

RESEARCH ARTICLE

Epiprofin orchestrates epidermal keratinocyte proliferation and differentiation

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ABSTRACT

The basal layer of the epidermis contains stem cells and transit amplifying cells that rapidly proliferate and differentiate further into the upper layers of the epidermis. A number of molecules have been identified as regulators of this process, including p63 (also known as tumor protein 63) and Notch1. However, little is known about the mechanisms that regulate the transitions from stem cell to proliferating or differentiating transit amplifying cell. Here, we demonstrate that epiprofin (Epf, also known as Sp6) plays crucial distinct roles in these transition stages as a cell cycle regulator and a transcription factor. *Epf* knockout mice have a thickened epidermis, in which p63-expressing basal cells form multiple layers owing to the accumulation of premature transit amplifying cells with reduced proliferation and a reduction in the number of differentiating keratinocytes expressing Notch1. We found that low levels of Epf expression increased the proliferation of human immortalized keratinocyte (HaCaT) cells by increasing EGF responsiveness and superphosphorylation of Rb. By contrast, high levels of Epf expression promoted cell cycle exit and differentiation, by reducing E2F transactivation and inducing Notch1 expression. Our findings identify multiple novel functions of Epf in epidermal development.

KEY WORDS: Sp transcription factor, Skin development, Proliferation, Differentiation, Keratinocyte, Transit amplifying cell, Stem cell, p63, Notch

INTRODUCTION

Skin self-renews throughout life by constantly replenishing keratinocytes in the outermost layer of the epidermis in a process that involves balancing the production of stem cells and transit amplifying cells, which are the committed keratinocyte precursors (Jones and Watt, 1993). The epidermis arises from a single ectodermal layer and renews by highly coordinated regulatory programs controlling proliferation and differentiation. The epidermis consists of four distinct layers that are mostly composed of keratinocytes at different stages of maturation. The

basal layer of the epidermis contains stem cells and transit amplifying cells. Transit amplifying cells divide a limited number of times (three to five) before commitment to terminal differentiation. Basal keratinocytes attach to the underlying basement membrane through integrins, such as $\alpha 3 \beta 1$ and $\alpha 6 \beta 4$. Other integrins, such as $\alpha 2 \beta 1$ and $\alpha 5 \beta 1$, required for collagen/laminin and fibronectin binding, respectively, are also involved in basal keratinocyte interaction with the basement membrane (Burgeson and Christiano, 1997). When transit amplifying cells eventually stop proliferating and begin to differentiate, the expression of these integrins is reduced, resulting in detachment of keratinocytes from the basement membrane and cell movement towards the upper layers of the epidermis (Fuchs, 2008).

Proliferation of transit amplifying cells is stimulated by the mitogenic epidermal growth factor (EGF) through the EGF receptor (EGFR)–ERK–MAPK signaling pathway. Although the EGFR is also expressed by stem cells, these cells generally respond poorly to EGF (Jensen and Watt, 2006; Powell et al., 2012; Wong et al., 2012). The difference in EGF responsiveness is due in part to the reduced levels of the Lrig proteins in transit amplifying cells. These proteins negatively regulate EGF-induced signaling by promoting the ubiquitylation and degradation of EGFR (Jensen and Watt, 2006; Laederich et al., 2004; Watt and Jensen, 2009). Expression of Lrig1 is essential for epidermal homeostasis; *Lrig1*-knockout mice show epidermal hyperplasia, and knockdown of Lrig1 expression in cultured human keratinocytes induces stem cell expansion (Jensen and Watt, 2006; Suzuki et al., 2002). Several cell cycle regulators have been implicated in skin morphogenesis. For example, retinoblastoma protein (Rb) and the associated pocket proteins (p107 and p130; also known as RBL1 and RBL2, respectively) regulate epidermal differentiation and maintenance (Ruiz et al., 2004).

Coordinated epidermal differentiation programs are regulated by a series of genes. Among them, Notch1 and the deltaN isoform of p63 (also known as tumor protein 63) are crucial regulators of epidermal keratinocyte development (Blanpain et al., 2006). The transcription factor p63 is necessary for the proper development of skin, teeth, limbs and craniofacial tissues. In the epidermis, p63 is required to maintain the population of stem cells and transit amplifying cells and to ensure transit amplifying cell commitment to the epidermal cell lineage (Koster et al., 2004; Mills et al., 1999; Pellegrini et al., 2001; Romano et al., 2012; Truong et al., 2006; Yang et al., 1999). A deficiency of p63 results in the absence of keratinocyte stratification and differentiation (Mills et al., 1999; Yang et al., 1999). Loss of Notch signaling also disrupts epidermal differentiation (Lowell et al., 2000; Rangarajan et al., 2001), as evidenced by hyperkeratosis, whereas overexpression of activated Notch1 in basal keratinocytes induces the loss of proliferative capacity and induction of premature differentiation (Blanpain

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et al., 2006; Lefort et al., 2007; Nguyen et al., 2006). The expression of Notch1 and p63 in the epidermis is mutually exclusive; Notch1 is primarily expressed by differentiating keratinocytes, whereas p63 expression occurs in the basal layer, which mainly contains stem cells and maturing transit amplifying cells (Nguyen et al., 2006). A negative regulatory loop involving p63 and Notch signaling controls transit amplifying cell commitment to keratinocyte differentiation. Thus, the balance between p63 and Notch1 expression and signaling is crucial for the development and controlled renewal of the epidermis. However, it is not clear how transit amplifying cell cycle exit is promoted and how Notch1 transcription is induced during the transition from proliferation to differentiation.

We previously identified epiprofin (Epfm, also known as Sp6) based on mRNA analysis of tooth germs and demonstrated its expression in certain ectodermal tissues such as teeth, skin, hair follicles and limb buds (Nakamura et al., 2004). Epfm is a member of the Sp family of transcription factors, and it contains a transactivation/suppressor domain at the N-terminus and three C2H2 zinc finger motifs at the C-terminus for DNA binding (Suske et al., 2005). *Epfm* knockout (*Epfm*^{-/-}) mice display striking phenotypic features, including defects in tooth morphology, supernumerary tooth formation, digit fusion, skin abnormalities and hairlessness (Hertveldt et al., 2008; Ibarretxe et al., 2012; Nakamura et al., 2008; Talamillo et al., 2010). In addition to excess teeth, *Epfm*^{-/-} mice display severe enamel hypoplasia due to defects in dental epithelial differentiation into enamel-matrix-secreting ameloblasts (Nakamura et al., 2008). In the absence of *Epfm*, the inner dental epithelium loses its characteristic ability for rapid proliferation, leading to severe enamel hypoplasia (Nakamura et al., 2008). Therefore, Epfm might be a multifunctional regulator of both proliferation and differentiation, depending on the developmental stage.

In this study, we demonstrate that *Epfm*^{-/-} mice develop hyperplastic epidermis and hyperkeratosis of the skin. In the *Epfm*^{-/-} epidermis, p63-expressing basal keratinocytes form multiple cell layers and Notch1 expression is reduced. We found that, in the absence of *Epfm*, premature transit amplifying (pre-TA) cells with reduced proliferation activity accumulate and show partial commitment to differentiation. In cell culture, Epfm promotes proliferation of keratinocyte progenitors at low levels of Epfm expression, whereas high Epfm expression promotes cell cycle exit and induces differentiation through inducing Notch1. Our findings reveal that Epfm is a novel regulator of epidermal development.

RESULTS

Defects in skin development and in morphogenesis in *Epfm*^{-/-} mice

Homozygous epiprofin-knockout (*Epfm*^{-/-}) mice are smaller than normal and display defects in both skin development and hair formation (Fig. 1Ab), as well as the tooth dysmorphogenesis described previously (Hertveldt et al., 2008; Nakamura et al., 2008). By contrast, heterozygous littermates (*Epfm*^{+/-}) show no obvious phenotype (Fig. 1Aa). Knockout mice are hairless over most of their bodies and have short, thin, wrinkled whiskers (Fig. 1Ac,d). There are no apparent histological abnormalities in the epidermal and pelage hair follicles of *Epfm*^{-/-} mice until approximately embryonic day 16.5 (Fig. 1Ba,b). After this stage, however, abnormalities in the epidermis and hair follicles become progressively more severe. After postnatal day 7 (P7), *Epfm*^{-/-} epidermal layers are significantly thicker than those of

heterozygotes, owing to hyperkeratosis and hypercellularity (Fig. 1Bc–h).

We subsequently examined possible defects in epidermal differentiation at P10 by double-immunofluorescent staining using antibodies against differentiation markers, including the keratins K5 and K10, as well as involucrin, filaggrin and loricrin (Fig. 1C). In control *Epfm*^{+/-} littermates, K5 was expressed in the basal epidermal layer (Fig. 1Ca), whereas K10 was expressed primarily in the suprabasal layer of the epidermis (Fig. 1Ca,c,e,g). In *Epfm*^{-/-} mice, the K5- and K10-positive cells formed multiple cell layers in which the K5-positive cells were located in a lower layer of the thickened epidermis as compared with the K10-positive cells (Fig. 1Cb). Involucrin, filaggrin and loricrin were expressed in differentiating keratinocyte layers in the control epidermis (Fig. 1Cc,e,g), but the expression of these genes was reduced in the *Epfm*^{-/-} epidermis (Fig. 1Cd,f,h).

