non-epidermal genes in p63 knockdown keratinocytes and p63 knock-out skin explants. *(Following up the figure 16).(C) Immunoblotting analysis of total cell lysates collected from primary mouse keratinocytes treated as in B. The indicate antibodies were used to detect krt8 and Clu two non-epidermal genes and p-smad1,5,8 to determinate the activity of BMP signaling and Smad2 for TGF-β. Protein extracts were normalized using anti-ERK antibody, Smad1 to normalize p-smad1,5,8 and Smad2 for p-smad2.(D) Skin explants was isolated from E14.5 p63 null and control mice. Skin explants was cultured overnight in DMEM with or without 5% FBS and were treated with additional 24h with BMP7 (80ng/ml) or left untreated in the absence (WT1 and KO1) or in presence of FBS (WT2 and KO2). Krt8 and Krt14 were measured as in B and represented the average of two independent experiments.*

Figure 17

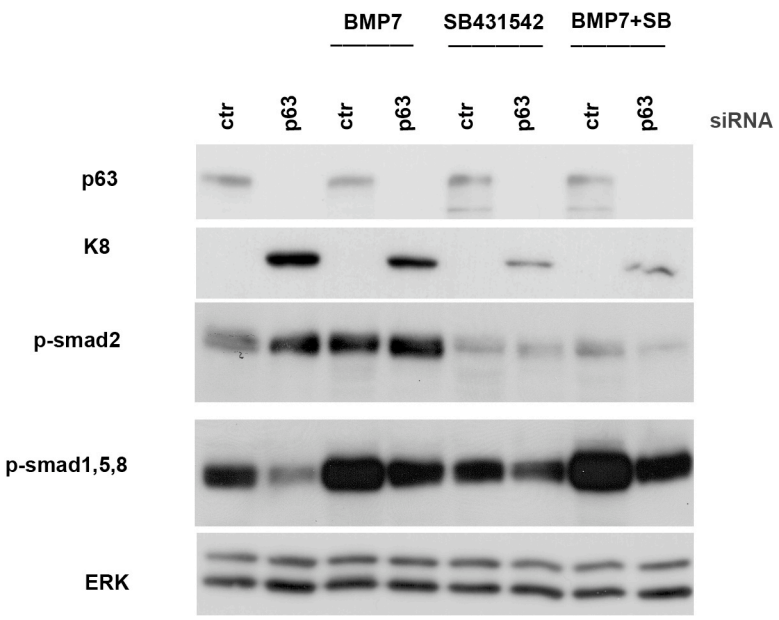


Figure 17. The expression of non-epidermal genes were rescued by blockage of TGF-β activity. *Immunoblotting analysis of protein lysates collected from primary mouse keratinocytes transfected for 48h with siRNA targeting p63 or control RNA. The cells were treated 24h after transfection with 20ng/ml of BMP7 or 2.5μM of SB431542 in presence of serum supplied with FBS. The immunoblotting was performed using the indicated antibodies. Protein extracts was normalized versus anti-ERK antibody.*

Figure 18

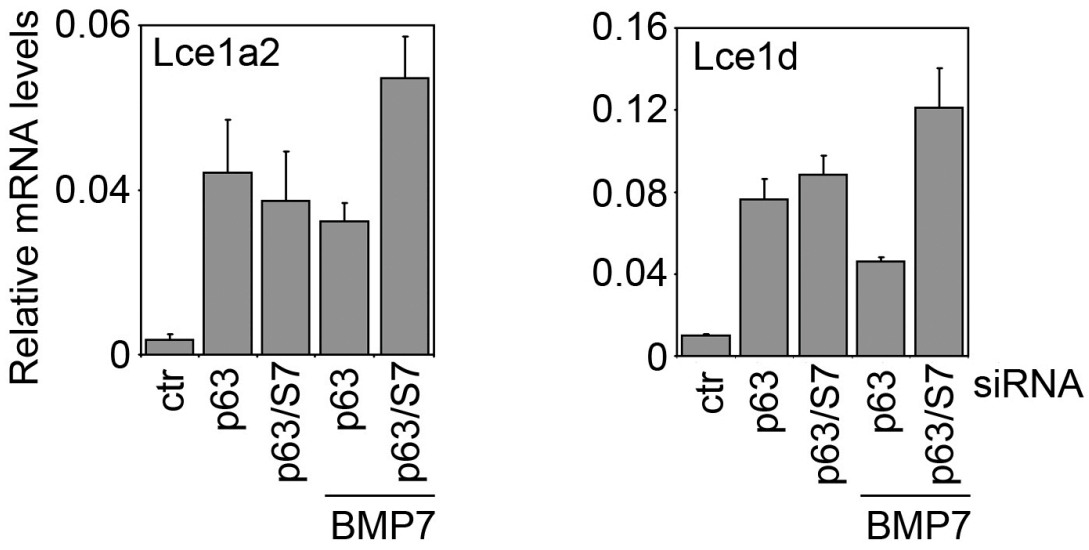


Figure 18. Loss of p63 induces late differentiation markers independently from BMP signaling. *mRNA expression levels of two late differentiation genes (Lce1a2 and Lce1d) were measured by real time RT-PCR in primary mouse keratinocytes transfected with p63, Smad7, p63 and Smad7 (p63/S7) or control siRNA. Cells were treated with BMP7 for at least 24h or left untreated and collected at 48h after transfection. mRNA values were normalized versus Actin-b mRNA levels and represented the means of independent experiments.*

Figure 19

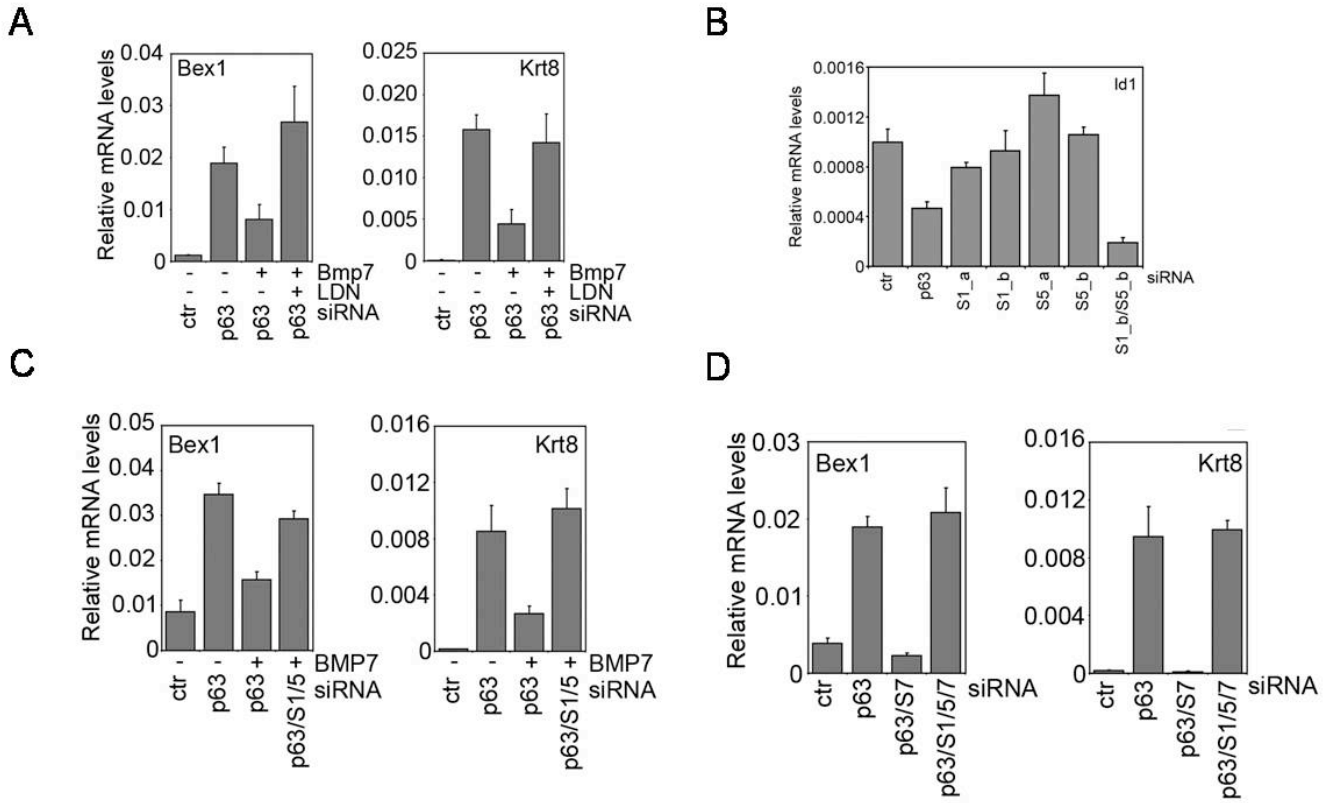


Figure 19. Canonical BMP/Smad pathway regulates the expression of non-epidermal genes *(A) mRNA expression levels of Bex1 and Krt8 were measured by real time RT- PCR in primary mouse keratinocytes transfected with siRNA targeting p63 and treated after 24h from transfection with BMP7 20ng/ml or BMP7 and LDN-193189 200nM until to 24h. mRNA values were normalized versus Actin-b mRNA levels. (B) mRNA expression levels of Id1 was measured by real time RT-PCR in primary mouse keratinocytes transfected respectively with p63, Smad1 (S1_a and S1_b), Smad5 (S5_a and S5_b) and Smad1 and Smad5 (S1_b/S5_B) siRNA for 48h. The values were normalized as in A (C) mRNA expression levels of Bex1 and Krt8, non-epidermal genes, were measured by real time RT- PCR in primary mouse keratinocytes transfected with siRNA targeting p63 or Smad5/Smad1 and p63siRNA . Cells were treated after 24h from transfection with BMP7 20ng/ml until to 24h. mRNA values were normalized as in A.(D) mRNA expression levels of Bex1 and Krt8 was measured by real time RT-PCR in primary mouse keratinocytes transfected respectively with p63, and Smad1 and Smad5 (S1/S5/p63) siRNA for 48h. The values were normalized as in .*

