

Transcriptional Repression of miR-34 Family Contributes to p63-Mediated Cell Cycle Progression in Epidermal Cells

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p63, a p53 family member, is highly expressed in the basal proliferative compartment of the epidermis and its expression has been correlated with the growth ability and regenerative capacity of keratinocytes. In this study we report a mechanism through which p63 maintains cell cycle progression by directly repressing miR-34a and miR-34c. In the absence of p63, increased levels of miR-34a and miR-34c were observed in primary keratinocytes and in embryonic skin, with concomitant G1-phase arrest and inhibition of the cell cycle regulators cyclin D1 and cyclin-dependent kinase 4 (Cdk4). p63 directly bound to p53-consensus sites in both miR-34a and miR-34c regulatory regions and inhibited their activity. Concomitant downregulation of miR-34a and miR-34c substantially restored cell cycle progression and expression of cyclin D1 and Cdk4. Our data indicate that specific miR-34 family members have a significant role downstream of p63 in controlling epidermal cell proliferation.

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INTRODUCTION

The epidermis, which is the outer layer of the skin, is a stratified epithelium that is continuously renewed by a finely tuned balance between proliferation and differentiation. p63 is expressed in epithelial cells with high clonogenic capacity (Yang *et al.*, 1998; Parsa *et al.*, 1999; Pellegrini *et al.*, 2001; Senoo *et al.*, 2007), and its expression has been correlated with their proliferative potential (Deyoung *et al.*, 2006; Truong *et al.*, 2006; Senoo *et al.*, 2007). Mice that are homozygous for a disrupted p63 gene have major defects in the development of various stratified epithelia (Yang *et al.*, 1998, 1999; Mills *et al.*, 1999), consistent with a crucial role of p63 in these tissues.

p63 encodes a tetrameric transcription factor characterized by six isoforms with different transactivation potentials sharing an identical DNA-binding domain (Yang *et al.*, 1999). The $\Delta Np63\alpha$, which is the most abundant isoform in cultured keratinocytes and epidermis (Yang *et al.*, 1998), contains both a transactivation domain and an inhibitory domain, and it has been shown to activate or repress gene transcription depending on the promoter context (Yang *et al.*, 1998; Serber

et al., 2002). The DNA-binding domain of p63 is highly homologous to that of p53, and—for this reason—p63 binds *in vitro* and *in vivo* to a consensus sequence that is very similar to the p53 binding site (Osada *et al.*, 2005; Yang *et al.*, 2006; Perez and Pietenpol, 2007; Della Gatta *et al.*, 2008), thus regulating a subset of known p53 target genes (Dohn *et al.*, 2001; Ellisen *et al.*, 2002; Westfall *et al.*, 2003; Ihrie *et al.*, 2005).

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 (for review, see 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 p21^{Cip1} (Cdkn1a) gene, encoding an inhibitor of G1 cyclin/CDK complexes (Harper *et al.*, 1993; el-Deiry *et al.*, 1993; Brugarolas *et al.*, 1995; 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 p21^{Cip1} by binding to the p53-consensus site (Westfall *et al.*, 2003; Goodheart *et al.*, 2005; Deyoung *et al.*, 2006; Nguyen *et al.*, 2006; Truong *et al.*, 2006). However, in human keratinocytes, downregulation of p21^{Cip1} 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).

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Abbreviations: miRNA, microRNA; siRNA, short interfering RNA

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MicroRNAs (miRNAs) form a class of small non-coding RNAs (~19–24 nucleotides) that have key roles in the regulation of gene expression in several cellular processes, including cell proliferation. 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 (for review, see 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; He *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; Tazawa *et al.*, 2007; Sun *et al.*, 2008).

In this study we 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.

RESULTS

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 also, 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 p63 knockdown keratinocytes versus control at 48 hours after transfection (Figure 1a, left panel, and data not shown), although a small fraction of p63-negative cells were still able to incorporate BrdU (see Supplementary Figure S1 online). Δ Np63 α 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 Δ Np63 α (Figure 1a, right panel), confirming the ability of Δ Np63 α to

promote cell proliferation. Flow cytometry analysis revealed that p63 knockdown resulted in a significant increase in 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 1b), 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 1c), 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 1d), 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.

