

C/EBP δ regulates cell cycle and self-renewal of human limbal stem cells

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Human limbal stem cells produce transit amplifying progenitors that migrate centripetally to regenerate the corneal epithelium. Coexpression of CCAAT enhancer binding protein δ (C/EBP δ), Bmi1, and Δ Np63 α identifies mitotically quiescent limbal stem cells, which generate holoclones in culture. Upon corneal injury, a fraction of these cells switches off C/EBP δ and Bmi1, proliferates, and differentiates into mature corneal cells. Forced expression of C/EBP δ inhibits the growth of limbal colonies and increases the cell cycle length of primary limbal cells through the activity of p27^{Kip1} and

p57^{Kip2}. These effects are reversible; do not alter the limbal cell proliferative capacity; and are not due to apoptosis, senescence, or differentiation. C/EBP δ , but not Δ Np63 α , indefinitely promotes holoclone self-renewal and prevents clonal evolution, suggesting that self-renewal and proliferation are distinct, albeit related, processes in limbal stem cells. C/EBP δ is recruited to the chromatin of positively (p27^{Kip1} and p57^{Kip2}) and negatively (p16^{INK4A} and involucrin) regulated gene loci, suggesting a direct role of this transcription factor in determining limbal stem cell identity.

Introduction

Stem cells have the unique capacity to self-renew and generate committed, transit amplifying (TA) progenitors that differentiate into the cell lineages of the tissue of origin (Niemann and Watt, 2002; Fuchs et al., 2004; Cotsarelis, 2006; Blanpain et al., 2007). The most important function of TA cells is to increase the number of differentiated progeny produced by each stem cell division, thus enabling stem cells to divide infrequently, at least under normal tissue homeostasis. The cornea provides an ideal experimental system for studying stem cells of human stratified epithelia (Lavker and Sun, 2003). Human corneal stem cells are segregated in the basal layer of the limbus, which is the vascularized zone encircling the cornea and separating it from the bulbar conjunctiva. The corneal epithelium lies on the avascular Bowman's membrane and is formed by TA keratinocytes that migrate millimeters away from their parental limbal stem cells (Schermer et al., 1986; Cotsarelis et al., 1989; Lehrer et al., 1998; Pellegrini et al., 1999a).

Clonal analysis of squamous human epithelia, including the cornea, has identified three types of clonogenic keratinocytes,

giving rise to holoclones, meroclones, and paraclones in culture (Barrandon and Green, 1987; Pellegrini et al., 1999a). Holoclone-forming cells have all the hallmarks of stem cells, including self-renewing capacity (Rochat et al., 1994; Claudinot et al., 2005), telomerase activity (Dellambra et al., 2000), and an impressive proliferative potential—a single holoclone can generate the entire epidermis of a human being (Rochat et al., 1994). Holoclone-forming cells generate all the epithelial lineages of the tissue of origin (Pellegrini et al., 1999a; Oshima et al., 2001; Blanpain et al., 2004; Claudinot et al., 2005), permanently restore massive epithelial defects (Gallico et al., 1984; Romagnoli et al., 1990; Pellegrini et al., 1997, 1999b; Ronfard et al., 2000), and can be retrieved from human epidermis regenerated from cultured keratinocytes years after grafting (De Luca et al., 2006). We have recently shown that a defined number of genetically corrected stem cells regenerate a normal epidermis in patients with genetic skin adhesion disorders (Mavilio et al., 2006). The paraclone is generated by a TA cell, whereas the meroclone has an intermediate clonal capacity and is a reservoir of TA cells (Barrandon and Green, 1987; Pellegrini et al., 1999a).

The p63 gene produces full-length (TAp63) and N-terminally truncated (Δ Np63) transcripts initiated by different promoters. Each transcript is alternatively spliced to encode three different p63 isoforms, designated α , β , and γ (Yang et al., 1998). The p63 gene products are essential for the morphogenesis and

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Abbreviations used in this paper: 4OHT, 4-hydroxytamoxifen; C/EBP, CCAAT enhancer binding protein; CFE, colony forming efficiency; ChIP, chromatin immunoprecipitation; ER, estrogen receptor; IRES, internal ribosomal entry site; NGFr, NGF receptor; PGK, phosphoglycerokinase; TA, transit amplifying.

The online version of this article contains supplemental material.

the regenerative proliferation of stratified epithelia (Mills et al., 1999; Yang et al., 1999). In particular, $\Delta Np63\alpha$ sustains the proliferative potential of basal epidermal keratinocytes (Parsa et al., 1999; Koster et al., 2004; McKeon, 2004; Nguyen et al., 2006). In the human corneal epithelium, high levels of $\Delta Np63\alpha$ identify limbal stem cells both in vivo and in vitro, whereas $\Delta Np63\beta$ and $\Delta Np63\gamma$ correlate with corneal regeneration and differentiation (Pellegrini et al., 2001; Di Iorio et al., 2005).

In mammary gland epithelial cells, the CCAAT enhancer binding protein δ (C/EBP δ) transcription factor regulates cell cycle by inducing a G₀/G₁ arrest. This effect is specific for epithelial cells and for the G₀/G₁ phase, as C/EBP δ expression does not increase in other types of G₀/G₁-arrested cells or in mammary cells arrested at other stages of the cell cycle (O'Rourke et al., 1999; Hutt et al., 2000). C/EBP δ is a member of a highly conserved family of leucine zipper transcription factors expressed in a variety of tissues and cell types and involved in the control of cellular proliferation and differentiation, metabolism, and inflammation (Ramji and Foka, 2002; Johnson, 2005). At least six members of the family have been isolated and characterized (C/EBP α –C/EBP ζ), with further diversity produced by the generation of different polypeptides by differential use of translational initiation sites, and extensive protein–protein interactions within the family and with other types of transcription factors (Ramji and Foka, 2002; Johnson, 2005).

In this paper, we show that C/EBP δ and $\Delta Np63\alpha$ are coexpressed by human limbal stem cells in vivo and in vitro and that the expression of C/EBP δ is restricted to a subset of mitotically quiescent $\Delta Np63\alpha^+$ /Bmi1⁺ cells. Forced expression of a constitutive C/EBP δ or of a tamoxifen-inducible estrogen

receptor (ER)–C/EBP δ fusion protein in human primary limbal keratinocytes shows that C/EBP δ is instrumental in regulating self-renewal and cell cycle length of limbal stem cells.

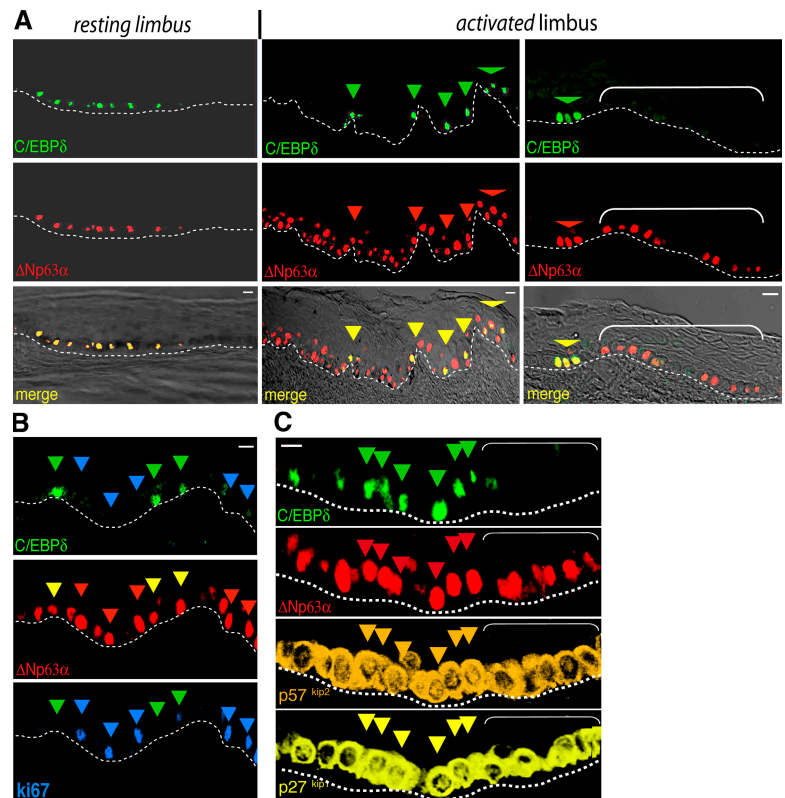
Results

Coexpression of C/EBP δ and $\Delta Np63\alpha$ in quiescent human limbal cells

Experiments were performed on four uninjured and five wounded corneas, referred to as resting and activated cornea, respectively (Di Iorio et al., 2005). We have previously shown that $\Delta Np63\alpha$ is expressed by 10% of resting limbal basal cells endowed with stem cell properties and that activated $\Delta Np63\alpha^+$ limbal cells contain $\Delta Np63\beta$ and $\Delta Np63\gamma$, proliferate, and migrate to the central cornea to restore a wounded epithelium (Di Iorio et al., 2005).