To address the molecular basis of the abnormal formation of multiple keratinocyte layers in the *Epfm*^{-/-} epidermis, we analyzed the expression of p63 and Notch1, which regulate cell fate and keratinocyte differentiation in the epidermis (Fig. 1Da,b). In the control P13 epidermis of heterozygous mice, p63 was expressed in the basal layer and Notch1 expression was observed in differentiating keratinocytes, in agreement with previous reports (Fig. 1Da,b) (Kurata et al., 2004; Mills et al., 1999; Rangarajan et al., 2001). In the *Epfm*^{-/-} epidermis, the p63-positive cells formed multiple layers (Fig. 1Db) and the number of p63-expressing cells was increased (Fig. 1E), whereas Notch1 expression was virtually undetectable in the epidermis (Fig. 1Db,E). Expression of Hes1, a transcription factor and downstream target of Notch, was also considerably lower in the *Epfm*^{-/-} epidermis compared with that of the heterozygotes (Fig. 1Dc,d,E), indicating perturbed Notch signaling in the *Epfm*^{-/-} epidermis. Finally, Epfm was expressed in basal layer keratinocytes and in differentiating keratinocytes in the epidermis during embryonic stages in the control epidermis but not in the *Epfm*^{-/-} epidermis (Fig. 1De,f,E). A schematic diagram of the expression pattern for p63, Notch1 and Epfm in the epidermis is shown in supplementary material Fig. S1.

Reduced keratinocyte proliferation and apoptosis in the *Epfm*^{-/-} epidermis

The epidermis of *Epfm*^{-/-} mice exhibited multiple layers of K5- and p63-expressing basal cells (Fig. 1C), suggesting dysregulation of both cell proliferation and apoptosis. We examined proliferation in the *Epfm*^{-/-} epidermis by immunostaining for proliferating cell nuclear antigen PCNA (a marker of late G1 and S phases) and Ki67, and by BrdU incorporation. Apoptosis was analyzed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining (Fig. 2A,B). In the P7 *Epfm*^{+/-} epidermis, the majority of the basal epidermal keratinocytes formed a single cell layer, and most of the cells were PCNA-positive (Fig. 2Aa,B). The number of PCNA-positive cells in the basal layer was significantly lower in the *Epfm*^{-/-} epidermis, but the total number of cells exhibiting some PCNA immunoreactivity was higher in the *Epfm*^{-/-} epidermis because of the long half-life of PCNA and the hypercellularity (Fig. 2Aa,b; Fig. 2B). Many Ki67-positive cells were detected in the basal layer of the P7 *Epfm*^{+/-} epidermis, whereas the number of Ki67-positive cells was reduced in the *Epfm*^{-/-} epidermis (Fig. 2Ac,d; Fig. 2B). Similarly, short-term incorporation of BrdU for 4 h to detect transit amplifying cells revealed that a significantly greater number of basal cells were proliferating in the control P7 *Epfm*^{+/-} epidermis compared with the *Epfm*^{-/-} epidermis

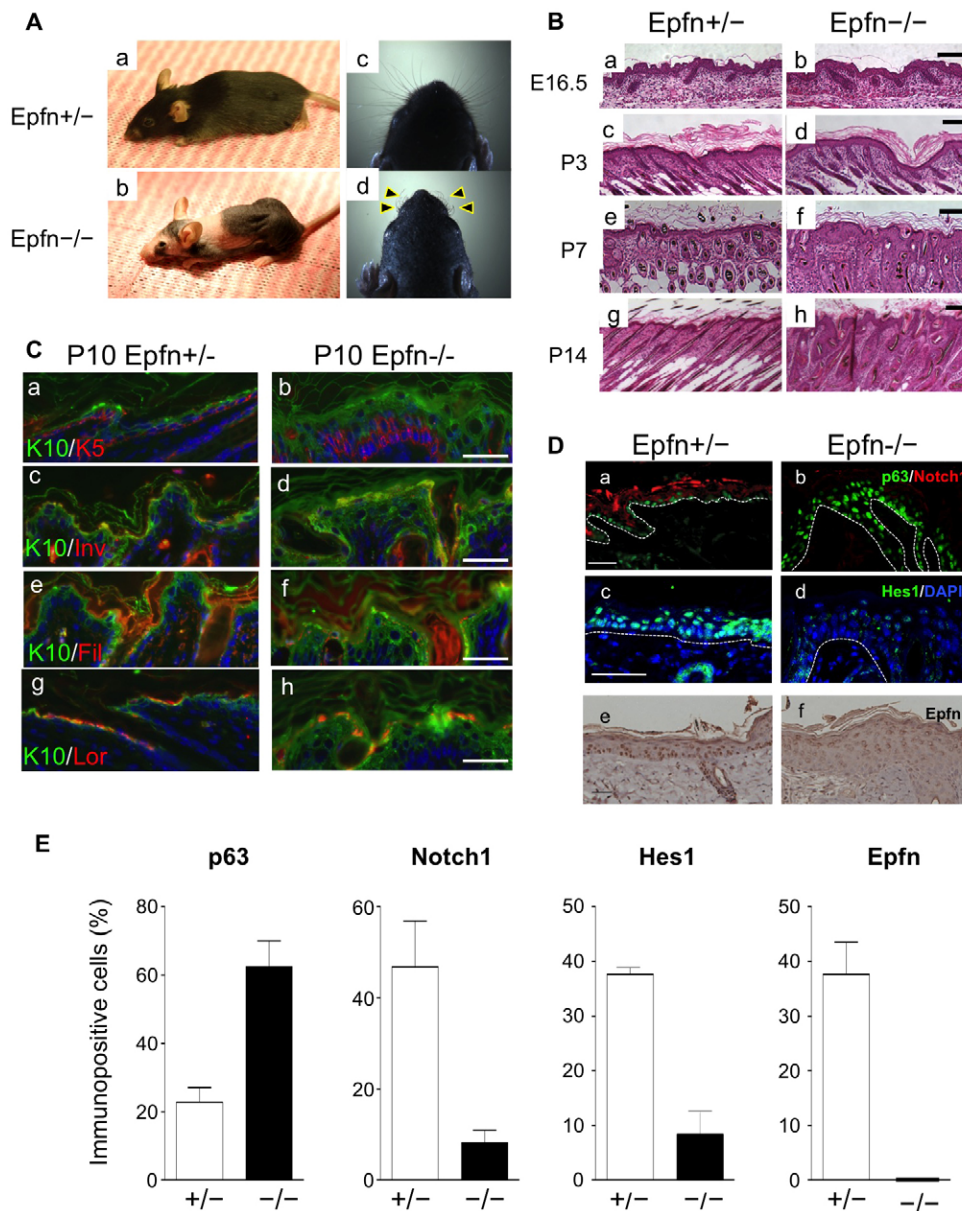


Fig. 1. Defects of skin and hair follicle development in *Epfn*^{-/-} mice.

(A) Defective skin, hair follicle and whisker formation (arrowheads) in 3-month-old *Epfn*^{+/-} (a,c) and *Epfn*^{-/-} (b,d) mice. (B) Defective skin morphogenesis and hair follicle formation. Histological analysis of developing skin and skin morphogenesis at E16.5 (a,b), P3 (c,d), P7 (e,f) and P14 (g,h) of *Epfn*^{+/-} (a,c,e,g) and *Epfn*^{-/-} (b,d,f,h) mice. Scale bars: 100 μ m. (C) Expression of epidermal keratinocyte differentiation marker proteins in P10 *Epfn*^{+/-} (a,c,e,g) and *Epfn*^{-/-} (b,d,f,h) mice. The expression patterns of K10 (green; a–h), K5 (red; a,b), involucrin (Inv; red; c,d), filaggrin (Fil; red; e,f) and loricrin (Lor; red; g,h) are shown. Hoechst 33258 (blue) was used to stain DNA (a–h). Scale bars: 100 μ m. (D) The expression of p63 (green) and Notch1 (red) (a,b) in P13 mice, and Hes1 (green; c,d) in P10 mice. In P10 mice, DNA was stained with DAPI (blue). The white dotted lines indicate the basement membrane of the epidermis. Also shown is *Epfn* expression (DAB) (e,f) in 3-month-old mice. Scale bars: 100 μ m (a,b,e,f), 50 μ m (c,d). (E) Ratios of cells immunopositive for p63, Notch1, Hes1 and *Epfn* in the epidermis of *Epfn*^{+/-} and *Epfn*^{-/-} mice. Data show the mean \pm s.e.m. (three independent experiments).

(Fig. 2Ae,f; Fig. 2B). These results suggest that transit amplifying cell proliferation is inhibited in the *Epfn*^{-/-} epidermis. However, these cells accumulate, resulting in hypercellularity. In addition, TUNEL staining analysis revealed that the number of apoptotic cells in P3 *Epfn*^{-/-} epidermis was significantly reduced compared with that in the *Epfn*^{+/-} epidermis (Fig. 2Ag,h,B). The lower rate of programmed cell death in the early postnatal mutant epidermis might have contributed to the observed increase in epidermal layer thickness and hypercellularity in *Epfn*^{-/-} mice.

Results thus far indicate that loss of *Epfn* disrupts the normal balance of transit amplifying cell proliferation and differentiation that is necessary for proper skin morphogenesis. To examine the effects of *Epfn* on cell proliferation under controlled conditions, we used primary keratinocytes isolated from the epidermis of newborn *Epfn*^{+/-} and *Epfn*^{-/-} mice. There were significantly fewer cells in cultures derived from *Epfn*^{-/-} mice compared with wild-type cultures after 4 days in serum-free and low-Ca²⁺ (differentiation-restricted) medium (KGM), suggesting that *Epfn* normally promotes keratinocyte proliferation (Fig. 2C). Flow cytometric

analysis revealed that approximately half of the keratinocytes from the *Epfn*^{+/-} epidermis were in the proliferating phases (G2/M and S), whereas the majority (~70%) of the keratinocytes from the *Epfn*^{-/-} epidermis were in the quiescent G0/G1 phase (Fig. 2D). Taken together, these results suggest that *Epfn* is essential for transit amplifying cell proliferation.