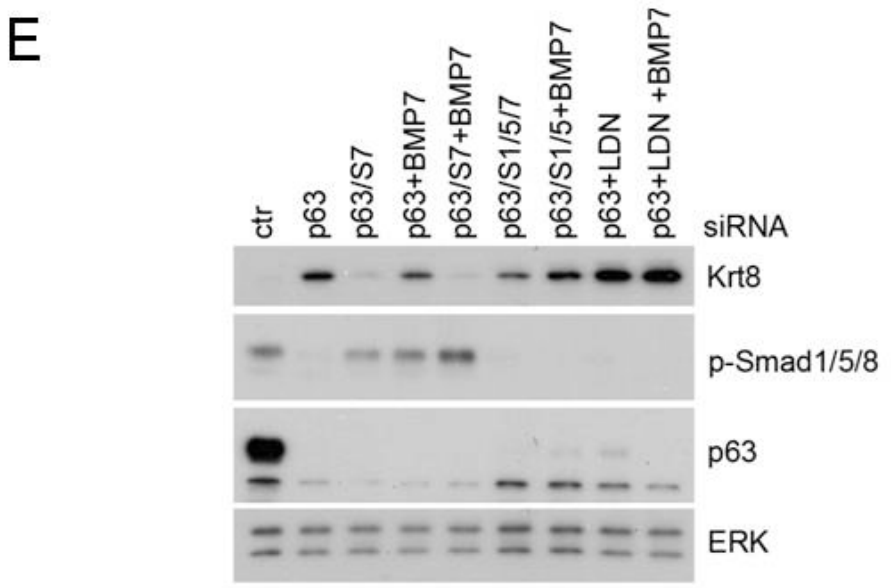


Figure 19. Canonical BMP/Smad pathway regulates the expression of non-epidermal genes. *(Following up the figure 19).(E) Expression of indicated proteins was measured by immunoblotting in primary mouse keratinocytes co-transfected with the indicated combinations of siRNA. Protein extracts were normalized using ERK antibody.*

Figure 20

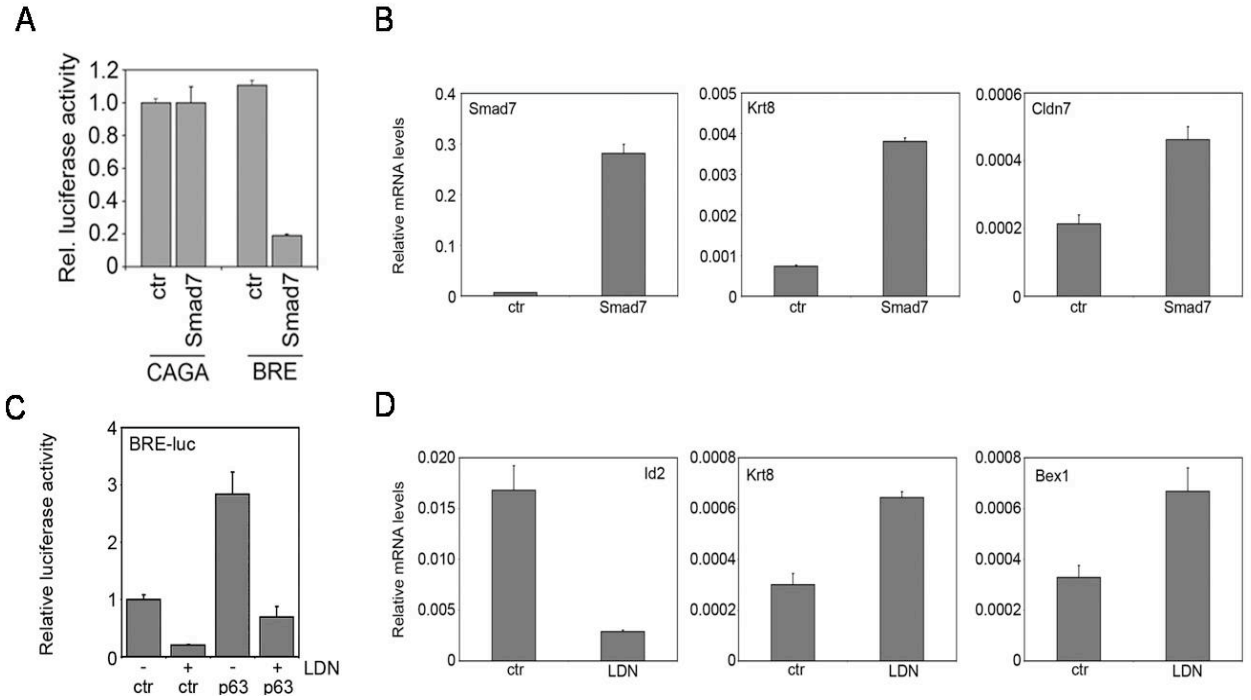


Figure 20. The canonical BMP/Smad signaling is able to induce non-epidermal genes *(A) Primary mouse keratinocytes were transfected with TGF-β (CAGA) or BMP (BRE) responsive elements and infected with retroviral vector carrying the inhibitory Smad7. The overexpression of Smad7 inhibited the activity of BMP responsive elements whereas no effects was observed on TGF-β one. Values of luciferase assay are normalized to Renilla luciferase activity and are expressed as fold-changes over the promoter activity in the presence of Smad7 overexpression. Values represent mean +/- S.E. of three independent experiment. (B) Primary mouse keratinocytes was infected with retrovirus vector carrying Smad7. mRNA levels of Smad7, Krt8 and Cldn7 was measured by real time RT-PCR. Values were normalized versus Actin-b mRNA levels. (C) Primary mouse keratinocytes were co-transfected with BMP responsive elements (BRE) and ΔNp63α isoform and treated with or without LDN-193189 200nM for 24h. Values of luciferase were normalized as in (A).(D) mRNA expression levels of non-epidermal genes and Id2 direct target of BMP signaling were measured in primary mouse keratinocytes treated with LDN-193189 200nM for 24h. Values were normalized versus Actin-b mRNA levels.*

Figure 21

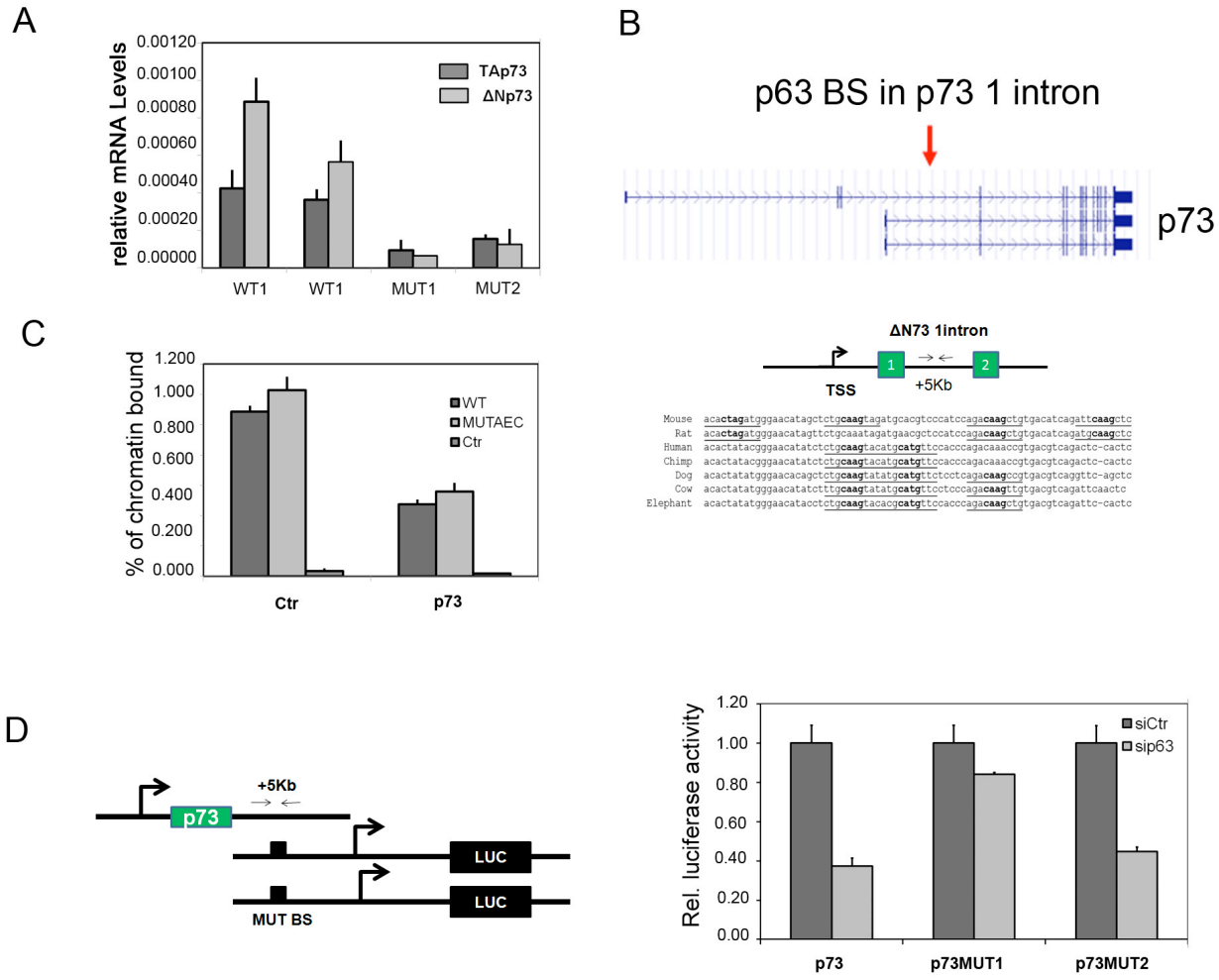


Figure 21. p73 a new target of p63 *(A) mRNA expression levels of Tap73 and ΔNp73were measured in primary mouse epidermis isolated from AEC mutant mice and WT mice. The values were normalized versus Hprt mRNA levels. (B) Predicted p63-binding hemi-sites located at +5Kb from the p73 TSS are indicated with their nucleotide sequence and phylogenetic conservation in multiple species. Bold nucleotides correspond to the core nucleotide sequence required for p63-binding, while underlined nucleotides are matches in the consensus sequence. (C) ChIP followed by real time PCR was performed on mouse p73 in first intron of p73 gene. Primary keratinocytes isolated from AEC mutant mice and wild-type mice were processed for ChIP with specific antibodies for p63 (grey bars), or unrelated anti-GFP antibodies as control (black bars), followed by real-time PCR amplification using different oligonucleotide primers for p73 and known target gene of p63 as control. The amount of precipitated DNA was calculated relative to the total input chromatin, and expressed as the percentage of the total DNA. (D) In the left, schema of p63BS in p73 gene cloned in pGL3TKluc with its mutant BS. In right, the activity of 0.5 kb of p73first intron was inhibited by p63 knockdown. Mutations in one canonical p63 binding hemi-sites lose the ability of p63 knockdown to inhibits promoter activity. Values of luciferase assay are normalized to Renilla luciferase activity and are expressed as fold-changes over the promoter activity in the absence of p63.*