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

To explore the mechanisms underlying cell cycle arrest as a consequence of the absence of p63, we tested the expression of a subset of G1-phase cell cycle regulators at different times upon p63 knockdown (Figure 1e). Low protein levels of cyclin D1 were detected at early times upon p63 knockdown. Interestingly, p21^{Cip1} was not induced by p63 knockdown in the CD1 keratinocytes in contrast to what was previously reported in human keratinocytes and in keratinocytes derived from other mouse strains (Westfall *et al.*, 2003; Deyoung *et al.*, 2006; Nguyen *et al.*, 2006; Truong *et al.*, 2006), further supporting the hypothesis that other mechanisms may be involved in mediating cell cycle arrest in the absence of p63. At 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, we speculated that p63 might regulate miR-34 family in keratinocytes. We 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

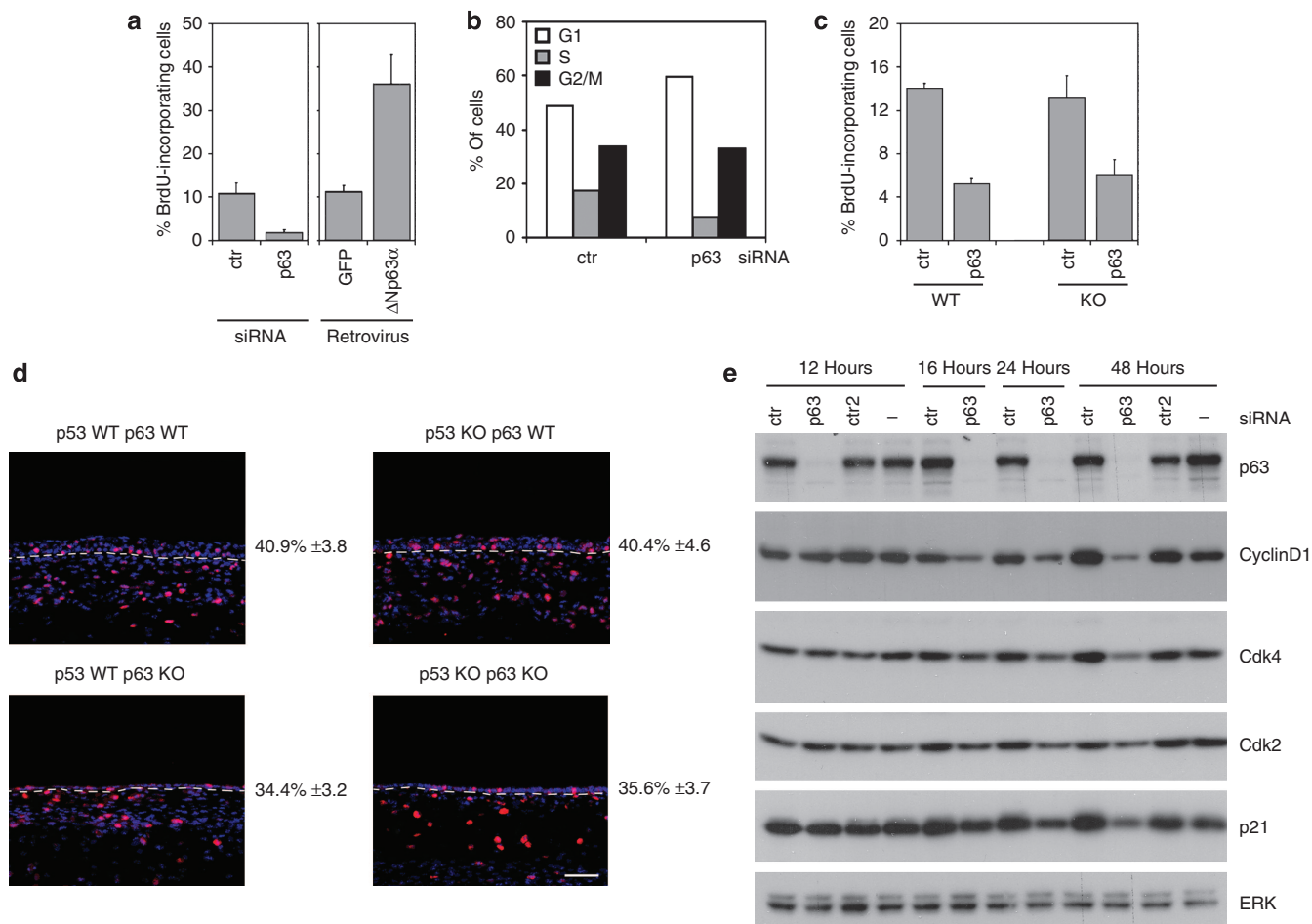


Figure 1. p63 regulates cell cycle progression of mouse epidermal cells independently of p53. (a) BrdU analysis performed in primary mouse keratinocytes transfected with p63 or with negative control short interfering RNA (siRNA; ctr; left panel), or in parallel infected with a retrovirus carrying $\Delta Np63\alpha$ or control (green fluorescent protein (GFP)) as indicated (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. (e) 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.

primary mouse keratinocytes, whereas miR-34c was expressed at lower levels, and miR-34b was undetectable (Figure 2a). 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 2c). In contrast, miR-34c was less expressed and mainly detected in the basal layer, whereas miR-34b was undetectable.

