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**“Role of p63 in skin biology: its function in cell
proliferation and differentiation”**

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ABSTRACT

p63, a p53 family member, plays an essential role in epidermal development controlling several biological functions, some of which remain poorly understood. Using a global gene expression analysis, I contributed to the identification of novel downstream targets and signaling pathways regulated by p63.

We firstly identified a set of genes not specifically expressed in epidermis (non-epidermal genes) that are indirectly suppressed by p63. In parallel, p63 sustains Bone Morphogenetic Protein (BMP) signaling by directly suppressing transcription of the inhibitory Smad7, and by inducing Bmp7. In the absence of p63, BMP signaling is compromised and leads to ectopic expression of the non-epidermal genes *in vitro* and *in vivo*. Reactivation of BMP signaling by exogenous stimuli suppresses ectopic expression of non-epidermal genes in the absence of p63. These data indicate that p63 prevents ectopic expression of non-epidermal genes by a mechanism involving activation of BMP signaling.

In our genome-wide analysis we also observed that many cell cycle genes were positively regulated by p63. I found that loss of p63 in keratinocytes causes cell cycle arrest both *in vitro* and *in vivo*. Thus, I investigated how cell cycle genes are affected by p63. I identified a new mechanism through which p63 favors cell cycle progression repressing two members of a microRNA family: miR-34a and miR-34c. In the absence of p63, I observed increased

levels of miR-34a and miR-34c in primary mouse keratinocytes and in mouse embryonic skin. p63 directly binds to p53-consensus sites in both miR-34a and miR-34c gene promoters and inhibits their activity. Keratinocytes are arrested in the G1-phase of the cell cycle in the absence of p63 and have reduced levels of two cell cycle regulators, cyclin D1 and cyclin-dependent kinase 4 (Cdk4), that are known targets of miR-34. Importantly, concomitant downregulation of miR-34a and miR-34c in the absence of p63 restores cell cycle progression and the expression of cyclin D1 and Cdk4. These data demonstrate that p63 sustains cell cycle progression in keratinocytes not only by previously defined mechanisms, such as repression of the CDK inhibitor p21 Cip1/Waf1, but also by directly repressing components of the miR-34 family.

In conclusion, during my PhD program I uncovered two novel mechanisms through which p63 regulates cell cycle progression and tissue identity in epidermis.

INTRODUCTION

1. Epidermal development

The epidermis, the outer most component of the skin, is the primary barrier that protects the body from dehydration, mechanical trauma, and microbial insults. The epidermis develops from an initial undifferentiated monolayer of epithelial cells that subsequently undergoes stratification starting from embryonic day 14.5 (E14.5) (Fuchs, 2007), it is separated from the underlying dermis by the basement membrane, which consists of proteins secreted by epidermal keratinocytes and by dermal fibroblasts (McMillan *et al.*, 2003). The two compartments of the skin, the dermis and the epidermis, function cooperatively and together are responsible for the development of epidermal appendages, including hair follicles and mammary glands (Chuong *et al.*, 2000). Therefore, a failure to properly develop either the dermis or the epidermis may result in defects in appendage development. This is, for example, illustrated by ectodermal dysplasias, in which primary defects in epidermal development are the cause of subsequent defects in epidermal appendages (Koster *et al.*, 2004; Priolo *et al.*, 2000). The barrier function of the epidermis is established during embryogenesis and is the result of a complex and precisely coordinated stratification program. In mice, the execution of this program occurs in a period of approximately 10 days, between E8.5 and E18.5, and initiates when cells of the surface ectoderm commit to an epidermal fate (Figure 1).

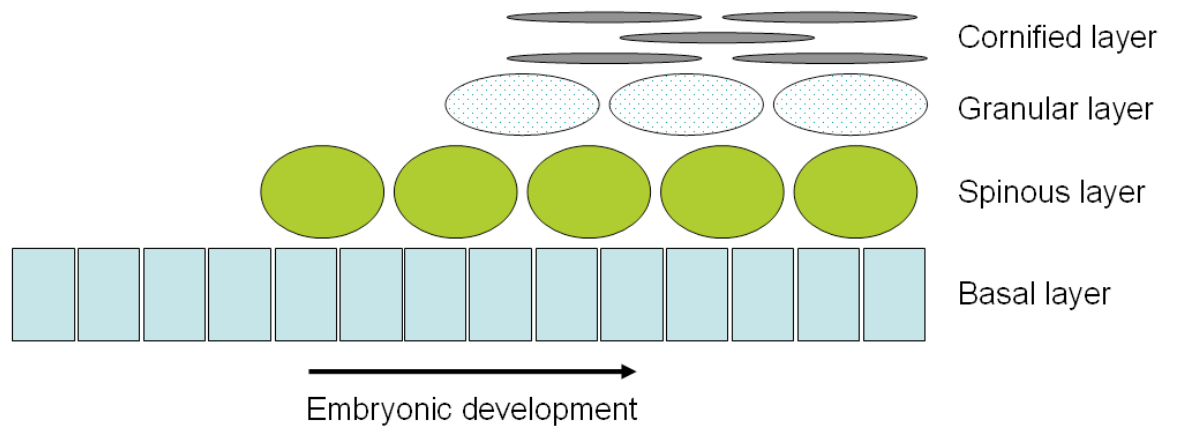


Figure 1: Schematic illustrating epidermal morphogenesis.

During epidermal morphogenesis, the single-layered surface ectoderm that initially covers the developing embryo initiates a stratification program culminating in the formation of the epidermal barrier.

After this initial commitment step, keratinocytes in the newly established embryonic basal layer give rise to a second layer of cells, the periderm (M'Boneko and Merker, 1988). The periderm is shed before birth in conjunction with the acquisition of epidermal barrier function (Hardman *et al.*, 1998). The next layer of the epidermis to form is the intermediate cell layer, which develops between the basal layer and the periderm. Development of this layer is associated with asymmetric cell division of embryonic basal keratinocytes (Lechler and Fuchs, 2005; Smart, 1970). Like basal keratinocytes, intermediate cells undergo proliferation, and the loss of this proliferative capacity is associated with the maturation of intermediate cells into spinous cells (Koster *et al.*, 2007). Spinous cells subsequently undergo further maturation into granular and cornified cells (Figure 2).

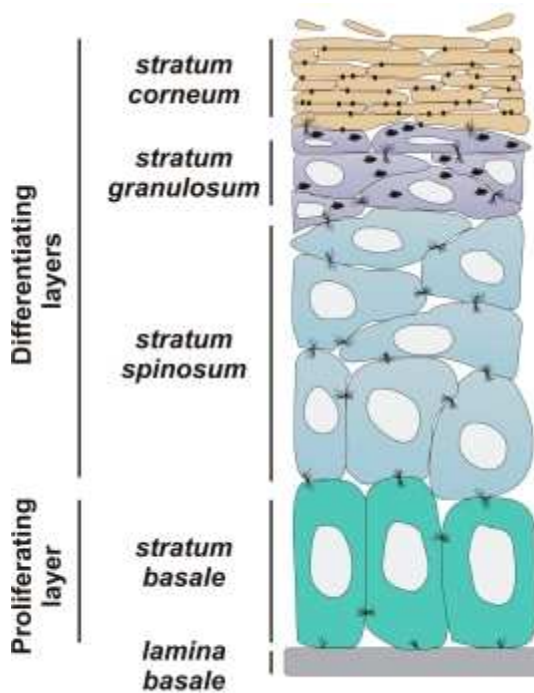


Figure 2. The epidermis is a pluristratified tissue.

The basal layer, or stratum basale, of the epidermis contains proliferating keratinocytes. Upon withdrawal from the cell cycle, basal keratinocytes detach from the basement membrane and undergo a terminal differentiation program to become corneocytes in the outer layers of the epidermis. In the intermediate stratum spinosum, the cells reinforce their keratin filament network, and adjacent cells interact via many desmosomes. In the stratum granulosum, keratinocytes become more flattened and express certain proteins such as filaggrin and loricrin, which aggregate to form the typical keratohyalin granules of the stratum granulosum.

The morphological changes that are a hallmark of epidermal stratification are associated with changes in the expression of keratin differentiation markers (Koster and Roop, 2004). The cells of surface ectoderm initiates the expression of Krt5 and Krt14, these cells commit to an epidermal fate (Byrne *et al.*, 1994). Subsequently, the initiation of terminal differentiation results in the induction of K1 and K10 expression in the newly formed suprabasal keratinocytes (Bickenbach *et al.*, 1995; Fuchs and Green, 1980). The final step in epidermal stratification involves the formation of the epidermal barrier. During normal development, barrier acquisition is patterned and initiates at the dorsal surface, spreading laterally to the ventral surface in approximately one day (Hardman *et al.*, 1998). The process of barrier formation is characterized by the formation of cornified cell envelopes, composed of proteins crosslinked into a rigid scaffold and of lipids covalently attached to the exterior surface (Rice and Green, 1977; Steven and Steinert, 1994). Although established in utero, the barrier function of the epidermis is maintained during postnatal life owing to the continuous selfrenewal of the epidermis, a process mediated by epidermal stem cells (Blanpain *et al.*, 2006). Under homeostatic conditions, the epidermal stem cells that are located in the interfollicular epidermis are responsible for the maintenance of this structure (Ito *et al.*, 2005). These stem cells represent a small proportion of basal keratinocytes and, through asymmetric cell division, give rise to a daughter stem cell and a transit-amplifying cell, which ultimately undergoes terminal

differentiation (Dunnwald *et al.*, 2003; Potten and Morris, 1988; Schneider *et al.*, 2003).

2. p63: structure and expression

p63 belongs to the p53 gene family consisting of three genes, p53, p63, and p73, that show significant sequence homology (Yang *et al.*, 2002). Each p53 family member contains a transactivation domain at the amino terminus, a DNA-binding domain, and an oligomerization domain. In addition, all family members share some functions and bind to a canonical p53-binding site, thus controlling the expression of a subset of p53 target genes (Yang *et al.*, 2002; Yang *et al.*, 2006) (Figure 3B).

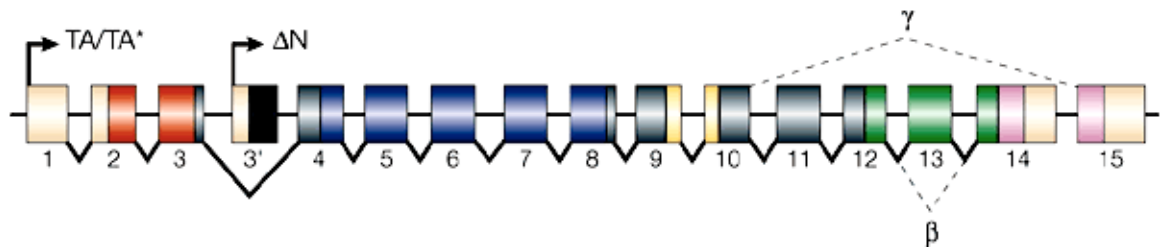
The p63 gene encodes a tetrameric transcription factor that can be expressed in at least six isoforms with widely different transactivation potential that share an identical DNA binding domain (Yang *et al.*, 1998). Alternative transcription start sites (TSS) give rise to transactivation (TA) isoforms, encoding proteins with a canonical transactivation domain similar to p53, and ΔN isoforms containing an alternative transactivation domain (Helton *et al.*, 2006; Yang *et al.*, 1998) (Figure 3A). $\Delta Np63\alpha$ contains both a transactivation domain and an inhibitory domain and has been shown to activate or repress gene transcription depending on the promoter context (Perez and Pietsenpol, 2007). $\Delta Np63\alpha$ 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 it continues to be expressed during skin development and in the basal proliferative layer in postnatal life (Koster *et al.*, 2004; Laurikkala *et al.*, 2006; Yang *et al.*, 1998). Whereas p63 is predominantly expressed in the

basal and spinous layers of the epidermis, it is downregulated upon keratinocyte differentiation both *in vitro* and *in vivo* (Bamberger and Schmale, 2001; Liefer *et al.*, 2000; Nguyen *et al.*, 2006; Parsa *et al.*, 1999; Pellegrini *et al.*, 2001; Westfall *et al.*, 2003; Yang *et al.*, 1999). In the basal layer, p63 is mainly involved in maintaining cell proliferation and cell adhesion (DeYoung *et al.*, 2006; Koster *et al.*, 2004; Sbisa *et al.*, 2006; Truong *et al.*, 2006). It has been proposed that p63 plays a dual role in keratinocyte differentiation, as it is required for initiating epithelial stratification (Koster *et al.*, 2004; Nguyen *et al.*, 2006; Truong *et al.*, 2006), whereas concurrently it inhibits the expression of some differentiation markers, at least in part through transcriptional repression of the Notch effector Hes1 (King *et al.*, 2003; Nguyen *et al.*, 2006). Interestingly, some p63 direct targets, such as Perp, whose expression in skin requires p63, are predominantly localized in the suprabasal layers (Ihrie *et al.*, 2005).

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 (Mills *et al.*, 1999; Yang *et al.*, 1999), 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 (Senoo *et al.*, 2007; Yang *et al.*, 1999), and/or altered epidermal stratification and cell differentiation associated with reduced expression levels of Krt5/Krt14 and Krt1/Krt10 (Koster *et al.*, 2004; Mills *et al.*, 1999; Romano *et al.*, 2009). 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* (Koster *et al.*, 2004; Truong *et al.*, 2006), suggesting that p63 may be involved in maintaining an epithelial gene expression program in mammalian cells.

A



B

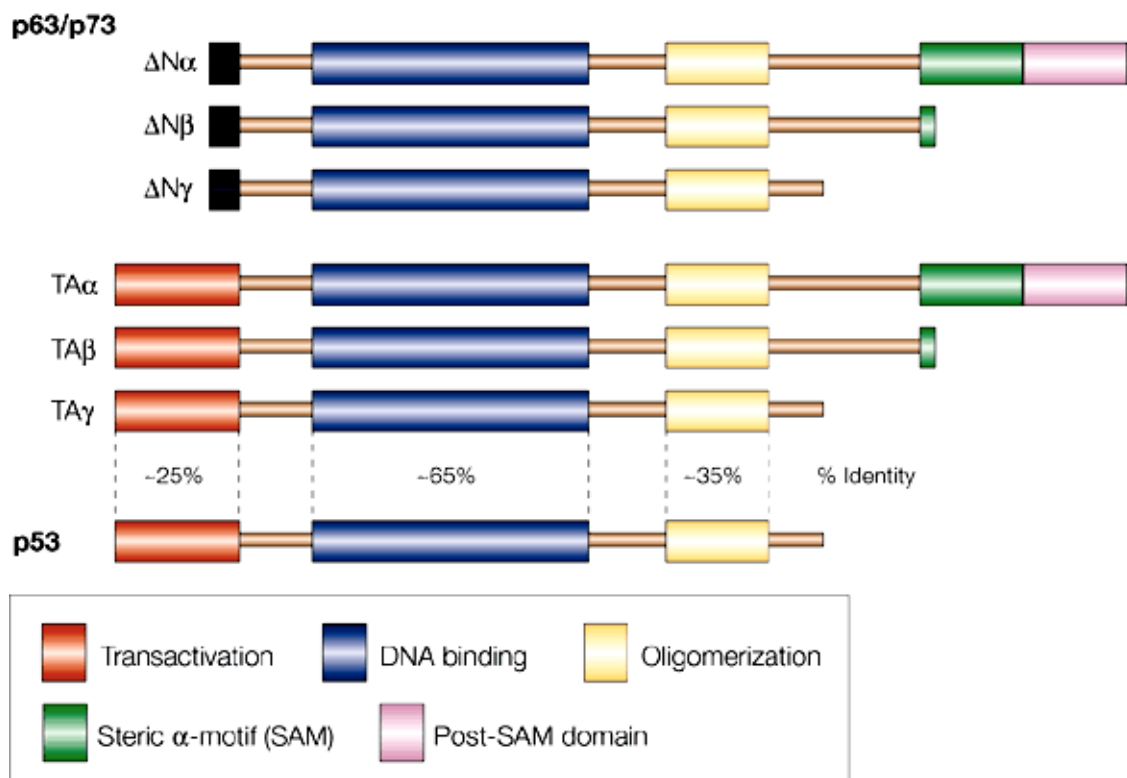


Figure 3. p63 gene and relative isoforms.

(A) Structure of the p63 gene. (B) Comparison between p53, p63 and p73 proteins. The schematic structure of TA α isoforms of p63 and p73 is shown, including the transactivation domain (TA), the DNA-binding domain and the oligomerization domain (oligo). SAM: sterile alpha motif; PS: post-SAM domain. The percentage of identity is indicated for each domain.

3. BMP Signaling

BMPs are part of the transforming growth factor (TGF) superfamily of morphogenetic proteins. Members of this family bind to a complex of transmembrane serine threonine kinase receptors type 1 and 2, triggering the phosphorylation and activation of the type 1 receptor by the type 2 receptor kinase. The activated type 1 receptor phosphorylates a receptor-associated SMAD that subsequently complexes with SMAD4 and translocates to the nucleus to regulate gene transcription (Massague, 1998). Although the TGF and BMP receptors have distinct receptor-associated SMADs, both need the common mediator SMAD4 to transduce a signal to the nucleus (Figure 4). A high level of complexity results from the existence of multiple ligands, multiple ligand-sequestering antagonists and multiple receptors, with considerable mixing and matching occurring both at the level of ligand–receptor interactions and type II–type I receptor interactions (Schmierer and Hill, 2007). Signaling then converges on three BMP-specific receptor-regulated SMADs — SMAD1, SMAD5 and SMAD8 — which complex with the co-SMAD SMAD4 and modulate target gene expression.

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 (Stern, 2006). In mammals the possible role of BMPs in epidermis development is still not definitively addressed. 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 (Bitgood and McMahon, 1995; Lyons *et al.*, 1989). Strong expression of Bmp7 mRNA is seen in mouse and rat epidermis during embryonic development from early stages (Laurikkala *et al.*, 2006; Takahashi and Ikeda, 1996), whereas Bmp6 is expressed in suprabasal layers of embryonic murine epidermis at E15.5 (Lyons *et al.*, 1989). Interestingly, Bmp7 transcripts are strongly downregulated in the ectoderm of p63 null mice (Laurikkala *et al.*, 2006), 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 RSmads for interaction with the activated receptors, ubiquitination and degradation of the receptors (Hoover and Kubalak, 2008). In addition Smad7 plays also noncanonical functions by regulating several other signaling proteins, including β -catenin (Han *et al.*, 2006; Hoover and Kubalak, 2008). During my PhD program I demonstrated that p63, and more specifically the Δ Np63 α 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.

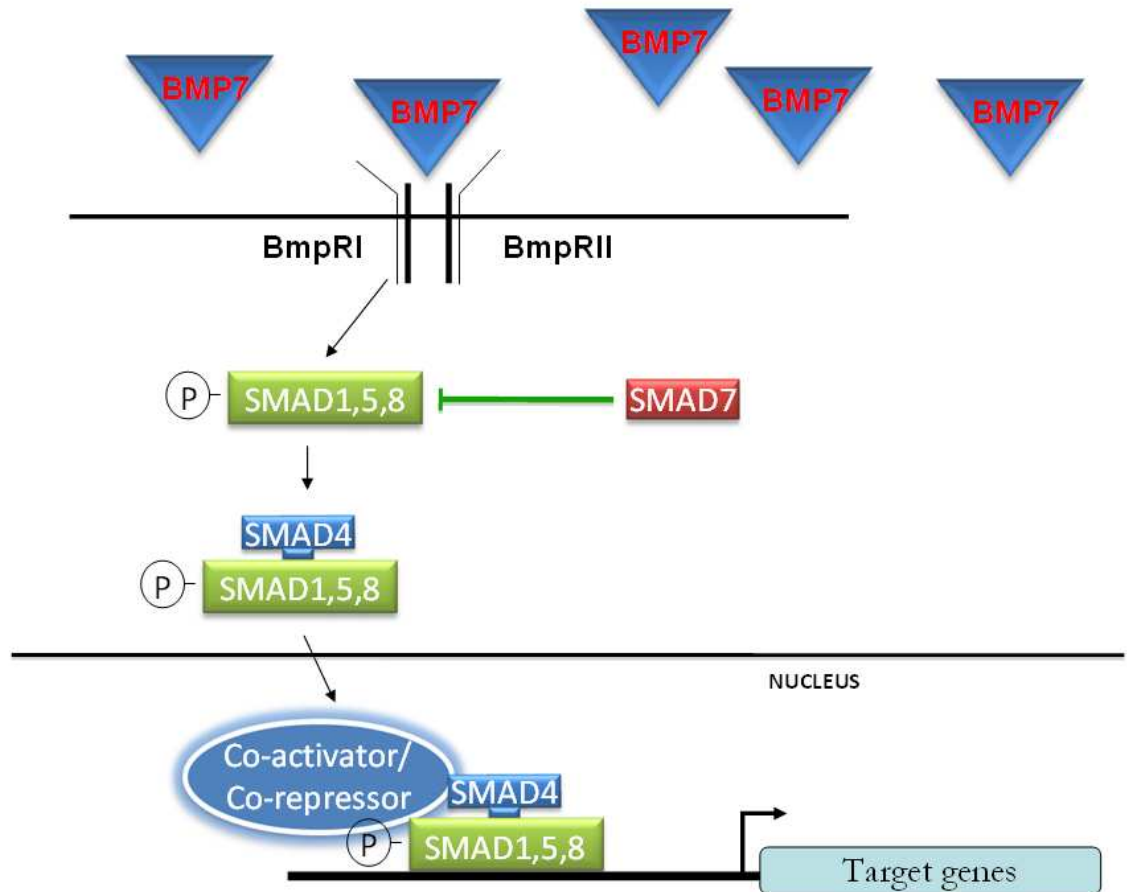


Figure 4: BMP signaling pathway.