To determine the molecular pathways leading to cell cycle disruption in keratinocytes from *Epfn*^{-/-} mice, we examined the expression and phosphorylation status of the cell cycle regulators Rb, p21 (also known as cyclin-dependent kinase inhibitor 1 or CDKN1A) and p107, and the cyclin-dependent kinases (CDKs) CDK4 and CDK6 (Fig. 2E). Cultured keratinocytes from the control *Epfn*^{+/-} epidermis expressed both the phosphorylated form of Rb (phos-Rb) and the unphosphorylated form of Rb. The expression of phos-Rb and p21 was reduced in *Epfn*^{-/-} keratinocytes, but the expression of p107 was not. CDK4 and CDK6 were expressed at similar levels in both cell types. These results suggest that *Epfn* promotes keratinocyte proliferation by regulating Rb phosphorylation and p21 expression (Fig. 2E).

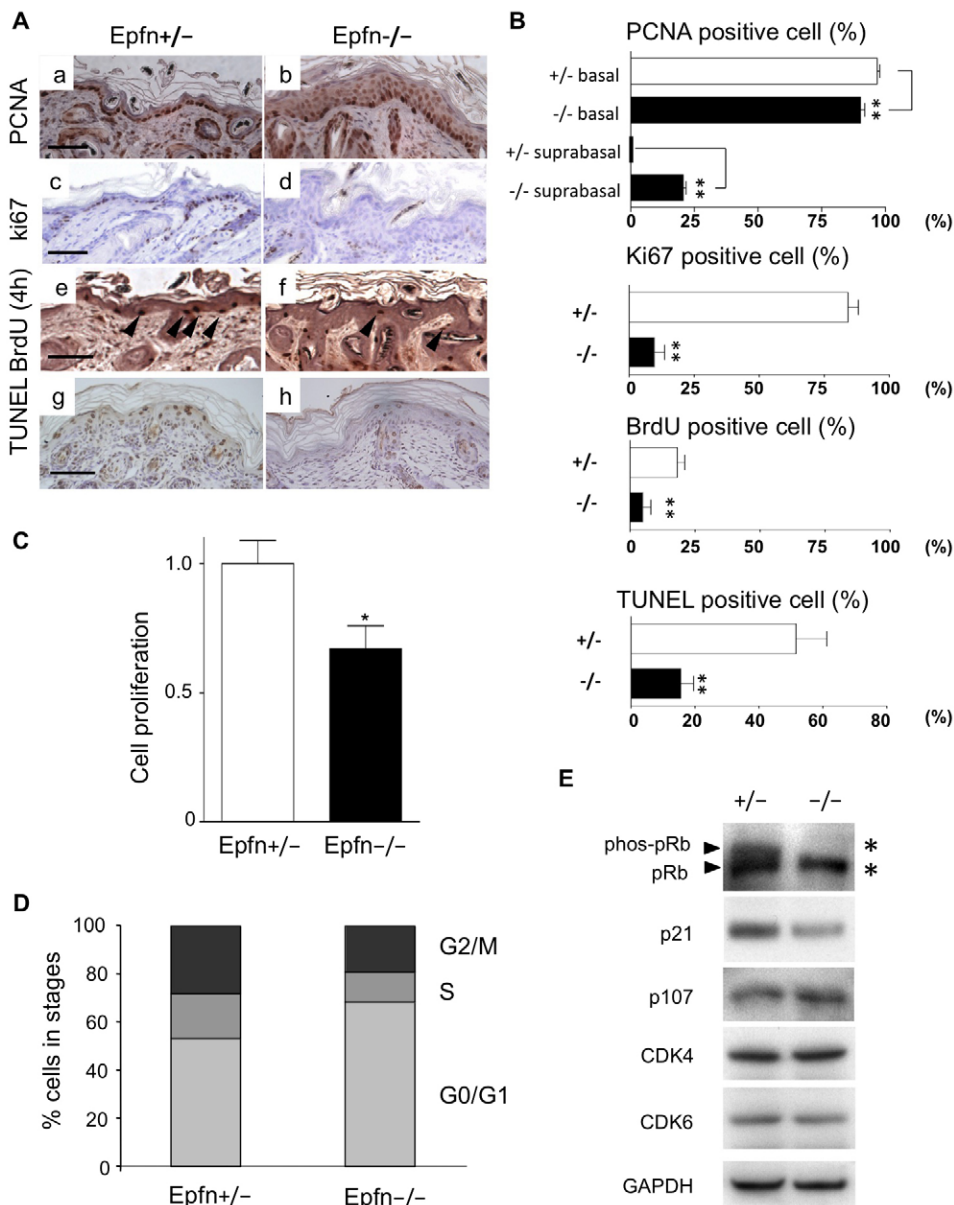


Fig. 2. Slower keratinocyte proliferation, reduced apoptosis and dysregulation of Rb phosphorylation in the *Epfm*^{-/-} epidermis. (A) PCNA (a,b), Ki67 (c,d) and BrdU (e,f) staining in the P7 epidermis, and TUNEL staining (g,h) in the P3 epidermis. Scale bars: 100 μ m. (B) Relative numbers of PCNA-, Ki67-, BrdU- and TUNEL-positive cells in *Epfm*^{+/-} and *Epfm*^{-/-} skin. Data show the mean+s.e.m. (three independent experiments); ***P*<0.01. (C) Proliferation of primary keratinocytes from newborn *Epfm*^{+/-} and *Epfm*^{-/-} mice. Data show the mean+s.e.m. (three independent experiments); **P*<0.05. (D) Flow cytometric analysis of cell cycle progression in primary keratinocytes from newborn *Epfm*^{+/-} and *Epfm*^{-/-} mice. (E) Expression of cell cycle regulators as revealed by western blotting. Reduced Rb phosphorylation (phos-pRb) and expression of cell cycle regulators in primary keratinocytes from newborn *Epfm*^{+/-} and *Epfm*^{-/-} mice. Upper arrowhead and asterisk, phos-Rb; lower arrowhead and asterisk, Rb protein.

Accumulation of premature transit-amplifying-cell-like keratinocytes in the *Epfm*^{-/-} epidermis

The basal epidermis of *Epfm*^{-/-} mice exhibited ectopic expression of keratins, and basal keratinocyte-like cells expressing K5 and p63 formed multiple cell layers (Fig. 1). Moreover, isolated keratinocytes from the *Epfm*^{-/-} epidermis proliferated more slowly compared with keratinocytes derived from the *Epfm*^{+/-} epidermis (Fig. 2C). To identify additional genes regulated by *Epfm*, we compared the expression of genes that are characteristic of normal stem cells and transit amplifying cells in *Epfm*^{-/-} and *Epfm*^{+/+} primary keratinocytes using microarray analysis (Fig. 3A). In *Epfm*^{-/-} keratinocytes, stem cell markers such as *Krt15* (cytokeratin 15) and the Notch ligands *Dll1* and *Jag2* were significantly downregulated compared with their expression in wild-type cells, whereas other markers, such as *Lrig3* and *p63*, were upregulated. The integrins $\alpha 3$ (*Itga3*), $\alpha 6$ (*Itga6*) and $\beta 1$ (*Itgb1*), which are markers for both stem cells and transit amplifying cells, and *Tfrc* (transferrin receptor, also known as CD71), a marker of transit amplifying cells, were upregulated in

Epfm^{-/-} keratinocytes. However, *Notch1* and *Notch3*, markers of differentiated keratinocytes, were downregulated in *Epfm*^{-/-} keratinocytes, consistent with immunohistochemical observations using the antibodies against Notch1 and Hes1 (Fig. 1D,E). These differences in gene expression between *Epfm*^{+/+} and *Epfm*^{-/-} keratinocytes were confirmed by quantitative PCR analysis using primer sets specific to individual genes (data not shown). Therefore, the premature transit-amplifying-like (pre-TA) cells that accumulated in the *Epfm*^{-/-} epidermis have an immature phenotype, retaining certain stem cell marker genes while expressing only some genes specific to transit amplifying cells. Moreover, accumulated pre-TA cells in the *Epfm*^{-/-} epidermis were not capable of rapid proliferation, which is a key characteristic of normal transit amplifying cells.