To test the possibility that miR-34 family may be a p63 target, we measured miR-34 expression levels in the presence or absence of p63 *in vitro* and *in vivo*. Induced expression

levels of miR-34a and miR-34c were observed in p63 knockdown when compared with control keratinocytes (Figure 2b). 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 2d), indicating that p63 negatively regulates miR-34a and miR-34c expression in epidermal cells *in vitro* and *in vivo*. 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 strongly bound to *miR-34a* and *miR-34b/c* promoters containing phylogenetically conserved p53-binding sites (Figure 3a and Supplementary Figure S2

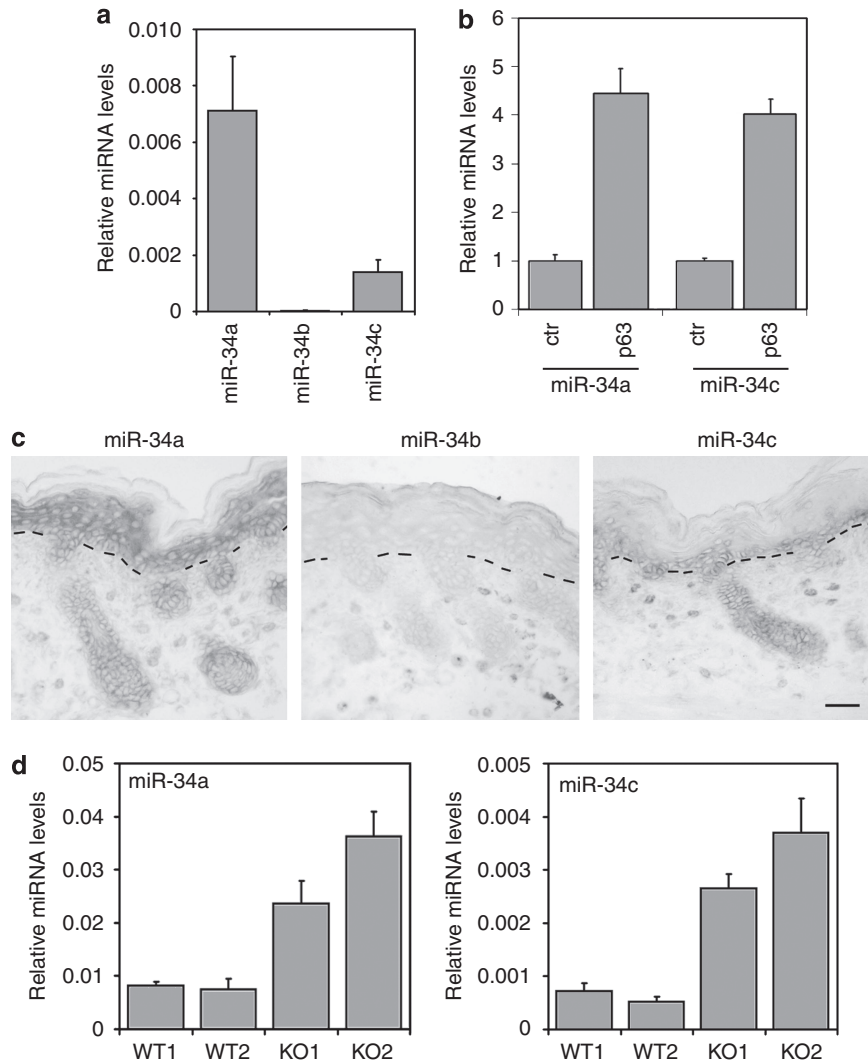


Figure 2. miR-34a and miR-34c are specifically expressed in mouse epidermis and are induced by loss of p63. (a) Expression levels of the indicated microRNA (miRNA) were measured using real-time quantitative reverse transcriptase-PCR (RT-PCR; Taqman) in primary mouse keratinocytes. miRNA samples were 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 primary mouse keratinocytes transfected with p63 or control short interfering RNAs (ctr siRNAs). (c) *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. (d) 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.

online). In addition, p63 overexpression significantly inhibited the activity of miR-34a and miR34b/c promoters, whereas p63 knockdown resulted in an enhancement of their activity (Figure 3b).