Immunofluorescence analysis on resting limbal sections revealed that C/EBP δ and $\Delta Np63\alpha$ were coexpressed in the same patches of basal cells (Fig. 1 A, left). Both transcription factors were undetectable in suprabasal cell layers (Fig. 1 A) and in the entire corneal epithelium (not depicted). Limbal cell nuclei were stained with DAPI to estimate the proportion of C/EBP δ^+ / $\Delta Np63\alpha^+$ cells in the basal layer. 1 mm of resting limbal epithelium contained a mean of 15 C/EBP δ^+ / $\Delta Np63\alpha^+$ cells, equivalent to \sim 10% of the basal layer. Upon corneal wounding and limbal activation, $\Delta Np63\alpha$ appeared in many basal and some suprabasal limbal cells, whereas C/EBP δ remained confined to \sim 10% of the basal layer (Fig. 1 A, middle). Of note, C/EBP δ^+ limbal cells invariably coexpressed $\Delta Np63\alpha$ (Fig. 1 A). In activated limbus, patches of C/EBP δ^+ / $\Delta Np63\alpha^+$

Figure 1. Expression of $\Delta Np63\alpha$, C/EBP δ , p27^{Kip1}, and p57^{Kip2} in the human limbus. (A) Double immunofluorescence of sections of resting (left) and activated (middle and right) limbus stained with C/EBP δ -specific (green) and p63 α -specific (red) purified IgG. Yellow in the merge frames indicates cells stained with both antibodies. $\Delta Np63\alpha$ and C/EBP δ were coexpressed by a discrete number of basal cells of a resting limbus (left). In the activated limbus, many basal and some suprabasal cells expressed $\Delta Np63\alpha$, whereas C/EBP δ was contained only in \sim 10% of $\Delta Np63\alpha^+$ basal cells (middle). Patches of C/EBP δ^+ / $\Delta Np63\alpha^+$ basal cells (right; arrowheads) were flanked by cells expressing $\Delta Np63\alpha$ but not C/EBP δ (right; brackets). (B) Sections of activated limbus were also stained with mAbs against the proliferation-associated nuclear antigen Ki-67 (blue). Proliferating Ki-67⁺ limbal cells (blue arrows) expressed $\Delta Np63\alpha$ (red arrows) but not C/EBP δ , whereas C/EBP δ^+ cells (green arrows) contained $\Delta Np63\alpha$ (yellow arrows) but not Ki-67. (C) Serial limbal sections stained with C/EBP δ -, p63 α -, p27^{Kip1}-, and p57^{Kip2}-specific IgG (as indicated in frames) showed that C/EBP δ -positive cells (green arrows) contained $\Delta Np63\alpha$ (red arrows) and nuclear p27^{Kip1} (yellow arrows) and p57^{Kip2} (orange arrows), whereas the two Cdk inhibitors were expressed only in the cytoplasm of C/EBP δ^- / $\Delta Np63\alpha^+$ cells (brackets). Bars, 10 μ m.



basal cells flanked by $C/EBP\delta^-/\Delta Np63\alpha^+$ cells were commonly observed (Fig. 1 A, right), whereas neither resting nor activated central corneal epithelium expressed $C/EBP\delta$ (not depicted). $C/EBP\delta^+/\Delta Np63\alpha^+$ resting limbal cells did not express Ki-67, a proliferation-associated nuclear antigen present throughout the cell cycle but absent in G_0/G_1 -arrested cells (not depicted). In activated limbus, proliferating Ki-67⁺ limbal cells expressed $\Delta Np63\alpha$, but not $C/EBP\delta$, whereas $C/EBP\delta^+$ cells contained $\Delta Np63\alpha$ but not Ki-67 (Fig. 1 B). Thus, $C/EBP\delta$ and $\Delta Np63\alpha$ are coexpressed by quiescent limbal basal cells, whereas $\Delta Np63\alpha$, but not $C/EBP\delta$, is expressed in proliferating limbal cells.

The cyclin/Cdk inhibitors $p27^{Kip1}$ and $p57^{Kip2}$ negatively regulate G_1 progression. Nuclear levels of $p27^{Kip1}$ are high in quiescent cells (Sherr and Roberts, 1999). Mitogenic and/or oncogenic signals activate different kinases that phosphorylate $p27^{Kip1}$ on serine and tyrosine residues, promoting its export from the nucleus and cytoplasmic proteolysis, thereby leading to cell proliferation (Rodier et al., 2001; Chu et al., 2007; Grimmier et al., 2007; Kaldis, 2007). Of note, $p57^{Kip2}$, which inhibits cyclin D–Cdk4/6 complexes (Yamazaki et al., 2006), is highly expressed in mouse epidermal stem, but not TA, cells (Dunnwald et al., 2003). Immunofluorescence analysis on limbal sections revealed that $C/EBP\delta$, $p27^{Kip1}$, and $p57^{Kip2}$ were coexpressed in the nucleus of the same patches of basal cells (Fig. 1 C, arrowheads). Such cells also expressed $\Delta Np63\alpha$ (Fig. 1 C, arrowheads). $C/EBP\delta^+$ cells were flanked by $C/EBP\delta^-/\Delta Np63\alpha^+$ cells containing cytoplasmic, but not nuclear, $p27^{Kip1}$ and $p57^{Kip2}$ (Fig. 1 C, brackets). Finally, $p27^{Kip1}$ and $p57^{Kip2}$ were never detected in a fully activated limbus or in the corneal epithelium (not depicted). These data are consistent with the notion that $p27^{Kip1}$ and $p57^{Kip2}$ are localized in the nucleus of quiescent cells, appear in the cytoplasm at the G_1 –S transition, and are not

expressed by actively proliferating cells (Rodier et al., 2001), and confirm that $C/EBP\delta$ is expressed only by quiescent limbal basal cells.

Expression of $C/EBP\delta$, $\Delta Np63\alpha$, and $Bmi1$ in limbal stem cells

Immunofluorescence analysis showed that $\Delta Np63\alpha$ is abundantly and uniformly expressed in holoclones (Fig. 2 A), is expressed in a subset of meroclone cells, and is not expressed in paraclones (Di Iorio et al., 2005). Western analysis showed that clonal evolution, i.e., the transition from holoclones to paraclones, is accompanied by a progressive disappearance of $\Delta Np63\alpha$ and a relative enrichment in $\Delta Np63\beta$ and $\Delta Np63\gamma$ (Fig. 2 B). Strikingly, $C/EBP\delta$ expression was detected exclusively in holoclones (Fig. 2 B) and confined to a subpopulation of $\Delta Np63\alpha^+$ cells (Fig. 2 A). $C/EBP\delta^+/\Delta Np63\alpha^+$ cells were not proliferating, as shown by the mutually exclusive expression of $C/EBP\delta$ and Ki-67 (Fig. 2 C). Of note, although Ki-67 and $C/EBP\delta$ were never expressed in the same cell (Fig. 2 C), large areas of the colony were formed by nonproliferating yet $C/EBP\delta$ -negative cells (Fig. 2 C, dots), suggesting that the expression of $C/EBP\delta$ was not merely related to the proliferative status of the limbal cell.

$C/EBP\alpha$ and $- \beta$ are the most commonly expressed and thoroughly studied isoforms of the C/EBP family (Ramji and Foka, 2002). In particular, $C/EBP\alpha$ and $- \beta$ are known to positively regulate the program of squamous differentiation in the epidermis (Oh and Smart, 1998; Zhu et al., 1999). Accordingly, we found that $C/EBP\alpha$ and $- \beta$ were contained in the suprabasal layers of both human limbal and corneal epithelium (unpublished data). Of note, however, although $C/EBP\beta$ was expressed in all limbal clonal types (Fig. 2 B), we could not detect $C/EBP\alpha$ in cultured limbal colonies.

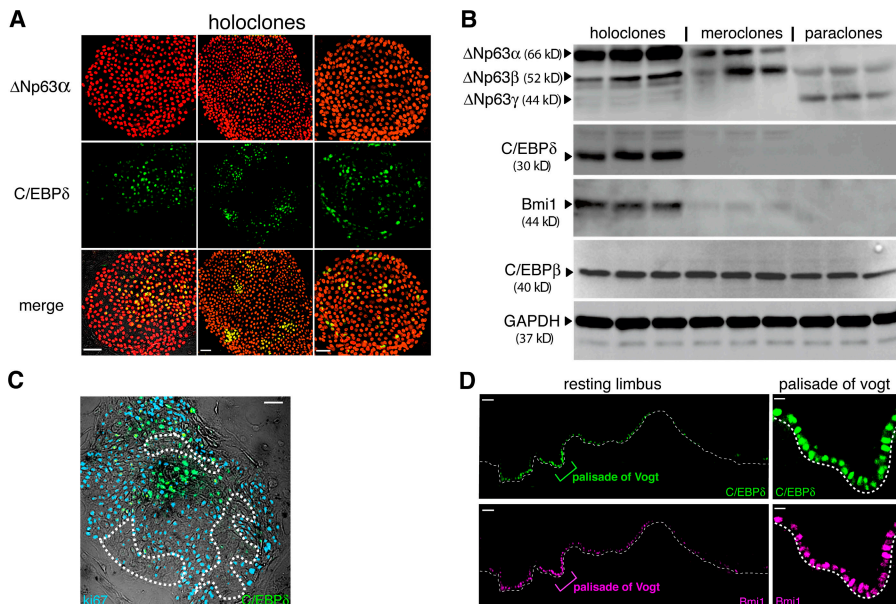


Figure 2. Expression of $\Delta Np63\alpha$, $C/EBP\delta$, and $Bmi1$ in limbal clones and resting limbus.

(A) Holoclone type colonies were isolated as described in Materials and methods. Double immunofluorescence was performed on PFA-fixed holoclones with $p63\alpha$ -specific (red) and $C/EBP\delta$ -specific (green) purified IgG. Yellow in the merge frames indicates cells stained with both antibodies. $C/EBP\delta^+$ was contained in a subpopulation of $\Delta Np63\alpha^+$ cells. Bars, 50 μ m. (B) Clonal analysis of subconfluent primary limbal cultures (Pellegrini et al., 2001). Cell extracts were prepared from cultures generated by holoclones, meroclones, and paraclones, run on SDS-polyacrylamide gels, and immunostained with 4A4 (pan- $p63$) and anti- $Bmi1$ mAbs and with anti- $C/EBP\delta$, anti- $C/EBP\beta$, and anti-GAPDH purified IgG. $C/EBP\delta$ and $Bmi1$ were detected exclusively in holoclones. Clonal evolution was characterized by a progressive disappearance of $\Delta Np63\alpha$ and an enrichment in $\Delta Np63\beta$ and $\Delta Np63\gamma$. $C/EBP\beta$ was uniformly expressed in all clonal types. (C) Selected holoclones were double stained with an anti-Ki-67 mAb (blue) and an anti- $C/EBP\delta$ IgG (green). The expression of $C/EBP\delta$ and Ki-67 was mutually exclusive. The dotted areas outline nonproliferating cells that do not express $C/EBP\delta$. (D) Double immunofluorescence of sections of resting limbus stained with anti- $C/EBP\delta$ IgG (green) and anti- $Bmi1$ mAb (violet). The two transcription factors were coexpressed by a defined number of basal limbal cells. Palisades of Vogt are indicated (left) and shown at higher magnification (right). Bars: (left) 100 μ m; (right) 10 μ m.

and Ki-67 was mutually exclusive. The dotted areas outline nonproliferating cells that do not express $C/EBP\delta$. (D) Double immunofluorescence of sections of resting limbus stained with anti- $C/EBP\delta$ IgG (green) and anti- $Bmi1$ mAb (violet). The two transcription factors were coexpressed by a defined number of basal limbal cells. Palisades of Vogt are indicated (left) and shown at higher magnification (right). Bars: (left) 100 μ m; (right) 10 μ m.