BMP ligands bind to the BMP receptors Bmpr1 and Bmpr2, and Bmpr2 then phosphorylates and activates Bmpr1. Phosphorylated Bmpr1 subsequently phosphorylates SMAD1, SMAD5 or SMAD8 (SMAD1,5,8), which associate with SMAD4 and enter the nucleus, where they regulate gene expression. The BMP signal can be blocked by extracellular antagonists, such as noggin, which bind BMPs and prevent their association with the BMP receptors, or by intracellular inhibitors, such as Smad7.

5. Cell Cycle

DNA replication starts as soon as mitosis ends, a gap named G1 phase is incorporated between nuclear division (M phase) and DNA synthesis (S phase) while another gap named G2 phase occurs between S and M. These gaps allow for the repair of DNA damage and replication errors. G1 is a period when many signals influence cell division and the deployment of a cell developmental program.

Compared to DNA replication and mitosis, which follow canonical steps that vary little from cell to cell, the steps controlling entry and progression through G1 are largely dependent on cell type and context. All proceed through G1 phase under different circumstances, different signals and different developmental programs and with different risks of malignant transformation. Definitively, however, to enter S phase all cells must activate cyclin-dependent kinases (CDKs).

CDKs are protein kinases that require binding to a cyclin subunit to become catalytically competent (Morgan, 1997; Murray, 2004). Different members of the CDK family, in association with different cyclins, turn on and off key switches throughout the cell cycle. Cyclin–CDK complexes are regulated by phosphorylation and protein interaction events that tightly control the timing and extent of CDK activation. The prototypic CDK, Cdk1, associates with cyclins A and B, and acts at the G2/M interface. The progressive accumulation of A and B cyclins during the cell cycle and their abrupt degradation at the onset of anaphase, mediates entry and exit from

mitosis, respectively. The drop in Cdk1 activity at the end of M phase allows DNA chromosomal sites known as replication origins to be loaded with a pre-replicative complex (PRC) (Kelly and Brown, 2000; Prasanth *et al.*, 2004). This complex contains ORC (origin of replication complex), the kinase Cdc6/18 and Cdt1 (Cdc10-dependent transcript 1), and loads MCM (mini-chromosome maintenance) proteins onto the DNA, licensing these sites for the initiation of replication.

The G1 CDKs include Cdk2, which combines with E-type cyclins (E1, E2) and cyclin A (Morgan, 1997; Murray, 2004). On Cdk2 activation, PRCs recruit DNA helicases, primases and polymerases, causing unwinding of the double helix and DNA replication (Kelly and Brown, 2000; Prasanth *et al.*, 2004). Cdk activity is essential for the unwinding step, and several components of the PRC become phosphorylated in the process. The newly replicated origins cannot reassemble new PRCs until CDK activity once again drops at the end of mitosis. Mitosis in turn will not proceed until DNA replication is completed. Together, these events ensure that DNA will be replicated once and only once per cell cycle (Kelly and Brown, 2000; Prasanth *et al.*, 2004).

Cyclin E expression is dependent on E2F transcription factors (Sears and Nevins, 2002; Stevaux and Dyson, 2002). In mitotically resting cells, and in cells that have just emerged from M phase, E2F factors are bound to the retinoblastoma protein (Rb) or its family members, p107 and p130 (Lipinski and Jacks, 1999). Rb binding turns E2Fs into repressors (in the case of E2F4

and E2F5) or inactive transactivators (in the case of E2F1, -2 and -3) (Stevaux and Dyson, 2002).

Mitogenic stimuli change this state by increasing the amount of D-type cyclins, which combine with Cdk4 and Cdk6 to phosphorylate and inactivate Rb (Morgan, 1997; Murray, 2004; Sherr and Roberts, 1999). Phosphorylation dissociates Rb from E2F, allowing E2F-dependent transcription. Along with cyclin E, the E2Fs activate transcription of a large set of components that support DNA replication (ORCs, MCMs, DNA polymerase) and subsequent events (cyclin B, Cdk1 and various DNA quality-control components) (Sears and Nevins, 2002; Stevaux and Dyson, 2002). Rb seems to be the only essential substrate of cyclin D–Cdk4/6, as cells lacking Rb no longer require cyclin D for proliferation (Sherr, 2004). Rb can also be phosphorylated by cyclin-E–Cdk2, creating a positive feedback loop that helps precipitate S-phase entry once enough Cdk2 has been activated.

The identities of the CDK substrates that directly trigger DNA replication remain unknown and a stinging reminder of how much we still do not know about how cell reproduction works.

A mechanism that prevents premature entry into S phase, and ties the G1/S transition to regulatory inputs, relies on inhibitory proteins that latch onto cyclin–CDK complexes and disrupt their catalytic centre (Pavletich, 1999). p21Cip1/WAF1 was the first CDK inhibitor to be identified as a mediator of p53-induced growth arrest in response to DNA damage, in cell

senescence, and as a direct CDK regulator (el-Deiry *et al.*, 1993; Gu *et al.*, 1993; Harper *et al.*, 1993; Noda *et al.*, 1994; Xiong *et al.*, 1993).

Infact, among other functions, p53 has a crucial role in negatively regulating cell cycle progression upon DNA damage and oncogene-induced senescence by transcriptional regulation of several cell cycle genes (Riley *et al.*, 2008). p53-dependent cell cycle arrest in G1 phase is mediated, at least in part, by transcriptional activation of the cyclin-dependent kinase inhibitor p21Cip1 gene (Cdkn1a), encoding an inhibitor of G1 cyclin/CDK complexes (Brugarolas *et al.*, 1995; el-Deiry *et al.*, 1993; Harper *et al.*, 1993; Waldman *et al.*, 1995). Conversely, p63 is required for cell cycle progression at least in cultured human epidermal cells and it has been shown to suppress the p21Cip1 gene by binding to the p53-consensus site (DeYoung *et al.*, 2006; Goodheart *et al.*, 2005; Nguyen *et al.*, 2006; Truong *et al.*, 2006; Westfall *et al.*, 2003). However, in human keratinocytes, downregulation of p21Cip1 is unable to fully restore cell cycle progression in the absence of p63 (Truong *et al.*, 2006), suggesting that other mechanisms may be involved. An involvement of p63 in controlling cell cycle progression in mouse keratinocytes has not been analyzed; however, p63 has been correlated to the proliferative potential of human and rat epidermal stem cells (Senoo *et al.*, 2007).

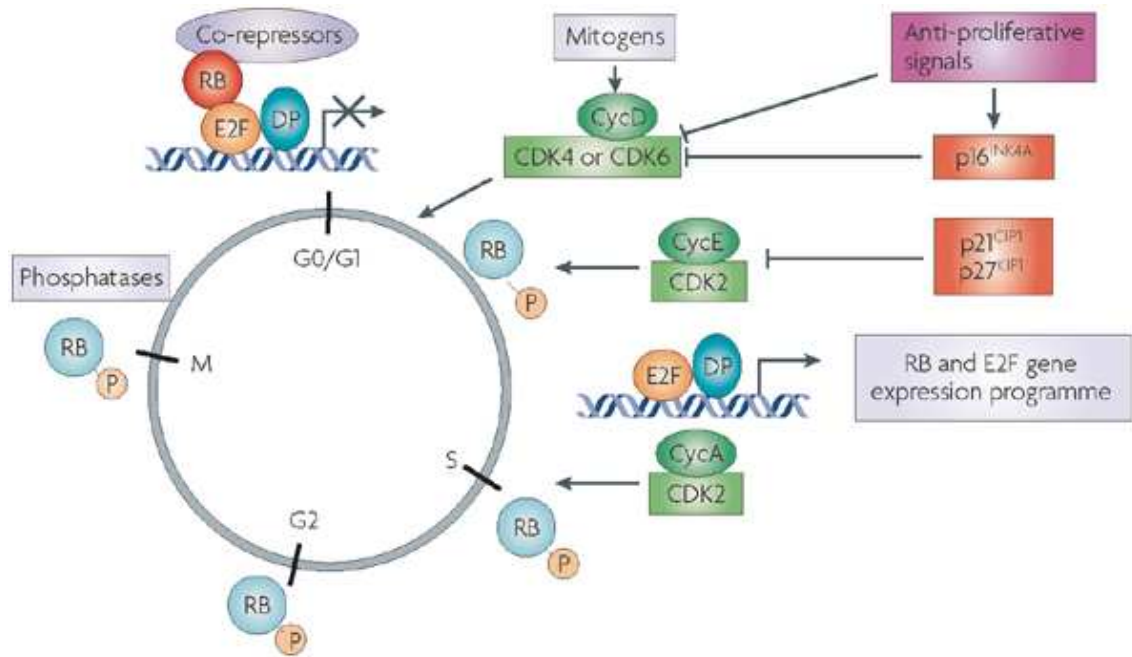


Figure 5: The cell cycle

Mitogenic signals stimulate the expression of D-type cyclins (Cyc) and a concomitant increase in cyclin-dependent kinase 4 (CDK4) and CDK6 activity. These factors initiate RB phosphorylation, which is augmented by the activity of CDK2 complexes with cyclins A and E. The phosphorylation of RB disrupts its association with E2F. The dissociation of p-Rb/E2F complex leads to release of E2F, which mediates the expression of E2F-sensitive genes involved in proliferation and progression through S-phase and mitosis. At the transition from mitosis to G1, RB is dephosphorylated through the action of phosphatases. Importantly, a large number of anti-mitogenic signals function to prevent RB phosphorylation either by limiting the activity of CDK4, CDK6 and CDK2 complexes or by inducing the activity of CDK inhibitors. (Figure taken from Nature Cancer, Erik et al 2008)

6. microRNA

MicroRNAs (miRNAs) form a class of small non-coding RNAs of 19–24 nucleotides (nt) that have key roles in the regulation of gene expression in several cellular processes, including cell proliferation.

MiRNAs are transcribed by RNA polymerases II or III as primary transcripts (pri-miRNAs), which are further processed by the nuclear RNase III enzyme Drosha to stem-loop-structured miRNA precursor molecules (pre-miRNAs). The pre-miRNAs are subsequently transported to the cytoplasm in a complex with exportin-5 and RAS-like nuclear protein (Ran)-GTP (Cullen, 2004). They are then further processed by Dicer, another RNase III enzyme, that cleaves off the double stranded (ds) portion of the hairpin and generates a short-lived dsRNA of about 20–25 nt in size. The duplex is subsequently unwound and only one strand gives rise to the mature miRNA, which is incorporated into RISC (RNA-induced silencing complex), where it associates with one of the highly conserved Argonaute proteins (Ambros, 2004; Bartel, 2004; Meister and Tuschl, 2004; Zamore and Haley, 2005). The miRNA-Argonaute complex interacts with the 3' untranslated region (UTR) of target mRNAs and inhibit their expression either by interfering with translation or by destabilizing the target mRNA (Meister and Tuschl, 2004; Pillai *et al.*, 2007).

Each miRNA is predicted to bind to more than 100 target mRNAs and to inhibit gene expression either by interfering with translation or by

destabilizing the target mRNA (Bartel, 2009). Recent studies have shown that members of the miR-34 family possess anti-proliferative potential and induce cell cycle arrest, senescence, and/or apoptosis (Bommer *et al.*, 2007; Chang *et al.*, 2007; Corney *et al.*, 2007; Raver-Shapira *et al.*, 2007; Tarasov *et al.*, 2007; Tazawa *et al.*, 2007). In mammals, the miR-34 family comprises three processed miRNAs that are encoded by two different genes: miR-34a is encoded by its own primary transcript, whereas miR-34b and miR-34c share common primary transcripts. In addition, miR-34a and miR-34b/c are direct transcriptional targets of p53. Transcriptional activation of miR-34a and miR-34b/c causes dramatic reprogramming of gene expression and contributes to p53-mediated cell cycle arrest and apoptosis (Chang *et al.*, 2007; Raver-Shapira *et al.*, 2007). miR-34 family mediates such functions through additive or synergistic effects on multiple targets, including cyclin-dependent kinase 4 (CDK4), CDK6, E2F3, cyclin D1, cyclin E2, Bcl2, and others (Bommer *et al.*, 2007; He *et al.*, 2007; Sun *et al.*, 2008; Tazawa *et al.*, 2007).

In this study I show that one of the mechanisms by which p63 regulates cell cycle progression in mouse epidermal cells is by directly repressing the expression of two members of the miR-34 family. In the absence of p63, high expression levels of miR-34a and miR-34c were observed both *in vitro* and *in vivo*. Moreover, concomitant suppression of miR-34a and miR-34c by specific antisense inhibitors induces reactivation of cell proliferation and transition from G1 to S phase in p63 knockdown keratinocytes, showing that

these miRNAs exert an effect downstream of p63 to regulate cell cycle progression.

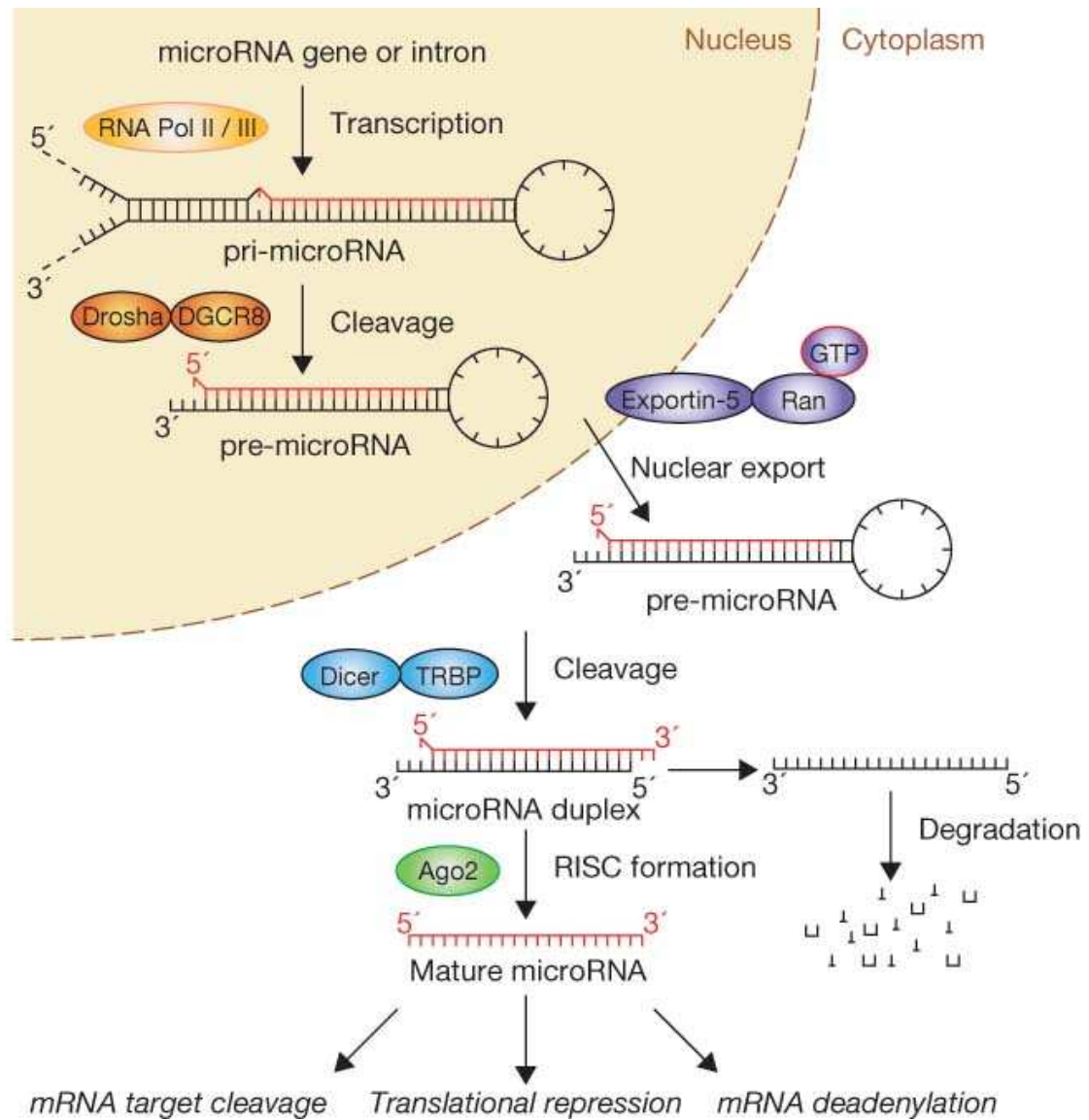


Figure 6: microRNA Biosynthesis

The miRNA processing pathway has long been viewed as linear and universal to all mammalian miRNAs. This canonical maturation includes the production of the primary miRNA transcript (pri-miRNA) by RNA polymerase II or III and cleavage of the pri-miRNA by the microprocessor complex Drosha-DGCR8 (Pasha) in the nucleus. The resulting precursor hairpin, the pre-miRNA, is exported from the nucleus by Exportin-5-Ran-GTP. In the cytoplasm, the RNase Dicer in complex with the double-stranded RNA-binding protein TRBP cleaves the pre-miRNA hairpin to its mature length.

The functional strand of the mature miRNA is loaded together with Argonaute (Ago2) proteins into the RNA-induced silencing complex (RISC), where it guides RISC to silence target mRNAs through mRNA cleavage, translational repression or deadenylation, whereas the passenger strand (black) is degraded. In this review we discuss the many branches, crossroads and detours in miRNA processing that lead to the conclusion that many different ways exist to generate a mature miRNA. (Figure taken from Nature Cell Biology, Winter et al 2009)

MATERIALS AND METHODS

1. Keratinocytes preparation and cell cultures

Primary mouse keratinocytes were isolated from skin of 2-day-old Swiss CD1 mice and cultivated in minimal essential medium with 4% FBS, EGF and 0.05 mM Calcium (low calcium medium). For all experiments, cells were used 5 days after plating. Cells were incubated at 34°C, 8% CO₂ and culture medium was changed every day.

2. Chromatin immunoprecipitation

3 x 10⁶ mouse keratinocytes were fixed with 1% formaldehyde in growth medium at 37°C for 10 min. Extracts were extensively sonicated on ice to obtain DNA fragments ranging from 400 to 800 bp in length. Chromatin was immunoprecipitated as in 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 7500 (Applied Biosystems).

Oligonucleotide Primers for ChIP:

Smad7 ctr (-0.2kb)

GCGAAACACAATCGCTTTTTT

CGTCACGTGGCCGTCTAGA

Smad7 (2) (-2.7kb)

ATCTGTTTTTACCCGGGCCT

CGTGAGTGGTGCTAATCCCCT

Smad7 (1) (-3.1kb)

GTGAGGCGAAAGAAGAGCCC

GCTCTGACTGGCTTGTATGCC

Bmp7 (1) (-1.7kb)

TGCTGTGGTGGGTGATCTGA

GTCATGTCTCCAGCTCGCAG

Bmp7 (2) (+2.2kb)

AGGCAAGGCGCTGAATTGT

TGTGATCTCCAGCAAGGCAGT

Bmp7 (3) (+2.5kb)

CCTCGCTGATTGAGAGGCTC

TCATGTTGAAAGGACAAAGCCTAG

3. Constructs, transfections and reporter assays.

Transfections were performed 5 days after plating using 2-3 μ l Lipofectamine 2000 (Invitrogen) and 1-1.5 μ g of DNA. Plasmids (250ng) were co-transfected with pCMV2-FLAG- Δ Np63 α or pCMV2-FLAG control. A 4.3kb Smad7 promoter (3.6 kb promoter region and 0.7kb 5'UTR upstream) 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). Luciferase activity was determined 48 hours (hrs) after transfection with the dual luciferase reporter assay kit (Promega). pCMVRenilla reporter (20ng; Promega) was used to normalize transfection efficiency. A total amount of 200nM siRNA (Stealth siRNA, Invitrogen) for pan-p63, for specific p63 isoforms, and/or for Smad7, Smad1, Smad5, Bmp7 and/or anti-miR-34a, anti-miR-34c (miRCURY, Exiqon, Vedbaek, Denmark), or scramble control (miRCURY, Exiqon 19900200) or control medium GC rich siRNA (Stealth siRNA, Invitrogen) were transfected by Lipofectamine 2000. 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.