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.

miR-34 downregulation counteracts cell cycle arrest in the absence of p63

To test the contribution of miR-34 family in the cell cycle arrest elicited by loss of p63, we co-transfected antisense inhibitors (anti-miR) for miR-34a, miR-34c, or both in the presence or absence of p63 (Figure 4a). 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 4b). Concomitant transfection of both anti-miR-34a and anti-miR-34c almost entirely rescued the proliferation defect in the absence of p63, whereas they had no effect 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 effect in the presence of p63 (Figure 4c).

The miR-34 family has been shown to regulate cell cycle progression in G1 phase by directly controlling a number of cell cycle regulators including cyclin D1 and Cdk4 (He *et al.*, 2007; Sun *et al.*, 2008). To test whether miR-34 family exerts an effect downstream of p63 to regulate the expression of cell

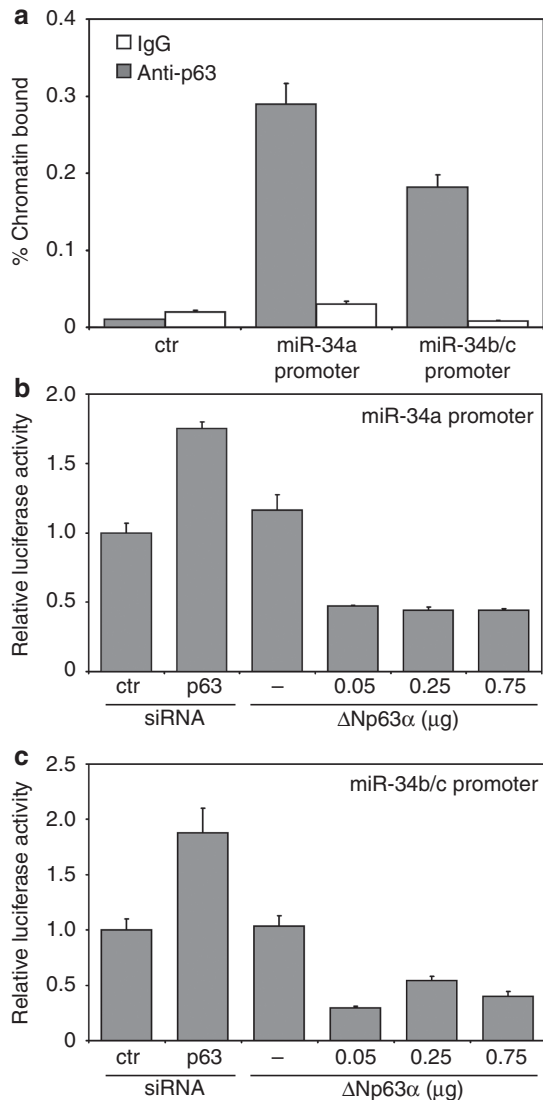


Figure 3. miR-34a and miR-34c are transcriptional targets of p63. (a) Chromatin immunoprecipitation in primary mouse keratinocytes using antibodies specific for p63 (gray bars), or rabbit IgG antibodies as control (white bars), followed by real-time PCR amplification using specific oligonucleotide primers 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 $\Delta Np63\alpha$ -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.

cycle proteins, we 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 5). Taken together, these data indicate that miR-34a and miR-34c are relevant targets of p63 in cell cycle regulation.