Gene profiling experiments have led to the identification of genes that are commonly expressed in adult stem cells. Among these genes, *Bmi1*, a member of the polycomb group of transcription factors, plays a crucial role in the renewal of hematopoietic and neural stem cells (Lessard and Sauvageau, 2003; Molofsky et al., 2003, 2005; Park et al., 2003) and is expressed in clonogenic, multipotent, and self-renewing murine hair follicle stem cells (Claudinot et al., 2005). Immunofluorescence performed on resting limbal sections revealed that C/EBP δ and *Bmi1* were coexpressed by the same limbal basal cells (Fig. 2 D). In particular, the basal layer of palisades of Vogt, where limbal stem cells are thought to be concentrated, was formed by C/EBP δ^+ /*Bmi1*⁺ cells (Fig. 2 D). Accordingly, Western blot analysis showed that *Bmi1* was expressed in holoclones but not in meroclones and paraclones (Fig. 2 B).

Collectively, these data indicate that C/EBP δ , Δ Np63 α , and *Bmi1* colocalize in limbal stem cells of the resting corneal epithelium in vivo and in limbal holoclone-forming cells in vitro and that expression of C/EBP δ is restricted to a subset of Δ Np63 α^+ cells that are mitotically quiescent both in vivo and in vitro.

C/EBP δ regulates the cell cycle of human limbal stem cells

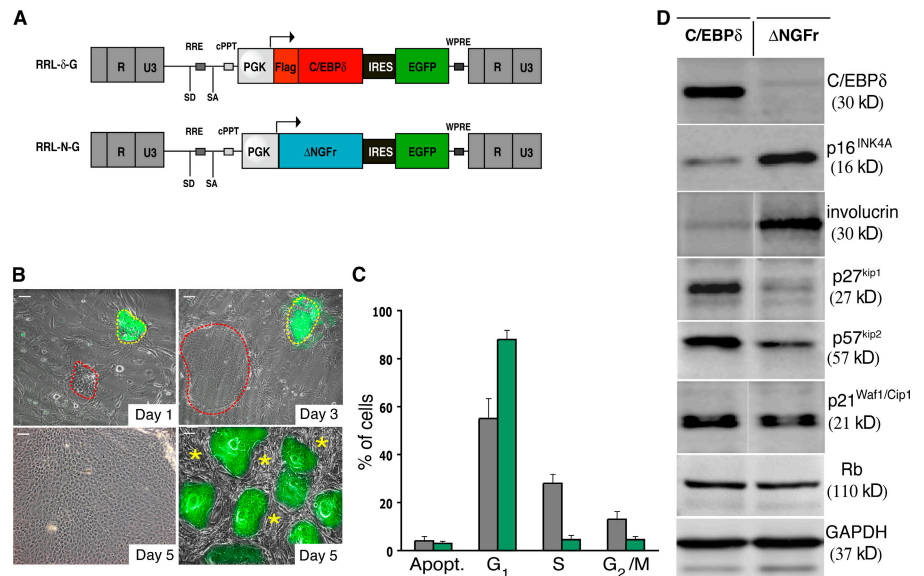
Primary limbal cultures were infected with a lentiviral vector expressing either an epitope-tagged human C/EBP δ or a control protein (a truncated form of the p75 low-affinity NGF receptor [Δ NGFr]) under the control of a constitutive phosphoglycerokinase (PGK) promoter. Both vectors expressed GFP under the control of an internal ribosomal entry site (IRES) element (Fig. 3 A, RRL- δ -G and RRL-N-G). Transduction efficiency on clonogenic cells was \sim 90%, as calculated by GFP expression. After 2 d of cultivation, the size of untransduced colonies increased

nearly threefold (Fig. 3 B, red circles), whereas the size of C/EBP δ^+ /GFP⁺ colonies increased only slightly (Fig. 3 B, yellow circles). Control cells reached confluency 5 d after plating (Fig. 3 B). In contrast, a 5-d culture of C/EBP δ^+ /GFP⁺ cells showed well-defined colonies composed of small, tightly packed cells (Fig. 3 B).

Replicative senescence and differentiation of keratinocytes are associated with increased levels of p16^{INK4A} and involucrin, which indicate irreversible exit from the cell cycle and onset of terminal differentiation, respectively (Dellambra et al., 2000). C/EBP δ -transduced cells contained threefold less p16^{INK4A} and involucrin than Δ NGFr-transduced cells (Fig. 3 D). C/EBP δ -transduced cells contained four- and threefold more p27^{Kip1} and p57^{Kip2} than control cells, respectively (Fig. 3 D). A cell cycle profile revealed that \sim 55, 35, and 10% of the control cells were in the G₁, S, and G₂-M phases, respectively (Fig. 3 C). In sharp contrast, most of the C/EBP δ -transduced cells were in the G₁ phase of the cell cycle (Fig. 3 C). The amount of apoptotic cells was negligible in both C/EBP δ -transduced and control cells (Fig. 3 C). Finally, C/EBP δ -dependent growth inhibition was associated with neither increase of p21^{Waf1/Cip1} or pRb expression (Fig. 3 D) nor activation of the p53 checkpoint pathway (not depicted). These data indicate that the growth inhibitory effect of C/EBP δ was not due to replicative senescence, terminal differentiation, or apoptosis.

To investigate whether the growth inhibitory effect of C/EBP δ was reversible, we transduced primary limbal cells with a lentiviral vector expressing an N-terminal fusion between C/EBP δ and a modified, 4-hydroxytamoxifen (4OHT)-inducible ligand binding domain of the human ER (Littlewood et al., 1995; Fig. 4 A, RRL-ER δ -G). In mock-transduced (RRL-ER-G) cells, C/EBP δ was found predominantly in the nucleus (Fig. 4 B, ER, middle).

Figure 3. Forced expression of C/EBP δ in human limbal keratinocytes. (A) Schematic map of the RRL- δ -G and RRL-N-G lentiviral vectors (proviral form), expressing C/EBP δ or Δ NGFr under the control of a constitutive, human PGK promoter. The vectors carry an inactivating deletion in the U3 region of the LTR. Splice donor (SD) and acceptor (SA) sites, Rev-responsive element (RRE), central poly-purine tract (cPPT), and the woodchuck hepatitis posttranscriptional regulatory element (WPRE) are indicated. In both vectors, EGFP is expressed under the control of an IRES. The Flag epitope fused to C/EBP δ is indicated. (B) C/EBP δ -transduced colonies are indicated by GFP expression (green). (top) The size of an untransduced colony (red circle) increased nearly threefold after 2 d of culture, whereas the size of a C/EBP δ -transduced colony contained in the same dish (yellow circle) did not increase considerably. (bottom) After 5 d of culture, untransduced cells reached confluency (left), whereas C/EBP δ -transduced colonies were well separated and composed of small tightly packed cells (right). Yellow asterisks indicate 3T3 feeder cells, which are not visible in untransduced cultures. Bars, 50 μ m. (C) Cell cycle analysis of Δ NGFr-transduced (control; gray bars) and C/EBP δ -transduced (green bars) limbal cells after 5 d of cultivation. Approximately 55, 35, and 10% of control cells were in the G₁, S, and G₂-M phases of the cell cycle, respectively, whereas most of the C/EBP δ -transduced cells were in the G₁ phase. Apoptotic cells were virtually undetectable in both cases. Error bars indicate SD. (D) Western blot analysis of the expression of C/EBP δ , p16, involucrin, p27^{Kip1}, p57^{Kip2}, p21^{Waf1/Cip1}, and Rb in cell extracts from C/EBP δ - and Δ NGFr-transduced limbal keratinocytes.



In the absence of 4OHT, the ER-C/EBP δ chimeric protein was found in the cytoplasm of transduced limbal cells, and nuclear translocation was observed within 12 h from the addition of 1 μ M 4OHT to the culture medium (Fig. 4, B [top], C, and D). In contrast, C/EBP β was present only in the nucleus, irrespective of the presence of 4OHT (Fig. 4 B [bottom] and Fig. D). Members of the C/EBP family are known to form homo- and heterodimers. In the absence of 4OHT, ER-C/EBP δ sequestered also the endogenous C/EBP δ in the cytoplasm, as indicated by the absence of nuclear immunofluorescent staining (Fig. 4 D, -4OHT), the absence of endogenous C/EBP δ in nuclear extracts, and the presence of C/EBP δ in the corresponding cytoplasmic extracts (Fig. 4 B, middle, -4OHT) of RRL-ER δ -G-transduced cells. Colonies of cells transduced with the control vector showed a progressive and linear increase in their size, irrespective of the presence of 4OHT (Fig. 4, E and F). In sharp contrast, the growth of ER-C/EBP δ ⁺/GFP⁺ colonies was strictly dependent on the

localization of the ER-C/EBP δ chimera (Fig. 4, E and F): (1) addition of 4OHT at day 1 considerably slowed the growth of transduced colonies; (2) removal of 4OHT at day 4 was promptly followed by a linear increase of the size of GFP⁺ colonies; and (3) readdition of 4OHT at day 6 again induced a growth arrest. Of note, untransduced, Δ NGFr- and ER-transduced primary limbal cells duplicated every 17–19 h, whereas C/EBP δ -transduced cells showed a doubling time of 41 h (Fig. 5 E, green). These data show that C/EBP δ lengthened the limbal cell cycle by forcing cells into the G₁ phase without altering their capacity for multiplication.