Table I

Probe set id	Gene title	Gene Symbol	p63KD[a]/ctr	FDR[b]	AVER.[c] ctr	AVER. p63KD
1418910_at	bone morphogenetic protein 7	Bmp7	0.44	0.067	305	130
1416953_at	connective tissue growth factor	Ctgf	3.88	0.052	292	1128
1423389_at	MAD homolog 7 (Drosophila)	Smad7	1.65	0.098	183	311
1420653_at	transforming growth factor, beta 1	Tgfb1	0.55	0.083	317	168
1417455_at	transforming growth factor, beta 3	Tgfb3	14.03	0.034	12	162

[a] p63KD: p63 knockdown
[b] FDR: False Discovery Rate
[c] AVER.: Average signal intensity for each probe in three independent biological replicates

Table I. TGF-β family in p63 Knockdown keratinocytes. *A subset of genes encoding for members of BMP/TGFβ family and their regulator were differentially expressed in p63 knockdown versus control keratinocytes. The microarray analysis was performed on three independent biological replicates using Affymetrix Mouse Genome 430A 2.0 chips.*

Table II

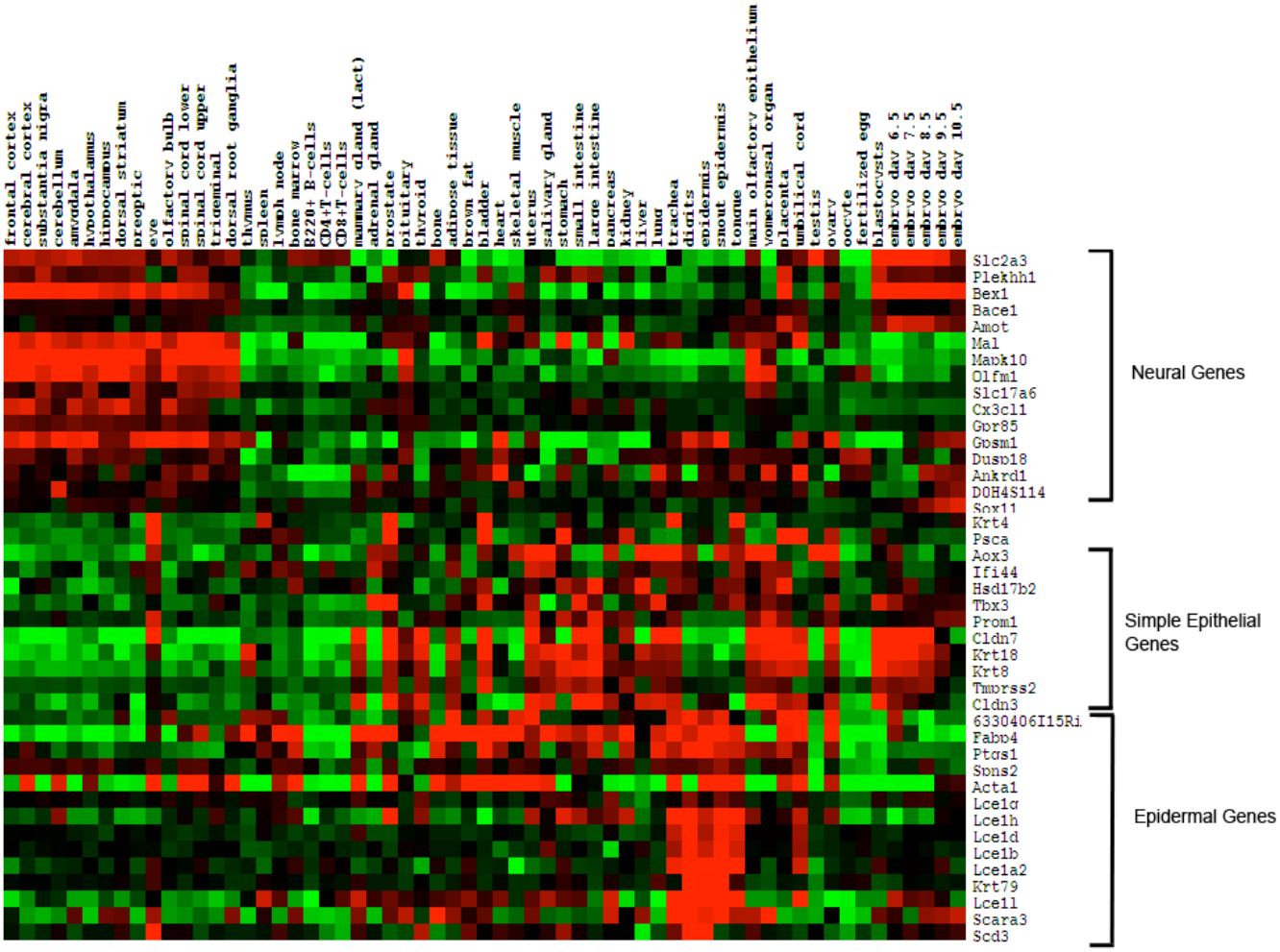


Table II. **Expression profile of genes induced in the absence of p63**. *Tissue expression profiling of up-regulated genes was obtained from a custom made mouse GNF1M (MAS5) GNF gene expression database (107) . In red high expression, in green low expression.*

7. Reference

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p63 Suppresses Non-epidermal Lineage Markers in a Bone Morphogenetic Protein-dependent Manner via Repression of Smad7*[S]

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p63, a p53 family member, plays an essential role in epidermal development by regulating its transcriptional program. Here we report a previously uncovered role of p63 in controlling bone morphogenetic protein (BMP) signaling, which is required for maintaining low expression levels of several non-epidermal genes. p63 represses transcription of the inhibitory Smad7 and activates Bmp7, thereby sustaining BMP signaling. In the absence of p63, compromised BMP signaling leads to inappropriate non-epidermal gene expression in postnatal mouse keratinocytes and in embryonic epidermis. Reactivation of BMP signaling by Smad7 knockdown and/or, to a lesser extent, by BMP treatment suppresses expression of non-epidermal genes in the absence of p63. Canonical BMP/Smad signaling is essential for control of non-epidermal genes as use of a specific inhibitor, or simultaneous knockdown of Smad1 and Smad5 counteract suppression of non-epidermal genes. Our data indicate that p63 prevents ectopic expression of non-epidermal genes by a mechanism involving Smad7 repression and, to a lesser extent, Bmp7 induction, with consequent enhancement of BMP/Smad signaling.

Mouse embryonic skin develops from an initial undifferentiated monolayer of epithelial cells that subsequently undergoes stratification beginning at embryonic day 14.5 (E14.5) (recently reviewed in Ref. 1). During embryogenesis, the surface ectoderm initiates the expression of gene products characteristic of the basal cells of the epidermis, including keratins Krt5 and Krt14. When stratification begins, suprabasal keratins Krt1 and Krt10 start to be expressed in the upper layers, followed by expression of late differentiation markers, including late cornified envelope proteins (Lce). *p63* is one of the first genes to be specifically expressed in the surface ectoderm prior to Krt5 and Krt14 expression at E7.5–E8, and continues to be expressed during embryonic skin development and in the basal proliferative layer in postnatal life (2–4). The *p63* gene encodes a tetrameric transcription factor that can be expressed in at least six isoforms with widely different transactivation potential that share an identical DNA binding domain (4). Alternative transcription start sites give rise to transactivation (TA)[6] isoforms, encoding proteins with a canonical transactivation domain similar to p53, and ΔN isoforms containing an alternative transactivation domain (4, 5). ΔNp63α, the most abundant isoform in isolated keratinocytes and in the epidermis (4), contains both a transactivation domain and an inhibitory domain, and has been shown to activate or repress gene transcription depending on the promoter context (reviewed in Ref. 6). Mice lacking the *p63* gene die from dehydration shortly after birth and display cleft palate, limb truncation, and absence of all stratified epithelia, including the epidermis (7, 8), suggesting that p63 plays a non-redundant role in these tissues. Defects in the surface epithelium of *p63*-null mice have been ascribed to loss of proliferative potential of keratinocyte stem cells (8, 9), and/or altered epidermal stratification and cell differentiation associated with reduced expression levels of Krt5/Krt14 and Krt1/Krt10 (2, 7, 10). In parallel with suppression of epidermal keratins, loss of p63 results in aberrant expression of the simple epithelial keratins Krt8 and Krt18 both *in vivo* and *in vitro* (2, 11), suggesting that p63 may be involved in maintaining an epithelial gene expression program in mammalian cells.