Mutagenesis of Smad7 promoter

Mut 166-167

GAATTA AACACCCACAATTACACCCGATGGGAACATAGCTCTG

Mut 187-188

GGGAACATAGCTCTGCCCGTAGATGCACGTCCC

Mut 212-213

GCACGTCCCATCCAGACCCGCTGTGACATCAGATTC

siRNA oligonucleotides

Smad7 AGTCAAGAGGCTGTGTTGCTGTGAA

Smad7_2 CCCATCACCTTAGTCGACTCTGTGA

Smad1_a CATATTGGGAAAGGAGTCCACCTTT

Smad1_b CAATCCTATTTTCATCCGTGTCTTAA

Smad5_a CAGAGATGTTCAGCCTGTGCGCCTAT

Smad5_b ATTCATAGTAGACAATCGAACACCA

Bmp7_a CCTGAAGGCTATGCTGCCTACTACT

Bmp7_b CAACCTAGTGGAACATGACAAAGAA

4. Retroviral infections.

Primary mouse keratinocytes were infected 2 x 2hr with high-titer pBABE-Smad7, GINCO-green-fluorescent protein or GINCO- Δ Np63 α retroviruses and were cultured for 4 additional days. in the presence of 8 μ g/mL polybrene (Sigma), selected with 2 μ g/ml puromycin for 48 hrs, and grown after selection for an additional 24 hrs in the absence of puromycin.

5. Analysis of gene expression

Gene expression profiling was obtained in p63 knockdown versus control keratinocytes 48hrs after transfection and using Affymetrix Mouse Genome 430A 2.0 chips and analyzed using the dChip algorithm. Among the genes affected by loss of p63, 106 genes were upregulated more than 5-fold by p63 knockdown (Table S1). Tissue expression profiling of upregulated genes was

obtained from a custom made mouse GNF1M (MAS5) GNF gene expression database (<http://symatlas.gnf.org>). 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.

6. Real-time RT-PCR.

Total RNA was extracted 48 hrs after transfection from primary 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 Actb expression.

Oligonucleotide Primers for Real Time RT-PCR:

Gapdh

GTATGACTCCACTCACGGCAA

TTCCATTCTCGGCCTTG

Actb

CTAAGGCCAACCGTGAAAAGAT

GCCTGGATGGCTACGTACATG

p63

CATGAGCTGAGCCGTGAGTTC

GGCTGTTCCCTTCTACTCGAA

Krt14

TGACGTCTCCACCCACCTG

ACCACGAGGAGGAAATGGC

Krt8

TGCTCATGTTCTGCATCCCA

GATCACCACTACCGCAAGC

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

Lce1d

TCGTCTTGCTCCAGAGCACTCACC

GGAGGCTGGCACTGCTGTTGG

Lce1a2

GTTCTGCTGGCAGGACATCT

GCCCAAGGATCTTGTACTGC

Id1

GAGCAGCAGGTGAACGTCCT

TCCTTGAGGCGTGAGTAGCA

Id2

TGATGCAGGCTGACGATAGTG

TCTTGGACCTGCAGATCGC

Smad1

ATTGAAAACACCAGGCGACATA

CCAACGTAATAAAGGTGGACTCCT

Smad5

ACTATTGAAAACACTAGGCGGCATA

CACCTCCCCACCAACGTAGTA

7. Immunostaining.

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. 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), Cldn 3 (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.

8. 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 in Laemmli sample buffer (10 % Glycerol, 0.01 % Bromophenol Blue, 0.0625 M Tris-HCl pH 6.8, 3 % SDS and 5 % β-mercaptoethanol (ME) for the other proteins. 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 immunoblotting analysis: p63 (4A4), cyclin D1 (72-13G), Cdk2 (M-2), Cdk4 (C-22), and ERK-1 (K-23), all from Santa Cruz Biotechnology (Heidelberg, Germany), Smad7 (MAB2029, R&D Systems), polyclonal anti-Bex1, β-actin (AC-15 Sigma).

9. in situ hybridization.

In situ hybridization was performed on frozen sections of P1 skin and E14.5 embryos as previously described (24). Digoxigenin (DIG)-labeled antisense

and sense RNA probes were transcribed from the SP6 and T7 promoters using a DIG labeling kit (Roche Applied Science). The Smad7 probe corresponding to a 485bp cDNA fragment was kindly provided by Xiao-Jing Wang. The Bmp6 probe was synthesized from a 893bp SacIEcorI 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 microRNA the hybridization was performed with miRCURY LNA Detection antisense Probes (Exiqon) specific for miR-34a, miR-34b, and miR-34c at 40 °C. Digoxigenin (DIG) labeling was monitored under a Zeiss Axioskop2 plus microscope using a Zeiss Plan-Neofluar 20X/0.50 objective.

10. Mice and Skin Explants

All experiments performed with mice were conducted under IACUC approval. p53-null mice in a C57/BL6 background were obtained from Alfredo Fusco (Federico II University, Napoli, Italy). p63-null mice (B6.129S7-Trp63tm1Brd/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% CO₂.

11. Flow cytometry and BrdU labeling

For cell cycle analysis, keratinocytes were harvested 48 hours after transfection and were incubated with a solution of propidium iodide (Sigma-Aldrich; St Louis, MO; 2.5 mgml⁻¹), RNase (1mgml⁻¹), and 0.15% NP-40 in phosphate-buffered saline overnight at 4 °C. For each sample, at least 10,000 events were acquired using FACSCanto II Flow Cytometry System (BD Biosciences, Erembodegem, Belgium), and analyzed using the ModFit LT 3.0 software (Verity Software House, Topsham, ME). For measurement of DNA synthesis, cells were labeled with BrdU (Zymed, San Francisco, CA; 1:100) for 3 h and subsequently fixed with 4% paraformaldehyde. After fixation, cells were permeabilized with NP-40 0.1%, and the DNA was denatured with 50 mM NaOH. BrdU was detected with mouse monoclonal antibodies (G3G4; Developmental Studies Hybridoma Bank, The University of Iowa) and rabbit anti mouse TRITC (Dako Cytomation). DNA was counterstained with DAPI (100 ng/mL). For *in vivo* analysis, BrdU was injected (1 ml per 100 g body weight) intraperitoneally in pregnant female mice and incubated for 3 hours. Embryos were taken at E14.5 and fixed in 4% paraformaldehyde, and embedded in paraffin. Sections, 7 μm thick, were permeabilized in 0.2% Triton X-100, and processed for immunofluorescence as described above.

12. miRNA expression analysis

Total RNA from primary keratinocytes or from total embryonic skin was extracted using TRIzol reagent (Invitrogen). The expression levels of mature miRNA species were quantified using TaqMan MicroRNA Assays (Applied Biosystems, Foster City, CA; miR-34a: no. 4373278; miR-34b: no. 4373343, and miR-34c: no. 4373036). The expression of each miRNA was normalized to the expression of U6 small nuclear RNA.

RESULTS

To explore the role of p63 in controlling gene expression in epidermal cells, I recently contributed to identify a large number of putative p63 target genes in primary mouse keratinocytes performing a global gene expression profiling.

We identified a large set of direct and functional p63 target genes by applying a novel reverse-engineering algorithm (TSNI: Time Series Network Identification) to dynamic gene expression profiles following p63 activation in primary keratinocytes (Della Gatta *et al.*, 2008). Validation of the identified p63 target genes was achieved by p63 knockdown and by ChIP-chip analysis.

Data obtained 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 (Koster *et al.*, 2007; Truong *et al.*, 2006). 67% of the genes upregulated 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 (Table 1). In addition we observed an induction of genes encoding for cell cycle regulators. Conversely, p63 knockdown inhibits most of these regulatory genes. According to global gene expression profiling, p63 induces cell cycle regulators indirectly. This conclusion is supported by the observation that none of the eight cell cycle genes analyzed by ChIP-chip was associated with a p63-bound region.

Table 1: Genes induced by p63 knockdown.

Probe Set ID	Gene Symbol	p63KD/ctr	FDR	GNF expression ^a
1451287_s_at	2810003C17Rik	9.6	0.142	U ^b
1436344_at	5830404H04Rik	6.0	0.039	ND ^c
1424838_at	A330049M08Rik	5.8	0.044	trigeminal
1455180_at	AA407270	7.3	0.024	ND
1422925_s_at	Acot3	6.8	0.094	liver, kidney
1427735_a_at	Acta1	5.6	0.090	skeletal muscle, epidermis, snout epidermis, tongue, prostate, bone, brown fat
1421172_at	Adam12	5.5	0.132	U
1450065_at	Adcy7	6.4	0.040	B cells, CD8+ positive cells
1454890_at	Amot	5.0	0.174	placenta, embryo day E6.5
1450325_at	Angpt4	5.4	0.248	snout epidermis
1420991_at	Ankrd1	10	0.234	heart, umbilical cord
1418858_at	Aox3	8.9	0.068	liver, lung, trachea
1455826_a_at	Bace1	7.4	0.155	U
1448595_a_at	Bex1	8.2	0.055	embryo E6.5, E7.5, E8.5
1448752_at	Car2	18.1	0.031	stomach, bone marrow, bone
1419609_at	Ccr1	6.4	0.076	U
1460569_x_at	Cldn3	17.5	0.039	intestin, prostate, blastocyst
1448393_at	Cldn7	7.4	0.145	small intestin, large intestin, blastocysts, uterus
1437689_x_at	Clu	22.0	0.028	bladder, liver, stomach, lymphonode, mammary gland, brain
1419872_at	Csf1r	5.2	0.051	U
1415803_at	Cx3cl1	6.0	0.178	cortex, dorsal striatum, olfactory bulb
1427289_at	Ddhd1	6.4	0.091	testis, ovary, trachea, small intestine, brain
1448619_at	Dhcr7	6.8	0.011	adrenal gland, snout epidermis
1418937_at	Dio2	7.9	0.068	uterus, brown fat
1451270_at	Dusp18	5.6	0.159	U
1417023_a_at	Fabp4	5.0	0.113	brown fat, adipose tissue, ovary
1424647_at	Gabrp	5.7	0.025	trachea
1437618_x_at	Gpr85	6.1	0.198	brain
1436917_s_at	Gpsm1	5.6	0.232	olfactory bulb, heart
1424553_at	Hhat1	5.9	0.181	heart, skeletal muscle
1418352_at	Hsd17b2	12.7	0.023	large intestin, liver, placenta
1427029_at	Htra3	10.1	0.037	epidermis, snout epidermis, digits, placenta, adipose tissue
1417141_at	Igtp	5.4	0.132	CD4+ cells
1435945_a_at	Kcnn4	7.1	0.100	CD4+ cells, CD8+ cells, thyroid
1448169_at	Krt18	17.0	0.058	blastocyst, placenta (stomach, large intestin)
1423691_x_at	Krt8	34.1	0.103	large intestin, blastocyst, stomach, small intestin
1420350_at	Lce1a2	6.7	0.249	digits, epidermis, snout epidermis, tongue
1419409_at	Lce1b	6.7	0.207	digits, epidermis, snout epidermis, tongue
1420332_x_at	Lce1d	5.1	0.210	digits, snout epidermis, tongue
1421316_at	Lce1g	16.2	0.118	epidermis, digits, pancreas
1449959_x_at	Lce1h	6.3	0.148	digits, snout epidermis, tongue
1417275_at	Mal	5.8	0.030	substantia nigra, eye, spinal cord, bladder, stomach, umbilical, cord
1430837_a_at	Mbd1	7.9	0.242	U
1417256_at	Mmp13	5.9	0.096	vomero nasal epithelium, bone
1460238_at	Msln	16.4	0.028	umbelical cord, trachea (pleura)
1426562_a_at	Olfm1	5.2	0.184	frontal cortex, cerebral cortex, hyppocampus, amygdala, dorsal striatum, bulb olfactory epithelium
1436851_at	Pkn1 /// Ptger1	7.5	0.024	B-cells
1452517_at	Plekhh1	6.3	0.071	placenta, blastocyst
1419700_a_at	Prom1	5.6	0.049	eye, uterus, large intestin, vomero nasal
1420352_at	Prss22	5.9	0.170	ND
1451258_at	Psca	6.3	0.121	bladder, stomach
1436448_a_at	Ptgs1	5.1	0.061	bladder, epidermis, digits
1422324_a_at	Pthlh	5.2	0.020	snout epidermis, vomeronasal epithelium
1451452_a_at	Rgs16	11.2	0.199	mammary gland
1427020_at	Scara3	10.9	0.035	trachea, digits, main olfactory epithelium
1450956_at	Scd3	6.5	0.050	eye, epidermis
1437052_s_at	Slc2a3	20.1	0.024	blastocyst, embryo E6.5, E7.5, E8.5, testis
1450032_at	Slco2a1	10.1	0.070	placenta
1420562_at	Slurp1	6.9	0.173	ND
1436790_a_at	Sox11	8.5	0.129	embryo E9.5, E10.5
1451601_a_at	Spns2	7.1	0.077	U
1420377_at	St8sia2	10.5	0.034	U
1439567_at	Tbx3	9.8	0.132	adrenal gland, prostate, stomach, lung
1417455_at	Tgfb3	13.8	0.034	mammary gland, umbilical cord
1417455_at	Tgfb3	14	0.034	mammary gland, umbilical cord
1450040_at	Timp2	11.0	0.050	U
1419089_at	Timp3	5.0	0.038	adrenal gland, kidney
1419154_at	Tmprss2	5.6	0.025	bladder
1438855_x_at	Tnfaip2	5.5	0.030	bladder, bone marrow, embryo, spleen, liver
1455812_x_at	Vasn	5.6	0.035	ND

^a See Supplementary Material and Methods for GNF expression

^b U: ubiquitous

^c ND: not determined

Chapter I

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

Aberrant expression of non-epidermal genes in p63 knockdown keratinocytes was confirmed at the RNA and protein levels for some relevant genes (Figure 7A and 7B). Knockdown of the $\Delta Np63$ and the p63 α isoforms strongly induced expression of non-epidermal genes and their gene products, whereas knockdown of TAp63 and p63 γ 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 at E14.5. Importantly, non-epidermal gene products were strongly expressed in p63-null epidermis but not in wild-type epidermis (Figure 7C and 7D). In addition, non-epidermal genes were not identified as early target genes upon activation of ERp63 (Della Gatta *et al.*, 2008), and their induction occurred at a late interval upon p63 knockdown, suggesting that they are not directly regulated by p63. 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.

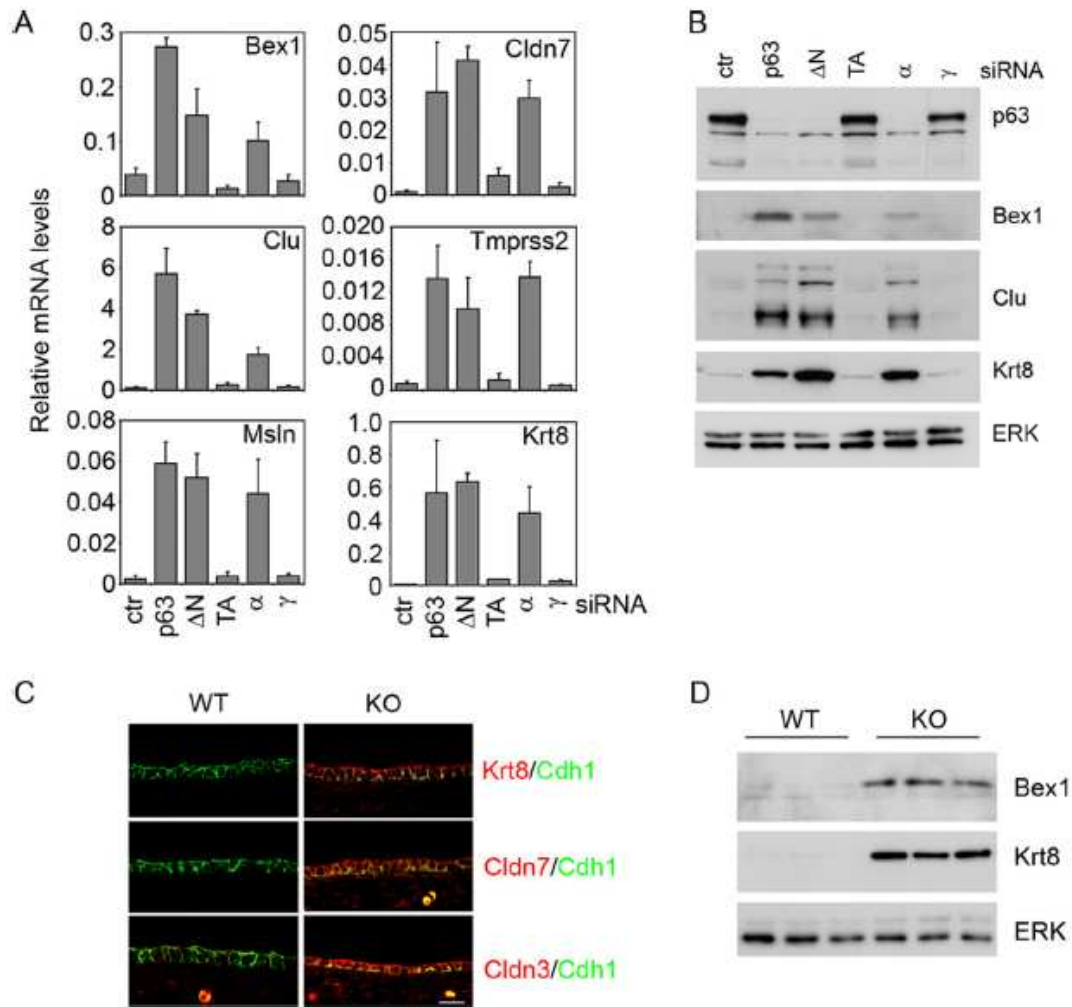


Figure 7: Aberrant expression of non-epidermal genes in the absence of p63.

(A) expression of the indicated non-epidermal genes was measured in primary mouse keratinocytes transfected with siRNA specific for pan-p63, Δ Np63, TAp63, p63 α , and p63 γ isoforms, or control (ctr). Values were expressed as Gapdh normalized mRNA levels. (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 μ m. (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.

2. p63 positively controls BMP signaling.

In addition, the global gene expression analysis (Della Gatta *et al.*, 2008) revealed that a subset of genes of the BMP family and their regulators were differentially expressed in p63 knockdown versus control keratinocytes. To establish whether p63 affected BMP or TGF- β signaling, I first co-transfected different amounts of a $\Delta Np63\alpha$ 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 (Figure 8A). The expression of p63 resulted in a dose-dependent induction of the BMP-responsive element, without affecting the TGF- β ones. In parallel, p63 knockdown, obtained transfecting p63-specific small interfering RNA, resulted in a significant reduction in Smad1/5/8 phosphorylation under basal conditions and upon BMP7 treatment without effect on Smad2 phosphorylation (Figure 8B). To assess whether p63 regulates BMP signaling during embryonic skin development, I measured Smad1/5/8 phosphorylation in p63-null and in wild-type skin. Immunoblotting analysis and immunofluorescence staining using anti phospho-Smad1/5/8 showed a strong activation of BMP signaling in the wild-type epidermis, whereas the phosphorylation of Smad1/5/8 was reduced in p63-null epidermis (Figure 8C, Figure 8D). In contrast Smad2 phosphorylation was similar in wild-type and in p63-null skin at this embryonic stage (Figure 8C). 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.