Basal keratinocytes express integrins such as $\alpha 3\beta 1$, $\alpha 6\beta 4$ and $\alpha 5\beta 1$ at the basal cell surface, and these act to anchor immature cells to the underlying basement membrane (Burgeson and Christiano, 1997). When transit amplifying cells differentiate, the expression of these integrins is reduced and the cells detach and

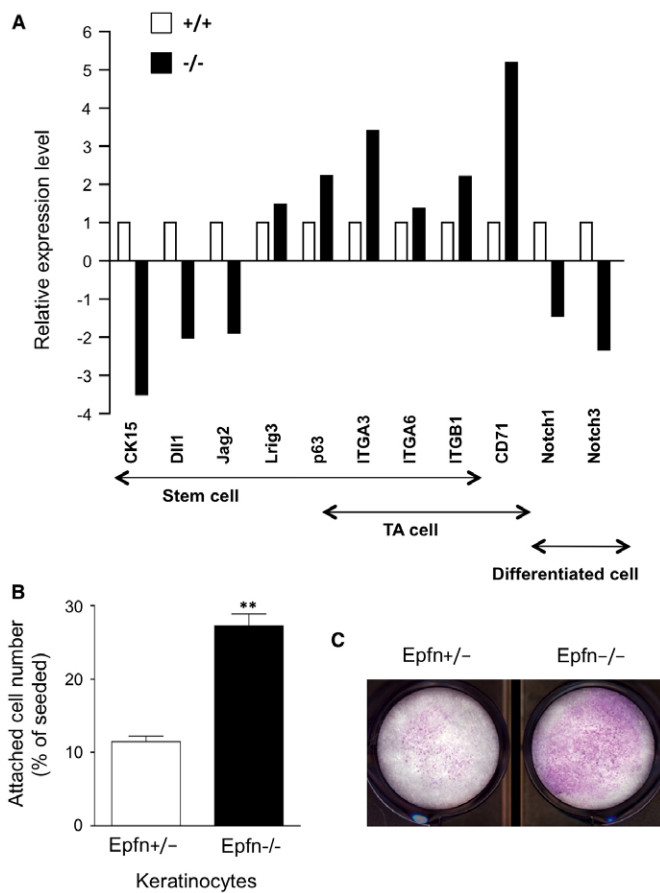


Fig. 3. Characteristics of keratinocytes from the *Epfn*^{-/-} epidermis. (A) Expression of various genes characteristic of stem cells, transit amplifying (TA) cells and mature keratinocytes in primary cultures derived from newborn *Epfn*^{+/+} and *Epfn*^{-/-} mouse epidermis as measured by microarray analysis. (B) Cell attachment and (C) colony formation assays were performed using primary keratinocytes from newborn *Epfn*^{+/+} and *Epfn*^{-/-} mice. Data in B show the mean±s.e.m. (four independent experiments); ***P*<0.01.

migrate towards the surface layers (Fuchs, 2008). However, immunostaining of the basal *Epfn*^{-/-} epidermis revealed integrin $\beta 6$ expression over the entire peripheral cell surface (data not shown), consistent with an immature phenotype. *In vitro*, stem cells rapidly attach to fibronectin-coated wells within 15 min of incubation because of strong integrin expression (Jones and Watt, 1993). To characterize the functional effects of integrin expression by pre-TA cells in the *Epfn*^{-/-} epidermis, we analyzed the attachment activity of keratinocytes from the *Epfn*^{-/-} epidermis to fibronectin (Fig. 3B). Approximately 30% of the keratinocytes from the *Epfn*^{-/-} epidermis attached to fibronectin-coated wells within 15 min of incubation, compared with only 10% of the keratinocytes from *Epfn*^{+/+} mice. Colony-forming assays confirmed that the *Epfn*^{-/-} keratinocytes formed more cell colonies than the *Epfn*^{+/+} keratinocytes (Fig. 3C), indicating that these *Epfn*^{-/-} keratinocytes retained certain stem-cell-like and immature cell properties.

Roles of *Epfn* in proliferation of HaCaT cells and keratinocytes

To address the mechanism of *Epfn* action in proliferation, we used the human epidermal keratinocyte cell line HaCaT, which proliferates in KGM but can be induced to differentiate in KGM

containing a high Ca^{2+} concentration (Boukamp et al., 1988). HaCaT cells express *Epfn* at a low level in the proliferation medium (Fig. 4A, left two lanes; no exogenous Ca^{2+} addition). Elevating the Ca^{2+} levels in the medium by the addition of 0.7 mM or 1.2 mM Ca^{2+} increased *Epfn* expression as well as that of markers of keratinocyte differentiation, such as filaggrin (Fig. 4A, right four lanes). Transfection of a targeted *Epfn* short hairpin (sh)RNA into HaCaT cells reduced the endogenous RNA and protein levels of the target (Fig. 4Ba). *Epfn* shRNA significantly reduced HaCaT cell proliferation (Fig. 4Bb) in the low- Ca^{2+} medium, consistent with the results in the primary keratinocyte cultures from the *Epfn*^{-/-} mice (Fig. 2C). We observed similar results using a different *Epfn* shRNA (data not shown).

One possible explanation for the reduced proliferation of *Epfn*^{-/-} keratinocytes might be the reduction in EGF-mediated proliferation. The *Lrig* family of proteins promotes EGFR degradation and negatively regulates EGF-mediated stem cell proliferation, thereby maintaining a slow proliferation (Cai et al., 2009; Laederich et al., 2004; Muller et al., 2013; Rondahl et al., 2013). The microarray analysis showed upregulation of *Lrig3* expression in *Epfn*^{-/-} keratinocytes (Fig. 3A), suggesting that *Epfn* normally inhibits *Lrig* expression and that *Epfn* deficiency leads to a loss of the EGF-mediated proliferation of keratinocytes. We explored these possibilities by testing the EGF response in HaCaT cells transfected with control shRNA or *Epfn* shRNA (Fig. 4C). The HaCaT cells were incubated in the absence or the presence of EGF for 3 days and the cell proliferation was analyzed by colorimetric assays using the WST-8 Kit. EGF increased proliferation of the HaCaT cells transfected with control shRNA, whereas those transfected with *Epfn* shRNA did not respond to EGF (Fig. 4C). The expression level of *Lrig1* was increased in the *Epfn* knockdown HaCaT cells (supplementary material Fig. S2). We confirmed the involvement of *Epfn* in EGF-mediated proliferation in primary human keratinocytes transfected with the same control shRNA and *Epfn* shRNA used for HaCaT cells (Fig. 4D). Two different *Epfn* shRNAs reduced the expression of endogenous *Epfn* in human keratinocytes (supplementary material Fig. S3). EGF treatment increased the proliferation of primary human keratinocytes, but it did not increase proliferation of the cells transfected with *Epfn* shRNA (Fig. 4D). Similarly, the primary mouse *Epfn*^{-/-} keratinocytes from the *Epfn*^{-/-} epidermis did not respond to EGF (Fig. 4E). These results suggest that *Epfn* promotes EGF-mediated proliferation of transit amplifying cells in part by suppressing *Lrig* expression.

Epfn promotes HaCaT cell proliferation by forming a complex with CDK4 and enhancing Rb phosphorylation

Next, we used HaCaT cells to examine whether *Epfn* promotes the proliferation of keratinocytes by interacting with cell cycle signaling molecules. We analyzed the dose-dependent effects of *Epfn* expression on HaCaT cell proliferation 2 days after transfection of HaCaT cells with a Halo-tagged *Epfn* expression vector under the control of CMV promoters containing various deletions (Fig. 5A). Western blotting using an anti-*Epfn* antibody revealed a gradual decrease in the expression of recombinant Halo-tagged *Epfn* with sequential deletions in the CMV promoter region (CMV, CMVd1; day3) (Fig. 5A). We found that low levels of *Epfn* expression (CMVd2, CMVd3) promoted more proliferation of HaCaT cells compared with that observed following high levels of *Epfn* expression (CMV, CMVd1)