C/EBP δ -dependent mitotic quiescence is mediated by p27^{Kip1} and p57^{Kip2}

Semiquantitative RT-PCR was performed on control and C/EBP δ -transduced cells using p27^{Kip1}- and p57^{Kip2}-specific primers. As shown in Fig. 5 (A and B), we observed a 5–10-fold

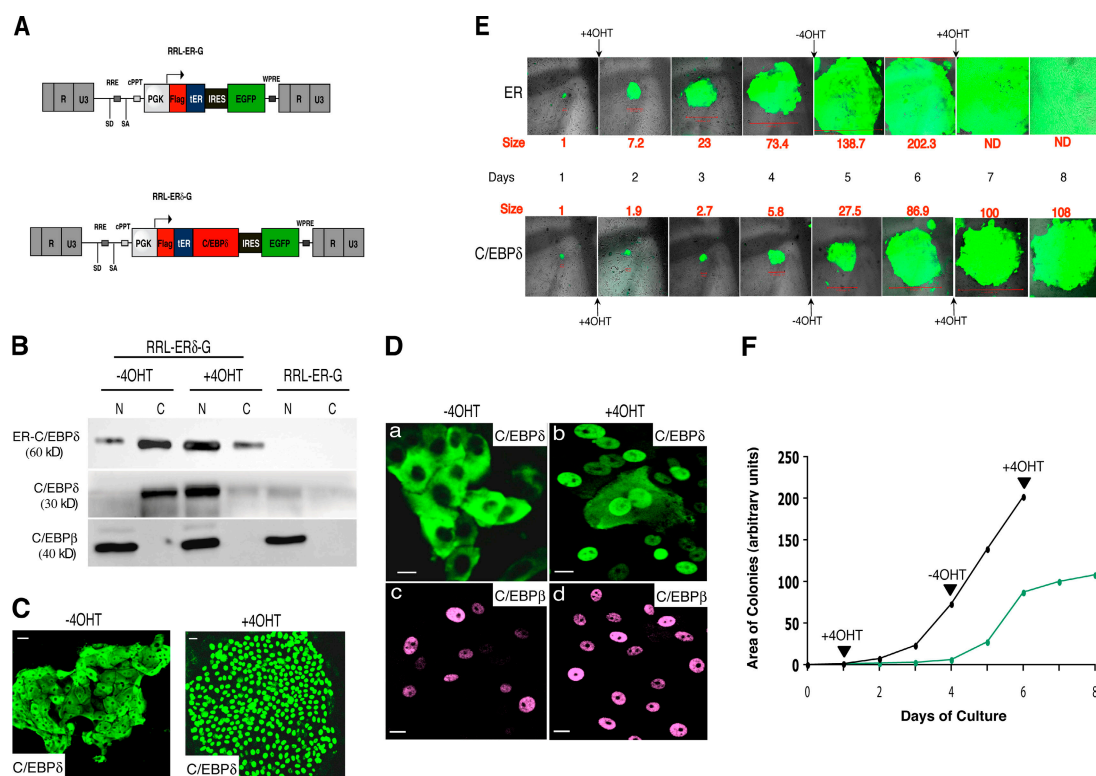


Figure 4. Expression of 4OHT-inducible C/EBP δ in limbal keratinocytes. (A) Schematic map of the RRL-ER δ -G and RRL-ER-G lentiviral vectors (proviral form), expressing an N-terminal fusion of C/EBP δ to a modified ER ligand binding domain, or the ER domain only, under the control of a PGK promoter. In both vectors, EGFP is expressed under the control of an IRES. SD, splice donor site; SA, splice acceptor site; RRE, Rev-responsive element; cPPT, central poly-purine tract; WPRE, woodchuck hepatitis posttranscriptional regulatory element. (B) Nuclear (N) and cytoplasmic (C) extracts were prepared from RRL-ER-G-transduced (ER) and RRL-ER δ -G-transduced cells, either treated (+4OHT) or untreated (-4OHT) with 1 μ M 4OHT, run on SDS-polyacrylamide gels, and immunostained with anti-C/EBP δ and anti-C/EBP β purified IgG. In mock-transduced cells, C/EBP δ was found exclusively in the nucleus (ER; middle). In the absence of 4OHT, the ER-C/EBP δ chimeric protein (top) was found predominantly in the cytoplasm (-4OHT). Nuclear translocation was observed within 12 h from the addition of 1 μ M 4OHT to the culture medium (+4OHT; top). Strikingly, in the absence of 4OHT, ER-C/EBP δ sequestered also the endogenous C/EBP δ in the cytoplasm, as indicated by the absence of any C/EBP δ species in nuclear extracts of -4OHT cells and the presence of C/EBP δ in the corresponding cytoplasmic extracts (middle). The exposure times of filters in top (ER-C/EBP δ) and middle (C/EBP δ) panels were 10 and 75 s, respectively. C/EBP β was present only in nuclear extracts, irrespective of the presence of 4OHT (bottom). (C and D) Cytoplasmic-nuclear translocation of the ER-C/EBP δ fusion protein in response to 4OHT treatment of RRL-ER δ -G-transduced limbal keratinocytes, stained with an anti-C/EBP δ antibody (green). Staining of C/EBP β is shown for comparison (pink). Note the absence of C/EBP δ in nuclei of untreated cells. Bars, 20 μ m. (E and F) Reversible growth inhibitory effect of C/EBP δ . In the presence of 4OHT (1–4 d of culture), the size of ER-C/EBP δ -transduced colonies did not increase considerably; removal of 4OHT at day 4 was followed by a linear increase of the size of GFP⁺ colonies, whereas readdition of 4OHT at day 6 again induced a growth arrest [E [bottom] and F [green circles]]. In contrast, RRL-ER-G-transduced colonies showed a progressive and linear increase of their size, irrespective of the presence of 4OHT [E [top] and F [black circles]]. Values of the size of colonies are in arbitrary units.

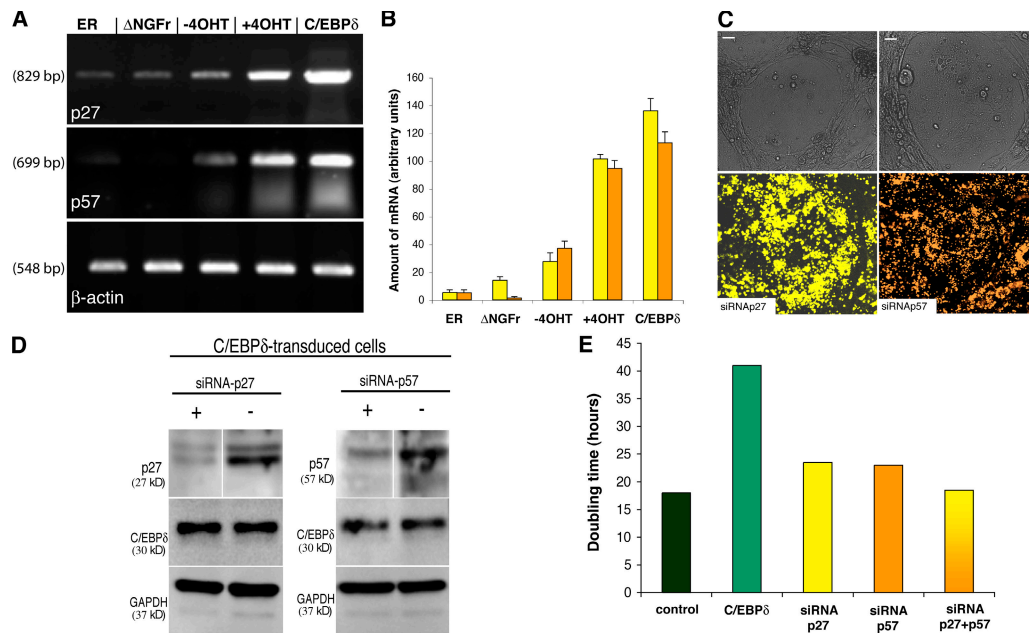


Figure 5. Role of p27^{Kip1} and p57^{Kip2} in C/EBPδ-induced mitotic quiescence. (A and B) Semiquantitative RT-PCR analysis was performed on limbal cells transduced with either control ER (ER), constitutive ΔNGFr (ΔNGFr), constitutive C/EBPδ (C/EBPδ), or inducible C/EBPδ in the absence (–4OHT) or in the presence (+4OHT) of tamoxifen, using p27^{Kip1}- and p57^{Kip2}-specific primers. A 5–10-fold increase of both p27^{Kip1} (yellow bars) and p57^{Kip2} (orange bars) transcripts was observed only in the presence of exogenous nuclear C/EBPδ. Error bars indicate SD. (C) C/EBPδ-transduced cells were then transfected with siRNA-p27^{Kip1} (left) and siRNA-p57^{Kip2} (right), with an efficiency of 84 ± 2 and 77 ± 3%, respectively. Bars, 20 μm. (D) Cell extracts were prepared from C/EBPδ-transduced cells transfected with siRNA-p27^{Kip1} (left) or siRNA-p57^{Kip2} (right), run on SDS-polyacrylamide gels, and immunostained with the indicated purified IgG. Note that siRNA-p27^{Kip1} and siRNA-p57^{Kip2} determined a strong decrease of the expression of p27^{Kip1} and p57^{Kip2}, respectively, but not of C/EBPδ and GAPDH. (E) Cell doubling time was calculated. Untransduced (black) and C/EBPδ-transduced cells, untransfected (green) or transfected with siRNA-p27^{Kip1} (yellow), siRNA-p57^{Kip2} (orange), or the combination of the two siRNA molecules (yellow + orange) showed a doubling time of 18, 41, 23.5, 23, and 18.5 h, respectively.

increase of both p27^{Kip1} and p57^{Kip2} transcripts in RRL-δ-G (C/EBPδ)-transduced limbal cells and RRL-ERδ-G-transduced cells treated with 4OHT (+4OHT), as compared with RRL-N-G (ΔNGFr)-, RRL-ER-G (ER)-, and RRL-ERδ-G-transduced cells not treated with 4OHT (–4OHT). To prove the role of p27^{Kip1} and p57^{Kip2} in mediating the effect of C/EBPδ on keratinocyte cell cycle, C/EBPδ-transduced limbal cells were transfected with siRNAs specifically targeted to the p27^{Kip1} and p57^{Kip2} mRNAs. Transfection efficiency was 84 ± 2 and 77 ± 3%, respectively (Fig. 5 C). Western blot analysis showed that siRNA-p27^{Kip1} and siRNA-p57^{Kip2} caused a strong decrease of the expression of p27^{Kip1} and p57^{Kip2}, but not of C/EBPδ and a control protein (Fig. 5 D).