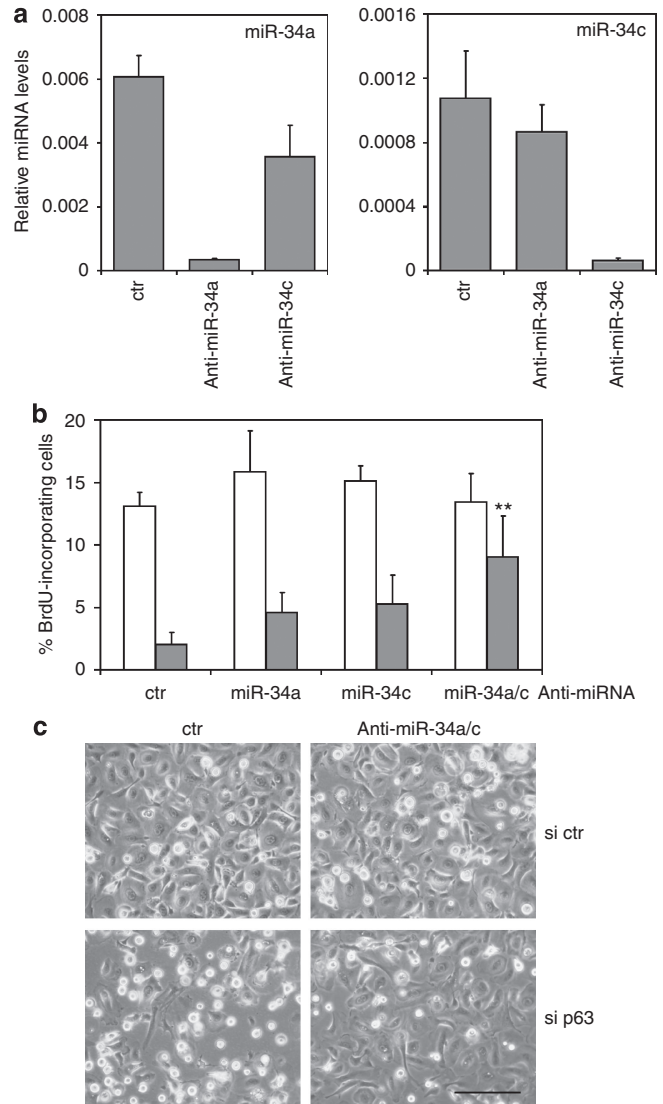


Figure 4. Concomitant downregulation of miR-34a and miR-34c expression reactivates cell cycle progression in p63 knockdown keratinocytes. (a) *miR-34a* and *miR-34c* levels measured in primary mouse keratinocytes at 48 hours after transfection with specific anti-miRNAs or a scramble control. (b) BrdU 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 with anti-miR-34a and miR-34c or scramble control, in the absence (gray bars) or in the presence (white bars) of p63. Results are representative of two independent experiments \pm SD (***P*-value < 0.01, *n* = 3). Scale bar = 50 μm.

DISCUSSION

We report that under physiological conditions 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

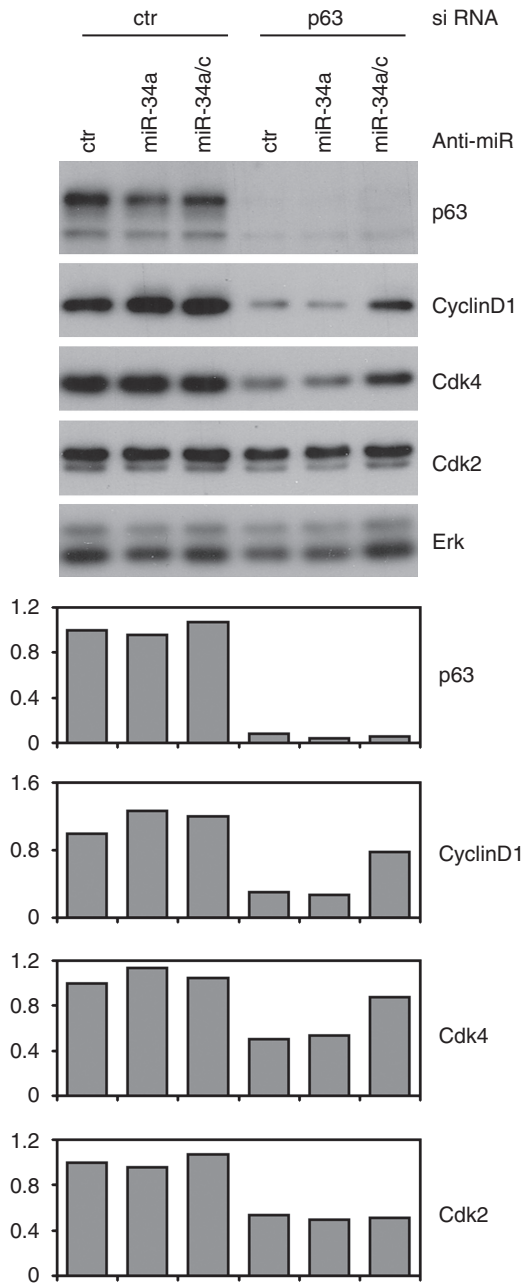


Figure 5. Concomitant downregulation of miR-34a and miR-34c expression restores expression levels of cyclin D1 and Cdk4 in p63 knockdown keratinocytes. Immunoblot of the indicated G1-phase regulators in primary mouse keratinocytes transfected as in Figure 4b. Protein levels were normalized for extracellular signal-regulated kinase (ERK) expression (lower panel). Similar results were obtained when normalizing for β -actin. Results are representative of two independent experiments.