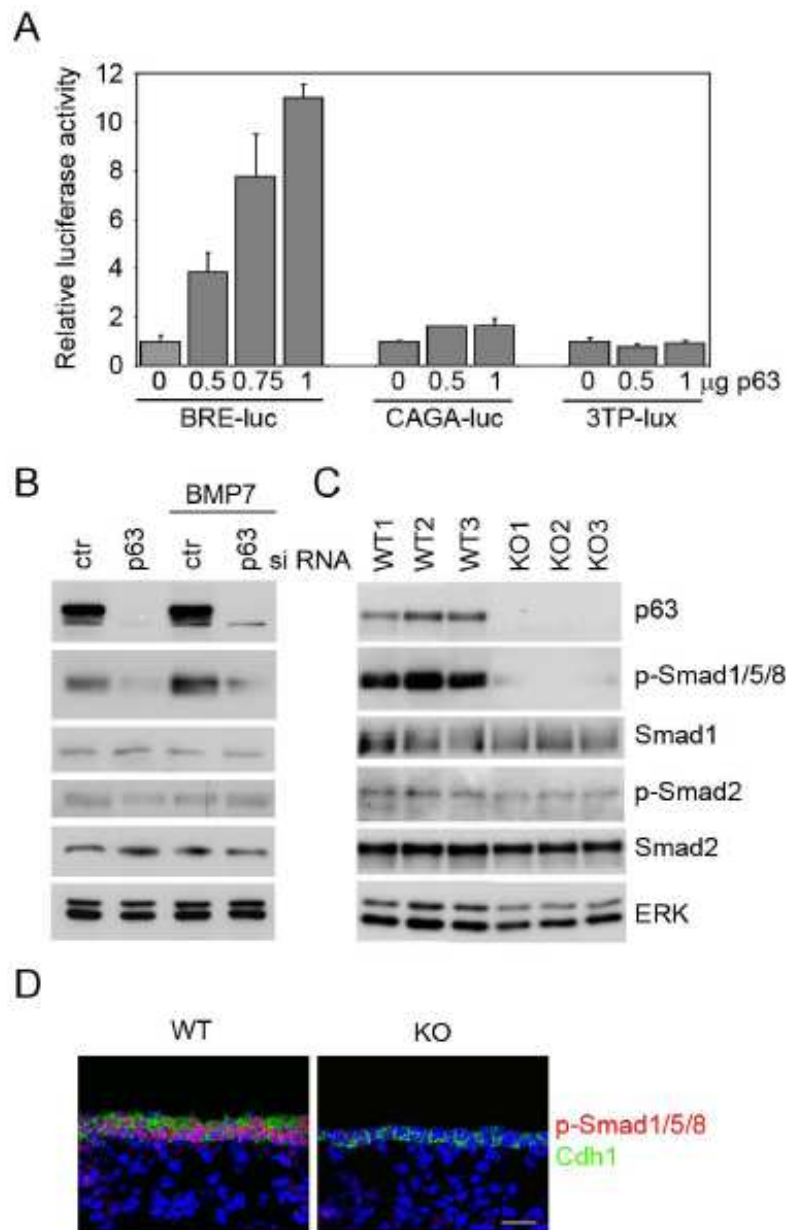


Figure 8: p63 positively regulates BMP signaling.

(A) Different amounts of the $\Delta Np63\alpha$ expression vector were co-transfected in primary mouse keratinocytes as indicated with the BMP-responsive reporter BREluc, or with the TGF- β responsive reporters CAGA-luc or 3TP-luc, and examined 48 hr after transfection. Values were normalized to renilla luciferase activity and are expressed as fold changes over the promoter activity in the absence of $\Delta Np63\alpha$, and represent mean \pm SE of three

independent experiments. (B) Immunoblotting of primary keratinocytes transfected with p63 siRNA or with control siRNA for 48 hrs, and either treated for 24 hrs with BMP7 (20ng/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 E-cadherin antibodies (Cdh1, green) in WT and p63 KO at E15.5, and detected by confocal microscopy. Nuclei were stained with DAPI (blue). Scale bar is 20 μ m.

3. p63 controls Smad7 gene expression.

Data obtained from previously global gene expression analysis indicated that p63 knockdown might affect Smad7 expression (Della Gatta *et al.*, 2008). Given that Smad7 has inhibitory role in phosphorylation of Smad1/5/8, I explored a possible crosstalk among p63, Smad7 and BMP signaling pathways. Smad7 mRNA and protein were induced in p63 knockdown keratinocytes, without affecting expression of the related gene Smad6 (Figure 9A and 9C). In addition, specific knockdown of $\Delta Np63$ and p63 α isoforms induced Smad7 expression, whereas knockdown of TAp63 and p63 γ isoforms had no effect (Figure 9B), indicating that the $\Delta Np63\alpha$ isoform is required for Smad7 repression. Supporting these data, Smad7 expression was significantly increased in p63-null embryonic epidermis while it was expressed at low levels in wild-type mice (Figure 9D and 9E).

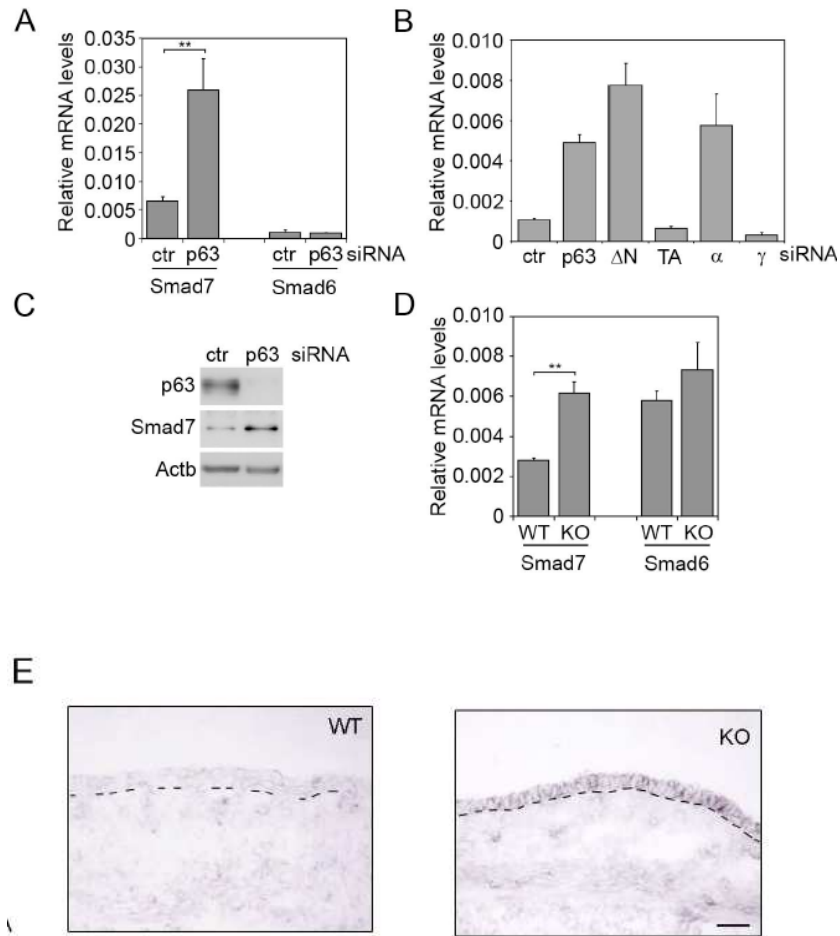


Figure 9: Smad7 is repressed by p63.

(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 48hrs after transfection. Values are expressed as Gapdh normalized mRNA levels, and represent the mean of independent experiments \pm SE (**P<0.005, n=8). (B) Smad7 expression was measured in primary mouse keratinocytes transfected with siRNA specific for pan-p63, Δ Np63, TAp63, p63 α , and p63 γ isoforms, or ctr. RNA levels were expressed as in (A). (C) Smad7 protein levels were measured in p63 knockdown and control

keratinocytes 48 hrs 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 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 p63KO using a DIG-labeled antisense probe for mouse Smad7. Scale bar is 50 μ m. A Smad7 sense probe gave no detectable signal under the same conditions. The dashed lines indicate the dermal–epidermal junction. Scale bar 60 μ m.

4. p63 directly regulates Smad7.

To check whether p63 might directly regulate Smad7, I tested a putative high affinity p63-binding region located at -2.8kb from the TSS, which emerged from our ChIP on-chip analysis (Della Gatta *et al.*, 2008).

The p63-binding region was centered on an evolutionary conserved sequence containing four canonical p63 binding hemi-sites (Figure 10A). ChIP with two independent sets of oligonucleotides confirmed that p63 specifically bound this genomic region (Figure 10B). 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 (Figure 10C, left panel). Conversely, p63 knockdown resulted in induction of the activity both of the Smad7 promoter and of the fragment containing the p63 binding sites (Figure 10C, right panel). Mutations in three canonical p63 binding hemi-sites abolished the responsiveness of the promoter to p63 knockdown. In conclusion, these data suggest that p63 directly represses Smad7 in primary mouse keratinocytes and in embryonic skin.

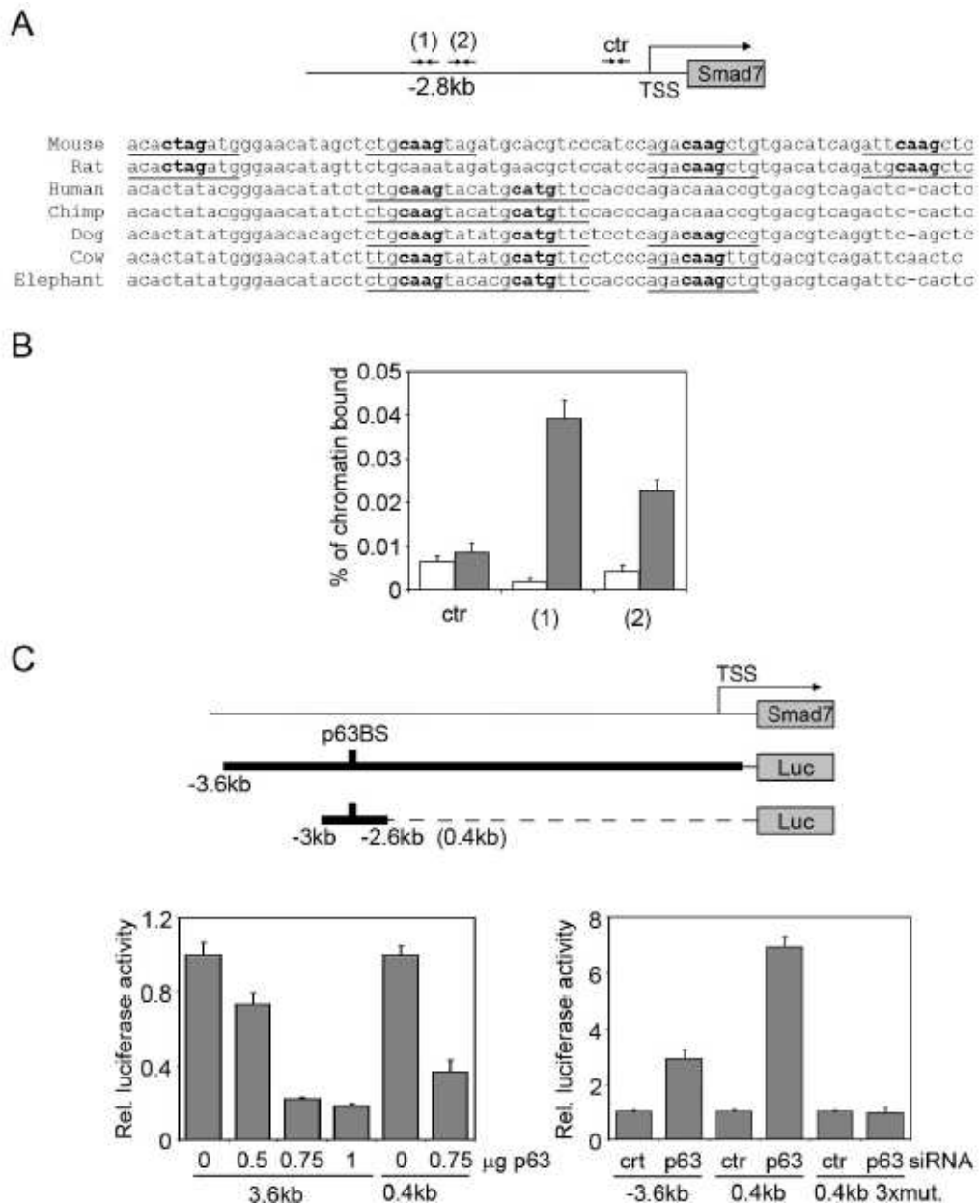


Figure 10: Smad7 is a transcriptional target of p63.

(A) A conserved p63-binding site is located at -2.8kb from the Smad7 transcription start site. The predicted p63-binding hemi-sites 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.

(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 TSS. The amount of precipitated DNA was calculated relative to the total input chromatin, and expressed as the percentage of the total DNA. (C) The activity of a 3.6kb Smad7 promoter region and of its 0.4kb fragment spanning the p63-binding sites (p63BS) were measured in the presence of the indicated amounts of a Δ Np63 α -expressing construct (p63) (left panel), or p63 or ctr siRNA (right panel). A 0.4kb fragment containing two base pair mutation in each of the first three binding hemisites indicated in (A) (0.4kb 3xmut.) was also co-transfected with p63 or ctr siRNA. Values were normalized to renilla luciferase activity and represent mean \pm SE of three independent experiments..

5. p63 controls Bmp7 gene expression.

Given a clear important role of p63 in the regulation of BMP signaling, I analyzed the expression of selected BMP family members known to be expressed in skin, in the presence or in the absence of p63, both *in vivo* and *in vitro*. Bmp2 and Bmp4 were poorly expressed in primary mouse keratinocytes and their expression was unaffected by p63 knockdown (Figure 110A). In p63-null embryonic skin at E14.5 Bmp2 was not significantly altered (Figure 11B). Moreover, 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 of BMP signaling observed in p63-null epidermis. 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 (Figure 11A and 11C) and in the developing murine epidermis (Lyons *et al.*, 1989; Wall *et al.*, 1993). Low levels of Bmp6 were modestly upregulated in p63-null embryonic skin at E14.5 in agreement with the reduction observed in p63 knockdown keratinocytes (Figure 11B). In contrast Bmp7 was the most highly expressed BMP family member in keratinocytes and its expression is specific of the basal layer of epidermis (Figure 11C). Bmp7 was inhibited approximately 50% by p63 knockdown (Figure 11A) and reduction of Bmp7 transcript was observed in p63-null versus wild-type skin at E14.5, as assessed by real time RT-PCR and by *in situ* hybridization (Figure 11B and 11D).

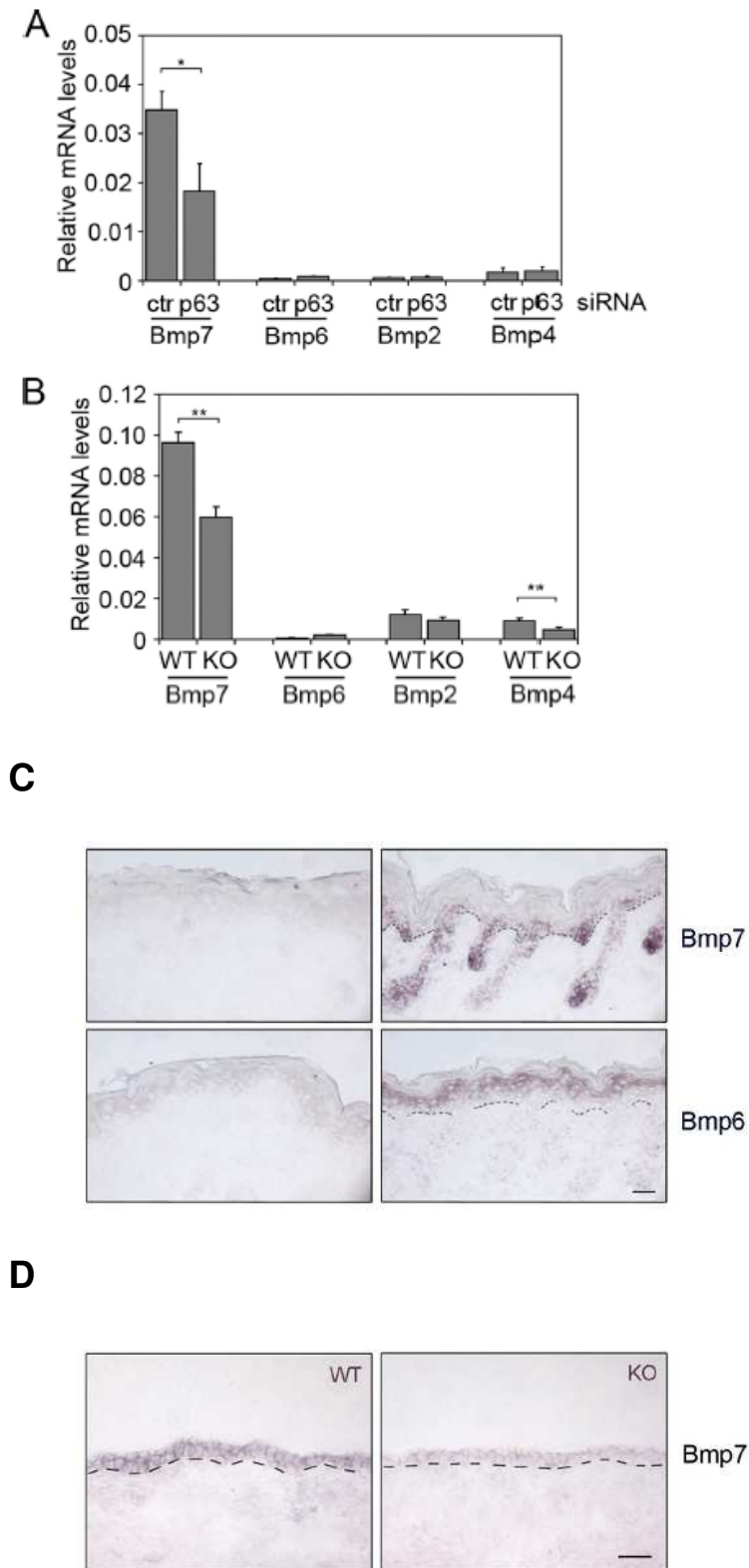


Figure 11: Bmp7 is the most abundant BMP family member in keratinocytes and embryonic skin and is down-regulated in the absence of p63.

(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. 2A. Values represent mean of independent experiments \pm 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). (C) Expression of Bmp7 and Bmp6 mRNA was determined by in situ hybridization in newborn mouse skin at P1. Frozen sections were hybridized with digoxigeninlabeled antisense probes. Corresponding sense cRNAs were used as control. The dashed lines indicate the dermal–epidermal junction. Scale bar 60 μ m. (D) 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 50 μ m.

6. p63 directly regulates Bmp7 gene expression.

Using ChIP analysis I revealed that p63 bound to an evolutionary conserved region in intron 1 (Figure 12), previously shown to bind all p53 family members in a breast cancer cell line (Yan and Chen, 2007). 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 regions proximal to TSS.

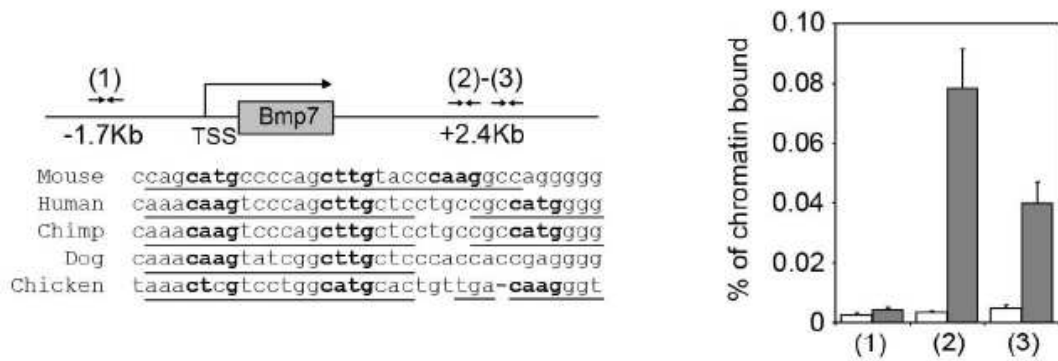


Figure 12: p63 binds to specific binding sites into the first intron of Bmp7 gene.

(A) The predicted p63-binding hemi-sites located at +2.4Kb from the Bmp7 gene TSS are indicated with their nucleotide sequence and phylogenetic conservation in various species. Bold nucleotides correspond to the core nucleotide sequence required for p63-binding, while underlined nucleotides are matches in the consensus sequence. (B) ChIP performed using 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 TSS. The amount of precipitated DNA was calculated relative to the total input chromatin and expressed as the percentage of total DNA.

7. p63 represses non-epidermal genes through a canonical BMP/Smad-dependent mechanism.

Since in lower vertebrates it has been demonstrated that BMP signaling was a crucial determinant of the epidermal cell fate during ectodermal development by suppressing alternative fates, I hypothesized that p63 could maintain low levels of non-epidermal genes activating the BMP signaling. To this end, I examined whether re-activation of BMP signaling in p63 knockdown keratinocytes by either Smad7 knockdown and/or by 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 (Figure 13A). Conversely, Smad7 overexpression inhibited BRE-Luc activity without affecting CAGA-Luc (Figure 13B).