Untransduced and C/EBPδ-transduced cells showed a doubling time of 18 and 41 h, respectively (Fig. 5 E). C/EBPδ-transduced cells transfected with either siRNA-p27^{Kip1} or siRNA-p57^{Kip2} showed a doubling time of 23.5 and 23 h, respectively. Of note, C/EBPδ-transduced cells transfected with both siRNAs simultaneously, showed a doubling time of 18.5 h, a value undistinguishable from that of untransduced control cells (Fig. 5 E). These data show that p27^{Kip1} and p57^{Kip2} mediate C/EBPδ-induced mitotic quiescence.

C/EBPδ binds to the p63, involucrin, p27^{Kip1}, p57^{Kip2}, and p16^{INK4A} loci in vivo

To provide evidence for a direct contribution of C/EBPδ in regulating the expression of ΔNp63α, involucrin, p27^{Kip1}, p57^{Kip2},

and p16^{INK4A}, we analyzed recruitment of C/EBPδ to these loci by a chromatin immunoprecipitation (ChIP) assay on cultured limbal keratinocytes three and five passages after transduction with either RRL-δ-G or the control, RRL-N-G vector. Protein–DNA complexes were immunoprecipitated with antibodies specific for C/EBPδ or the Flag epitope and with control IgGs. Immunoprecipitated chromatin DNA was analyzed by PCR with primers specific for different regions of the p63, involucrin, p27^{Kip1}, p57^{Kip2}, and p16^{INK4A} loci (Fig. 6 A, red arrowheads), containing evolutionarily conserved and/or putative C/EBP binding elements.

In C/EBPδ-transduced cells, vector-derived (Flag-tagged) C/EBPδ was found associated to the p63 locus in intron 3 (at position +147873 to +148041) and in an evolutionarily conserved, keratinocyte-specific enhancer in intron 5 (+202579 to +202761; Antonini et al., 2006). Primers designed to amplify other sequences from the p63 locus detected the correct fragment only in the input samples (Fig. 6, A and B). Binding of Flag-tagged C/EBPδ was also observed to a region upstream of the involucrin promoter (–421 to –119) containing a C/EBP responsive element previously characterized in keratinocytes (Agarwal et al., 1999; Balasubramanian and Eckert, 2004) and upstream of the p27^{Kip1} (–227 to +14), p57^{Kip2} (–622 to –398), and p16^{INK4A} (–1020 to –871) loci (Fig. 6 B). Binding to all these sites was observed specifically in C/EBPδ-transduced cells and was more pronounced at the fifth than at the third passage (Fig. 6 B). The signals obtained with the anti-Flag

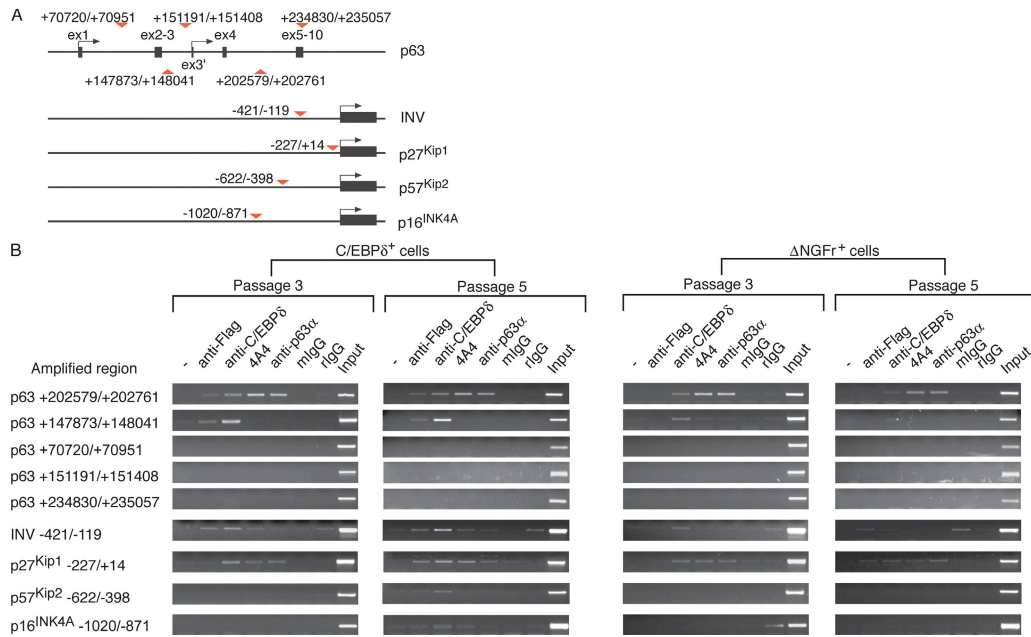


Figure 6. Recruitment of C/EBPδ and p63 on selected gene targets in primary limbal keratinocytes in vivo. (A) Schematic representation of the genomic target loci. Positions of amplified regions (red arrowheads) are indicated with respect to transcription start sites (arrows). INV, involucrin. (B) Chromatin from C/EBPδ- or ΔNGFr-transduced (control) limbal keratinocytes was cross-linked at the third and fifth passage in culture and immunoprecipitated without antibody (-) or with anti-C/EBPδ, anti-Flag, anti-p63 (4A4), and anti-p63α antibodies, and control IgGs, and analyzed by PCR using primers specific for the genomic regions indicated in A. Amplified fragments were run on an agarose gel and stained with ethidium bromide. The last lane on each gel corresponds to the input sample.

antibody were always weaker than those obtained with the anti-C/EBPδ antibody, probably reflecting a lower immunoprecipitation efficiency. In control, ΔNGFr-transduced cells, a weak but specific signal was observed at the p63, involucrin, and p27^{Kip1} loci in chromatin immunoprecipitated with the anti-C/EBPδ but not the anti-Flag antibody. Binding was observed at the third but not at the fifth passage (Fig. 6 B), most likely as a result of the presence of endogenous C/EBPδ activity in a subset of early passage cells, which is lost in later passages.

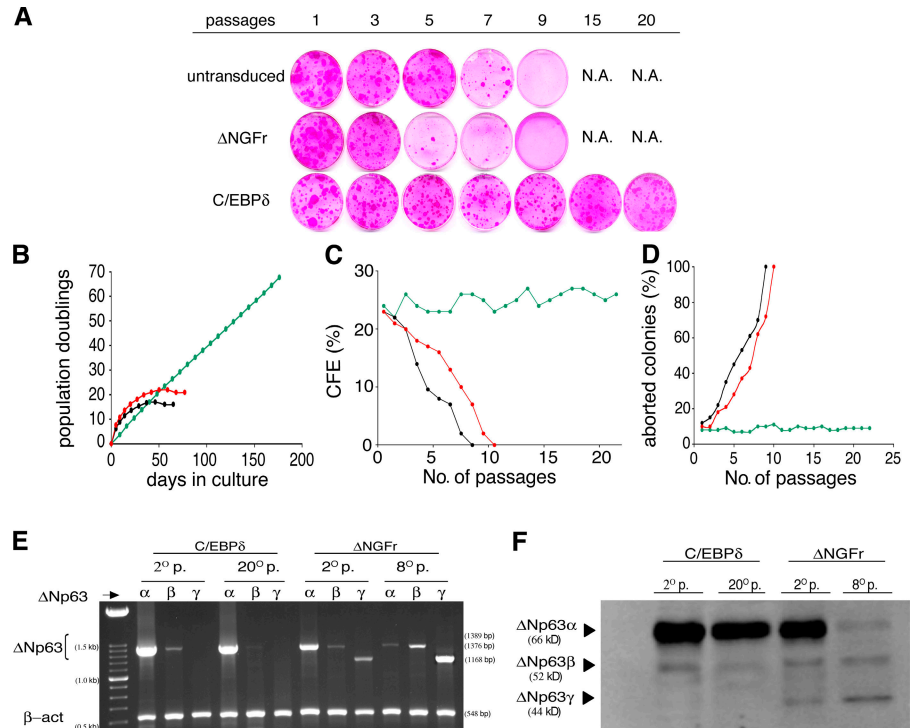
Chromatin from the same cells was also immunoprecipitated with antibodies specific for all isoforms or only the α isoforms of p63. Binding of ΔNp63α was observed in the intron 5 enhancer of the p63 locus (Antonini et al., 2006) in both C/EBPδ-transduced and control cells. Binding was more pronounced in C/EBPδ⁺ than in control cells, reflecting either an increased recruitment of ΔNp63α to the enhancer or simply the increased proportion of cells expressing ΔNp63α in these cultures. Interestingly, ΔNp63α and C/EBPδ appear to bind the same regions in the p63 and p27^{Kip1} loci (Fig. 6 B). These results suggest that the p63, involucrin, p27^{Kip1}, p57^{Kip2}, and p16^{INK4A} loci might be direct targets of C/EBPδ activity, in some cases in combination with ΔNp63α.

C/EBPδ promotes self-renewal of holoclone-forming cells

Clonogenic ability and proliferative potential are distinct properties of epithelial cells. Keratinocyte stem cells are endowed with high clonogenic and high proliferative capacity, and self-renewal occurs when both properties are maintained. Conversely, TA cells are clonogenic but have a limited capacity for multiplication.