In non-mammalian vertebrates BMP signaling is an important determinant of epidermal fate specification, acting as an epidermal inducer and suppressing neural fate in early development (reviewed in Ref. 12). In mammals a putative BMP function in regulating epidermal fate or specific gene expression has not been demonstrated consistent with a possible redundant function among BMP family members or with other signaling pathways. The BMP family consists of more than 20

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[6] The abbreviations used are: TA, transactivation; TGF-β, transforming growth factor-β; BMP, bone morphogenetic protein; siRNA, small interfering RNA; RT, reverse transcriptase; ChIP, chromatin immunoprecipitation; ERK, extracellular signal-regulated kinase.

secreted proteins that belong to the transforming growth factor-β (TGF-β) family. Members of the BMP family are differentially expressed in embryonic skin. Expression of Bmp2 and Bmp4 transcripts in developing murine skin is restricted to the hair follicle epithelium and mesenchyme, respectively (13, 14). Strong expression of Bmp7 mRNA is seen in mouse and rat epidermis during embryonic development from early stages (3, 15), whereas Bmp6 is expressed in suprabasal layers of the embryonic murine epidermis at E15.5 (14). Interestingly, Bmp7 transcripts are strongly down-regulated in the ectoderm of *p63*-null mice (3), although the functional significance of this finding has not been addressed.

An important role in BMP/TGF-β signaling is played by the inhibitory Smads, Smad6 and Smad7, which block signaling by several mechanisms including competition with R-Smads for interaction with the activated receptors, ubiquitination, and degradation of the receptors (for a review, see Ref. 16). In addition Smad7 also plays noncanonical functions by regulating several other signaling proteins, including β-catenin (16, 17).

Here we report that p63, and more specifically the ΔNp63α isoform, activates BMP signaling both *in vitro* and *in vivo*. p63 directly binds to an evolutionary conserved regulatory region on the *Smad7* promoter thereby repressing its expression. At the same time, p63 sustains Bmp7 expression in the epidermis and indirectly controls Bmp4 expression in the dermis. Induction of BMP signaling maintains physiological levels of non-epidermal genes downstream of p63 in a Smad1/5-dependent manner. Taken together, these findings reveal a previously uncovered role of BMP/Smad signaling downstream of p63 in suppressing non-epidermal gene expression in keratinocytes.

EXPERIMENTAL PROCEDURES

Cell Cultures, Constructs, Transfections, Reporter Assays, and Retroviral Infections—Primary mouse keratinocytes were isolated from 2-day-old Swiss CD1 mice and cultured as previously described (18). Transfections were performed 5 days after plating using Lipofectamine 2000 (Invitrogen). Reporter plasmids (250 ng) were co-transfected with pCMV2-FLAG-ΔNp63α or pCMV2-FLAG control (18). A 4.3-kb *Smad7* promoter (3.6-kb promoter region and 0.7-kb 5′-untranslated region upstream) (19) was cloned into the XhoI-HindIII sites in the pGL3 reporter plasmid (Promega). The *Smad7* fragment (−3.0/−2.6) was generated by deletion of the *Smad7* promoter using NheI-PstI and cloned into a pGL3-TKLuc reporter plasmid (20). Mutations in the p63 binding sites were generated using the QuikChange Site-directed mutagenesis kit (Stratagene). Luciferase activity was determined 48 h after transfection with the dual-luciferase reporter assay kit (Promega). pCMV-*Renilla* reporter (20 ng; Promega) was used to normalize transfection efficiency. A total amount of 200 nM siRNA (Stealth siRNA, Invitrogen) for pan-p63, for specific p63 isoforms (18), and/or for Smad7, Smad1, Smad5, Bmp7, or control medium GC-rich siRNA (Stealth siRNA, Invitrogen) were transfected by Lipofectamine 2000. In some experiments cells were treated with BMP7 (20 ng/ml) (R&D Systems) 24 h after transfection. The BMP type I receptor inhibitor LDN-193189 (200 nM) was given to the cells 30 min before BMP7 addition. High titer retrovirus production was obtained in human embryonic kidney-293T cells by transient transfection of the pBABE-Smad7 (21) using Lipofectamine 2000 as previously described (20). Primary keratinocytes were infected in the presence of 8 μg/ml Polybrene (Sigma), selected with 2 μg/ml puromycin for 48 h, and grown after selection for an additional 24 h in the absence of puromycin.

Analysis of Gene Expression, Real Time RT-PCR, and ChIP—Gene expression profiling was obtained in p63 knockdown *versus* control keratinocytes 48 h after transfection using Affymetrix Mouse Genome 430A 2.0 chips and analyzed as reported (22). Among the genes affected by loss

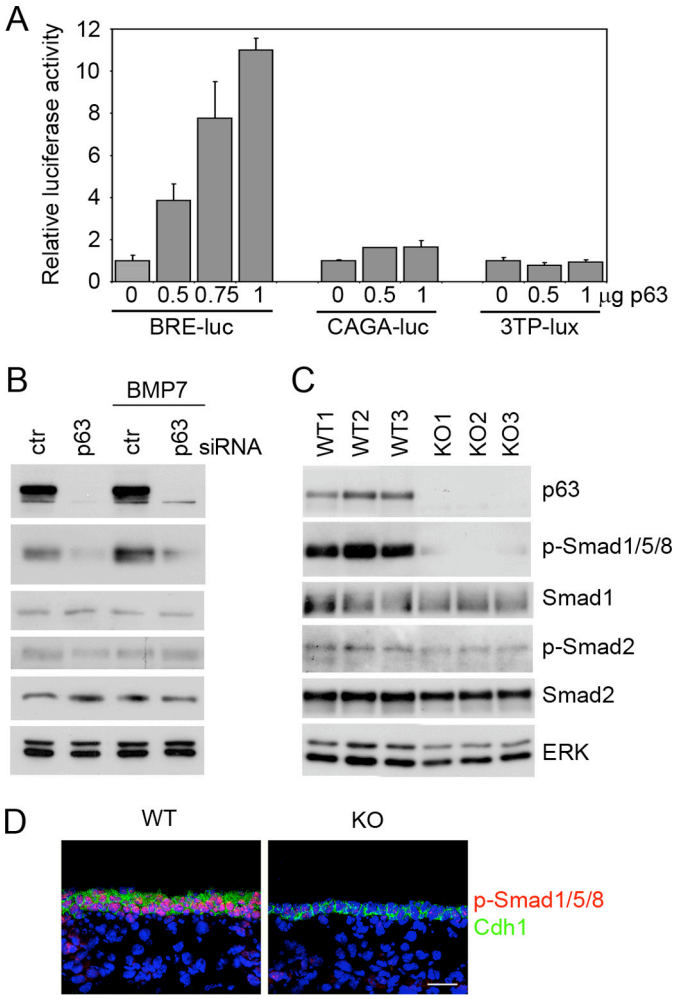

FIGURE 1. **p63 positively regulates BMP signaling.** *A*, different amounts of the ΔNp63α expression vector were co-transfected in primary mouse keratinocytes as indicated with the BMP-responsive reporter BRE-luc (50), or with TGF-β responsive reporters CAGA-luc (51) or 3TP-lux (52), and examined 48 h after transfection. Values are normalized to *Renilla* luciferase activity and are expressed as fold-changes over the promoter activity in the absence of ΔNp63α, and represent mean ± S.E. of three independent experiments. *B*, immunoblotting of primary keratinocytes transfected with p63 siRNA or control siRNA for 48 h, and either treated for 24 h with BMP7 (20 ng/ml) or left untreated, and probed with the indicated antibodies. Activation of BMP and TGF-β signaling was measured using phosphorylation-specific antibodies for Smad1/5/8 (*p-Smad1/5/8*) and Smad2 (*p-Smad2*), respectively. Total Smad1 and Smad2 expression is shown. ERK was used as loading control. *C*, immunoblotting of total skin extracts isolated from wild-type (*WT*) and *p63*-null embryos (*KO*) at E14.5, probed with the indicated antibodies. *D*, immunofluorescence staining was performed with anti-*p*-Smad1/5/8 (*red*) and anti-E-cadherin antibodies (*Cdh1*, *green*) in WT and *p63* KO at E15.5, and detected by confocal microscopy. Nuclei were stained with 4′,6-diamidino-2-phenylindole (*blue*). *Scale bar* is 20 μm.

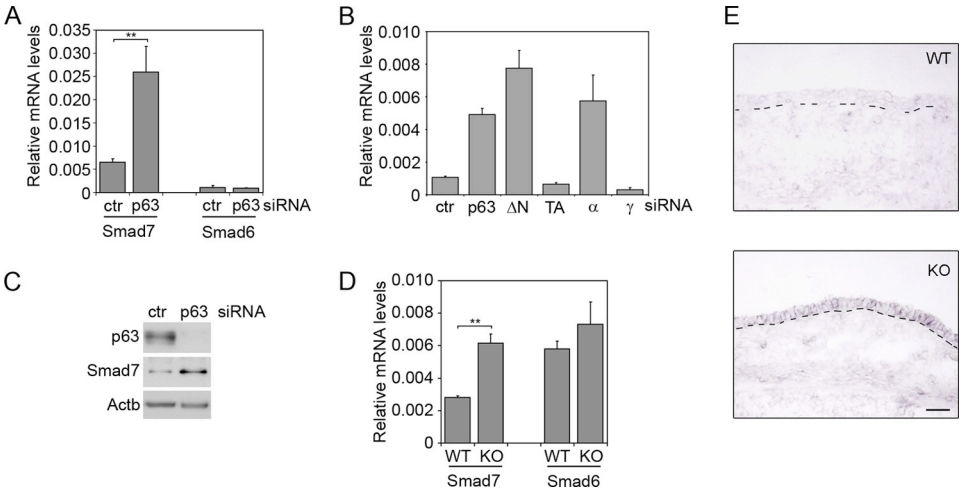

FIGURE 2. **Smad7 is repressed by p63.** *A*, Smad7 and Smad6 mRNA levels were measured by real time RT-PCR in primary keratinocytes transfected with p63 (*p63*) or control siRNA (*ctr*). Cells were collected 48 h after transfection. Values are expressed as glyceraldehyde-3-phosphate dehydrogenase-normalized mRNA levels, and represent the mean of independent experiments ± S.E. (**, $p < 0.005$, $n = 8$). *B*, Smad7 expression was measured in primary mouse keratinocytes transfected with siRNA specific for pan-p63, ΔNp63, TAp63, p63α, and p63γ isoforms, or control. RNA levels were expressed as in *A*. Isoform-specific siRNA specifically inhibited the corresponding isoforms (data not shown). *C*, Smad7 protein levels were measured in p63 knockdown and control keratinocytes 48 h after transfection. Protein extracts were normalized using anti-β-actin (*Actb*) antibodies. *D*, Smad7 and Smad6 mRNA levels were measured by real time RT-PCR in *p63* KO and wild type (*WT*) skin at E14.5 (**$p < 0.005$, $n = 5$ embryos). *E*, RNA *in situ* hybridization of mouse embryonic skin sections at E14.5 from WT and *p63* knock-out (*KO*) using a digoxigenin-labeled antisense probe for mouse Smad7. The *dashed line* indicates the dermal-epidermal junction. A Smad7 sense probe gave no detectable signal under the same conditions (data not shown). *Scale bar* is 50 μm.