(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. We show 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 p21^{Cip1} and 14-3-3 σ (Westfall et al., 2003; Nguyen et al., 2006; Truong et al., 2006), are repressed by p63 and are likely to contribute to p63-mediated cell cycle progression. Thus, p63—more specifically its Δ Np63 α 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). Our data indicate 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, p21^{Cip1} 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. We previously reported upregulation of p21^{Cip1} upon p63 downregulation in Sencar keratinocytes (Nguyen et al., 2006), whereas in the CD1 background p21^{Cip1} expression is not induced, further reinforcing the notion that p21^{Cip1} is unlikely to be the only player in cell cycle arrest downstream of p63. Similar to other cell cycle regulators, the downregulation of p21^{Cip1} 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 (Vigano et al., 2006; Yang et al., 2006; Della Gatta et al., 2008). Among the cyclin-dependent kinase inhibitors, p16^{Ink4a} 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 p16^{Ink4a} with partial restoration of keratinocyte proliferation (Su et al., 2009). Future experiments will need to address the contribution of miR-34 to cell cycle regulation by p63 *in vivo*, and in human keratinocytes.

Our data 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; Tazawa et al., 2007; Sun et al., 2008). 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.

We find 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. More puzzling is the prevalent expression of miR-34c in the basal layer of the epidermis, 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, we find that 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. Post-transcriptional regulation for a large fraction of miRNAs has been shown at the Drosha processing step. 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.*, 2009). Interestingly, p53 binds p68 through a carboxy terminal portion of the DNA-binding domain, which is highly homologous to the corresponding region in p63. Thus, an outstanding question for future studies will be whether p63 shares with p53 the ability to bind p68 and interfere with miRNA maturation.

An important role of miRNA in skin has been previously uncovered. 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 *et al.*, 2006, 2009; Yi and Fuchs, 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.

MATERIALS AND METHODS

Cell cultures, transfections, retroviral preparation, and mice

Primary mouse keratinocytes were isolated from 2-day-old Swiss ICR(CD-1) mice (Harlan Laboratories, Udine, Italy) and cultured under low Ca^{2+} conditions as previously described (Antonini *et al.*, 2006). Transfections were performed 5 days after plating. A total amount of 200 nM siRNA (Stealth siRNA, Invitrogen) for p63 (Antonini *et al.*, 2008), or control medium GC-rich siRNA (Stealth siRNA, Invitrogen, Milano, Italy), and/or anti-miR-34a, anti-miR-34c (miRCURY, Exiqon, Vedbaek, Denmark), or scramble control (miRCURY, Exiqon 19900200) were transfected using Lipofectamine 2000 (Invitrogen) as described earlier (Antonini *et al.*, 2006). Stealth RNA interference negative control medium GC (Invitrogen) and an alternative RNA interference (5'-ACAGCTACATCGCGCTCATCGC TAT-3') were used as negative controls as indicated. Primary keratinocytes were infected with high-titer GINCO-green-fluorescent

protein or GINCO- Δ Np63 α retroviruses (Della Gatta *et al.*, 2008) and were cultured for 4 additional days. p63-null mice (B6.129S7-*Trp63*^{tm1BrdJ}) were obtained from Jackson Laboratory (Bar Harbor, ME). p53-null mice (Donehower *et al.*, 1992) in a C57/BL6 background were obtained from Alfredo Fusco (Federico II University, Napoli, Italy). All experiments performed with mice were conducted under the approval institutional animal care and use committee.

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 mg ml⁻¹), RNase (1 mg ml⁻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 hours and stained as previously described (Della Gatta *et al.*, 2008). 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 μ m thick, were permeabilized in 0.2% Triton X-100, and processed for immunofluorescence as described above.

miRNA expression analysis and *in situ* hybridization

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.

In situ hybridization was performed essentially as previously described (Brancaccio *et al.*, 2004). Hybridization was performed with miRCURY LNA Detection antisense Probes (Exiqon) specific for miR-34a, miR-34b, and miR-34c at 40 °C. Digoxigenin labeling was monitored under a Zeiss Axioskop2 plus microscope using a Zeiss Plan-Neofluar (Zeiss, Arese, Italy) \times 20/0.50 objective.

Immunoblotting

Cells were lysed in Laemmli sample buffer and protein extracts were run on SDS-PAGE gels, transferred on Immobilon-P transfer membranes (Millipore, Milano, Italy), probed with the indicated antibodies and detected using enhanced chemiluminescence (GE Healthcare Life Sciences, Milano, Italy). 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). For quantification of immunoblotting, exposed films were scanned using ChemiDoc (Bio-Rad, Hercules, CA) and quantified using ImageJ software (<http://rsbweb.nih.gov/ij/>).