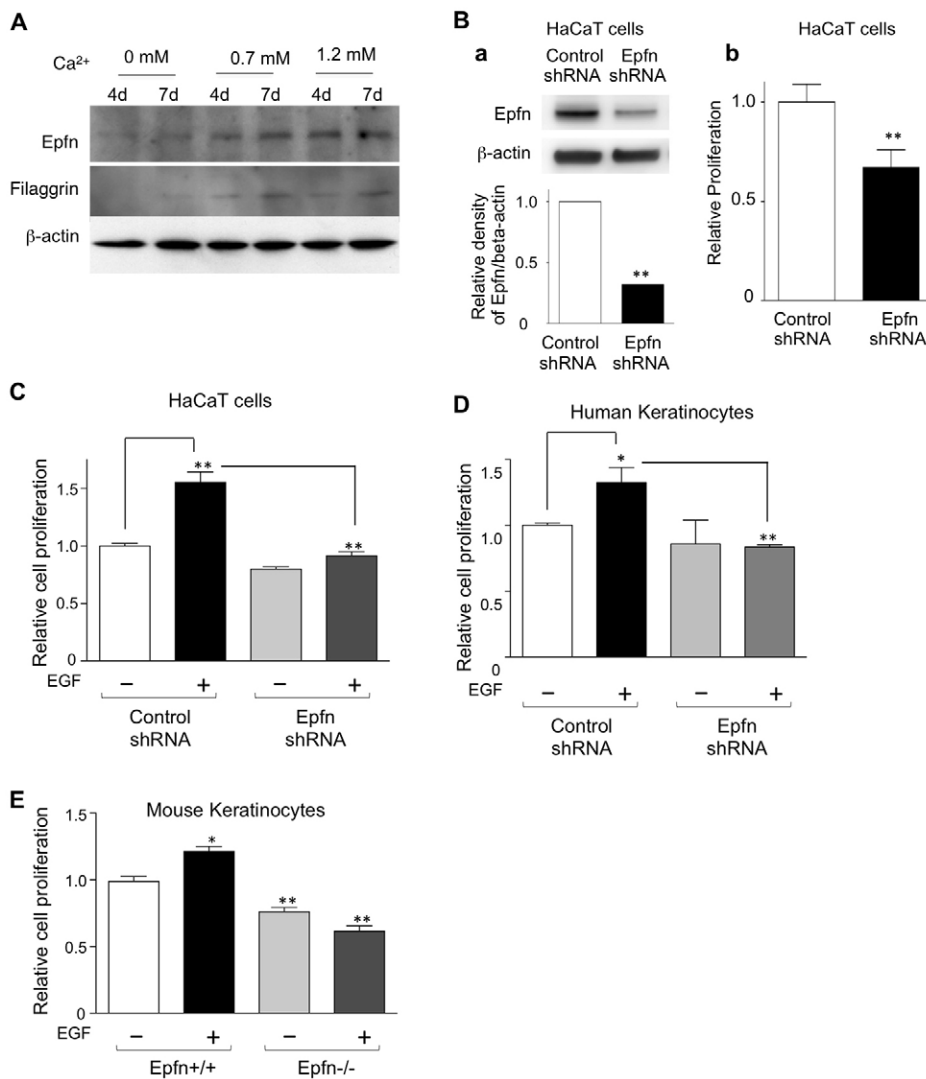


Fig. 4. Epfn expression in differentiated HaCaT cells and the role of Epfn in EGF-mediated cell proliferation. (A) Immunoblot analysis of Epfn and filaggrin expression in differentiating HaCaT cells cultured in a low-Ca²⁺ medium (KGM) with the addition of 0 mM, 0.7 mM or 1.2 mM Ca²⁺ for 4 days (4d) or 7 days (7d). (B) HaCaT cell proliferation. (a) Upper panel shows western blot analysis of endogenous Epfn expression in HaCaT cells transfected with control shRNA and *Epfn* shRNA. Lower panel shows densitometric analysis of the western blot assays using NIH ImageJ. (b) Proliferation of HaCaT cells transfected with control shRNA and *Epfn* shRNA. (C) EGF-mediated proliferation of HaCaT cells transfected with control shRNA and *Epfn* shRNA. (D) EGF-mediated proliferation of human epidermal keratinocytes transfected with control shRNA and *Epfn* shRNA. (E) EGF-mediated proliferation of keratinocytes from the wild-type (*Epfm*^{+/+}) and *Epfm*^{-/-} epidermis. Primary keratinocytes were cultured in KGM for 4 days with or without recombinant EGF (10 ng/ml). Quantitative data show the mean±s.e.m. (three independent experiments); **P*<0.05; ***P*<0.01.

(Fig. 5B). These results suggest that low levels of *Epfn* expression are involved in promoting proliferation.

To identify the molecular mechanism of Epfn-mediated cell proliferation, we examined the expression of cell cycle regulators in HaCaT cells transfected with a His-tagged Epfn expression vector (Fig. 5C). *Epfn* expression increased the phosphorylation of Rb (Fig. 5C) but had no effect on Rb protein levels (data not shown) and did not affect the expression of E2F1, a transcription factor that binds to Rb in a cell-cycle-dependent manner and is a crucial regulator of cell cycle control. Epfn-induced Rb phosphorylation is consistent with decreased amounts of phos-Rb in *Epfn*^{-/-} keratinocytes (Fig. 2E). Rb phosphorylation by the cyclin-D-CDK complex from the G1 to S phase of the cell cycle results in the release of active E2F1 from the E2F1-Rb complex. Consequently, E2F1 activates the transcription of genes that drive cell cycle progression. Because Epfn promoted Rb phosphorylation in HaCaT cells (Fig. 5C), we speculated that Epfn might also interact with the cyclin-D-CDK complex. As CDK4 is a crucial regulator of Rb phosphorylation, we assessed possible interactions between Epfn and CDK4 by co-immunoprecipitation assays (Fig. 5Da). HEK293T cells were co-transfected with Myc-tagged Epfn and CDK4 expression vectors, and the extracts were immunoprecipitated with an anti-Myc antibody and analyzed by

western blotting using an anti-CDK4 antibody. The CDK4 antibody recognized the protein complex pulled down by anti-Myc, strongly suggesting that Epfn directly or indirectly complexes with CDK4 (Fig. 5Da). Moreover, the isolated Epfn-CDK4 complex strongly enhanced Rb phosphorylation *in vitro* (Fig. 5Db). These results suggest that Epfn complexes with CDK4 and that this Epfn-CDK4 complex promotes cell proliferation by enhancing the phosphorylation of Rb (schematic in Fig. 7B).

Epfn promotes cell cycle exit and differentiation

Transit amplifying cells rapidly proliferate, but only for a limited number of divisions before cell cycle exit for terminal differentiation. The factor involved in controlling this transition is not clear. *Epfn* is continuously expressed in basal and differentiating keratinocytes in the epidermis, and in the *Epfn*^{-/-} epidermis, keratinocyte differentiation is reduced. In cell culture, Epfn expression was induced during HaCaT cell differentiation in the high-Ca²⁺ medium (Fig. 4A). Therefore, Epfn might play a pivotal role in promoting cell cycle exit and triggering differentiation processes. To address this hypothesis, we first examined the ability of Epfn to promote the differentiation of HaCaT cells in low-Ca²⁺ medium. We found that Epfn overexpression in HaCaT cells increased the mRNA

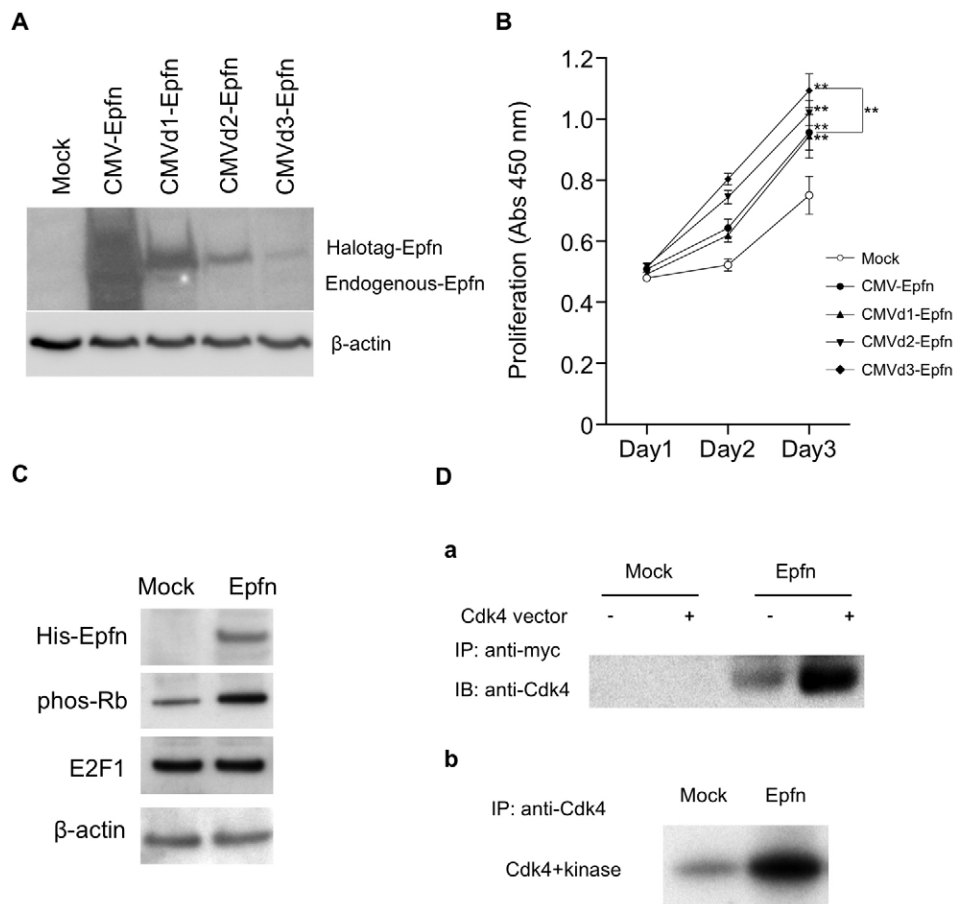


Fig. 5. Epfn promotes HaCaT cell proliferation through enhancing Rb phosphorylation and Epfn-Cdk4 interaction. (A) Immunoblot analysis of Epfn expression in HaCaT cells transfected with various Halo-tagged Epfn expression vectors driven by either the full-length CMV promoter (CMV-Epfn) or versions of the CMV promoter containing deletions (CMVd1-Epfn, CMVd2-Epfn, CMVd3-Epfn). β -actin is shown as a loading control. (B) The relationship between Epfn expression levels and HaCaT cell proliferation. HaCaT cells were transfected with one of four Halo-tagged Epfn expression vectors. The proliferation was analyzed at various time-points. Data show the mean \pm s.e.m. (four independent experiments); ** $P < 0.01$. (C) Immunoblot analysis of cell cycle proteins. HaCaT cells were transfected with a His-tagged Epfn expression vector, and lysates were analyzed by western blotting using antibodies against the His tag, phos-Rb, E2F1 and β -actin (the loading control). (D) Co-immunoprecipitation assay of Epfn and Cdk4 (a) and an Rb phosphorylation assay (b) in HEK293 cells transfected with Mock or Epfn (Myc tag) expression vectors. IP, immunoprecipitated; IB, immunoblotted.

expression of Notch1, K10 and involucrin compared with that of HaCaT cells transfected with a control vector (Fig. 6A). These results suggest that overexpression of *Epfn* promotes the expression of keratinocyte differentiation markers.

E2F transcription factors activate genes that promote cell cycle progression, and therefore they can induce cell cycle re-entry. In addition, E2F1 forms a heterocomplex with Sp family transcription factors (Lin et al., 1996; Rotheneder et al., 1999). Therefore, we examined the interactions between Epfn and E2F1 by co-transfection of HaCaT cells with the VSV-G-tagged Epfn and HA-tagged E2F1 expression vectors. The extracts were immunoprecipitated with an anti-HA antibody and western blotted using anti-E2F1 and anti-VSV antibodies. We found that Epfn coprecipitated with E2F1 (Fig. 6B), suggesting that Epfn forms a protein complex with E2F1. We subsequently examined E2F transactivation activity using an E2F reporter construct in HaCaT cells transfected with the E2F1 and Epfn expression vectors (Fig. 6C). Epfn and E2F1 expression reduced E2F-mediated transcriptional activity, suggesting that Epfn inhibited E2F1 activity and cell cycle progression by sequestering the protein within an Epfn-E2F1 complex. Complex formation between Epfn and E2F1 when Epfn expression is elevated might explain why maturing transit amplifying cells exit the cell cycle after only a limited number of cell divisions (Fig. 7).