of p63, 106 genes were up-regulated more than 5-fold by p63 knockdown (supplemental Table S1). Tissue expression profiling of up-regulated genes was obtained from a custom made mouse GNF1M (MAS5) GNF gene expression data base (23). Among 72 genes that were up-regulated more than 5-fold by p63 knockdown (false discovery rate < 0.25), 48 (67%) were not expressed in normal epidermis, but rather in other tissues. Total RNA was extracted 48 h after transfection from primary keratinocytes using TRIzol reagent (Invitrogen), and from mouse embryonic skin (E14.5) using the RNAspin Mini RNA isolation kit (GE Healthcare) according to the manufacturer's instruction. RNA samples were treated with RNase-free DNase I (Promega), and cDNA was synthesized using SuperScript Vilo (Invitrogen). Two-step real time RT-PCR was performed using the SYBR Green PCR master mixture in an ABI PRISM 7500 (Applied Biosystems). Levels of the target genes were quantified using specific oligonucleotide primers and normalized for glyceraldehyde-3-phosphate dehydrogenase or anti-β-actin expression as indicated in the figure legends. Approximately 3×10^6 primary keratinocytes were fixed with 1% formaldehyde and ChIP was performed using anti-p63 antibodies (H-137, Santa Cruz Biotechnology), and anti-ERK1 antibodies as negative control. ChIP and real time PCR were performed as previously described (18). For oligonucleotide sequences, see supplemental Table S2.

Immunostaining, Immunoblotting, and in Situ Hybridization—Embryos were fixed in 4% paraformaldehyde and either embedded in OCT (Sakura) or paraffin. Fluorescent signals were monitored under a Zeiss confocal microscope LSM510meta using a Zeiss EC Plan-Neofluar ×40/1.3 oil immersion objective. For immunoblotting cells were lysed in sample buffer or in 1% Triton X-100 lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA) for Smad7, and protein extracts were run on SDS-PAGE gels, transferred on Immobilon-P transfer membranes (Millipore), probed with the indicated antibodies, and detected by chemiluminescence (ECL, GE Healthcare). For a full list of antibodies, see the supplemental data. *In situ* hybridization was performed on frozen sections of P1 skin and E14.5 embryos as previously described (24). Digoxigenin-labeled antisense and sense RNA probes were transcribed from the SP6 and T7 promoters using a digoxigenin labeling kit (Roche Applied Science) according to manufacturer's instructions. The *Smad7* probe corresponding to a 485-bp cDNA fragment was kindly provided by Xiao-Jing Wang (17). The Bmp6 probe was synthesized from a 893-bp SacI-EcoRI cDNA fragment kindly provided by Dr. M. Mikkola. The Bmp7 probe corresponds to a 440-bp long cDNA fragment generated by PCR and inserted in the pCR®II vector (Invitrogen). For PCR oligonucleotide sequences, see supplemental Table S2. Digoxigenin labeling was monitored under a Zeiss Axioskop2 plus microscope using a Zeiss Plan-Neofluar ×20/0.50 objective.

Mice and Skin Explants—All experiments performed with mice were conducted under IACUC approval. *p63*-null mice (B6.129S7-*Trp63*^{tm1Brd}/J) were obtained from the Jackson Laboratory. For skin explants, dorsal skins from embryos at E14.5 were laid on a culture plate insert (Millipore) and cultured in the presence or absence of BMP7 in Dulbecco's modified Eagle's medium with or without 5% fetal bovine serum, overnight at 37 °C and 5% CO_2.

RESULTS

p63 Positively Controls BMP Signaling—To gain insight into the role of p63 in controlling gene expression in epidermal cells, we recently identified a large number of putative p63 target genes in primary mouse keratinocytes (22). A subset of genes encoding for members of the BMP family and their regulators were differentially expressed in p63 knockdown *versus* control keratinocytes.

To assess whether p63 affected BMP or TGF-β signaling, we first co-transfected varying amounts of a ΔNp63α-expressing plasmid together with a luciferase reporter gene under the control of either BMP (BRE) or TGF-β (CAGA and 3TP) responsive elements in primary mouse keratinocytes. As shown in Fig. 1*A*, expression of p63 resulted in a dose-dependent induction of the BMP-responsive element, without affecting the TGF-β

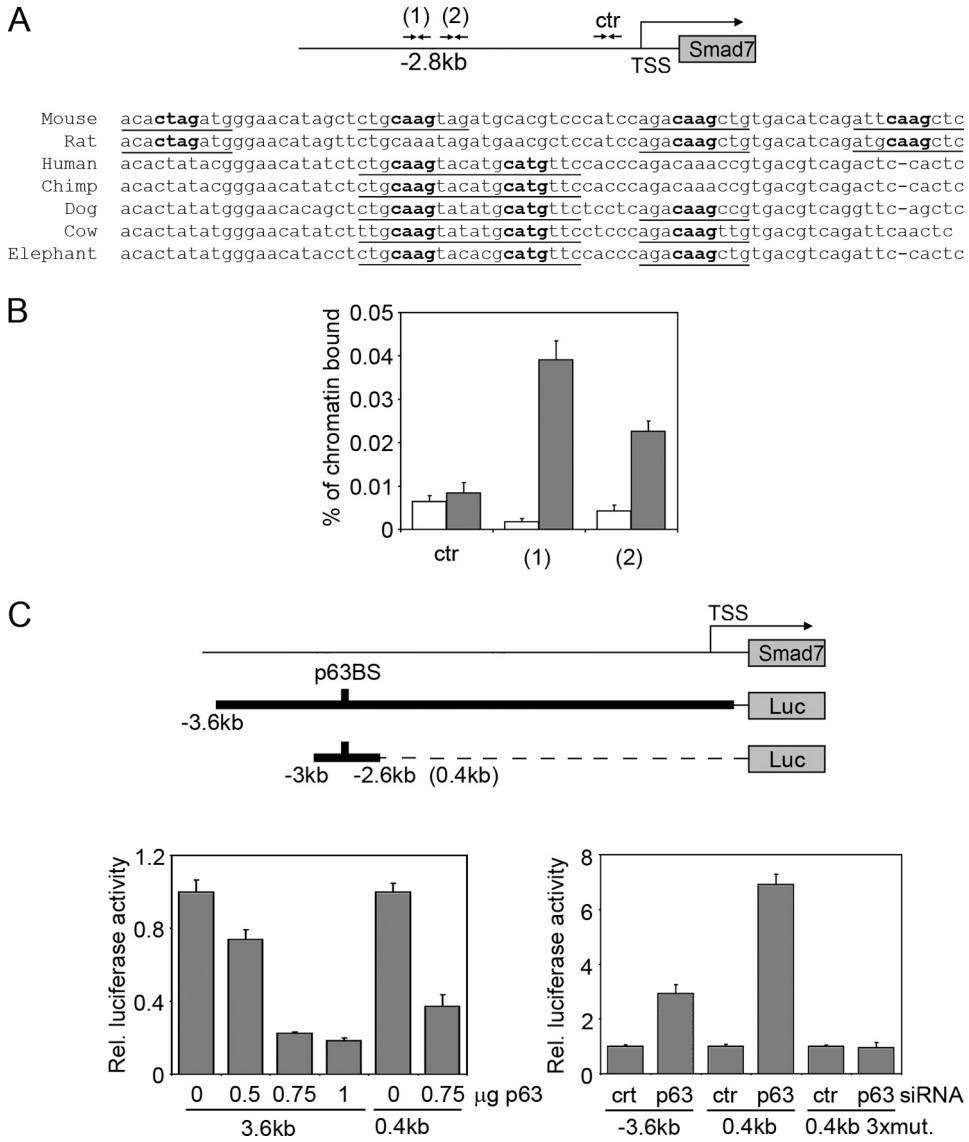

FIGURE 3. **Smad7 is a transcriptional target of p63.** *A*, a conserved p63-binding site is located at −2.8 kb from the *Smad7* transcription start site. The predicted p63-binding hemisites are indicated with their nucleotide sequence and phylogenetic conservation in multiple species. *Bold nucleotides* correspond to the core nucleotide sequence required for p63 binding, whereas *underlined nucleotides* are matches in the consensus (22, 53). *B*, specific binding of endogenous p63 to the mouse *Smad7* promoter. Primary keratinocytes were processed for ChIP with antibodies specific for p63 (*gray bars*), or unrelated anti-ERK antibodies as control (*white bars*), followed by real-time PCR amplification using oligonucleotide primers designed at the indicated position from the transcription start site. The amount of precipitated DNA was calculated relative to the total input chromatin, and expressed as the percentage of the total DNA, as previously described (54). *C*, the activity of a 3.6-kb *Smad7* promoter region and its 0.4-kb fragment spanning the p63-binding sites (*p63BS*) were measured in the presence of the indicated amounts of a ΔNp63α-expressing construct (*p63*) (*left panel*), or p63 or control (*ctr*) siRNA (*right panel*). A 0.4-kb fragment containing 2-bp mutations in each of the first three binding hemisites indicated in *A* (*0.4kb 3xmut*) was also co-transfected with p63 or control siRNA. Values are expressed as described in Fig. 1*A*.

ones. In parallel, p63 knockdown resulted in a significant reduction in Smad1/5/8 phosphorylation under basal conditions and upon BMP7 treatment (Fig. 1*B*). In contrast, p63 knockdown elicited no effect on Smad2 phosphorylation, a direct TGF-β effector, either under basal conditions or upon TGF-β1 stimulation (Fig. 1*B* and data not shown).