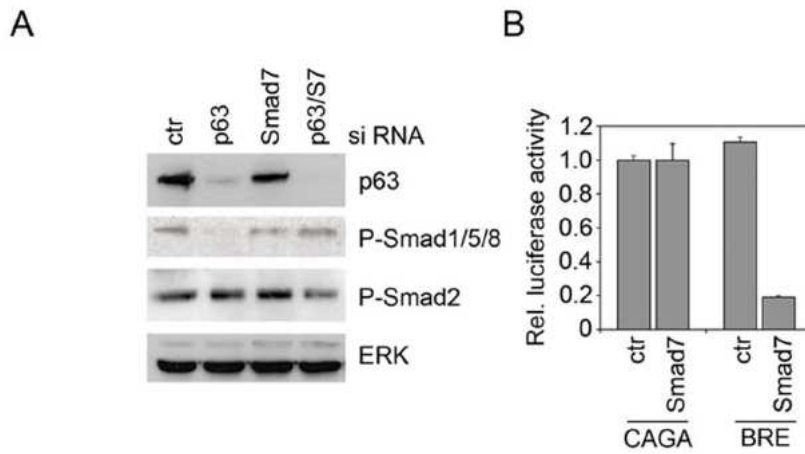


Figure 13: Smad7 controls activation of Smad1/5/8 signaling.

(A) Keratinocytes were transfected with p63, Smad7 and ctr siRNA, and the indicated proteins were detected by immunoblotting. (B) Keratinocytes were transfected with pBABESmad7 (Smad7) or with an empty pBABE vector (ctr) and the indicated reporters. Luciferase activity was measured 48 hrs after transfection and values were normalized to renilla and are expressed as fold changes over the promoter activity in the absence of Smad7, and represent mean \pm SE of three independent experiments.

In parallel, Smad7 knockdown resulted in strong downregulation of non-epidermal gene expression in p63 knockdown keratinocytes both at the RNA and protein levels (Figure 14A and 14B), suggesting that Smad7 depletion counteracted the effect of p63 knockdown. A similar effect was also observed with a second siRNA oligonucleotide targeting Smad7 (Figure 14C), confirming the specificity of this effect. Inhibition of non-epidermal genes was also observed by treating p63 knockdown keratinocytes with BMP7 (Figure 14A and 14B). Concomitant Smad7 knockdown and BMP treatment had little additional effect on the expression of non-epidermal markers as compared to each treatment alone, suggesting that these treatments repressed non-epidermal genes through overlapping mechanisms.

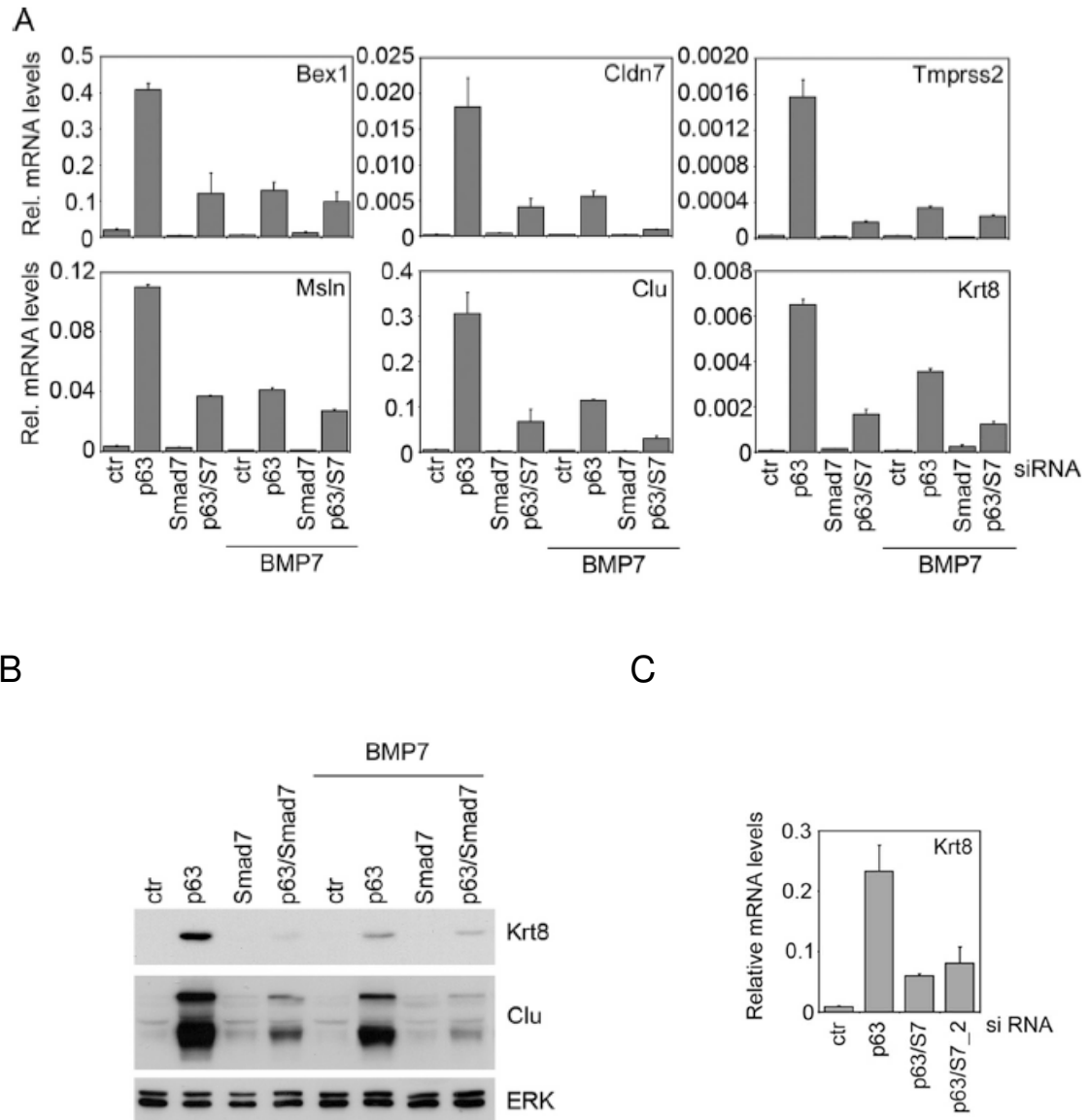


Figure 14: Re-activation of BMP signaling restores low levels of non-epidermal genes *in vitro* in p63 knockdown keratinocytes.

(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. Values are expressed as Gapdh normalized mRNA levels, and represent the mean of three independent experiments \pm SE. (B) Total protein extracts from primary keratinocytes transfected with p63, Smad7, p63 and Smad7 (p63/S7), or control (ctr)

siRNA, and cultured for 48h, were immunoblotted with the indicated specific antibodies. Protein extracts were normalized using anti-ERK polyclonal antibodies.

8. Activation of BMP Signaling specifically affects non epidermal genes.

To test whether BMP signaling functions specifically downstream of p63 to regulate non epidermal genes, or it has a broader compensatory role on p63 downstream targets, I measured expression of the Lce genes (or Sprr1), which encode epidermal markers involved in late differentiation and are upregulated by p63 knockdown (Table 1). Neither BMP treatment nor Smad7 knockdown rescued the effect of p63 knockdown on Lce genes (Figure 15), indicating that BMP signaling selectively restored low levels of non-epidermal genes.

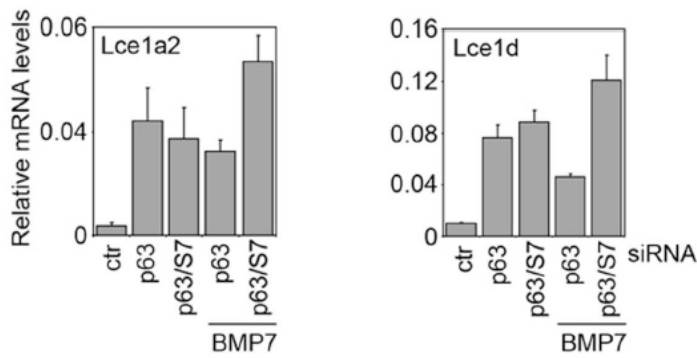


Figure 15: BMP signaling doesn't affect the expression of late differentiation marker of epidermis.

mRNA of Lce1a2 and Lce1d were measured by real time RT-PCR in primary keratinocytes transfected with p63, Smad7, p63 and Smad7 (p63/S7), or ctr siRNA 48 hrs after transfection. Cells were either treated with BMP7 for the last 24 hrs or left untreated. Values are normalized for Gapdh mRNA expression.

9. BMP signaling represses non-epidermal genes in embryonic epidermis.

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 downregulated Krt8 expression in p63-null skin (Fig. 16), 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.

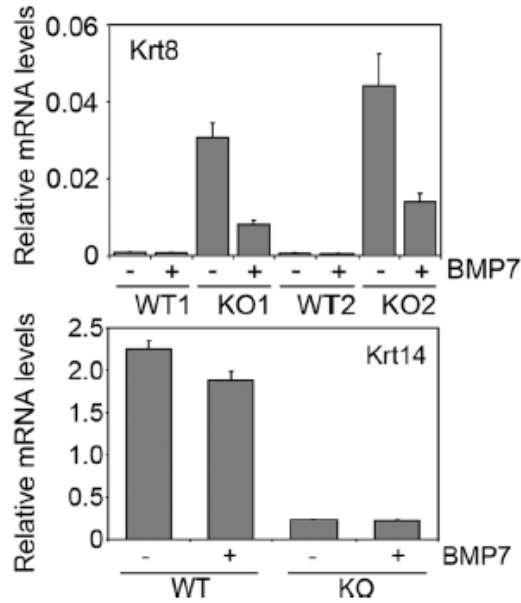


Figure 16: Re-activation of BMP signaling restores low levels of Krt8 gene in p63 KO skin explants.

Skin explants at E14.5 were cultured overnight and then treated with BMP7 (80 ng/ml) or left untreated for 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. Data presented are the average of two independent experiments and relative S.E is indicated. KO, knockout.

10. Loss of BMP signaling may be sufficient to cause aberrant expression of non-epidermal genes.

Smad7 overexpression upregulated non-epidermal genes in primary keratinocytes (Figure 17A), while inhibited 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 (Yu *et al.*, 2008), similarly induced expression of non-epidermal genes and repressed Id2 (Figure 17B). However, in embryonic epidermis Smad7 overexpression (Han *et al.*, 2006) or homozygous deletion of Bmp7 (Dudley *et al.*, 1995) did not induce non-epidermal genes. 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.

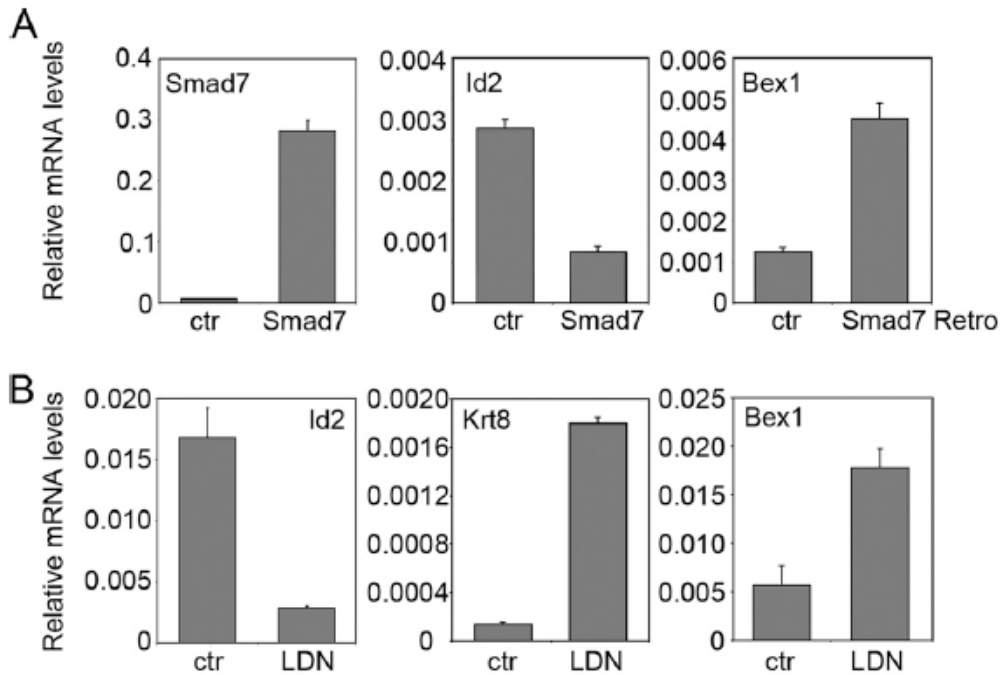


Figure 17: Canonical BMP/Smad pathway regulates the expression of non-epidermal genes.

(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.

11. Blocking BMP signaling using Smad1/5 specific siRNA abolished the rescue.

BMP-mediated receptor activation leads to induction of a canonical signaling pathway mediated by Smad1/5/8 and to the activation of other signaling molecules, including p38 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. Concomitant Smad1/5 knockdown using specific siRNA inhibited Id1 expression (Figure 18A and 18B), consistent with a decreased BMP/Smad signaling, whereas knockdown of either Smad1 or Smad5 alone was insufficient to elicit any effect (Figure 18B). I 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 (Figure 18C and 18D). Thus the canonical BMP/Smad signaling pathway is required for suppression of non-epidermal genes downstream of p63.

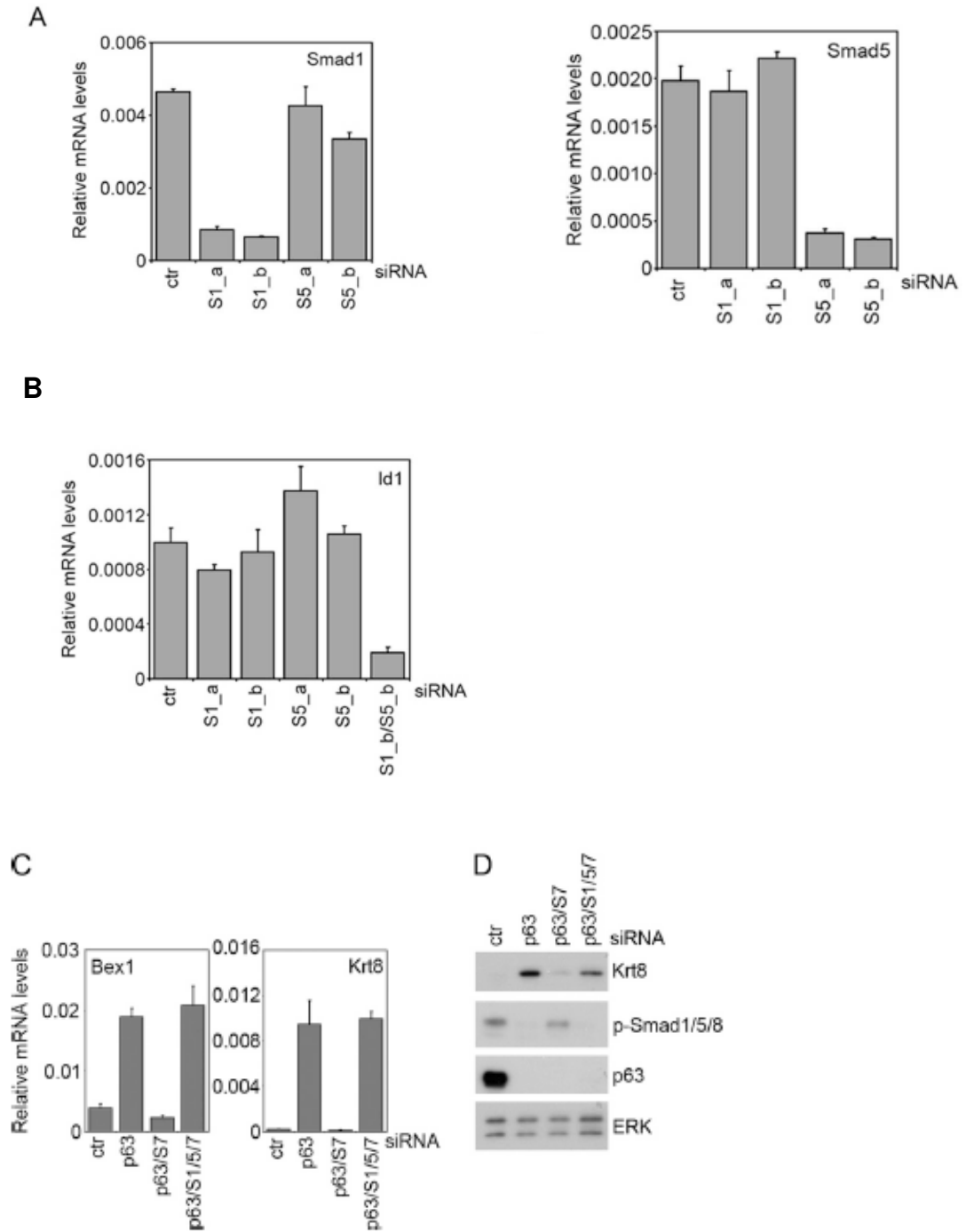


Figure 18: Concomitant Smad1/Smad5/Smad7 knockdown in the absence of p63 abolishes the rescue of ectopic expression of non epidermal genes.

(A) Smad1 (left panel) and Smad5 (right panel) expression levels were measured by real time RT-PCR in primary keratinocytes transfected with two

unrelated Smad1 (S1_a and S1_b), two unrelated Smad5 (S5_a and S5_b), or ctr siRNA. Values are normalized for β -actin expression. (B) Id1 expression was measured by real time RT-PCR in primary mouse keratinocytes transfected with siRNA for ctr, Smad1 (S1_a), Smad5 (S5_a), or both Smad1 and Smad5. (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 indicated proteins was measured by immunoblotting in primary mouse keratinocytes co-transfected with the indicated combinations of siRNA. Protein extracts were normalized using ERK antibodies.

Chapter II

12. p63 positively regulates cell proliferation of mouse epidermal cells in a p53-independent manner

p63 is required for human keratinocyte proliferation with a mechanism that remains poorly understood. To test whether a similar requirement for p63 in cell cycle progression occurs in mouse too, primary murine keratinocytes were transfected with two independent p63-specific small interfering RNA (siRNAs), and their rate of DNA synthesis was detected using BrdU incorporation. Loss of p63 resulted in cell proliferation defect as BrdU incorporation was strongly reduced in p63knockdown keratinocytes versus control, 48 hours after transfection (Figure 19A, left panel). $\Delta Np63\alpha$ is the predominant p63 isoform expressed in basal epidermal cells both *in vitro* and *in vivo*. An increased number of cells incorporating BrdU was detected in mouse keratinocytes infected with a retrovirus carrying $\Delta Np63\alpha$ (Figure 19A, right panel), confirming the ability of $\Delta Np63\alpha$ to promote cell proliferation. Flow cytometry analysis revealed that p63 knockdown resulted in a significant increase the percentage of cells in the G1 phase and a concomitant reduction of cells in S phase, whereas the G2/M phase remained largely unaffected (Figure 19B), consistent with a G1-phase arrest in the absence of p63. To determine whether the cell cycle arrest in the absence of p63 was dependent on p53, DNA synthesis was measured in p53-null primary keratinocytes in the presence or absence of p63. p63 knockdown elicited a

similar negative effect on DNA synthesis in p53-null keratinocytes and in their wild-type counterparts (Figure 19C), indicating that p53 is not required for p63-mediated cell cycle arrest at least in mouse keratinocytes. To analyze the relevance of these findings in a more physiological context, the role of p63 and p53 in cell proliferation was assessed in embryonic epidermis. BrdU *in vivo* labeling was performed in embryos carrying a deletion of p63, p53, or both, and compared with wild-type embryos. A significant reduction in DNA synthesis was observed in the epidermis of p63-null embryos at embryonic day 14.5 (E14.5) (Figure 19D), whereas p53 depletion did not affect the rate of DNA synthesis in either the presence or absence of p63. Taken together, these data reveal that p63 positively regulates progression through the G1 phase in a p53-independent manner in both cultured murine keratinocytes and embryonic skin.