Serially cultivated, untransduced, or ΔNGFr-transduced limbal cells showed a progressive decrease of their clonogenic capacity (Fig. 7, A and C) and ceased to proliferate after 60–75 d (or 9–11 passages) in culture (Fig. 7 B). Replicative senescence occurs because of clonal evolution, as indicated by the progressive increase of aborted, paraclone-type colonies (Fig. 7 D) and by the replacement of ΔNp63α with ΔNp63β and ΔNp63γ expression (Fig. 2 B and Fig. 7, E and F). In sharp contrast, both clonogenic ability (Fig. 7, A and C) and proliferative capacity (Fig. 7 B) of C/EBPδ-transduced cells were maintained indefinitely. This effect was due to the capacity of enforced C/EBPδ expression to promote self-renewal and halt clonal evolution in holoclones, as indicated by the following evidence: (1) serially cultivated C/EBPδ-transduced cells showed no increase in the number of paraclones (Fig. 7 D) or replacement of ΔNp63α with ΔNp63β and ΔNp63γ (Fig. 7, E and F); (2) statistical analysis of cell size (Di Iorio et al., 2006), a major marker of clonogenic stem cells (Barrandon and Green, 1985), showed that C/EBPδ-transduced cells were nearly 10-fold smaller than control cells (325.93 vs. 3,035.25 μm³); (3) clonal analysis revealed that the percentage of holoclone-forming cells decreased and eventually set to zero in serially cultivated control cells but remained constant in C/EBPδ-transduced cells (10–15% of inoculated cells); and (4) ER-C/EBPδ was able to fully sequester also endogenous C/EBPδ in the cytoplasm of limbal cells in the absence of 4OHT (Fig. 4 B). Such cells ceased to express ΔNp63α (not depicted) and underwent replicative senescence in only two passages as compared with 9–11 passages of control untransduced cells (Fig. 7 B).

Figure 7. C/EBP δ halts clonal evolution and promotes self-renewal of human limbal keratinocytes. (A) Clonogenic capacity of untransduced and Δ NGFr- and C/EBP δ -transduced limbal cells was evaluated at the cell passages indicated by numbers. NA (not available) indicates cultures that reached replicative senescence. (B–D) Number of cumulative population doublings (B), CFE values (number of colonies/inoculated cells ratio; C), and aborted colonies values (aborted colonies/total colonies ratio; D) are indicated. Untransduced and Δ NGFr- and C/EBP δ -transduced cells are indicated by red, black, and green circles, respectively. Note that (1) control cells underwent senescence, whereas C/EBP δ -transduced cells proliferated indefinitely, and (2) control cells showed a progressive decrease of their CFE and a progressive increase of the percentage of aborted colonies, whereas both the number of clonogenic cells and aborted colonies remained constant during serial cultivation of C/EBP δ -transduced cells. (E) Semiquantitative RT-PCR analysis was performed using primers specific for each of the p63 Δ N isoforms (Di Iorio et al., 2005) on C/EBP δ - and Δ NGFr-transduced cells at different passages (indicated by numbers). β -Actin (β act) has been used as a control. (F) Western blot analysis of cell extracts prepared from the same cultures used for RT-PCR. Upon serial cultivation, control cells showed a progressive disappearance of Δ Np63 α and enrichment of Δ Np63 β and Δ Np63 γ , whereas C/EBP δ -transduced cultures expressed high levels of Δ Np63 α and low amounts of Δ Np63 β and Δ Np63 γ .



To investigate whether C/EBP δ was able to rescue TA cells from their terminal fate, we transduced different single cell-derived clones. As expected, holoclone, meroclone, and paraclone type clones displayed a progressive decrease in clonogenicity and Δ Np63 α content (Fig. 8). Forced expression of C/EBP δ was able to sustain the self-renewal of holoclones and meroclones and, hence, of clones still containing Δ Np63 α ⁺ cells, but not that of Δ Np63 α ⁻ paraclones (Fig. 8). All cells in transduced holoclones and meroclones expressed Δ Np63 α (not depicted), further suggesting that C/EBP δ is able to foster self-renewal only of Δ Np63 α ⁺ cells and to maintain expression of Δ Np63 α in such cells.

These data prompted us to investigate whether forced expression of Δ Np63 α was sufficient to sustain limbal cell self-renewal. Primary limbal cultures and single cell-derived clones were infected with a lentiviral vector expressing the Δ Np63 α isoform (Fig. 8, RRL- Δ N α -G). Δ Np63 α -transduced holoclones underwent regular clonal evolution and ceased to proliferate after 11 passages, a value identical to control untransduced cells (Figs. 7 and 8). Δ Np63 α was therefore unable to sustain limbal stem cell self-renewal both in primary cultures (unpublished data) and in clones. Finally, simultaneous infection with lentiviral vectors expressing C/EBP δ and Δ Np63 α was unable to rescue clonogenic ability and self-renewal in paraclones, suggesting that loss of self-renewal is an irreversible process, at least in limbal keratinocytes.

Discussion

Exceptional progress has been made in understanding the molecular mechanisms regulating keratinocyte stem cells. The role

of transcription factors, such as p63, tcf3, CCAAT displacement protein, and GATA-3, and of adhesion and signaling molecules, such as integrins, Wnt/ β -catenin, c-Myc, Notch, hedgehog, Sgk3, and bone morphogenic proteins, in controlling hair follicle and epidermal development and stem cell fate has been highlighted (Niemann and Watt, 2002; Fuchs et al., 2004; Cotsarelis, 2006; Blanpain et al., 2007). Molecular phenotyping of some of the keratinocyte stem cell niches helped explain how stem cells interact with the microenvironment to maintain their properties (Morris et al., 2004; Tumber et al., 2004). Little is known, however, on the regulation of perhaps the most important property of epithelial stem cells, that is, their capacity to self-renew. It has been shown that the Rho guanosine triphosphatase Rac1 sustains murine epidermal stem cell renewal and human epidermal stem cell clonogenicity by negatively regulating MYC (Benitah et al., 2005). However, differences exist between different lining epithelia and among animal species. For instance, Rac1 stimulates differentiation and not self-renewal in the intestinal epithelium (Stappenbeck and Gordon, 2000), whereas the CD34 antigen identifies murine but not human hair follicle stem cells (Cotsarelis, 2006).

We took advantage of the availability of human corneas to carry out genetic manipulation experiments on primary, clonogenic limbal stem cells and show that C/EBP δ plays a key role in regulating their cell cycle and self-renewal properties. Our findings are graphically summarized in Fig. 9. According to this model, a defined number of mitotically quiescent limbal stem cells coexpress Bmi1, Δ Np63 α , and C/EBP δ under normal homeostasis. Coexpression of Bmi1, Δ Np63 α , and C/EBP δ therefore

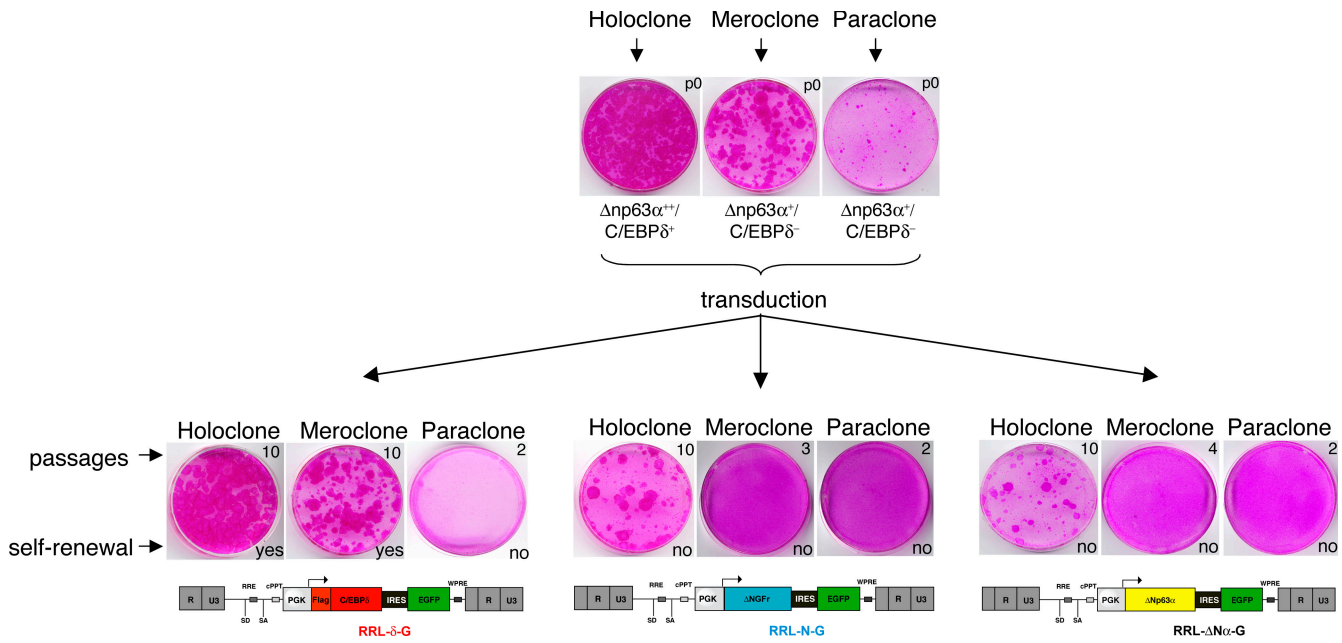


Figure 8. **C/EBP δ promotes self-renewal of holoclones but not paraclones.** Immunofluorescence analysis was performed on clone-derived cytopins using anti- Δ Np63 α and anti-C/EBP δ purified IgG. Representative clonal types are shown, and their Δ Np63 α and C/EBP δ content is expressed in arbitrary values. Immediately after their isolation (p0), daughter cells from each clone were transduced with lentiviral vectors expressing C/EBP δ (RRL- δ -G), Δ NGFr (RRL-N-G), or Δ Np63 α (RRL- Δ N α -G). CFEs performed at selected passages (p) are shown. Enforced C/EBP δ expression sustained self-renewal of holoclones and meroclones indefinitely (CFEs at p10 are shown), but not that of paraclones, which ceased proliferation after p2. Enforced Δ Np63 α and Δ NGFr expression were unable to foster self-renewal, irrespective of the clonal type. SD, splice donor site; SA, splice acceptor site; RRE, Rev-responsive element; cPPT, central poly-purine tract; WPRE, woodchuck hepatitis posttranscriptional regulatory element.