Epfn promotes *Notch1* transcription in HaCaT cell differentiation

Notch1 promotes keratinocyte differentiation (Rangarajan et al., 2001). It is therefore likely that Epfn overexpression promotes

HaCaT cell differentiation through Notch1 activation. To examine whether or not *Epfn* induces Notch signaling, we used an RBP-Jk (also known as RBPJ) reporter construct, because RBP-Jk is a DNA-binding factor and interacts with Notch as a canonical Notch target protein (de la Pompa et al., 1997). We found that Epfn transfection significantly increased RBP-Jk reporter activity (Fig. 6D), suggesting that the Epfn-mediated increase in *Notch1* mRNA led to the activation of Notch1 signaling. We further examined Epfn-induced *Notch1* transcription by assaying Epfn binding to the *Notch1* promoter using chromatin immunoprecipitation (ChIP) assays. The human *Notch1* promoter contains two distinct putative Sp-binding regions (Sp site A at -3.0 kb and Sp site B at -50 bp), both of which have multiple potential Sp-binding motifs upstream of the *Notch1* gene transcriptional start site (Fig. 6E, upper panel). ChIP analysis revealed that Epfn bound to Sp site A, but not to Sp site B (site B) (Fig. 6E, lower panel) or to other promoter regions (data not shown). Immunoprecipitation of nuclear extracts using a rabbit IgG antibody as a negative control resulted in no genomic amplification. These results suggest that Epfn overexpression promotes *Notch1* transcription and concomitant activation of Notch-dependent gene transcription cascades that are necessary for the differentiation of transit amplifying cells into keratinocytes (Fig. 7).

DISCUSSION

Our *in vivo* and cell culture results revealed that Epfn is essential for epidermal keratinocyte development by promoting both proliferation and differentiation of transit amplifying cells

through distinct mechanisms (Fig. 7). In the *Epf^{n-/-}* epidermis, p63 was expressed in multiple cell layers (Fig. 1Da,b) rather than in just a single basal layer of the normal epidermis. These p63-expressing basal-layer-like cells lost their rapid proliferation activity, which is a characteristic of transit amplifying cells. In addition, the expression of Notch1 and other differentiation marker genes was severely inhibited (Fig. 1Db,E; supplementary material Fig. S1).

Significant reductions in the number of BrdU-positive cells were observed, and weak PCNA-positive cells were found in the multi-cell layers, likely corresponding to p63-positive cells in the multi-cell layer in the *Epf^{n-/-}* epidermis (Fig. 2A,B). In addition, primary keratinocytes from *Epf^{n-/-}* epidermis showed reduced proliferation activity (Fig. 2C) and accumulated in the G1/G0 phase (Fig. 2D). Knockdown of endogenous *Epfⁿ* reduced the proliferation of HaCaT cells (Fig. 4B). These results suggest that *Epfⁿ* is necessary for rapid proliferation of transit amplifying cells. The proliferation of HaCaT cells by forced expression of *Epfⁿ* at a low level further supports the positive regulation of keratinocyte proliferation by *Epfⁿ*.

Rb binds to and inactivates members of the E2F transcription factor family that normally serve to promote cell cycle progression to S phase. Phosphorylation of Rb by CDKs releases E2F from the inactive E2F–Rb complex, allowing E2Fs to activate the genes that are necessary for cell cycle

progression (Hanahan, 2000; Harbour et al., 1999). *Epfⁿ* deficiency reduces Rb phosphorylation in keratinocytes (Fig. 2E) and dental epithelial cells (Nakamura et al., 2008), whereas *Epfⁿ* enhances Rb phosphorylation by forming a complex with CDK4, leading to the proliferation of HaCaT cells (Fig. 5C,D). Our data demonstrate the novel mechanism by which *Epfⁿ* regulates the G1/S transition and is therefore involved in the rapid phase of transit amplifying cell proliferation. The Rb proteins also participate in cell lineage specification during adipogenesis, cardiogenesis, hematopoiesis and myogenesis (Bergh et al., 1999; Classon et al., 2000; Li et al., 2000; Papadimou et al., 2005). In myogenesis, Rb and Sp1 (the most ubiquitously expressed Sp family protein) interact with tissue-specific transcription factors to regulate cell fate and muscle cell differentiation, suggesting that phosphorylation of Rb family proteins by the Sp family proteins might be a common mechanism for controlling progenitor proliferation (Guo et al., 2003). However, it is not clear whether *Epfⁿ* interacts with either Rb or Sp1 during interfollicular epidermal formation.

Lrig is a marker of epidermal stem cells and maintains stem cell quiescence by suppressing EGF–EGFR signaling in the basal epidermis (Jensen et al., 2009). Stem cells and transit amplifying cells express similar levels of EGFR and are equally exposed to EGF. Lrig1 or Lrig3 interacts with EGFR and leads to EGFR degradation through ubiquitylation (Gur et al., 2004; Laederich

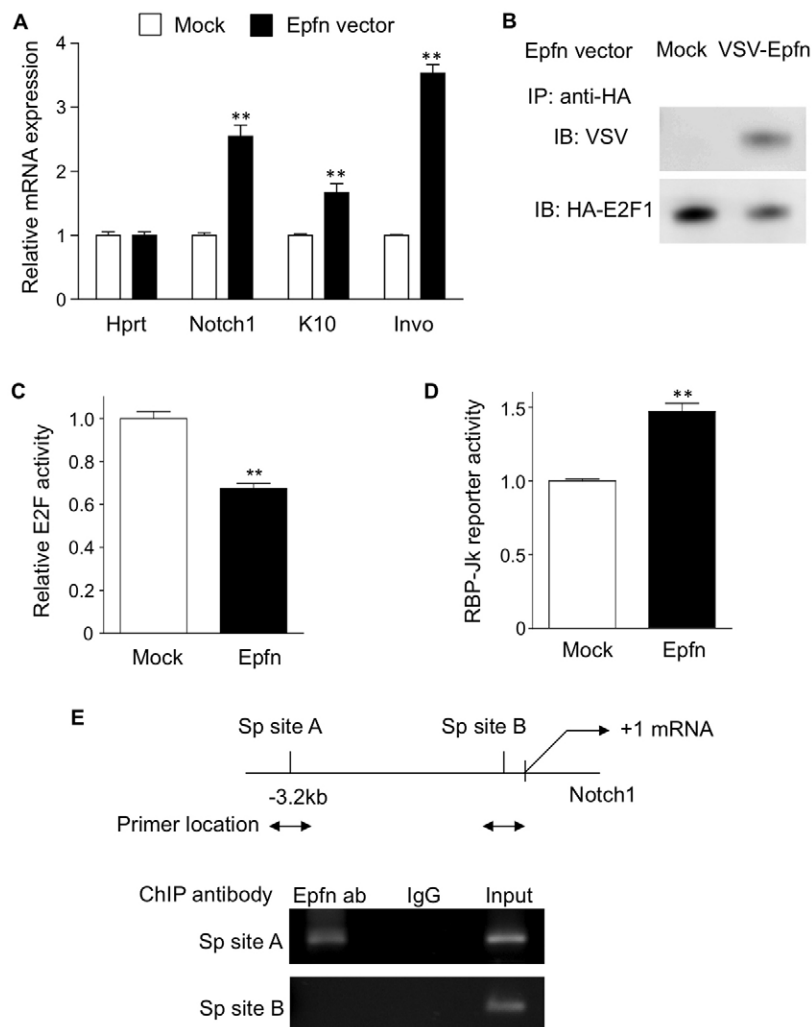


Fig. 6. *Epfn* promotes HaCaT cell differentiation through regulating Notch1.

(A) Expression of keratinocyte marker genes as revealed by qPCR in HaCaT cells transfected with either Mock or *Epfn* expression vectors. (B) Co-immunoprecipitation assay of *Epfn* and E2F1 in HaCaT cells transfected with E2F1 (HA tag) and *Epfn* (VSV tag) expression vectors. IP, immunoprecipitated; IB, immunoblotted. (C) E2F activity assay in HaCaT cells transfected with Mock and *Epfn* expression vectors. (D) RBP-Jk reporter activity in HaCaT cells transfected with either Mock or *Epfn* expression vectors. Quantitative data in A,C,D show the mean±s.e.m. (three independent experiments); ** $P < 0.01$. (E) ChIP analysis of the *Notch1* promoter using the anti-VSV antibody in HaCaT cells transfected with either Mock or *Epfn* (VSV tag) expression vectors. Upper panel, *in silico* prediction of potential Sp transcription factor binding sites (Sp sites A and B) in the human *Notch1* promoter. Lower panel, nuclear extracts from either VSV–*Epfn*- or Mock-transfected HaCaT cells were used for ChIP analysis with primer sets for Sp sites A and B. ab, antibody.