To assess whether p63 regulates BMP signaling during embryonic skin development, we measured Smad1/5/8 phosphorylation in *p63*-null and in wild-type skin. Immunoblotting analysis and immunofluorescence staining using anti-phospho-Smad1/5/8 revealed strong activation of BMP signaling in the developing wild-type epidermis, whereas the Smad1/5/8 signal was dramatically reduced in the *p63*-null epidermis (Fig. 1, *C* and *D*). In contrast Smad2 phosphorylation was similar in wild-type and *p63*-null skin at this embryonic stage (Fig. 1*C*). Taken together, these data indicate that p63 positively regulates BMP signaling in primary mouse keratinocytes and in embryonic epidermis, without significantly affecting TGF-β signaling.

p63 Transcriptionally Controls Smad7 and Bmp7 Gene Expression—Our previous gene expression profiling indicated that p63 knockdown might affect Smad7 expression (22). Given the key role of Smad7 in regulating BMP signaling, we sought to confirm and expand this finding. p63 knockdown in keratinocytes caused a significant induction of Smad7 both at the RNA and at the protein levels, without affecting expression of the related gene Smad6 (Fig. 2, *A* and *C*). In addition, specific knockdown of ΔNp63 and p63α isoforms induced Smad7 expression, whereas knockdown of TAp63 and p63γ isoforms had no effect (Fig. 2*B*), indicating that the ΔNp63α isoform is required for Smad7 repression. In parallel with these findings, Smad7 was expressed at low levels in wild-type embryonic and newborn epidermis (Fig. 2, *D* and *E*, and supplemental Fig. S1*A*), whereas its expression was significantly increased in the *p63*-null epidermis (Fig. 2, *D* and *E*).

To test the possibility that p63 might directly regulate Smad7, we tested a putative high affinity p63-binding region located at −2.8 kb from the transcription start site, which emerged from our previous ChIP-on-ChIP experiment in a 48.5-kb genomic sequence spanning the *Smad7* locus (22). The p63-binding region was centered on an evolutionary conserved genomic sequence containing four canonical p63 binding hemisites (Fig. 3*A*). ChIP followed by real time PCR with two independent sets of oligonucleotides confirmed that p63 specifically bound this genomic region (Fig. 3*B*). In addition, p63 overexpression significantly inhibited the activity of a 3.6-kb *Smad7* promoter, as well as the activity of a 0.4-kb fragment containing the p63

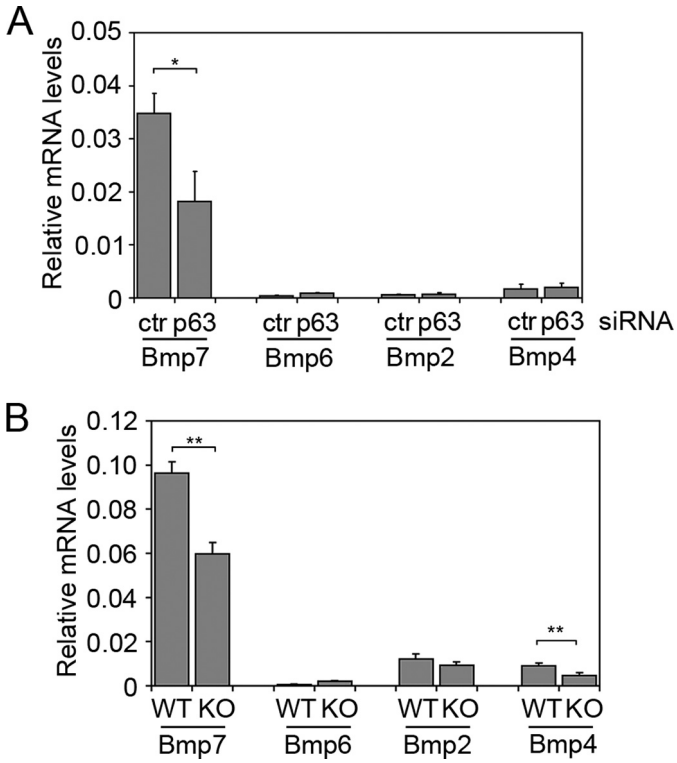

FIGURE 4. **Bmp7 is the most abundant BMP family member in keratinocytes and embryonic skin and is down-regulated in the absence of p63.** *A*, expression levels of the indicated BMPs were measured by real time RT-PCR in primary keratinocytes transfected with p63 or control (*ctr*) siRNA as described in the legend to Fig. 2*A*. Values represent mean of independent experiments ± S.E. (*, $p < 0.05$, $n = 4$). *B*, BMP mRNA levels were measured in *p63* knockout (*KO*) and wild-type (*WT*) skin at E14.5. Bmp7 and Bmp4 are significantly down-regulated in *p63* KO skin (**, $p < 0.005$, $n = 4$ embryos).

binding sites (Fig. 3*C*, *left panel*). Conversely, p63 knockdown resulted in enhancement of the activity both of the *Smad7* promoter and the fragment containing the p63 binding sites (Fig. 3*C*, *right panel*). Mutations in three canonical p63 binding hemisites in the promoter fragment abolished the ability of p63 knockdown to enhance promoter activity. Taken together, these data strongly suggest that p63 directly represses Smad7 in primary mouse keratinocytes and in embryonic skin.

Next we analyzed the expression of selected BMP family members known to be expressed in skin, in the presence or absence of p63. Bmp2 and Bmp4 were poorly expressed in primary mouse keratinocytes and their expression was unaffected by p63 knockdown (Fig. 4*A*). Bmp6 was modestly induced by p63 knockdown, however, its expression was very low under non-differentiating conditions, consistent with its suprabasal expression in newborn skin (supplemental Fig. S1*A*) and in the developing murine epidermis (14, 25). In contrast Bmp7 was by far the most highly expressed BMP family member in keratinocytes, and its expression was inhibited ~50% by p63 knockdown. Consistent with this observations, low levels of Bmp6 were modestly up-regulated in *p63*-null embryonic skin at E14.5, whereas Bmp2 was not significantly altered (Fig. 4*B*). Interestingly, Bmp4, which is mainly expressed in the mesenchymal component of the skin, was reduced in *p63*-null skin, suggesting that p63 may indirectly control Bmp4 expression in the adjacent dermis, thus possibly contributing to the reduction in BMP signaling observed in the *p63*-null epidermis. Strong signaling for Bmp7 mRNA was detected by *in situ* hybridization both in the basal layer of newborn epidermis and in embryonic epidermis (supplemental Fig. S1, *A* and *B*). Bmp7 was previously reported to be absent in *p63*-null skin at E14 by *in situ* hybridization, although no quantitative data were provided (3). We observed a similar, although more modest, reduction of Bmp7 transcript in *p63*-null *versus* wild-type skin at E14.5 as assessed by real time RT-PCR and *in situ* hybridization (Fig. 4*B* and supplemental Fig. S1*B*), in agreement with the reduction observed in p63 knockdown keratinocytes. ChIP analysis revealed that p63 bound to an evolutionary conserved genomic region in intron 1 (supplemental Fig. S1*C*), previously shown to bind all p53 family members in a breast cancer cell line (26). Taken together these data demonstrate that p63 regulates Smad7 and Bmp7 in epidermal cells, inducing one and repressing the other, by direct binding to specific consensus sequences in highly conserved genomic regions proximal to their transcription start sites.

Loss of p63 Induces Expression of Non-epidermal Genes in Primary Keratinocytes and in Embryonic Epidermis—Global gene expression profiling of p63 knockdown *versus* control keratinocytes (22) revealed that p63 negatively regulates a set of genes that are preferentially expressed in other tissues (non-epidermal genes), including the previously reported Krt8 and Krt18 (11, 27). 67% of the genes up-regulated more than 5 times in the absence of p63 were expressed at very low levels in wild-type keratinocytes, and were enriched in genes expressed in early embryonic development, in simple epithelia, or in neural tissues (supplemental Table S1).