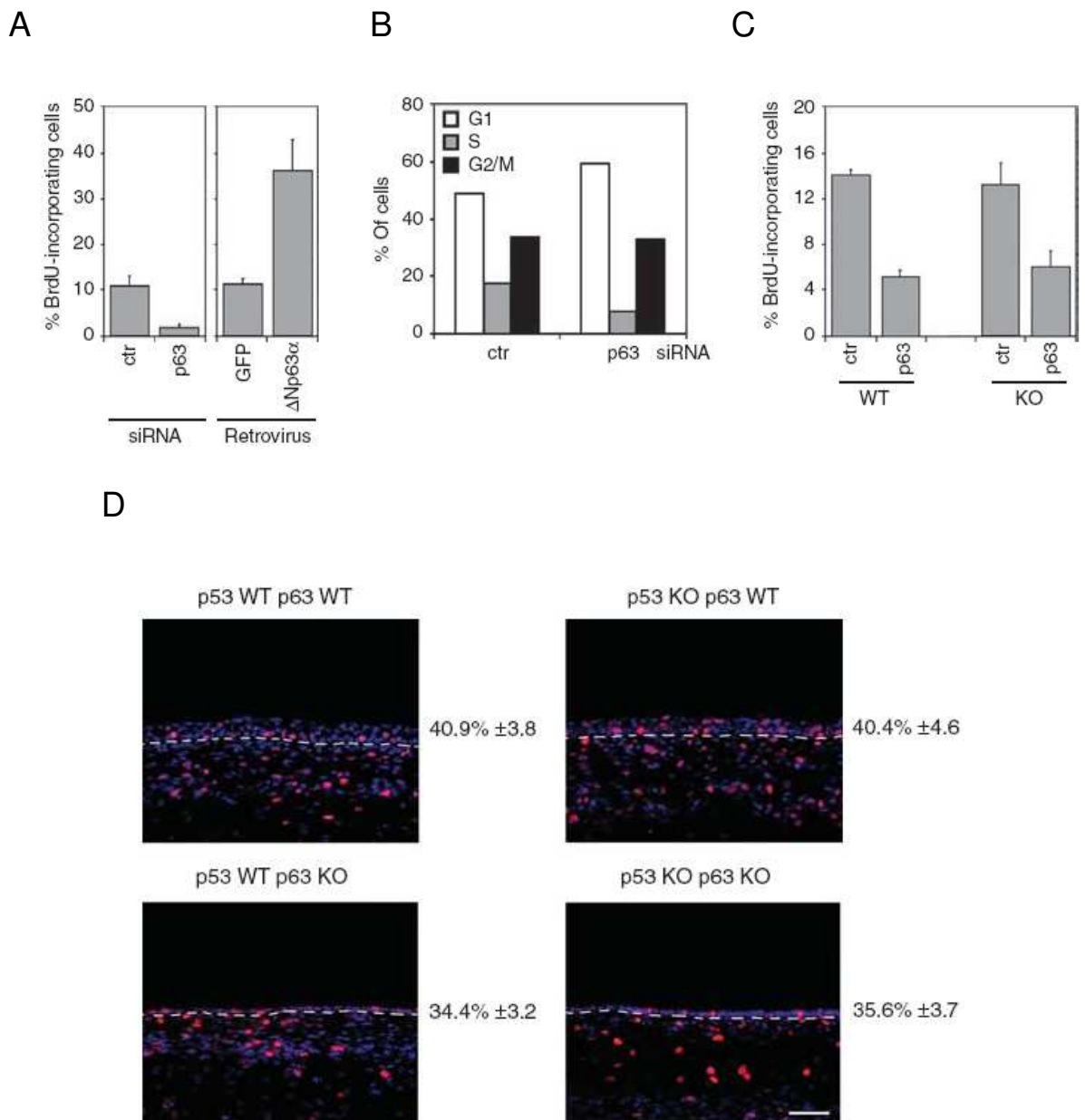


Figure 19: p63 regulates cell cycle progression of mouse epidermal cells independently of p53.

(A) BrdU analysis performed in primary mouse keratinocytes transfected with p63 or with negative control short interfering RNA (siRNA; ctr; left panel), or in primary mouse keratinocytes infected with a retrovirus carrying $\Delta Np63\alpha$ or green fluorescent protein (GFP) as control (right panel). (B) Cell cycle profiles generated by flow cytometry analysis of propidium iodide-stained

primary mouse keratinocytes 48 hours after transfection with p63 or ctr siRNA. White bars represent the G1 phase of the cell cycle, gray bars the S phase, and black bars the G2/M phase. (C) BrdU analysis performed in p53 knockout (KO) and wild-type (WT) primary mouse keratinocytes transfected with p63 or negative control siRNA. (D) Immunofluorescence analysis using antibodies specific for BrdU (in red) in dorsal skin of p63 and/or p53 KO and WT embryos at E14.5. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; blue). The percentage of BrdU-incorporating cells compared with the total amount of nuclei in the epidermis and relative SD are indicated. Scale bar = 50 μ m. Results are representative of at least two independent experiments \pm SD.

13. Absence of p63 represses G1 phase cell cycle genes.

To explore the mechanisms underlying cell cycle arrest as a consequence of the absence of p63, I tested the expression of a subset of G1-phase cell cycle regulators at different times upon p63 knockdown (Figure 20). Low levels of cyclin D1 were detected at early times upon p63 knockdown. Interestingly, p21Cip1 was not induced by p63 knockdown in the CD1 keratinocytes in contrast to what previously reported in human keratinocytes and in keratinocytes derived from other mouse strains (DeYoung *et al.*, 2006; Nguyen *et al.*, 2006; Truong *et al.*, 2006; Westfall *et al.*, 2003), further supporting the hypothesis that other mechanisms may be involved in mediating cell cycle arrest in the absence of p63. 48 hours after transfection, all tested cell cycle regulators were downregulated in the absence of p63, possibly as a consequence of cell cycle arrest. Recent studies have shown that cyclin D1 protein expression is regulated by several miRNAs, including miR-34a (Sun *et al.*, 2008). As miR-34 family is directly regulated by p53 in many cell types (Chang *et al.*, 2007; He *et al.*, 2007; Raver-Shapira *et al.*, 2007; Tarasov *et al.*, 2007) and p63 can bind to p53-consensus sequences, I hypothesized that p63 might regulate miR-34 family in keratinocytes.

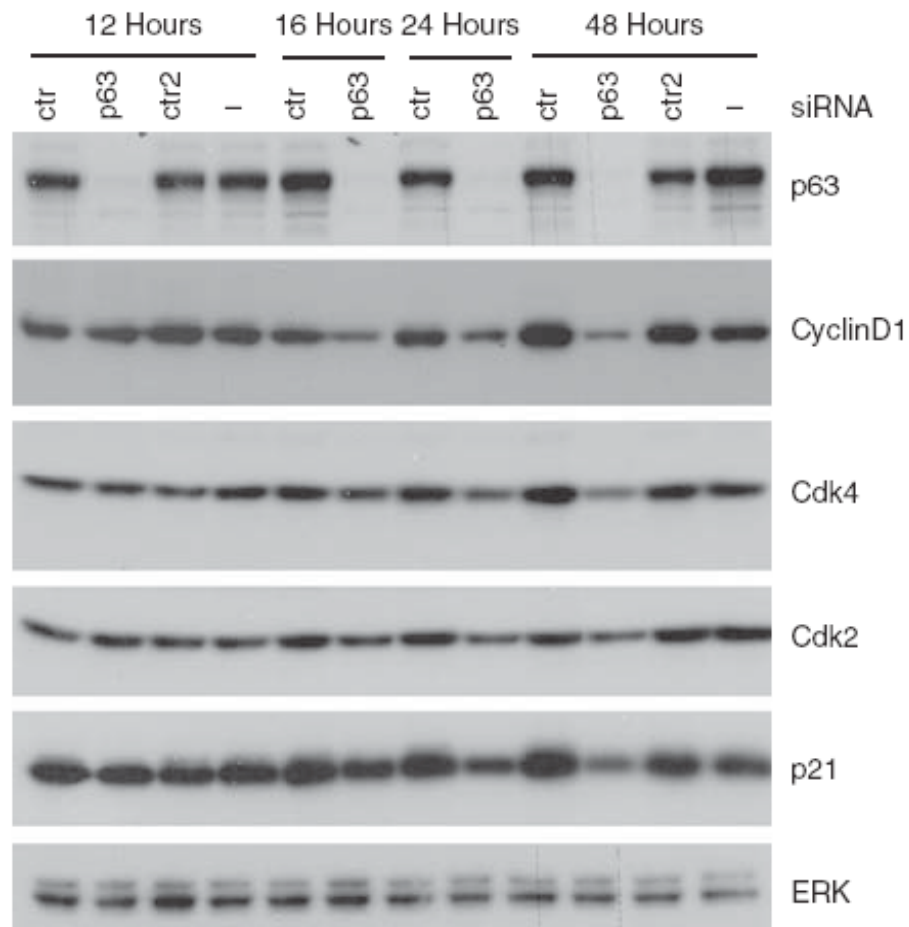


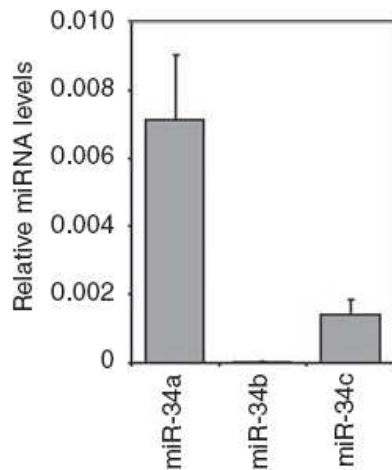
Figure 20: p63 knockdown alters the expression of a subset of cell cycle regulators.

Total protein extracts were collected from primary mouse keratinocytes at the indicated time points upon transfection of p63, control (ctr), or unrelated control (ctr2) siRNAs. Extracts from untransfected cells (-) were also loaded as control. Immunoblots were incubated with the indicated antibodies. Equal loading was confirmed by extracellular signal-regulated kinase (ERK) expression. Results are representative of two independent experiments.

14. miR-34a and miR-34c are expressed in epidermis.

I first measured the expression of miR-34 family in newborn primary keratinocytes and skin. miR-34a was previously reported to be highly represented in a small RNA library from mouse epidermis at E17.5 (Yi *et al.*, 2006), whereas no expression studies were performed for miR-34b and miR-34c in the skin. Expression of miR-34 family members was measured by TaqMan reverse transcriptase-PCR using specific miRNA probes. miR-34a was strongly expressed in primary mouse keratinocytes, whereas miR-34c was expressed at lower levels, and miR-34b was undetectable (Figure 21A). miR-34 expression in keratinocytes paralleled the expression in newborn skin, as assessed using in situ hybridization. miR-34a was readily detectable in the epidermis and to a lesser extent in the hair follicle with the signal being stronger in the suprabasal layers than in the basal layer (Figure 21B). In contrast, miR-34c was less expressed and mainly detected in the basal layer, whereas miR-34b was undetectable.

A



B

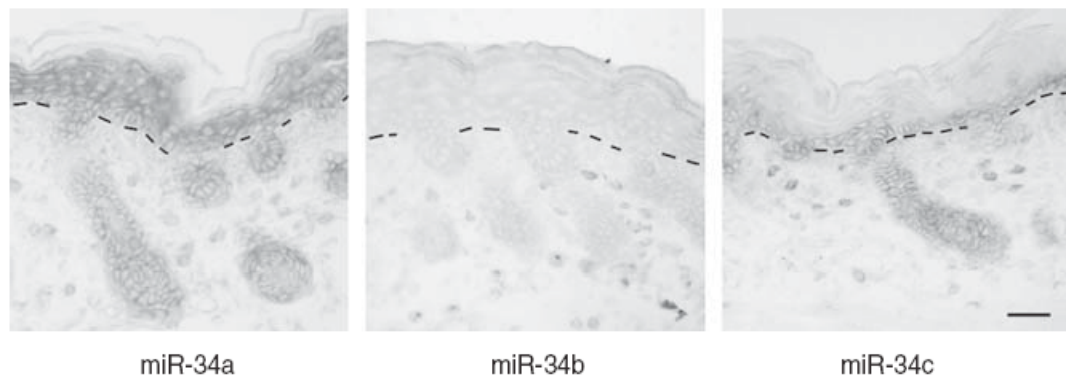


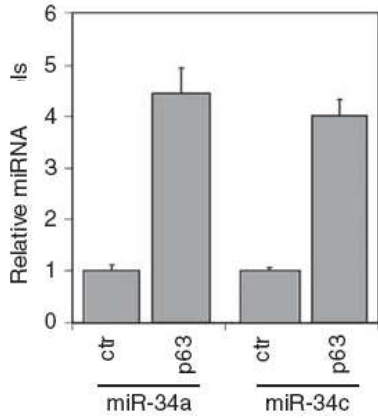
Figure 21: miR-34a and miR-34c are expressed in mouse epidermis.

(A) Expression levels of the indicated miRNA were measured using real-time quantitative reverse transcriptase-PCR (RT-PCR; Taqman) in primary mouse keratinocytes. miRNA measurements were normalized to U6 small nuclear RNA (snRNA) expression levels. (B) In situ hybridization performed in mouse newborn skin sections using digoxigenin (DIG)-labeled anti-miR probes specific for miR-34a, miR-34b, and miR-34c. Scale bar = 50 μ m.

15. miR-34a and miR-34c are transcriptionally repressed by p63.

To test the possibility that miR-34 family may be a p63 target, I measured miR-34 expression levels in the presence or absence of p63 *in vitro* and *in vivo*. miR-34a and miR-34c were increased in p63 knockdown when compared with control keratinocytes (Figure 22A). Similarly, higher expression levels of miR-34a and miR-34c were found in p63-null embryonic skin at E14.5 when compared with wild-type littermates (Figure 22B), indicating that p63 negatively regulates miR-34a and miR-34c expression in epidermal cells *in vitro* and *in vivo*.

A



B

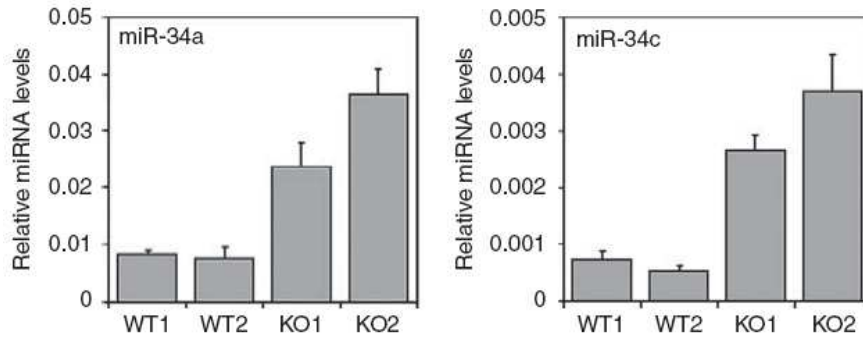


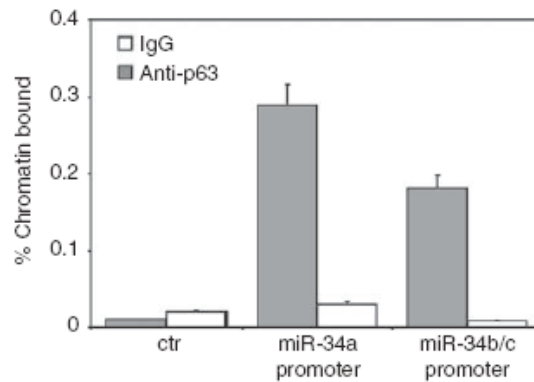
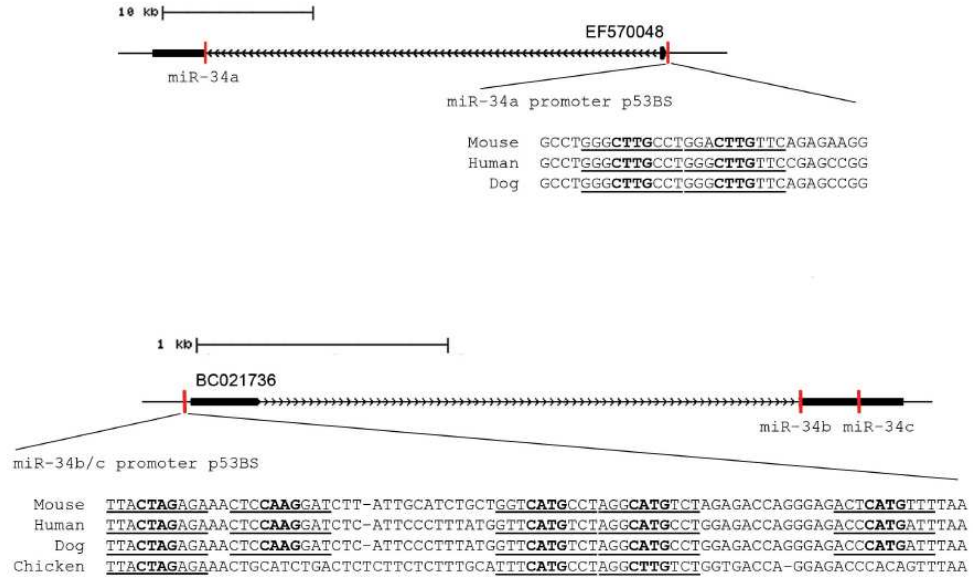
Figure 22: miR-34a and miR-34c are induced by loss of p63.

(A) Expression levels of the indicated microRNA (miRNA) were measured using real-time quantitative reverse transcriptase-PCR (RT-PCR; Taqman) in primary mouse keratinocytes transfected with p63 or with negative control short interfering RNA. miRNA samples were normalized to U6 small nuclear RNA (snRNA) expression levels. (B) miR-34a and miR-34c levels were measured using real-time quantitative RT-PCR in E14.5 skin of wild-type (WT) and p63 knockout (KO) embryos.

16. miR-34a and miR-34c are transcriptional targets of p63

To explore the possibility that p63 may directly regulate miR-34a and miR-34b/c, chromatin immunoprecipitation analysis using p63-specific antibodies was performed in primary mouse keratinocytes. p63 bound to miR-34a and miR-34b/c promoters containing phylogenetically conserved p53-binding sites (Figure 23A). In addition, p63 overexpression significantly inhibited the activity of miR-34a and miR-34b/c promoters, whereas p63 knockdown resulted in an enhancement of their activity (Figure 23B). Taken together, these data indicate that miR-34a and miR-34c are expressed in a spatially discrete manner in the epidermis, and are transcriptionally repressed by p63.

A



B

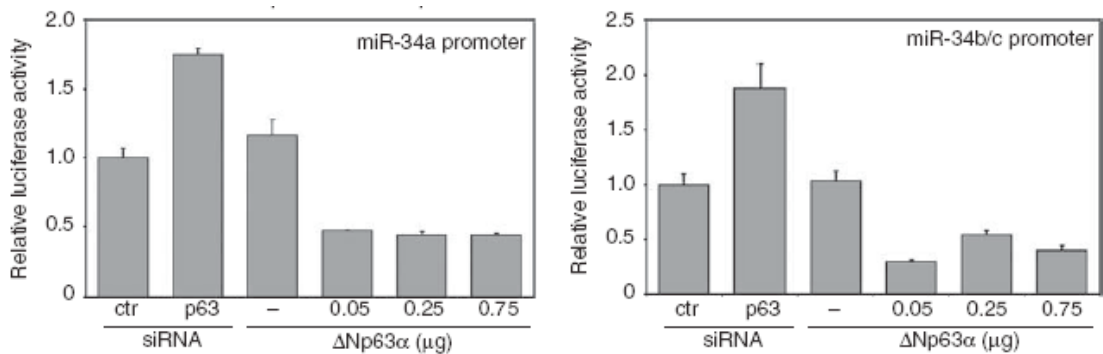


Figure 23: p63 binds to miR-34a and miR-34c promoters inhibiting their expression.

(A) Predicted structures of human miR-34a and miR-34b/c genes. Sequence conservation between several species are represented. The promoter regions

of miR-34a and miR-34b/c contain a sequences that matches the canonical p53 binding hemisite. the p53 binding site and the miR-34 are indicated as red bars. kb, kilobase. In the lower panel, chromatin immunoprecipitation has been performed in primary mouse keratinocytes using antibodies specific for p63 (gray bars), or rabbit IgG antibodies as control (white bars), followed by real-time PCR amplification using specific oligonucleotide primers designed in miR-34a and miR-34b/c promoters. (B) The activity of miR-34a and miR-34b/c promoters (He *et al.*, 2007) was measured in the presence of the indicated amounts of a Δ Np63 α -expressing construct, or p63 or control short interfering RNA (ctr siRNA). Values are expressed as luciferase activity relative to Renilla. Results are representative of at least three independent experiments \pm SD.

17. Concomitant downregulation of miR-34a and miR-34c expression reactivates cell cycle progression in p63 knockdown keratinocytes.

To test the contribution of miR-34 family in the cell cycle arrest elicited by loss of p63, I co-transfected antisense inhibitors (anti-miR) for miR-34a, miR-34c, or both in the presence or absence of p63 (Figure 24A). Consistent with previous experiments, DNA synthesis was strongly impaired in p63 knockdown keratinocytes, whereas transfection with anti-miR-34a or anti-miR-34c partially restored DNA synthesis (Figure 24B). Concomitant transfection of both anti-miR-34a and anti-miR-34c almost entirely rescued the proliferation defect provoked by p63 knockdown, whereas they had no effects on DNA synthesis in the presence of p63. Interestingly, concomitant downregulation of miR-34a and miR-34c also resulted in a decreased number of cells with morphological alterations due to the absence of p63, whereas it had no effects in the presence of p63 (Figure 24C).