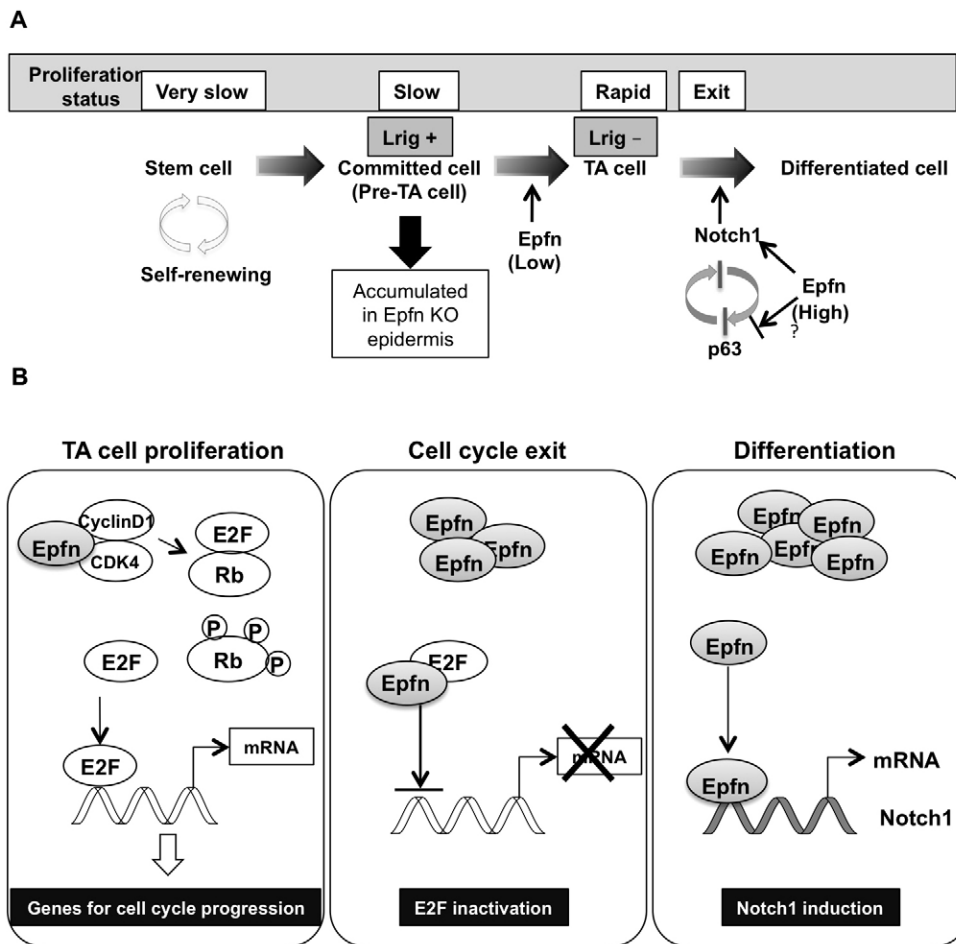


Fig. 7. Schematic diagram of Epfn roles in keratinocyte proliferation and differentiation. (A) Epfn promotes rapid transit amplifying (TA) cell proliferation. In the absence of Epfn, pre-TA cells accumulate in the epidermis. Pre-TA cells are committed to differentiation but retain some stem cell characteristics, such as slow proliferation in part due to elevated Lrig expression. As Epfn expression increases, Epfn promotes cell cycle exit and activates Notch1 expression, triggering differentiation. Epfn-induced Notch expression suppresses p63 expression. In addition, Epfn might directly inhibit p63 expression. Epfn regulates the p63 and Notch signaling pathways that are essential for epidermal development, maintenance and renewal. KO, knockout. (B) Multiple functions of Epfn during keratinocyte proliferation and differentiation. In transit amplifying cells, Epfn enhances Rb phosphorylation by interacting with CDK4 to promote proliferation. As Epfn expression increases, Epfn binds to E2F, which inhibits cell progression and promotes cell cycle exit by inhibiting E2F transactivation activity. Epfn also promotes keratinocyte differentiation by inducing *Notch1* transcription.

et al., 2004). The *Epfn*^{-/-} keratinocytes and *Epfn*-knockdown HaCaT cells showed increased Lrig expression and reduced EGF-mediated proliferation (Fig. 3A; supplementary material Fig. S2; Fig. 4C–E). These results reveal the crucial roles of Epfn in controlling the proliferation of transit amplifying cells, which it does by acting as both a positive regulator through Rb phosphorylation and a negative regulator by suppressing *Lrig* expression.

Epfn is expressed in the basal layer keratinocytes and proliferating HaCaT cells, and its expression levels are increased during the differentiation of keratinocytes and HaCaT cell differentiation. These expression patterns suggest that Epfn is involved in keratinocyte differentiation processes. Indeed, we found that *Epfn* transfection into HaCaT cells induced the expression of keratinocyte differentiation markers such as Notch1 and K10, even when cultured in a low-Ca²⁺ medium. Therefore, in addition to proliferation, Epfn is required for the differentiation of epidermal keratinocytes. This result is in agreement with our previous report on tooth development, in which we show that Epfn is crucial for both proliferation of the inner dental epithelium – an ameloblast progenitor similar to the transit amplifying cells of the basal layer epidermis – and its differentiation into the enamel-secreting ameloblast during amelogenesis (Nakamura et al., 2008).

The Notch signaling pathway regulates cell fate determination during the development of many tissues (Artavanis-Tsakonas et al., 1999; Blanpain et al., 2006; Boulter et al., 2012; Fuchs, 2008; Moriyama et al., 2008; Xiong et al., 2013). In the epidermis,

Notch1 signaling is involved in cell cycle exit and commitment to differentiation (Breunig et al., 2007; Georgia et al., 2006; Rangarajan et al., 2001). A conditional Notch1 deficiency in the basal epidermal layer of newborn mice results in the formation of multiple p63-expressing cell layers, delayed cell cycle exit and premature keratinocyte differentiation (Devgan et al., 2005; Rangarajan et al., 2001). In the *Epfn*^{-/-} epidermis, Notch1 expression was significantly reduced and multiple p63-expressing cell layers were formed (Fig. 1Db,E), similar to the Notch1-deficient epidermis. In addition, expression of the cell cycle inhibitor p21, one of the targets of Notch1 in keratinocytes (Devgan et al., 2005; Dotto, 2008; Nicolas et al., 2003; Rangarajan et al., 2001), was reduced in the *Epfn*^{-/-} epidermis and gave similar phenotypes to that of the Notch-deficient epidermis (Fig. 2E). Epfn induced Notch1 expression and promoted its transactivation activity as well as HaCaT cell differentiation (Fig. 6A,D). These results strongly suggest that Epfn is upstream of Notch1 and regulates *Notch1* expression. Epfn binds to the *Notch1* promoter, suggesting its involvement in the transcriptional activation of the *Notch1* gene (Fig. 6E). The expression of p63 and Notch is mutually exclusive because of a negative regulatory loop (Nguyen et al., 2006). Therefore, Epfn-induced Notch1 expression probably also suppresses p63 expression. Our results reveal that Epfn promotes the commitment to keratinocyte differentiation through the induction of *Notch1* transcription and promotion of the Notch1–p63 negative regulatory loop (Fig. 7).

In normal organogenesis, cell cycle exit is a prerequisite for differentiation. How does Epfn act as a positive regulator of both cell

proliferation and differentiation? One possible explanation for this apparent contradiction is that *Epf*n expression levels might regulate the switch from proliferation to differentiation. Low levels of *Epf*n expression promoted proliferation of HaCaT cells, whereas high levels did not (Fig. 5B). *Epf*n interacted with E2F and reduced E2F transactivation activity in HaCaT cells (Fig. 6B,C), suggesting that *Epf*n inhibits the DNA binding of E2F by sequestration in a multiprotein complex. The inhibition of E2F activity reduced the expression of genes required for cell cycle progression and promoted cell cycle exit. We propose that low *Epf*n levels increase the amount of free active E2F through Rb phosphorylation and concomitant release of E2F from E2F–Rb complexes (which results in proliferation), whereas high *Epf*n expression increases the formation of inactive E2F–*Epf*n complexes, resulting in cell cycle exit (Fig. 7B). In addition, post-translational modifications of *Epf*n, such as phosphorylation and sumoylation, might also be involved in this functional switch in *Epf*n activity from promoting proliferation to promoting differentiation.

In summary, our data provide evidence for a novel mechanism that sequentially regulates the proliferation and differentiation of transit amplifying cells through multiple distinct functions of *Epf*n as a cell cycle regulator and a transcription factor (Fig. 7B). The switch from proliferation to differentiation is regulated in part by the level of *Epf*n expression. Our *in vitro* and *in vivo* studies suggest that *Epf*n promotes rapid proliferation of transit amplifying cells by enhancing Rb phosphorylation and suppressing expression of the EGFR antagonist *Lrig1*, and that *Epf*n promotes keratinocyte differentiation by inducing *Notch1* transcription. Our findings suggest that *Epf*n regulates the p63–Notch axis in interfollicular epidermal formation. Thus, *Epf*n plays multiple roles in orchestrating keratinocyte proliferation and differentiation that are crucial to epidermal homeostasis and morphogenesis (Fig. 7).

MATERIALS AND METHODS

Mice and cells

*Epf*n^{−/−} mice were generated as described previously (Nakamura et al., 2008). The animal protocol was approved by the National Institute of Dental and Craniofacial Research (NIDCR) Animal Care and Use Committee. All mice were housed in an animal facility that was approved by the American Association for the Accreditation of Laboratory Animal Care.