Aberrant expression of non-epidermal genes in p63 knockdown keratinocytes was confirmed at the RNA and protein levels for some relevant genes (Fig. 5, *A* and *B*, and data not shown). Knockdown of the ΔNp63 and the p63α isoforms strongly induced expression of non-epidermal genes and their gene products and at the same time inhibited Smad1/5/8 phosphorylation, whereas knockdown of TAp63 and p63γ isoforms had no effect. To test whether the effect elicited by p63 knockdown in primary keratinocytes correlated with p63 function *in vivo*, we analyzed expression of non-epidermal genes in total embryonic skin of *p63*-null embryos and their wild-type counterparts at E13.5 and E14.5. Importantly, non-epidermal gene products were strongly expressed in the *p63*-null epidermis but not in wild-type epidermis (Fig. 5, *C* and *D*). Taken together these data indicate that p63 is required to suppress several non-epidermal genes during embryonic skin development and in postnatal keratinocytes, and that this regulation is unlikely to occur directly.

p63 Represses Non-epidermal Genes through a Canonical BMP/sSmad-dependent Mechanism—Non-epidermal genes were not identified as early target genes upon p63 activation (22), and their induction occurred at a late interval upon p63 knockdown (data not shown), suggesting that they are not directly regulated by p63. Because in lower vertebrates BMP signaling has been shown to be a crucial determinant of the epidermal cell fate during ectodermal development by suppressing alternative fates, we hypothesized that p63 could maintain low levels of non-epidermal genes via a BMP-dependent mechanism. To this end we

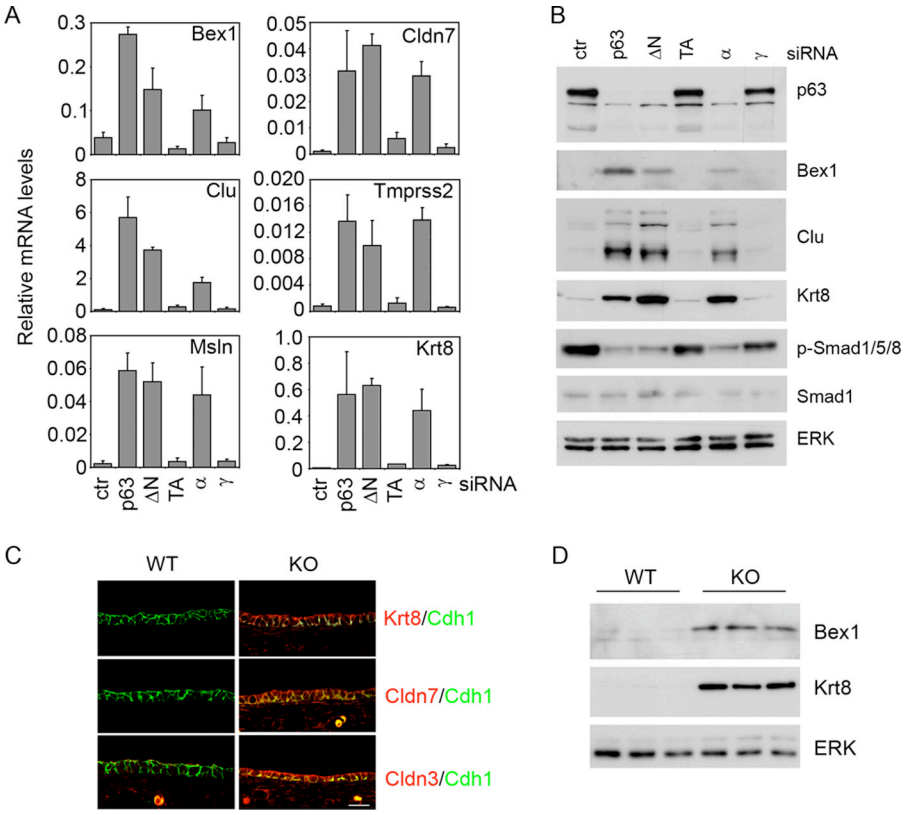

FIGURE 5. **Aberrant expression of non-epidermal genes in the absence of p63.** *A,* expression of the indicated non-epidermal genes was measured in primary mouse keratinocytes transfected with siRNA specific for pan-p63, ΔNp63, TAp63, p63α, and p63γ isoforms, or control (*ctr*). RNA levels were expressed as described in the legend to Fig. 2*A*. Efficient knockdown for the specific isoforms was demonstrated at the mRNA level (data not shown). *B,* immunoblotting of total cell lysates prepared from primary keratinocytes transfected with siRNA as in *A*. *C,* immunofluorescence analysis of the indicated non-epidermal proteins (in *red*) and E-cadherin (*Cdh1*, in *green*) in dorsal skin of *p63* knockout (KO) and wild-type (*WT*) embryos at E13.5. Nonspecific staining in the dermis is due to some autofluorescent cells. *Scale bar* is 30 μm. *D,* immunoblotting of total cell lysates prepared from the dorsal skin of *p63* KO and WT embryos at E14.5 and probed with the indicated antibodies.

examined whether re-activation of BMP signaling in p63 knockdown keratinocytes by either Smad7 knockdown and/or BMP treatment could restore physiological levels of non-epidermal genes. Smad7 knockdown resulted in a significant re-activation of Smad1/5/8 signaling in the absence of p63, without affecting Smad2 activation (supplemental Fig. S2*A*). Conversely, Smad7 overexpression inhibited BRE-Luc activity without affecting CAGA-Luc (supplemental Fig. S2*B*). In parallel, Smad7 knockdown resulted in strong down-regulation of non-epidermal gene expression in p63 knockdown keratinocytes both at the RNA and protein levels (Fig. 6, *A* and *B*), suggesting that Smad7 depletion counteracted the effect of p63 knockdown. A similar counteracting effect was also observed with a second distinct siRNA oligonucleotide against Smad7 (supplemental Fig. S2*C*), confirming the specificity of this effect. Inhibition of non-epidermal genes was also observed by treating p63 knockdown keratinocytes with BMP7, BMP4, or BMP6 (Fig. 6, *A* and *B*, and data not shown). Concomitant Smad7 knockdown and BMP treatment had little additional effect on the expression of non-epidermal markers as compared with each treatment alone, suggesting that these treatments repressed non-epidermal genes through overlapping mechanisms. Neither Smad7 knockdown nor BMP treatment affected p63 expression (data not shown), excluding a feedback loop mechanism. TGF-β treatment under the same experimental conditions did not suppress non-epidermal genes (data not shown).

To test whether BMP signaling functions specifically downstream of p63 to regulate non-epidermal genes, or has a broader compensatory role on p63 downstream targets, we measured expression of the Lce genes (or Sprrl), which encode epidermal markers involved in late differentiation and are up-regulated by p63 knockdown (supplemental Table S1). Neither BMP treatment nor Smad7 knockdown rescued the effect of p63 knockdown on Lce genes (supplemental Fig. S2*D*), indicating that BMP signaling selectively restored low levels of non-epidermal genes.

To demonstrate that loss of BMP signaling contributes to the expression of non-epidermal genes in *p63*-null epidermis, skin explants were isolated at E14.5 from *p63*-null and wild-type mice and cultured with or without BMP7. BMP7 treatment significantly down-regulated Krt8 expression in *p63*-null skin (Fig. 6*C*), consistent with a role of BMP signaling in repressing non-epidermal genes in the embryonic epidermis. In contrast, BMP7 treatment was insufficient to rescue loss of Krt14 expression in *p63*-null skin explants, indicating that BMP7 elicited a selective effect on non-epidermal genes in embryonic epidermis.

We then explored the possibility that loss of BMP signaling may be sufficient to cause aberrant expression of non-epidermal genes. Smad7 overexpression up-regulated non-epidermal genes in primary keratinocytes (Fig. 7*A* and data not shown), although to a lesser extent than p63 knockdown, while inhibiting the expression of the BMP direct target gene *Id2* as expected. Treatment with LDN-193189, a selective small molecule inhibitor of the BMP type I receptor kinases (28), similarly induced expression of non-epidermal genes and repressed *Id2* (Fig. 7*B*), whereas SB431542, a selective inhibitor of the TGF-β type I receptor did not (data not shown). However, in the embryonic epidermis *Smad7* overexpression (17) or homozygous deletion of *Bmp7* (29) were insufficient to induce non-epidermal genes (data not shown). Taken together, these data indicate that p63 represses non-epidermal genes in a BMP-dependent manner, and that, at least in isolated keratinocytes, loss of BMP signaling by itself leads to induction of non-epidermal genes.

BMP-mediated receptor activation leads to induction of a canonical signaling pathway mediated by Smad1/5/8 and to