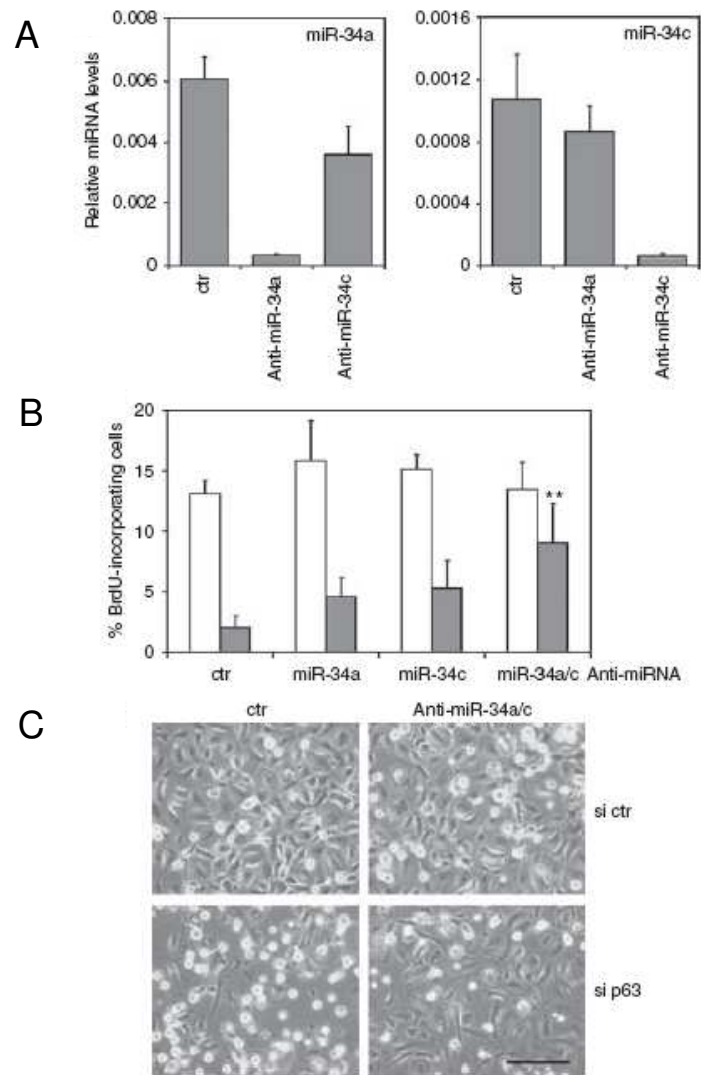


Figure 24: miR-34a and miR34c knockdown in the absence of p63 restores cell cycle progression and cell morphology.

(A) miR-34a and miR-34c levels measured in primary mouse keratinocytes at 48 hours after transfection with specific anti-miRNAs or a scramble control. (B) BrdU analysis performed in primary mouse keratinocytes transfected with anti-miR-34a and 34c or scramble control, in the absence (gray bars) or in the presence (white bars) of p63. (C) Morphology of primary mouse keratinocytes transfected as in (B). Results are representative of three independent experiments \pm SD (** p-value <0.01, n=3). Scale bar = 50 μ m.

18. Concomitant downregulation of miR-34a and miR-34c expression restores expression levels of cyclin D1 and Cdk4 in p63 knockdown keratinocytes.

To test whether miR-34 family exerts an effect downstream of p63 to regulate the expression of cell cycle proteins, I measured the protein levels of a subset of cell cycle regulators in the presence or absence of p63 and miR-34 family. p63 knockdown inhibited the expression of cyclin D1, CDK4, and to a lesser extent CDK2. Concomitant downregulation of both miR-34a and miR-34c resulted in a significant rescue of the expression of cyclin D1 and CDK4, the two known miR-34 targets, whereas CDK2 remained unaffected (Figure 25). Taken together, these data indicate that miR-34a and miR-34c are relevant targets of p63 in cell cycle regulation.

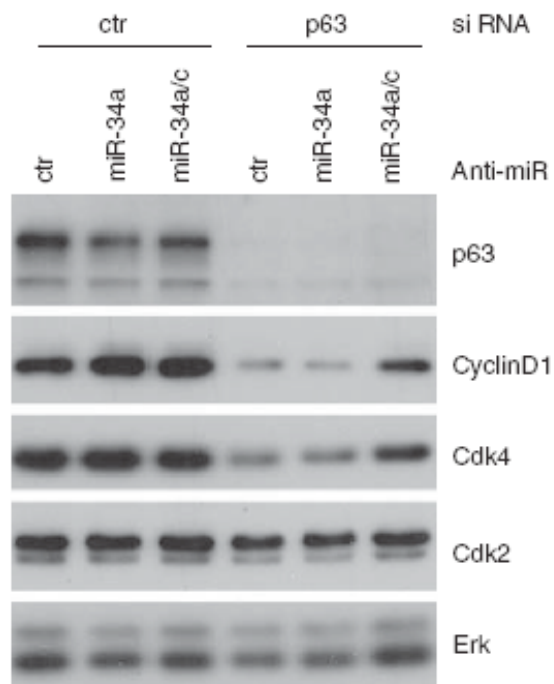


Figure 25: miR-34a and miR34c knockdown in the absence of p63 restores expression levels of G1 phase cell cycle genes.

Immunoblot of G1-phase regulators in primary mouse keratinocytes transfected as in Figure 4b. Protein levels were normalized for extracellular signal-regulated kinase (ERK) expression.

DISCUSSION

p63 is an important regulator of gene expression in the epidermis and it is essential for epidermal development, however, its interaction with signaling pathways is still poorly understood. In this thesis I establish a novel function of p63 in repressing non-epidermal genes through a mechanism that involves BMP/Smad signaling and a new mechanism through which p63 controls cell cycle progression in keratinocytes. I observe that many non-epidermal genes are induced by loss of p63 in epidermal cells both *in vitro* and *in vivo*, further reinforcing the notion that p63 may participate in maintaining an epithelial gene expression program in mammalian cells (Barbieri *et al.*, 2006).

BMP7 was recently described as a novel direct target of the p53 family in breast cancer cells (Yan and Chen, 2007). 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, I 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.

BMP signaling is activated by p63 both in isolated keratinocytes as well as in the embryonic epidermis and p63 directly suppresses the inhibitory Smad7 by binding to a highly conserved genomic region in the promoter. Smad7 is poorly expressed in the developing epidermis and is induced in the p63-null epidermis. I observed 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. Loss of BMP signaling by itself induces aberrant expression of non-epidermal genes in cultured keratinocytes, whereas *Bmp7* deletion or *Smad7* overexpression do not induce non-epidermal genes in embryonic epidermis.

Lack of 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 (Andl *et al.*, 2004; Kobiela *et al.*, 2003; Kobiela *et al.*, 2007). However, *Bmpr1B/ALK6* and *Acvr1/ALK2* are also expressed in embryonic epidermis (Dewulf *et al.*, 1995; He *et al.*, 2002; Verschueren *et al.*, 1995), and *Acvr1/ALK2* is the main receptor for BMP7 (Macias-Silva *et al.*, 1998; ten Dijke *et al.*, 1994; Yamashita *et al.*, 1995), suggesting that depletion of multiple receptors may be required to observe an epidermal phenotype.

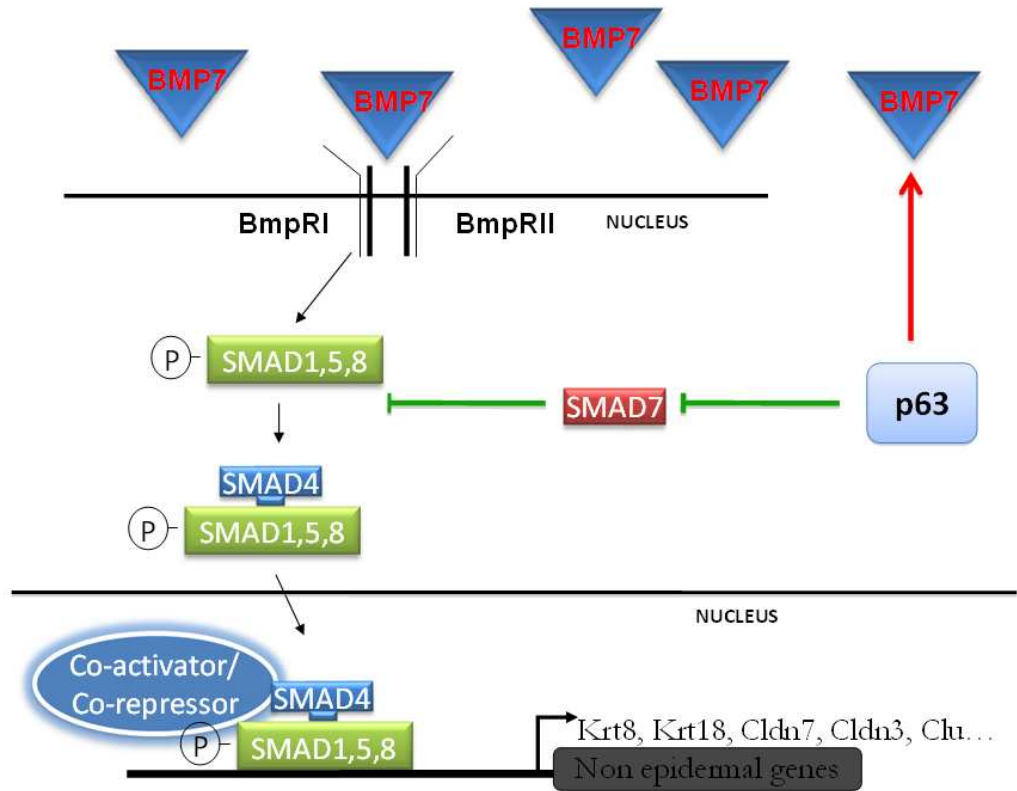
In addition to decreased *Bmp7* and to the concomitant induction of *Smad7*, I observed that *Bmp4* is down-regulated in the p63-null skin at E14.5. *Bmp4* is poorly expressed in keratinocytes and is unaffected by p63 knockdown, indicating that unidentified indirect signals derived from the p63-null 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 (Aono *et al.*, 1995), 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 (Suzuki *et al.*, 2008), whereas in the developing epidermis multiple signaling molecules may be involved in this function.

Moreover, BMP7 promotes cell survival in p53-deficient breast carcinoma cells, at least in part, through *Id2* (Yan and Chen, 2007), and has been demonstrated to promote cell survival in other contexts and tissues (Mitu *et al.*, 2007; Vukicevic *et al.*, 1998; Yabe *et al.*, 2002; Yang *et al.*, 2008). Survival of epidermal cells is compromised in the absence of p63 (Keyes *et al.*, 2005). 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.

These 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 mechanisms. Further assessment of the role of BMP signaling in the embryonic epidermis will be a key subject for subsequent investigations.

Figure 26: p63 activates BMP signaling pathway.



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On the other hand, I found a new mechanism through which p63 maintains cell cycle progression in mouse basal epidermal cells in a p53-independent manner, consistent with a strong $\Delta Np63\alpha$ expression and very low p53 levels in basal keratinocytes. The reduced rate of DNA synthesis in p63-null embryonic epidermis is not as dramatic as the one observed *in vitro*, suggesting that other compensatory mechanisms are likely to overcome the proliferation defect in the context of the skin tissue. Beside impaired proliferation, defective stratification (Koster *et al.*, 2004; Mills *et al.*, 1999), and possibly cell adhesion (Carroll *et al.*, 2006), significantly contribute to severe epidermal phenotype that is observed at birth in p63-null mice, I observed that a relevant mechanism through which p63 regulates G1-to-S-phase transition is by direct repression of the previously reported p53 targets miR-34a and miR-34c. Other p53 target genes, such as p21Cip1 and 14-3-3 σ (Nguyen *et al.*, 2006; Truong *et al.*, 2006; Westfall *et al.*, 2003), are repressed by p63 and are likely to contribute to p63-mediated cell cycle progression. Thus p63, more specifically its $\Delta Np63\alpha$ isoform, and p53 regulate a set of target genes involved in cell proliferation in an opposing manner, by which p63 binding represses genes that cause cell cycle arrest and p53 activates them. The role of p53 in p63-mediated cell cycle progression in keratinocytes is controversial. Simultaneous p63 and p53 knockdown in human keratinocytes rescued the cell proliferation defect in the absence of p63 in one study, possibly due to high p53 expression levels in that setting (Truong *et al.*, 2006), whereas regulation of cell cycle progression by p63 was found to be

independent of p53 and p73 in another study (DeYoung *et al.*, 2006). I found that in mouse epidermal cells, p53 does not contribute to cell cycle arrest due to loss of p63 at least in the absence of DNA damage. In human keratinocytes, p21Cip1 expression was elevated (DeYoung *et al.*, 2006; Truong *et al.*, 2006), but its downregulation was insufficient to restore cell proliferation in the absence of p63. I contributed previously in a work where we reported upregulation of p21Cip1 upon p63 downregulation in Sencar mouse keratinocytes (Nguyen *et al.*, 2006), whereas in the CD1 background p21Cip1 expression is not induced, further reinforcing the notion that p21Cip1 is unlikely to be the only player in cell cycle arrest downstream of p63.

Similar to other cell cycle regulators, the downregulation of p21Cip1 observed at late time points upon p63 knockdown is likely to be a consequence of cell cycle arrest. Other p63 target genes are likely to contribute to cell cycle progression as p63 transcriptionally regulates a large number of downstream targets (Della Gatta *et al.*, 2008; Vigano *et al.*, 2006; Yang *et al.*, 2006). Among the cyclin-dependent kinase inhibitors, p16Ink4a is induced in p63-null skin (Keyes *et al.*, 2005; Su *et al.*, 2009), and the skin phenotype of p63-null mice is ameliorated by loss of p16Ink4a with partial restoration of keratinocyte proliferation (Su *et al.*, 2009).

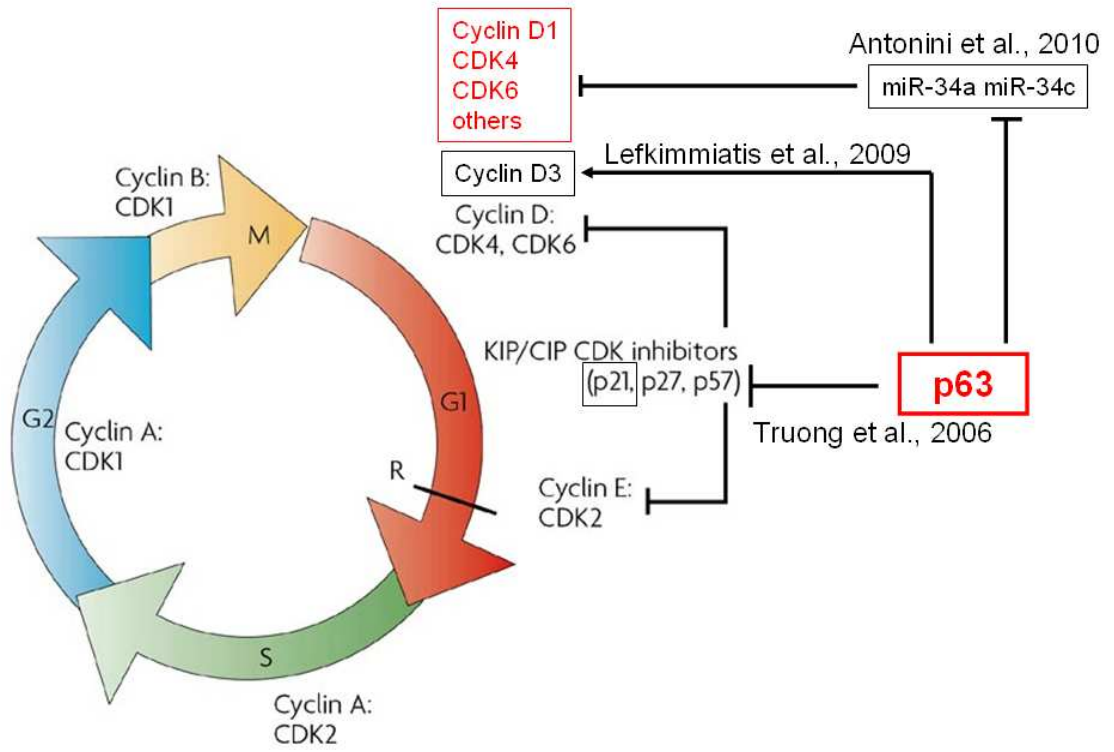
Data obtained indicate that miR-34 downregulation leads to induced expression of cyclin D1 and CDK4 in the absence of p63. Both cyclin D1 and CDK4 are direct targets of miR-34; however, previously reported G1-phase regulators whose expression is controlled by miR-34 also include CDK6,

E2F3, cyclin D3, and cyclin E2 (Bommer *et al.*, 2007; He *et al.*, 2007; Sun *et al.*, 2008; Tazawa *et al.*, 2007). Given that each miRNA is predicted to bind to more than 100 target mRNAs, p63 is likely to control a large set of cell cycle regulators by directing repressing miR-34 family. I found a strong and specific expression of miR-34a in the epidermis and in the hair follicle, with a more intense signal in the suprabasal layers than in the basal layer, consistent with the reciprocal expression of p63.

The prevalent expression of miR-34c in the basal layer of the epidermis is controversial, which is consistent with the notion that other epidermal-specific transcription factors beside p63 are likely to regulate miR-34 expression in the epidermis. In addition to its elevated expression in the epithelial component of the skin, miR-34a is highly expressed in the brain, with variable expression in other tissues (Bommer *et al.*, 2007). In contrast, miR-34c is most highly expressed in lung, with low expression in brain and very low to undetectable expression in other tissues. Expression of miR-34b in normal mouse tissues parallels miR-34c expression (Bommer *et al.*, 2007), consistent with the fact that they share the same precursor transcript. Interestingly, miR-34c is expressed in skin whereas miR-34b is not, as shown using Taqman assay and in situ hybridization, suggesting that their maturation may be different. In fact, it has been recently shown that p53 modulates miRNA processing by functional interaction with the Drosha processing complex through binding of the DEAD box RNA helicases p68 (Suzuki *et al.*, 2008). p53 binds p68 through a carboxy terminal portion of the DNA-binding

domain, which is highly homologous to the corresponding region in p63. In addition, it has been demonstrated that TAp63 binds to and transactivates the Dicer promoter, demonstrating direct transcriptional regulation of Dicer by TAp63, supporting a roles of p63 in regulation of microRNAs maturation (Su *et al.*, 2010). Moreover, conditional targeting of Dicer or of Dgcr8 in skin epithelium results in a severe phenotype, including altered barrier function of epidermis and hair follicle that fail to invaginate (Andl *et al.*, 2006; Yi and Fuchs, 2009; Yi *et al.*, 2006; Yi *et al.*, 2009). In addition, inducible conditional knockout of Dicer after hair follicle maturation leads to epidermal thickening (Yi *et al.*, 2008). miRNA-203 is induced in the skin concomitantly with stratification and differentiation, and promotes epidermal differentiation by restricting proliferative potential and inducing cell cycle exit in both human and mouse keratinocytes (Lena *et al.*, 2008; Yi *et al.*, 2008). miR-203 directly represses the expression of p63, which fails to switch off suprabasally when either Dicer1 or miR-203 is absent. p63 becomes repressed basally when miR-203 is prematurely expressed. This study suggest the possibility that a regulatory axis exists in the skin, by which miR-203 and miR-34 family are, respectively, upstream and downstream of p63 to control cell proliferation.

Figure 27: p63 controls cell cycle progression directly repressing miR-34a and miR-34c.



REFERENCES

- Ambros V (2004) The functions of animal microRNAs. *Nature* 431:350-355.
- Andl T, Ahn K, Kairo A, Chu EY, Wine-Lee L, Reddy ST, *et al.* (2004) Epithelial Bmpr1a regulates differentiation and proliferation in postnatal hair follicles and is essential for tooth development. *Development* 131:2257-2268.
- Andl T, Murchison EP, Liu F, Zhang Y, Yunta-Gonzalez M, Tobias JW, *et al.* (2006) The miRNA-processing enzyme dicer is essential for the morphogenesis and maintenance of hair follicles. *Curr Biol* 16:1041-1049.
- Aono A, Hazama M, Notoya K, Taketomi S, Yamasaki H, Tsukuda R, *et al.* (1995) Potent ectopic bone-inducing activity of bone morphogenetic protein-4/7 heterodimer. *Biochem Biophys Res Commun* 210:670-677.
- Bamberger C, Schmale H (2001) Identification and tissue distribution of novel KET/p63 splice variants. *FEBS Lett* 501:121-126.
- Barbieri CE, Tang LJ, Brown KA, Pietenpol JA (2006) Loss of p63 leads to increased cell migration and up-regulation of genes involved in invasion and metastasis. *Cancer Res* 66:7589-7597.
- Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116:281-297.
- Bartel DP (2009) MicroRNAs: target recognition and regulatory functions. *Cell* 136:215-233.
- Bickenbach JR, Greer JM, Bundman DS, Rothnagel JA, Roop DR (1995) Loricrin expression is coordinated with other epidermal proteins and the appearance of lipid lamellar granules in development. *J Invest Dermatol* 104:405-410.
- Bitgood MJ, McMahon AP (1995) Hedgehog and Bmp genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo. *Dev Biol* 172:126-138.
- Blanpain C, Lowry WE, Pasolli HA, Fuchs E (2006) Canonical notch signaling functions as a commitment switch in the epidermal lineage. *Genes Dev* 20:3022-3035.
- Bommer GT, Gerin I, Feng Y, Kaczorowski AJ, Kuick R, Love RE, *et al.* (2007) p53-mediated activation of miRNA34 candidate tumor-suppressor genes. *Curr Biol* 17:1298-1307.
- Brugarolas J, Chandrasekaran C, Gordon JI, Beach D, Jacks T, Hannon GJ (1995) Radiation-induced cell cycle arrest compromised by p21 deficiency. *Nature* 377:552-557.
- Byrne C, Tainsky M, Fuchs E (1994) Programming gene expression in developing epidermis. *Development* 120:2369-2383.
- Carroll DK, Carroll JS, Leong CO, Cheng F, Brown M, Mills AA, *et al.* (2006) p63 regulates an adhesion programme and cell survival in epithelial cells. *Nat Cell Biol* 8:551-561.