Chromatin immunoprecipitation

Primary keratinocytes were fixed with 1% formaldehyde and chromatin immunoprecipitation/real-time PCR was performed using anti-p63 antibodies (H-137, Santa Cruz Biotechnology), and

non-specific rabbit IgG antibodies as negative control. Chromatin immunoprecipitation and real time PCR were performed as previously described (Antonini *et al.*, 2008). Oligonucleotide primers used for real-time PCR amplifications were: miR-34a promoter: F: 5'-CAGCCTGGAGGAGGATCGA-3', R: 5'-TTCTCTGAACAAGTCCAGGCAA-3'; miR-34b/c promoter F: 5'-CTGTTGATCCTGCCACAGTT-3', R: 5'-GCATGACCAGCAGATGCAAT-3'. Luciferase assay was performed as previously described (Antonini *et al.*, 2008).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

REFERENCES

- Andl T, Murchison EP, Liu F *et al.* (2006) The miRNA-processing enzyme dicer is essential for the morphogenesis and maintenance of hair follicles. *Curr Biol* 16:1041–9
- Antonini D, Dentice M, Mahtani P *et al.* (2008) Tprg, a gene predominantly expressed in skin, is a direct target of the transcription factor p63. *J Invest Dermatol* 128:1676–85
- Antonini D, Rossi B, Han R *et al.* (2006) An autoregulatory loop directs the tissue-specific expression of p63 through a long-range evolutionarily conserved enhancer. *Mol Cell Biol* 26:3308–18
- Bartel DP (2009) MicroRNAs: target recognition and regulatory functions. *Cell* 136:215–33
- Bommer GT, Gerin I, Feng Y *et al.* (2007) p53-mediated activation of miRNA34 candidate tumor-suppressor genes. *Curr Biol* 17:1298–307
- Brancaccio A, Minichiello A, Grachtchouk M *et al.* (2004) Requirement of the forkhead gene Foxe1, a target of sonic hedgehog signaling, in hair follicle morphogenesis. *Hum Mol Genet* 13:2595–606
- Brugarolas J, Chandrasekaran C, Gordon JI *et al.* (1995) Radiation-induced cell cycle arrest compromised by p21 deficiency. *Nature* 377:552–7
- Carroll DK, Carroll JS, Leong CO *et al.* (2006) p63 regulates an adhesion programme and cell survival in epithelial cells. *Nat Cell Biol* 8:551–61
- Chang TC, Wentzel EA, Kent OA *et al.* (2007) Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. *Mol Cell* 26:745–52
- Corney DC, Flesken-Nikitin A, Godwin AK *et al.* (2007) MicroRNA-34b and microRNA-34c are targets of p53 and cooperate in control of cell proliferation and adhesion-independent growth. *Cancer Res* 67:8433–8
- Della Gatta G, Bansal M, Ambesi-Impiombato A *et al.* (2008) Direct targets of the TRP63 transcription factor revealed by a combination of gene expression profiling and reverse engineering. *Genome Res* 18:939–48
- Deyoung MP, Johannessen CM, Leong CO *et al.* (2006) Tumor-specific p73 up-regulation mediates p63 dependence in squamous cell carcinoma. *Cancer Res* 66:9362–8
- Dohn M, Zhang S, Chen X (2001) p63alpha and DeltaNp63alpha can induce cell cycle arrest and apoptosis and differentially regulate p53 target genes. *Oncogene* 20:3193–205
- Donehower LA, Harvey M, Slagle BL *et al.* (1992) Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* 356:215–21
- el-Deiry WS, Tokino T, Velculescu VE *et al.* (1993) WAF1, a potential mediator of p53 tumor suppression. *Cell* 75:817–25
- Ellisen LW, Ramsayer KD, Johannessen CM *et al.* (2002) REDD1, a developmentally regulated transcriptional target of p63 and p53, links p63 to regulation of reactive oxygen species. *Mol Cell* 10:995–1005
- Goodheart MJ, Ritchie JM, Rose SL *et al.* (2005) The relationship of molecular markers of p53 function and angiogenesis to prognosis of stage I epithelial ovarian cancer. *Clin Cancer Res* 11:3733–42
- Harper JW, Adami GR, Wei N *et al.* (1993) The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* 75:805–16
- He L, He X, Lim LP *et al.* (2007) A microRNA component of the p53 tumour suppressor network. *Nature* 447:1130–4
- Ihrie RA, Marques MR, Nguyen BT *et al.* (2005) Perp is a p63-regulated gene essential for epithelial integrity. *Cell* 120:843–56
- Keyes WM, Wu Y, Vogel H *et al.* (2005) p63 deficiency activates a program of cellular senescence and leads to accelerated aging. *Genes Dev* 19:1986–99
- Koster MI, Kim S, Mills AA *et al.* (2004) p63 is the molecular switch for initiation of an epithelial stratification program. *Genes Dev* 18:126–31
- Lena AM, Shalom-Feuerstein R, Rivetti di Val Cervo P *et al.* (2008) miR-203 represses “stemness” by repressing DeltaNp63. *Cell Death Differ* 15:1187–95
- Mills AA, Zheng B, Wang XJ *et al.* (1999) p63 is a p53 homologue required for limb and epidermal morphogenesis. *Nature* 398:708–13
- Nguyen BC, Lefort K, Mandinova A *et al.* (2006) Cross-regulation between Notch and p63 in keratinocyte commitment to differentiation. *Genes Dev* 20:1028–42
- Osada M, Nagakawa Y, Park HL *et al.* (2005) p63-specific activation of the BPAG-1e promoter. *J Invest Dermatol* 125:52–60
- Parsa R, Yang A, McKeon F *et al.* (1999) Association of p63 with proliferative potential in normal and neoplastic human keratinocytes. *J Invest Dermatol* 113:1099–105
- Pellegrini G, Dellambra E, Golisano O *et al.* (2001) p63 identifies keratinocyte stem cells. *Proc Natl Acad Sci USA* 98:3156–61
- Perez CA, Pietenpol JA (2007) Transcriptional programs regulated by p63 in normal epithelium and tumors. *Cell Cycle (Georgetown, TX)* 6:246–54
- Raver-Shapira N, Marciano E, Meiri E *et al.* (2007) Transcriptional activation of miR-34a contributes to p53-mediated apoptosis. *Mol Cell* 26:731–43
- Riley T, Sontag E, Chen P *et al.* (2008) Transcriptional control of human p53-regulated genes. *Nat Rev Mol Cell Biol* 9:402–12
- Senoo M, Pinto F, Crum CP *et al.* (2007) p63 is essential for the proliferative potential of stem cells in stratified epithelia. *Cell* 129:523–36
- Serber Z, Lai HC, Yang A *et al.* (2002) A C-terminal inhibitory domain controls the activity of p63 by an intramolecular mechanism. *Mol Cell Biol* 22:8601–11
- Su X, Cho MS, Gi YJ *et al.* (2009) Rescue of key features of the p63-null epithelial phenotype by inactivation of Ink4a and Arf. *EMBO J* 28:1904–15
- Sun F, Fu H, Liu Q *et al.* (2008) Downregulation of CCND1 and CDK6 by miR-34a induces cell cycle arrest. *FEBS Lett* 582:1564–8
- Suzuki HI, Yamagata K, Sugimoto K *et al.* (2009) Modulation of microRNA processing by p53. *Nature* 460:529–33
- Tarasov V, Jung P, Verdoodt B *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 (Georgetown, TX)* 6:1586–93
- Tazawa H, Tsuchiya N, Izumiya M *et al.* (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 USA* 104:15472–7
- Truong AB, Kretz M, Ridky TW *et al.* (2006) p63 regulates proliferation and differentiation of developmentally mature keratinocytes. *Genes Dev* 20:3185–97