identifies limbal holoclones and is part of the genetic program maintaining stem cell identity. Bmi1 fosters self-renewal of haematopoietic and neural stem cells through regulation of the p16^{INK4A} and p19^{ARF} pathways (Lessard and Sauvageau, 2003; Molofsky et al., 2003, 2005; Park et al., 2003; Walkley et al., 2005) and might play a similar role also in limbal stem cells. Δ Np63 α sustains the proliferative potential of stem cells in several stratified epithelia, including the cornea (Parsa et al., 1999; Pellegrini et al., 2001; Koster et al., 2004; McKeon, 2004; Di Iorio et al., 2005; Nguyen et al., 2006). We show here that C/EBP δ regulates mitotic quiescence of limbal keratinocytes by forcing cells in the G₀/G₁ phase of the cell cycle. Even under culture conditions specifically designed to promote keratinocyte proliferation, forced C/EBP δ expression greatly increases the cell cycle length through activation of the cell cycle inhibitors p27^{Kip1} and p57^{Kip2}. The growth inhibitory effect of C/EBP δ is not due to replicative senescence or terminal differentiation, as confirmed by the down-regulation of p16^{INK4A} and involucrin. Perhaps more important, C/EBP δ promotes the self-renewal of Δ Np63 α ⁺ limbal stem cells, as suggested by the block of clonal evolution and the indefinite maintenance of the number of holoclones during serial cultivation of C/EBP δ -transduced limbal keratinocytes.

Stem cells are capable of shifting from a homeostatic state of relative quiescence to rapid proliferation under specific conditions (activation). In the ocular surface, this shift occurs upon central corneal wounding (Lehrer et al., 1998; Di Iorio et al., 2005). This explains the apparently opposing actions of C/EBP δ and Δ Np63 α . On one hand, C/EBP δ induces mitotic quiescence

(through a positive regulation of p27^{Kip1} and p57^{Kip2}) and self-renewal of limbal stem cells; on the other, it preserves their proliferative potential (essential for stem cell-dependent tissue regeneration) through a positive regulation of Δ Np63 α . In this way, when some limbal stem cells are released from

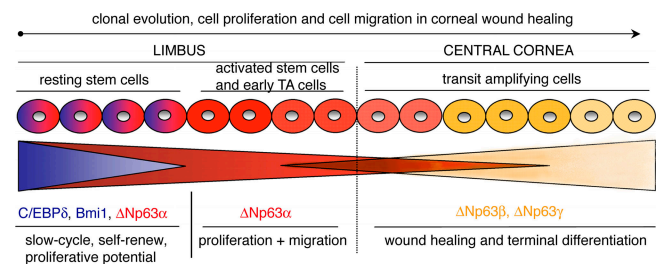


Figure 9. **Schematic description of a model for human corneal regeneration.** Under normal homeostasis, quiescent stem cells localized in the basal layer of the limbus (Fig. 1 and Fig. 2 D) coexpress C/EBP δ and Bmi1 (blue), which are responsible for mitotic quiescence and self-renewal properties, and Δ Np63 α (red), which sustains the stem cell proliferative potential (Parsa et al., 1999; Yang et al., 1999; Pellegrini et al., 2001; Koster et al., 2004; McKeon, 2004; Di Iorio et al., 2005; Nguyen et al., 2006). These cells give rise to holoclones in culture (Fig. 2, A and C). Under stress conditions, such as those induced by a corneal damage, a fraction of such stem cells switches off C/EBP δ (and Bmi1) but maintains Δ Np63 α (red). Activated Δ Np63 α ⁺ stem cells actively proliferate and migrate to the central cornea to restore and regenerate the corneal epithelium (Fig. 2 A; Di Iorio et al., 2005). Activated stem cells, however, lose their self-renewal properties, enter into the TA compartment, and progressively lose Δ Np63 α expression. TA cells switch on Δ Np63 β and Δ Np63 γ , which might regulate terminal differentiation and stratification during the regeneration of the damaged corneal epithelium (Di Iorio et al., 2005).

C/EBP δ -dependent mitotic constraints, as in a corneal damage, they can unchain their remarkable p63-dependent proliferative capacity, multiply, and migrate to repair a corneal wound. This process is, however, irreversible and leads to limbal stem cell terminal differentiation (Fig. 9). Our data therefore strengthen the notion that proliferation and self-renewal capabilities are two related, albeit distinct, processes. At least in human limbal stem cells, proliferation potential relies on the expression of Δ Np63 α , whereas self-renewal requires also C/EBP δ . Similarly, Bmi1 is essential for the self-renewal of neural stem cells but does not influence the proliferative capacity of their committed progeny (Molofsky et al., 2003). The notion that Δ Np63 α induces the expression of growth factor receptors and adhesion molecules regulating survival and motility of epithelial cells (Carroll et al., 2006) is consistent with our proposed model.

Our data establish an interesting parallel with the hematopoietic system, where quiescence and self-renewal of stem cells have been recently shown to be linked and regulated by p27^{Kip1}, p57^{Kip2}, and Mad1 (Scandura et al., 2004; Walkley et al., 2005; Yamazaki et al., 2006). Indeed, loss of p27^{Kip1} allows relatively quiescent hematopoietic stem cells to rapidly enter the cell cycle to restore haematopoiesis (Walkley et al., 2005). Finally, we show that C/EBP δ is directly associated *in vivo*, alone or in combination with Δ Np63 α , to chromatin-surrounding promoters or regulatory elements of the p63, p27^{Kip1}, p57^{Kip2}, and p16^{INK4A} loci, suggesting a direct role of this transcription factor in determining the genetic program of self-renewing stem cells.

The role of C/EBP δ described here is intriguing. Indeed, C/EBPs have been mainly related to cellular differentiation. C/EBP α , β , and δ are instrumental in regulating adipogenesis, whereas C/EBP α , ϵ , and β orchestrate myeloid differentiation into mature neutrophils, atypical neutrophils, and macrophages (Rosen et al., 2000; Ramji and Foka, 2002), and C/EBP δ regulates learning and long-term memory in the central nervous system (Sterneck et al., 1998; Taubenfeld et al., 2001). The importance of the C/EBP family in cellular differentiation also extends to other cell types, including hepatocytes, ovarian luteal cells, intestinal epithelial cells, and epidermal keratinocytes. For instance, it has been shown that C/EBP α and β induces cell cycle exit in normal keratinocytes and positively regulates the program of squamous differentiation in the epidermis (Oh and Smart, 1998; Zhu et al., 1999). However, C/EBP β promotes keratinocyte proliferation and skin tumor formation in the presence of oncogenic Ras or in response to carcinogens (Zhu et al., 2002; Sterneck et al., 2006) and fosters hepatocyte proliferation during liver regeneration after partial hepatectomy (Greenbaum et al., 1998). Mammary epithelial cells from C/EBP β -deficient mice have a proliferation defect that leads to impaired ductal morphogenesis and a failure to lactate (Robinson et al., 1998; Seagroves et al., 1998), and ectopic C/EBP β expression in human mammary epithelial cells induces hyperproliferation and a partially transformed phenotype (Bundy and Sealy, 2003). Finally, C/EBP δ induces late differentiation events in epidermal keratinocytes (Smith et al., 2004) and is indeed detected in the subbasal layers of the human epidermis (unpublished data). Therefore, the biological effects of C/EBPs appear to be highly

species and cell context specific, suggesting that role that C/EBP δ exerts in the human corneal epithelium might not necessarily be observed in other squamous epithelia.

The mechanisms controlling C/EBP δ expression and function in the limbus, as well as the downstream mediators of C/EBP δ activity in controlling stem cell quiescence and self-renewal, remain to be determined. The expression of the C/EBPs has been found to change markedly during several physiological and pathophysiological conditions through the action of extracellular signals. C/EBPs are subject to extensive species- and tissue-specific posttranscriptional regulation and phosphorylation-mediated changes in DNA binding activity and nuclear localization (Ramji and Foka, 2002). Furthermore, the different C/EBP proteins are able to form heterodimers in all intrafamilial combinations and to associate with other factors (Ramji and Foka, 2002). A combination of biochemical, cellular, and genetic experiments is necessary to acquire a more comprehensive description of upstream regulators and downstream targets of C/EBP δ and to elucidate the networks of protein interactions and regulatory pathways that control its activity in human limbal stem cells.

Materials and methods

Human specimens, cell culture, and cell cycle analysis

Corneas taken from organ donors and considered unsuitable for transplantation (solely because of hepatitis seropositivity of the donor) were examined with a slit lamp immediately before retrieval and classified as resting corneas, which did not show epithelial defect, dehydration, edema, or inflammation, or activated corneas, which had central corneal epithelial defects and/or abrasions, usually as a result of incomplete closure of the eyelids after death. Resting and activated corneas were taken 3.93 ± 0.69 and 6.79 ± 2.9 h from death, respectively. Corneas were provided by D. Ponzin and A. Ruzza (The Veneto Eye Bank Foundation, Venice, Italy).