Chang TC, Wentzel EA, Kent OA, Ramachandran K, Mullendore M, Lee KH, *et al.* (2007) Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. *Mol Cell* 26:745-752.

Chuong CM, Patel N, Lin J, Jung HS, Widelitz RB (2000) Sonic hedgehog signaling pathway in vertebrate epithelial appendage morphogenesis: perspectives in development and evolution. *Cell Mol Life Sci* 57:1672-1681.

Corney DC, Flesken-Nikitin A, Godwin AK, Wang W, Nikitin AY (2007) MicroRNA-34b and MicroRNA-34c are targets of p53 and cooperate in control of cell proliferation and adhesion-independent growth. *Cancer Res* 67:8433-8438.

Cullen BR (2004) Transcription and processing of human microRNA precursors. *Mol Cell* 16:861-865.

Della Gatta G, Bansal M, Ambesi-Impiombato A, Antonini D, Missero C, di Bernardo D (2008) Direct targets of the TRP63 transcription factor revealed by a combination of gene expression profiling and reverse engineering. *Genome Res* 18:939-948.

Dewulf N, Verschueren K, Lonnoy O, Moren A, Grimsby S, Vande Spiegle K, *et al.* (1995) Distinct spatial and temporal expression patterns of two type I receptors for bone morphogenetic proteins during mouse embryogenesis. *Endocrinology* 136:2652-2663.

DeYoung MP, Johannessen CM, Leong CO, Faquin W, Rocco JW, Ellisen LW (2006) Tumor-specific p73 up-regulation mediates p63 dependence in squamous cell carcinoma. *Cancer Res* 66:9362-9368.

Dudley AT, Lyons KM, Robertson EJ (1995) A requirement for bone morphogenetic protein-7 during development of the mammalian kidney and eye. *Genes Dev* 9:2795-2807.

Dunnwald M, Chinnathambi S, Alexandrunas D, Bickenbach JR (2003) Mouse epidermal stem cells proceed through the cell cycle. *J Cell Physiol* 195:194-201.

el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, *et al.* (1993) WAF1, a potential mediator of p53 tumor suppression. *Cell* 75:817-825.

Fuchs E (2007) Scratching the surface of skin development. *Nature* 445:834-842.

Fuchs E, Green H (1980) Changes in keratin gene expression during terminal differentiation of the keratinocyte. *Cell* 19:1033-1042.

Goodheart MJ, Ritchie JM, Rose SL, Fruehauf JP, De Young BR, Buller RE (2005) The relationship of molecular markers of p53 function and angiogenesis to prognosis of stage I epithelial ovarian cancer. *Clin Cancer Res* 11:3733-3742.

Gu Y, Turck CW, Morgan DO (1993) Inhibition of CDK2 activity in vivo by an associated 20K regulatory subunit. *Nature* 366:707-710.

Han G, Li AG, Liang YY, Owens P, He W, Lu S, *et al.* (2006) Smad7-induced beta-catenin degradation alters epidermal appendage development. *Dev Cell* 11:301-312.

- Hardman MJ, Sisi P, Banbury DN, Byrne C (1998) Patterned acquisition of skin barrier function during development. *Development* 125:1541-1552.
- Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ (1993) The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* 75:805-816.
- He L, He X, Lim LP, de Stanchina E, Xuan Z, Liang Y, *et al.* (2007) A microRNA component of the p53 tumour suppressor network. *Nature* 447:1130-1134.
- He W, Li AG, Wang D, Han S, Zheng B, Goumans MJ, *et al.* (2002) Overexpression of Smad7 results in severe pathological alterations in multiple epithelial tissues. *EMBO J* 21:2580-2590.
- Helton ES, Zhu J, Chen X (2006) The unique NH₂-terminally deleted (DeltaN) residues, the PXXP motif, and the PPXY motif are required for the transcriptional activity of the DeltaN variant of p63. *J Biol Chem* 281:2533-2542.
- Hoover LL, Kubalak SW (2008) Holding their own: the noncanonical roles of Smad proteins. *Sci Signal* 1:pe48.
- Ihrle RA, Marques MR, Nguyen BT, Horner JS, Papazoglu C, Bronson RT, *et al.* (2005) Perp is a p63-regulated gene essential for epithelial integrity. *Cell* 120:843-856.
- Ito M, Liu Y, Yang Z, Nguyen J, Liang F, Morris RJ, *et al.* (2005) Stem cells in the hair follicle bulge contribute to wound repair but not to homeostasis of the epidermis. *Nat Med* 11:1351-1354.
- Kelly TJ, Brown GW (2000) Regulation of chromosome replication. *Annu Rev Biochem* 69:829-880.
- Keyes WM, Wu Y, Vogel H, Guo X, Lowe SW, Mills AA (2005) p63 deficiency activates a program of cellular senescence and leads to accelerated aging. *Genes Dev* 19:1986-1999.
- King KE, Ponnampertuma RM, Yamashita T, Tokino T, Lee LA, Young MF, *et al.* (2003) deltaNp63alpha functions as both a positive and a negative transcriptional regulator and blocks in vitro differentiation of murine keratinocytes. *Oncogene* 22:3635-3644.
- Kobielak K, Pasolli HA, Alonso L, Polak L, Fuchs E (2003) Defining BMP functions in the hair follicle by conditional ablation of BMP receptor IA. *J Cell Biol* 163:609-623.
- Kobielak K, Stokes N, de la Cruz J, Polak L, Fuchs E (2007) Loss of a quiescent niche but not follicle stem cells in the absence of bone morphogenetic protein signaling. *Proc Natl Acad Sci U S A* 104:10063-10068.
- Koster MI, Dai D, Marinari B, Sano Y, Costanzo A, Karin M, *et al.* (2007) p63 induces key target genes required for epidermal morphogenesis. *Proc Natl Acad Sci U S A* 104:3255-3260.
- Koster MI, Kim S, Mills AA, DeMayo FJ, Roop DR (2004) p63 is the molecular switch for initiation of an epithelial stratification program. *Genes Dev* 18:126-131.
- Koster MI, Roop DR (2004) Genetic pathways required for epidermal morphogenesis. *Eur J Cell Biol* 83:625-629.

Laurikkala J, Mikkola ML, James M, Tummers M, Mills AA, Thesleff I (2006) p63 regulates multiple signalling pathways required for ectodermal organogenesis and differentiation. *Development* 133:1553-1563.

Lechler T, Fuchs E (2005) Asymmetric cell divisions promote stratification and differentiation of mammalian skin. *Nature* 437:275-280.

Lena AM, Shalom-Feuerstein R, Rivetti di Val Cervo P, Aberdam D, Knight RA, Melino G, *et al.* (2008) miR-203 represses 'stemness' by repressing DeltaNp63. *Cell Death Differ* 15:1187-1195.

Liefer KM, Koster MI, Wang XJ, Yang A, McKeon F, Roop DR (2000) Down-regulation of p63 is required for epidermal UV-B-induced apoptosis. *Cancer Res* 60:4016-4020.

Lipinski MM, Jacks T (1999) The retinoblastoma gene family in differentiation and development. *Oncogene* 18:7873-7882.

Lyons KM, Pelton RW, Hogan BL (1989) Patterns of expression of murine Vgr-1 and BMP-2a RNA suggest that transforming growth factor-beta-like genes coordinately regulate aspects of embryonic development. *Genes Dev* 3:1657-1668.

M'Boneko V, Merker HJ (1988) Development and morphology of the periderm of mouse embryos (days 9-12 of gestation). *Acta Anat (Basel)* 133:325-336.

Macias-Silva M, Hoodless PA, Tang SJ, Buchwald M, Wrana JL (1998) Specific activation of Smad1 signaling pathways by the BMP7 type I receptor, ALK2. *J Biol Chem* 273:25628-25636.

Massague J (1998) TGF-beta signal transduction. *Annu Rev Biochem* 67:753-791.

McMillan JR, Akiyama M, Shimizu H (2003) Epidermal basement membrane zone components: ultrastructural distribution and molecular interactions. *J Dermatol Sci* 31:169-177.

Meister G, Tuschl T (2004) Mechanisms of gene silencing by double-stranded RNA. *Nature* 431:343-349.

Mills AA, Zheng B, Wang XJ, Vogel H, Roop DR, Bradley A (1999) p63 is a p53 homologue required for limb and epidermal morphogenesis. *Nature* 398:708-713.

Mitu GM, Wang S, Hirschberg R (2007) BMP7 is a podocyte survival factor and rescues podocytes from diabetic injury. *Am J Physiol Renal Physiol* 293:F1641-1648.

Morgan DO (1997) Cyclin-dependent kinases: engines, clocks, and microprocessors. *Annu Rev Cell Dev Biol* 13:261-291.

Murray AW (2004) Recycling the cell cycle: cyclins revisited. *Cell* 116:221-234.

Nguyen BC, Lefort K, Mandinova A, Antonini D, Devgan V, Della Gatta G, *et al.* (2006) Cross-regulation between Notch and p63 in keratinocyte commitment to differentiation. *Genes Dev* 20:1028-1042.

- Noda A, Ning Y, Venable SF, Pereira-Smith OM, Smith JR (1994) Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. *Exp Cell Res* 211:90-98.
- Parsa R, Yang A, McKeon F, Green H (1999) Association of p63 with proliferative potential in normal and neoplastic human keratinocytes. *J Invest Dermatol* 113:1099-1105.
- Pavletich NP (1999) Mechanisms of cyclin-dependent kinase regulation: structures of Cdk, their cyclin activators, and Cip and INK4 inhibitors. *J Mol Biol* 287:821-828.
- Pellegrini G, Dellambra E, Golisano O, Martinelli E, Fantozzi I, Bondanza S, *et al.* (2001) p63 identifies keratinocyte stem cells. *Proc Natl Acad Sci U S A* 98:3156-3161.
- Perez CA, Pietenpol JA (2007) Transcriptional programs regulated by p63 in normal epithelium and tumors. *Cell Cycle* 6:246-254.
- Pillai RS, Bhattacharyya SN, Filipowicz W (2007) Repression of protein synthesis by miRNAs: how many mechanisms? *Trends Cell Biol* 17:118-126.
- Potten CS, Morris RJ (1988) Epithelial stem cells in vivo. *J Cell Sci Suppl* 10:45-62.
- Prasanth SG, Mendez J, Prasanth KV, Stillman B (2004) Dynamics of pre-replication complex proteins during the cell division cycle. *Philos Trans R Soc Lond B Biol Sci* 359:7-16.
- Priolo M, Silengo M, Lerone M, Ravazzolo R (2000) Ectodermal dysplasias: not only 'skin' deep. *Clin Genet* 58:415-430.
- Raver-Shapira N, Marciano E, Meiri E, Spector Y, Rosenfeld N, Moskovits N, *et al.* (2007) Transcriptional activation of miR-34a contributes to p53-mediated apoptosis. *Mol Cell* 26:731-743.
- Rice RH, Green H (1977) The cornified envelope of terminally differentiated human epidermal keratinocytes consists of cross-linked protein. *Cell* 11:417-422.
- Riley T, Sontag E, Chen P, Levine A (2008) Transcriptional control of human p53-regulated genes. *Nat Rev Mol Cell Biol* 9:402-412.
- Romano RA, Ortt K, Birkaya B, Smalley K, Sinha S (2009) An active role of the DeltaN isoform of p63 in regulating basal keratin genes K5 and K14 and directing epidermal cell fate. *PLoS One* 4:e5623.
- Sbisa E, Mastropasqua G, Lefkimmiatis K, Caratozzolo MF, D'Erchia AM, Tullo A (2006) Connecting p63 to cellular proliferation: the example of the adenosine deaminase target gene. *Cell Cycle* 5:205-212.
- Schmierer B, Hill CS (2007) TGFbeta-SMAD signal transduction: molecular specificity and functional flexibility. *Nat Rev Mol Cell Biol* 8:970-982.
- Schneider TE, Barland C, Alex AM, Mancianti ML, Lu Y, Cleaver JE, *et al.* (2003) Measuring stem cell frequency in epidermis: a quantitative in vivo functional assay for long-term repopulating cells. *Proc Natl Acad Sci U S A* 100:11412-11417.

- Sears RC, Nevins JR (2002) Signaling networks that link cell proliferation and cell fate. *J Biol Chem* 277:11617-11620.
- Senoo M, Pinto F, Crum CP, McKeon F (2007) p63 Is essential for the proliferative potential of stem cells in stratified epithelia. *Cell* 129:523-536.
- Sherr CJ (2004) Principles of tumor suppression. *Cell* 116:235-246.
- Sherr CJ, Roberts JM (1999) CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev* 13:1501-1512.
- Smart IH (1970) Variation in the plane of cell cleavage during the process of stratification in the mouse epidermis. *Br J Dermatol* 82:276-282.
- Stern CD (2006) Neural induction: 10 years on since the 'default model'. *Curr Opin Cell Biol* 18:692-697.
- Stevaux O, Dyson NJ (2002) A revised picture of the E2F transcriptional network and RB function. *Curr Opin Cell Biol* 14:684-691.
- Steven AC, Steinert PM (1994) Protein composition of cornified cell envelopes of epidermal keratinocytes. *J Cell Sci* 107 (Pt 2):693-700.
- Su X, Chakravarti D, Cho MS, Liu L, Gi YJ, Lin YL, *et al.* (2010) TAp63 suppresses metastasis through coordinate regulation of Dicer and miRNAs. *Nature* 467:986-990.
- Su X, Cho MS, Gi YJ, Ayanga BA, Sherr CJ, Flores ER (2009) Rescue of key features of the p63-null epithelial phenotype by inactivation of Ink4a and Arf. *EMBO J* 28:1904-1915.
- Sun F, Fu H, Liu Q, Tie Y, Zhu J, Xing R, *et al.* (2008) Downregulation of CCND1 and CDK6 by miR-34a induces cell cycle arrest. *FEBS Lett* 582:1564-1568.
- Suzuki K, Haraguchi R, Ogata T, Barbieri O, Alegria O, Vieux-Rochas M, *et al.* (2008) Abnormal urethra formation in mouse models of split-hand/split-foot malformation type 1 and type 4. *Eur J Hum Genet* 16:36-44.
- Takahashi H, Ikeda T (1996) Transcripts for two members of the transforming growth factor-beta superfamily BMP-3 and BMP-7 are expressed in developing rat embryos. *Dev Dyn* 207:439-449.
- Tarasov V, Jung P, Verdoodt B, Lodygin D, Epanchintsev A, Menssen A, *et al.* (2007) Differential regulation of microRNAs by p53 revealed by massively parallel sequencing: miR-34a is a p53 target that induces apoptosis and G1-arrest. *Cell Cycle* 6:1586-1593.
- Tazawa H, Tsuchiya N, Izumiya M, Nakagama H (2007) Tumor-suppressive miR-34a induces senescence-like growth arrest through modulation of the E2F pathway in human colon cancer cells. *Proc Natl Acad Sci U S A* 104:15472-15477.
- ten Dijke P, Yamashita H, Sampath TK, Reddi AH, Estevez M, Riddle DL, *et al.* (1994) Identification of type I receptors for osteogenic protein-1 and bone morphogenetic protein-4. *J Biol Chem* 269:16985-16988.

- Truong AB, Kretz M, Ridky TW, Kimmel R, Khavari PA (2006) p63 regulates proliferation and differentiation of developmentally mature keratinocytes. *Genes Dev* 20:3185-3197.
- Verschueren K, Dewulf N, Goumans MJ, Lonnoy O, Feijen A, Grimsby S, *et al.* (1995) Expression of type I and type IB receptors for activin in midgestation mouse embryos suggests distinct functions in organogenesis. *Mech Dev* 52:109-123.
- Vigano MA, Lamartine J, Testoni B, Merico D, Alotto D, Castagnoli C, *et al.* (2006) New p63 targets in keratinocytes identified by a genome-wide approach. *EMBO J* 25:5105-5116.
- Vukicevic S, Basic V, Rogic D, Basic N, Shih MS, Shepard A, *et al.* (1998) Osteogenic protein-1 (bone morphogenetic protein-7) reduces severity of injury after ischemic acute renal failure in rat. *J Clin Invest* 102:202-214.
- Waldman T, Kinzler KW, Vogelstein B (1995) p21 is necessary for the p53-mediated G1 arrest in human cancer cells. *Cancer Res* 55:5187-5190.
- Wall NA, Blessing M, Wright CV, Hogan BL (1993) Biosynthesis and in vivo localization of the decapentaplegic-Vg-related protein, DVR-6 (bone morphogenetic protein-6). *J Cell Biol* 120:493-502.
- Westfall MD, Mays DJ, Sniezek JC, Pietenpol JA (2003) The Delta Np63 alpha phosphoprotein binds the p21 and 14-3-3 sigma promoters in vivo and has transcriptional repressor activity that is reduced by Hay-Wells syndrome-derived mutations. *Mol Cell Biol* 23:2264-2276.
- Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D (1993) p21 is a universal inhibitor of cyclin kinases. *Nature* 366:701-704.
- Yabe T, Samuels I, Schwartz JP (2002) Bone morphogenetic proteins BMP-6 and BMP-7 have differential effects on survival and neurite outgrowth of cerebellar granule cell neurons. *J Neurosci Res* 68:161-168.
- Yamashita H, ten Dijke P, Huylebroeck D, Sampath TK, Andries M, Smith JC, *et al.* (1995) Osteogenic protein-1 binds to activin type II receptors and induces certain activin-like effects. *J Cell Biol* 130:217-226.
- Yan W, Chen X (2007) Targeted repression of bone morphogenetic protein 7, a novel target of the p53 family, triggers proliferative defect in p53-deficient breast cancer cells. *Cancer Res* 67:9117-9124.
- Yang A, Kaghad M, Caput D, McKeon F (2002) On the shoulders of giants: p63, p73 and the rise of p53. *Trends Genet* 18:90-95.
- Yang A, Kaghad M, Wang Y, Gillett E, Fleming MD, Dotsch V, *et al.* (1998) p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Mol Cell* 2:305-316.
- Yang A, Schweitzer R, Sun D, Kaghad M, Walker N, Bronson RT, *et al.* (1999) p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature* 398:714-718.

- Yang A, Zhu Z, Kapranov P, McKeon F, Church GM, Gingeras TR, *et al.* (2006) Relationships between p63 binding, DNA sequence, transcription activity, and biological function in human cells. *Mol Cell* 24:593-602.
- Yang S, Pham LK, Liao CP, Frenkel B, Reddi AH, Roy-Burman P (2008) A novel bone morphogenetic protein signaling in heterotypic cell interactions in prostate cancer. *Cancer Res* 68:198-205.
- Yi R, Fuchs E (2009) MicroRNA-mediated control in the skin. *Cell Death Differ* 17:229-235.
- Yi R, O'Carroll D, Pasolli HA, Zhang Z, Dietrich FS, Tarakhovskiy A, *et al.* (2006) Morphogenesis in skin is governed by discrete sets of differentially expressed microRNAs. *Nat Genet* 38:356-362.
- Yi R, Pasolli HA, Landthaler M, Hafner M, Ojo T, Sheridan R, *et al.* (2009) DGCR8-dependent microRNA biogenesis is essential for skin development. *Proc Natl Acad Sci U S A* 106:498-502.
- Yi R, Poy MN, Stoffel M, Fuchs E (2008) A skin microRNA promotes differentiation by repressing 'stemness'. *Nature* 452:225-229.
- Yu PB, Deng DY, Lai CS, Hong CC, Cuny GD, Buxsein ML, *et al.* (2008) BMP type I receptor inhibition reduces heterotopic [corrected] ossification. *Nat Med* 14:1363-1369.
- Zamore PD, Haley B (2005) Ribo-gnome: the big world of small RNAs. *Science* 309:1519-1524.