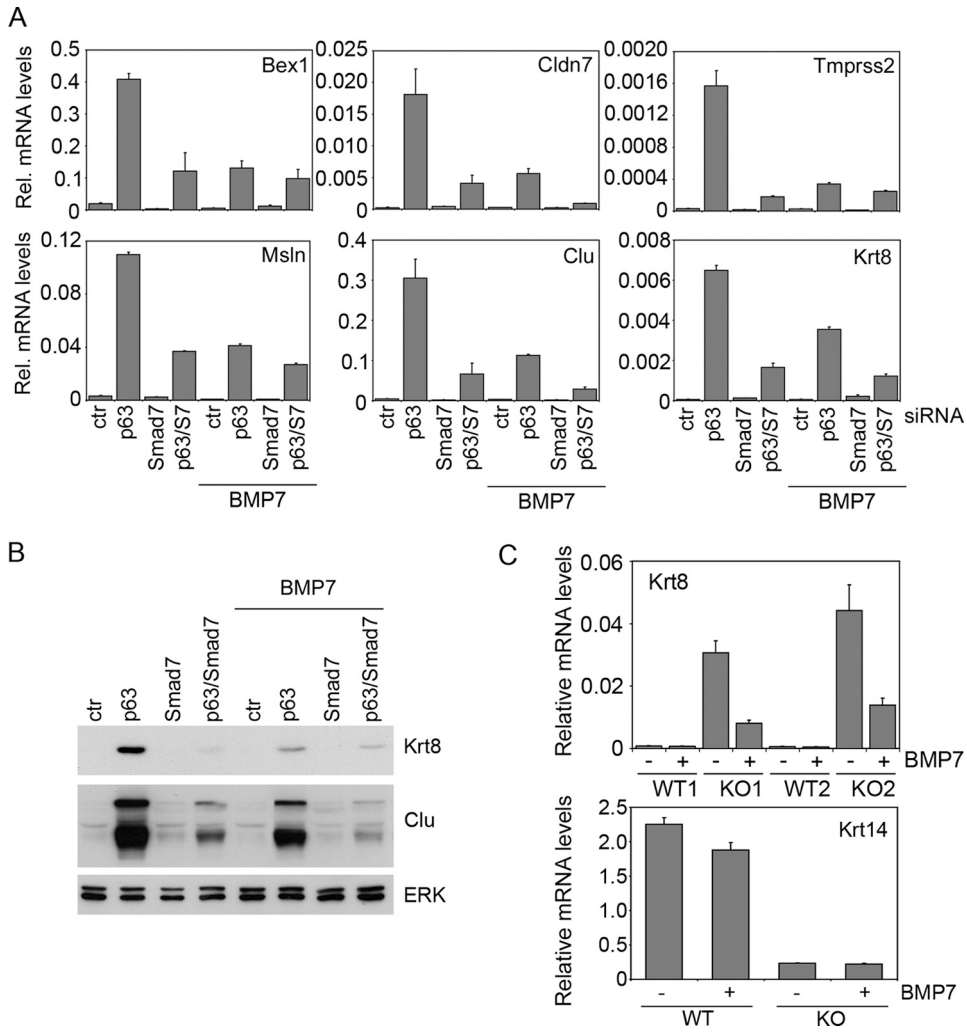

FIGURE 6. **Re-activation of BMP signaling restores low levels of non-epidermal genes in *p63* knockdown keratinocytes and *p63* KO skin explants.** *A*, expression of the indicated non-epidermal genes was measured in primary keratinocytes transfected with p63, Smad7, p63 and Smad7 (*p63/S7*), or control (*ctr*) siRNA, and cultured for 48 h. Cells were either treated with BMP7 for the last 24 h or left untreated. Relative mRNA levels was measured as described in the legend to Fig. 2*A*. *B*, total protein extracts from primary keratinocytes treated as in *A* were subjected to immunoblotting with the indicated specific antibodies. Protein extracts were normalized using anti-ERK polyclonal antibodies. *C*, skin explants at E14.5 were cultured overnight and then treated with BMP7 (80 ng/ml) or left untreated for an additional 24 h in the absence (*WT1* and *KO1*) or presence of fetal bovine serum (*WT2* and *KO2*). Krt8 and Krt14 mRNA levels were evaluated by real time RT-PCR and normalized as in *A*. Data presented are the average of two independent experiments and the S.E. is indicated. *KO*, knockout.

activation of other signaling molecules, including p38 mitogen-activated protein kinase (MAPK). Similarly, Smad7 has also been shown to regulate R-Smad independent pathways as discussed above. Among the BMP-responsive Smads, Smad1 and Smad5 were highly expressed in primary mouse keratinocytes (supplemental Fig. S3, *A* and *B*). Concomitant Smad1/5 knockdown inhibited Id1 and Id2 expression, consistent with decreased BMP/Smad signaling, whereas knockdown of either Smad1 or Smad5 alone was insufficient to elicit any effect (supplemental Fig. S3*C* and data not shown). We then tested whether activation of Smad1/5 is required for suppression of non-epidermal genes. Concomitant Smad1/5 depletion completely restored high levels of non-epidermal genes in the absence of p63 and Smad7 (Fig. 7, *C* and *D*). A requirement for Smad1/5 was similarly obtained upon treatment with BMP7 (data not shown). Thus the canonical BMP/Smad signaling pathway is required for suppression of non-epidermal genes downstream of p63.

DISCUSSION

p63 acts as a crucial regulator of gene expression in the epidermis and is essential for epidermal development, however, its interaction with signaling pathways involved in epidermal development is still poorly understood. We previously demonstrated a cross-talk between p63 and Notch, in which p63 transcriptionally represses the Notch effector Hes1, thus regulating keratinocyte differentiation (30). Here we establish a novel function of p63 in repressing non-epidermal genes through a mechanism that involves BMP/Smad signaling. We show that besides the previously reported Krt8 and Krt18, many other non-epidermal genes are induced by loss of p63 in epidermal cells both *in vitro* and *in vivo*. Interestingly, loss of ΔNp63α expression in human keratinocytes also induces the expression of a subset of non-epidermal genes (31), further reinforcing the notion that p63 may participate in maintaining an epithelial gene expression program in mammalian cells.

BMP7 was recently described as a novel direct target of the p53 family in breast cancer cells (26). BMP7 is induced by p53, p63, and p73, and all three members of the family directly bind to the p53 responsive element located in the BMP7 intron 1. In keratinocytes, we find that Bmp7 is inhibited in the absence of p63, although the remaining levels of Bmp7 are still fairly high, possibly suggesting that other p53 family members are responsible for its basal expression in keratinocytes. BMP7 promotes cell survival in p53-deficient breast carcinoma cells, at least in part, through Id2 (26), and has been demonstrated to promote cell survival in other contexts and tissues (32–35). Survival of epidermal cells is compromised in the absence of p63 (36). Thus, besides playing a role in maintaining epidermal identity, an interesting possibility that will require further studies is that Bmp7 may cooperate with other p63 downstream target genes to promote epidermal cell survival.

We demonstrate that p63 positively regulates BMP signaling both in isolated keratinocytes as well as in the embryonic epidermis. We provide entirely novel and compelling evidence that p63 directly suppresses the inhibitory Smad7 by binding to

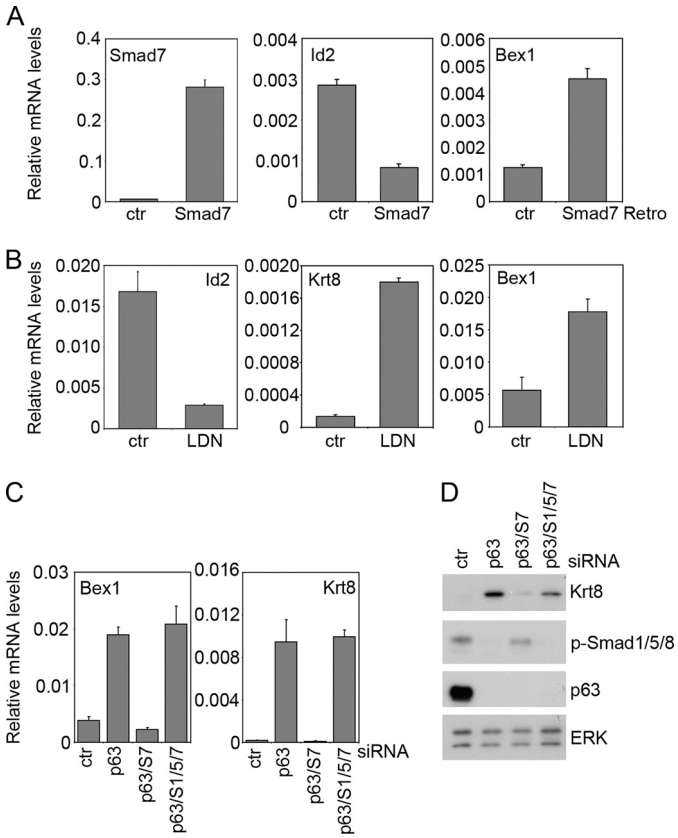

FIGURE 7. **Canonical BMP/Smad pathway regulates the expression of non-epidermal genes.** *A*, expression of the indicated genes was measured by real time RT-PCR in primary keratinocytes infected with a Smad7 expressing or a control retrovirus. *B*, expression of the indicated genes was measured by real time RT-PCR in primary keratinocytes treated with 200 nM LDN-193189 for 24 h. *C*, expression of the indicated non-epidermal genes was measured by real time RT-PCR in p63 knockdown primary keratinocytes in the presence or absence of siRNA targeting Smad7 (*S7*), Smad1 (*S1*), and Smad5 (*S5*) in the indicated combinations. *D*, expression of the indicate proteins was measured by immunoblotting in primary mouse keratinocytes co-transfected with the indicated combinations of siRNA. Protein extracts were normalized using ERK antibodies.

a highly conserved genomic region in the *Smad7* promoter. Consistent with this notion, Smad7 is poorly expressed in the developing epidermis and is induced in the *p63*-null epidermis. It has been previously reported that Smad7 induces expression of Cripto-1, and that both Smad7 and Cripto-1 induce Krt8 expression in mouse keratinocytes (21, 37). Loss of p63 does not affect Cripto-1 expression,[7] suggesting that other molecules downstream of Smad7 are required for repressing non-epidermal genes. Accordingly, we show that aberrant expression of non-epidermal genes seen in p63-deficient cells can be reversed by Smad7 knockdown or, to a lesser extent, by BMP7 treatment and that this effect is dependent on the Smad1/5 canonical pathway. The lack of synergy between BMP7 treatment and Smad7 knockdown suggests a threshold response, for which a critical level of BMP signaling is necessary, but additional signaling does not further increase the response.

Loss of BMP signaling by itself induces aberrant expression of non-epidermal genes in cultured keratinocytes, whereas *Bmp7* deletion or Smad7 overexpression are insufficient to induce non-epidermal genes in embryonic epidermis. A role for BMP signaling in epidermal development has remained elusive despite the strong Smad1/5/8 phosphorylation observed in the interfollicular epidermis during embryogenesis (present work and Ref. 38). Lack of an epidermal phenotype in mice carrying a deletion of single components of the pathway may be due at least in part to functional redundancy within the pathway. Among the BMP receptors, Bmpr1A/ALK3 plays a crucial role in hair follicle development with little phenotype in the epidermis (39–41). However, Bmpr1B/ALK6 and Acvr1/ALK2 are also expressed in the embryonic epidermis (42–44), and Acvr1/ALK2 is the main receptor for BMP7 (45–47), suggesting that depletion of multiple receptors may be required to observe an epidermal phenotype.

In addition to decreased Bmp7 and to the concomitant induction of Smad7, we observed that Bmp4 is down-regulated in the *p63*-null skin at E14.5. Bmp4 is poorly expressed in keratinocytes and is unaffected by p63 knockdown, indicating that as yet unidentified indirect signals derived from the *p63*-null epidermis inhibit Bmp4 expression in the dermis. BMP7 and BMP4 are capable of forming heterodimers 3–10 times more active than either BMP4 or BMP7 homodimers (48), suggesting that the concomitant reduction in both molecules observed in the *p63*-null skin could have more severe consequences on BMP signaling in the epidermis than loss of BMP7 alone. Interestingly, *Bmp7* deletion in the urethra is by itself sufficient to induce Krt8 expression (49), whereas in the developing epidermis multiple signaling molecules may be involved in this function. Thus our data indicate that BMP signaling plays a crucial and selective role in suppressing the expression of non-epidermal genes downstream of p63 in embryonic and postnatal epidermal cells, possibly in conjunction with other as yet unidentified mechanisms. Further assessment of the role of BMP signaling in the embryonic epidermis will be a key subject for subsequent investigations.

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