- Vigano MA, Lamartine J, Testoni B *et al.* (2006) New p63 targets in keratinocytes identified by a genome-wide approach. *EMBO J* 25:5105–16
- Waldman T, Kinzler KW, Vogelstein B (1995) p21 is necessary for the p53-mediated G1 arrest in human cancer cells. *Cancer Res* 55:5187–90
- Westfall MD, Mays DJ, Sniezek JC *et al.* (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* 23:2264–76
- Yang A, Kaghad M, Wang Y *et al.* (1998) p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Mol Cell* 2:305–16
- Yang A, Schweitzer R, Sun D *et al.* (1999) p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature* 398:714–8
- Yang A, Zhu Z, Kapranov P *et al.* (2006) Relationships between p63 binding, DNA sequence, transcription activity, and biological function in human cells. *Mol Cell* 24:593–602
- Yi R, Fuchs E (2009) MicroRNA-mediated control in the skin. *Cell Death Differ*; e-pub ahead of print 17 July 2009
- Yi R, O'Carroll D, Pasolli HA *et al.* (2006) Morphogenesis in skin is governed by discrete sets of differentially expressed microRNAs. *Nat Genet* 38:356–62
- Yi R, Pasolli HA, Landthaler M *et al.* (2009) DGCR8-dependent microRNA biogenesis is essential for skin development. *Proc Natl Acad Sci USA* 106:498–502
- Yi R, Poy MN, Stoffel M *et al.* (2008) A skin microRNA promotes differentiation by repressing "stemness". *Nature* 452:225–9