Swiss mouse 3T3-J2 cells (a gift from H. Green, Harvard Medical School, Boston, MA) were grown in DME supplemented with 10% calf serum. Keratinocytes were cultivated on a feeder layer of lethally irradiated 3T3-J2 cells, and colony forming efficiency (CFE) assays and calculation of the number of cell generations and population doublings were performed as described previously (Pellegrini et al., 1999a; Dellambra et al., 2000). Clonal analysis was performed from subconfluent primary cultures as described previously (Pellegrini et al., 1999a, 2001). In brief, single cells were inoculated onto multiwell plates containing a feeder layer of 3T3 cells. Clones were identified after 7 d of culture under an inverted microscope and transferred to replicate dishes. One dish (1/4 of the clone) was fixed 9–12 d later and stained with rhodamine B for clonal type classification (Barrandon and Green, 1987; Pellegrini et al., 1999a). The second dish was used for further experiments and analyses. In selected experiments, 100 limbal cells were plated in 100-cm dishes and cultured for 1 wk. Colonies were then examined under a microscope (Axiovert 200 M; Carl Zeiss MicroImaging, Inc.): large round colonies with smooth and regular borders and formed entirely by small cells with scarce cytoplasm were classified as holoclones (Di Iorio et al., 2005) and were subjected to immunofluorescence.

For cell cycle analysis, subconfluent keratinocyte cultures were trypsinized and fixed in 70% ethanol at 4°C. Samples (10^6 cells) were rehydrated in PBS/1% FCS at room temperature for 10 min and stained with 20 μ g/ml propidium iodine for 30 min at 4°C. Flow cytometry was performed using a LSR II FACSscan (Becton Dickinson).

Immunofluorescence and Western analysis

The following antibodies were used: rabbit anti-C/EBP δ , anti-Ras^{GAP}, anti-Rb, and p57^{Kip2} purified IgG (Santa Cruz Biotechnology, Inc.); 4A4 pan-p63 mAb (BD Biosciences); p16^{INK4A}, p21^{Waf1/Cip1}, and p27^{Kip1} mAbs (Exalpha Biologicals, Inc.); involucrin and Ki67 mAbs (Novocastra); Bmi1 mAb (Upstate Biotechnology); rabbit anti-p63 α unconjugated and FITC-conjugated purified IgG raised against a synthetic peptide (NH₂-DFNFDMDARRNKQQRKEEGE-COOH) comprising the C terminus post-SAM domain

of p63 α (Primm; Di Iorio et al., 2005). Secondary rhodamine- or FITC-labeled antibodies were obtained from Santa Cruz Biotechnology, Inc. For immunofluorescence analysis, keratinocyte colonies were fixed (3% paraformaldehyde/2% sucrose in PBS, pH 7.6), permeabilized (0.5% Triton X-100 in PBS), and coated with 0.5% BSA/PBS for 1 h at RT. Paraformaldehyde-fixed corneal samples were embedded in OCT, frozen, and sectioned. Immunofluorescence was performed on fixed colonies and 5–7- μ m corneal sections as described previously (Di Iorio et al., 2005). Confocal analyses were done with a confocal analyzer (LSM510-META; Carl Zeiss Microimaging, Inc.). Multitrack analysis was used for image acquisition. For immunoblots, mass or clonal cultures were extracted on ice with RIPA buffer (0.15 mM NaCl/0.05 mM Tris/HCl, pH 7.5/1% Triton X-100/1% sodium deoxycholate/0.1% SDS). Nuclear and cytoplasmic protein extraction was performed using the NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce Chemical Co.) following conditions supplied by the manufacturer. Equal amounts of samples were electrophoresed on 7.5% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride filters (Immobilon-P; Millipore). Immunoreactions were performed as described previously (Pellegrini et al., 2001) using antibodies at a 1:500 dilution. Immobilized antibodies were detected by chemiluminescence with ECL (GE Healthcare).

Semiquantitative RT-PCR

Total RNA was extracted from keratinocyte cultures, purified with RNase Micro kit (QIAGEN), and quantified by spectrophotometry. RT-PCR was performed using the One Step RT-PCR kit (QIAGEN). cDNAs were synthesized from 0.5–2 μ g of total RNA, and PCR reactions were performed using 20, 24, 28, 32, 36, and 40 cycles. β -Actin was used for normalization. Ethidium bromide-stained agarose gels were visualized with an Image Station 440 CF (Kodak). Quantification was performed using 1D 3.5 software (Kodak). Primer sequences for p63 isoforms and annealing temperatures were as described previously (Di Iorio et al., 2005). The following primers and annealing temperatures were used for p27, p57, and β -actin RT-PCR: p27^{Kip1}, 5'-AGTGTCTAACGGGAGCCCTA-3' and 3'-GTCCATTC-CATGAAGTCAGC-5' (annealing temperature 60°C, 829 bp); p57^{Kip2}, 5'-CAGCATGGAGCGCTCTTGTC-3' and 3'-CTTCTCAGGCGCTGATCTCT-5' (annealing temperature 60°C, 699 bp); and β -actin, 5'-GAGCGCAAGT-ACTCCGTGT-3' and 3'-ACGAAGGCTCATTCAAA-5' (annealing temperature 58°C, 548 bp).

Lentiviral vectors

The human C/EBP δ cDNA was cloned by RT-PCR from total RNA extracted from the THP1 cell line using specifically designed primers containing EcoRI recognition sequences. To generate a N-terminal Flag epitope-tagged C/EBP δ sequence, the EcoRI C/EBP δ cDNA fragment was subcloned into the pFlagCMV-2 plasmid (Sigma-Aldrich), after inserting an EcoRV restriction site at position 913 by PCR (for all primers, see Table S1, available at <http://www.jcb.org/cgi/content/full/jcb.200703003/DC1>). To generate a Flag-tagged ER-C/EBP δ fusion sequence, a MfeI-EcoRI PCR-amplified fragment containing the modified ligand binding domain of the human ER (Littlewood et al., 1995) was fused to the C terminus of the Flag epitope before inserting the C/EBP δ cDNA. The Flag-C/EBP δ , Flag-ER-C/EBP δ , and Flag-ER cassettes were extracted as EcoRV fragments and cloned downstream the human PGK promoter and upstream an IRES-EGFP cassette into the blunted SmaI-BamHI sites of the pRRL.ppt.PGK.IRES.GFP.WPRE lentiviral vector (Urbini et al., 2005), to obtain the RRL- δ -G, RRL-ER δ -G, and RRL-ER-G vectors. The control RRL-N-G vector was generated by cloning an NcoI-EcoRV fragment encoding a truncated form of the low-affinity, p75 NGFr (Δ NGFr; Bonini et al., 1997) into the same vector backbone. The RRL- Δ N α -G vector was obtained by inserting the cDNA of Δ Np63 α isoform (Yang et al., 1998) into the same vector backbone.

Lentiviral stocks pseudotyped with the vesicular stomatitis G protein (VSV-G) were prepared by transient cotransfection of 293T cells using a three-plasmid system (the transfer vector and the helper plasmids pCMV Δ R8.74, encoding Gag, Pol, Tat, and Rev, and pMD.G, encoding VSV-G), as previously described (Dull et al., 1998). Viral titers were determined by transduction of HeLa cells with serial dilution of the vector stocks and ranged from 10⁷ to 10⁸ TU/ml. Transduction efficiency was evaluated by scoring GFP and/or Δ NGFr transgene expression by flow cytometry.

Transduction of limbal keratinocytes

Subconfluent primary or clonal limbal cultures were trypsinized. 2 \times 10⁴ cells were resuspended in 1 ml of culture medium containing 8 mg/ml polybrene and transduced with lentiviral vector stocks at a MOI of 25, overnight at 37°C. Gene transfer efficiency was assessed 4 d after transduction

by scoring GFP⁺ cells by confocal fluorescence microscopy (LSM510-META; Carl Zeiss Microimaging, Inc.). In selected experiments (Fig. 4), 1 μ M 4OHT was added every 12 h for 3 d to RRL-ER δ -G-transduced cultures. 4OHT was then removed from the culture medium for 2 d and readded until control cultures reached confluency.

Transfection of siRNA-p27 and siRNA-p57

These experiments were performed using the siRNA-p27 and siRNA-p57 RNAi Human/Mouse Starter kit (QIAGEN). The siRNA duplexes were designed using the HiPerformance Design Algorithm licensed from Novartis AG, integrated with a stringent in-house homology analysis tool. Double-stranded RNAs were synthesized by QIAGEN. Primary cultured human limbal epithelial cells previously transduced with a lentiviral vector carrying the C/EBP δ cDNA (RRL- δ -G) were plated into 24-well plates at 4 \times 10⁴ cells/cm². 48 h later, cells were transfected using fluorescently labeled p27^{Kip1} (Alexa Fluor) and p57^{Kip2} (Cy3) siRNAs at a final concentration of 67 nM (siRNA to HiPerfect reagent ratio 1:6) and incubated under normal growth conditions (37°C and 5% CO₂). Transfection efficiency was determined 24 h after siRNA addition through laser-scanning confocal microscope (LSM510-META) analysis. Nonsilencing siRNAs (Alexa Fluor) were used as negative controls at the same conditions of transfection (67 nM; 1:6 ratio) and cell density.

ChIP assay

ChIP assays were performed essentially as described previously (Testa et al., 2005). Chromatin was prepared from 10⁷ limbal keratinocytes at the third and the fifth passage after transduction with either the RRL- δ -G or the RRL-N-G vector. Nuclear extracts were sonicated to obtain DNA fragments ranging from 400 to 800 bp in length. The equivalent of \sim 5 \times 10⁵ cells was immunoprecipitated with rabbit anti-CEBP δ (Santa Cruz Biotechnology, Inc.), mouse anti-Flag (Sigma-Aldrich), mouse anti-p63 (4A4 pan63; BD Biosciences), and rabbit anti-p63 α antibodies. Immunoprecipitations with mouse and rabbit IgGs (BD Biosciences) were included as controls. Immunoprecipitated DNA was analyzed by PCR with primers spanning regions containing known or putative CEBP δ and p53/p63 binding motifs within the genomic loci of p63 (from position +70720 to +70951, +147873 to +148041, +151191 to +151408, +202579 to +202761, and +234830 to +235057 from the transcription start site), involucrin (–421 to –119), p27^{Kip1} (–227 to +14), p57^{Kip2} (–622 to –398), and p16^{INK4A} (–1020 to –871). Specific primers are listed in Table S1.

Online supplemental material

Table S1 gives the primers used in this study. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200703003/DC1>.

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