Primary keratinocytes were prepared from newborn *Epf*n^{+/+}, *Epf*n^{+/-} and *Epf*n^{-/-} mice as described previously (Hennings et al., 1980), with minor modifications. Briefly, the mouse skin was floated on a layer of dispase (5 mg/ml) in Ca²⁺ and Mg²⁺-free PBS at 4°C overnight. The dermis and epidermis were separated and keratinocytes were collected by mincing the epidermis, placing it in 0.25% trypsin/0.5 mM EDTA and incubating for 15 min at 37°C. After resuspending in Ca²⁺ and Mg²⁺-free PBS, the keratinocytes were filtered through a 100- μ m cell strainer (BD Dickinson, Franklin Lakes, NJ) and centrifuged at 100 *g* for 10 min. The keratinocytes were cultured in a serum-free and low-Ca²⁺ keratinocyte growth medium (KGM) (Invitrogen, Carlsbad, CA).

HaCaT cells, a spontaneously transformed human epithelial cell line from adult skin (Boukamp et al., 1988), were obtained from Silvio Gutkind (NIDCR, NIH). Primary human keratinocytes and HEK293T cells were obtained from Life Technologies (Invitrogen, Carlsbad, CA). Primary human keratinocytes were cultured in Epilife™ medium (Invitrogen, Carlsbad, CA) and HEK293T cells were cultured in DMEM (Invitrogen, Carlsbad, CA).

Transfection and proliferation assay

For transfection, exponentially growing HaCaT cells were electroporated with the *Epf*n expression vector (*Epf*n-pcDNA3.1/myc-His vector; Nakamura et al., 2004) or Halo-tagged *Epf*n vectors (Promega,

Madison, WI) using Amaxa Nucleofector Solution V (Lonza, Walkersville, MD). For the gene knockdown studies, HaCaT cells or primary human keratinocytes were transfected with either the *Epf*n shRNA vector (ID: TRCN0000017806, Open Biosystems, Inc., Huntsville, AL) or control pLKO vector, and the resulting puromycin-resistant colonies were pooled and used for proliferation analysis. We also used another *Epf*n shRNA (ID: TRCN0000017807, Open Biosystems) and obtained similar results.

Cell proliferation was assessed using the Cell Counting Kit-8 containing WST-8, according to the manufacturer's manual (Dojindo Laboratories, Kumamoto, Japan). EGF (Sigma, St Louis, MO) was added to the cell cultures as a mitogenic cytokine (10 ng/ml). The cells were cultured in the absence or presence of EGF for three days and then the proliferation activities were analyzed. To test HaCaT cell proliferation associated with various *Epf*n expression levels, we prepared Halo-tag *Epf*n expression vectors driven by full-length CMV promoter or CMV promoters with various deletions (CMV, CMV-d1, -d2 and -d3) (Promega, Madison, WI). In these experiments, we used the reverse transfection method with Lipofectamine LTX reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The transfection mixtures for individual vectors and HaCaT cells (10,000 cells/well) were incubated with KGM. The cell proliferation assay was performed on days 0, 1 and 2 after the medium change to KGM. The protein expression of Halo-tagged *Epf*n in HaCaT cells was examined on day 1 by western blot analysis with anti-*Epf*n and anti- β -actin antibodies. For transfection of primary human keratinocytes, ViaFect™ (Promega, Madison, WI) was employed with the reverse-transfection method.

Tissue sections, immunohistochemistry and antibodies

Tissues were fixed with 4% paraformaldehyde in PBS at 4°C overnight. For histological analysis, sections were stained with Harris hematoxylin and eosin Y. For immunohistochemistry, sections were boiled with a targeted retrieval solution (Dako, Carpinteria, CA) and incubated in 1% bovine serum albumin (BSA) in PBS (blocking reagent) for 1 h before incubation with the primary antibody. We used antibodies against cytokeratin 10 (Sigma, St Louis, MO), cytokeratin 5, involucrin, filaggrin and loricrin (all from Covance, Berkeley, CA), p63 (BD Pharmingen, San Jose, CA), Notch1 (Cell Signaling Technology, Danvers, MA; BD Pharmingen, San Jose, CA), Hes1 (Millipore, Billerica, MA), PCNA (Zymed, Carlsbad, CA), Ki67 (Abcam, Cambridge, MA) and BrdU (Roche, Indianapolis, IN). The affinity-purified rabbit polyclonal antibody against *Epf*n was as described previously (Nakamura et al., 2008). Primary antibodies were detected with Cy-3-conjugated and Cy-5-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA). Nuclear staining was performed with Hoechst 33258 or DAPI (Sigma, St Louis, MO). All the immunopositive cells within the epidermis grid (600 μ m \times 600 μ m) were counted and the positive cell ratio was calculated by dividing by the total number of cells in the grid. To calculate the ratio, at least four independent regions were counted.

Western blotting, immunoprecipitation assay and kinase assay

Total cellular proteins were prepared from exponentially growing HaCaT cells cultured in KGM with either 0 mM, 0.7 mM or 1.2 mM Ca²⁺ using NP-40 lysis buffer with a protease inhibitor cocktail (Roche, Indianapolis, IN). Protein extracts were separated on 4–12% NuPAGE gels (Invitrogen, Carlsbad, CA), followed by western blotting. Kinase assays were performed using subconfluent 293T cells transfected with both HA-tagged CDK4 and *Epf*n expression vectors. Cells were washed in cold PBS and incubated on ice for 10 min with 500 μ l of immunoprecipitation buffer (20 mM HEPES pH 7.5, containing 10 mM EGTA, 40 mM β -glycerophosphate, 1 mM DTT and 1% NP-40), supplemented with a protease inhibitor cocktail (Roche, Indianapolis, IN) and 2 mM orthovanadate. Protein extracts were incubated with an anti-HA-antibody (Thermo Scientific, Rockford, IL) at 4°C for an additional 1 h. Immune complexes were pulled down with Protein-G–Sepharose beads and washed twice with immunoprecipitation buffer. Immune complexes on the beads were equilibrated with Rb kinase buffer (50 mM HEPES pH 7.5, containing 2.5 mM EGTA, 10 mM β -glycerophosphate, 10 mM MgCl₂,

5 mM MnCl₂, 1 mM DTT, 0.1 mM sodium orthovanadate, 1 mM NaF and 2 μCi of [γ -³²P]ATP). The beads were resuspended in Rb kinase buffer containing 2 μg of GST-Rb (Millipore, Billerica, MA) and were subsequently incubated at 30°C for 30 min. To reduce the background signal, 5 mM cold ATP was added to each reaction mixture. The reaction was stopped by adding SDS sample buffer. The samples were boiled for 5 min and resolved by SDS-10% PAGE. The gels were dried and visualized by using autoradiography on BioMAX MR film. The co-immunoprecipitation assay was performed with an HA-Tag Co-IP kit (Thermo Scientific, Rockford, IL) using subconfluent HaCaT cells transfected with both the HA-tagged E2F1 expression vector, which was kindly gifted from Kristian Helin (University of Copenhagen, Denmark), and the VSV-tagged E2F1 expression vector. The immune complexes on the beads were resuspended in lysis buffer and the samples were boiled for 5 min and resolved using NuPage 4–12% gradient gels (Invitrogen, Carlsbad, CA). Immunoblotting was performed with antibodies against phosphorylated Rb, Rb, CDK4 (Cell Signaling Technology, Danvers, MA), VSV (Sigma-Aldrich, St Louis, MO), E2F1 (Santa Cruz Biotechnology, Dallas, TX), GAPDH (Santa Cruz Biotechnology, Dallas, TX) and β -actin (Cell Signaling Technology, Danvers, MA).

BrdU and TUNEL assays

BrdU (100 mg/kg body weight) was intraperitoneally injected into P13 mice. The mice were euthanized 4 h later, and the skin was dissected and fixed with 4% paraformaldehyde. BrdU incorporation was visualized with diaminobenzidine (DAB) using the BrdU detection kit (Roche, Indianapolis, IN). TUNEL assays (Roche, Indianapolis, IN) were performed on paraffin-embedded skin sections from P3 and P7 mice. The sections were counterstained with hematoxylin. The ratios of the BrdU- and TUNEL-positive cells within the grid in the epidermis (600 μm×600 μm) were calculated by dividing by the total number of cells in the grid.

Flow cytometry

Primary keratinocytes (2×10⁶ cells) from either *Epfⁿ^{+/-}* or *Epfⁿ^{-/-}* mice were cultured for 3 days in type I collagen-coated dishes (BD Bioscience, Franklin Lakes, NJ) and washed three times in PBS, followed by fixation with ice-cold 70% ethanol for 30 min. After fixative removal, cells were centrifuged (100 g for 10 min), and the pellet was resuspended in 500 μl of propidium iodide solution (50 μg/ml in PBS) together with RNase A (10 mg/ml; Invitrogen, Carlsbad, CA) and incubated on ice for 30 min before cell cycle analysis using the FACSCalibur (BD Biosciences, Franklin Lakes, NJ).

Real-time quantitative PCR and microarray analysis

Primary keratinocytes or HaCaT cells were transiently transfected with E2F1 vectors. After 48 h, total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA). After DNase I (Sigma) treatment, 2 μg of total RNA was used for reverse transcription to generate cDNA, which was subsequently used as a template for PCR reactions with gene-specific primers (supplementary material Table S1). For expression analysis, real-time quantitative (q)PCR was performed using the 2XSYBR green supermixture (BioRad, Hercules, CA) and the Chromo4 thermocycler (MJ Research, Waltham, MA). For microarray analysis, total RNA was extracted from the primary keratinocytes of newborn *Epfⁿ^{+/+}* and *Epfⁿ^{-/-}* mice. Affymetrix mouse 430 2.0 array chips were used to analyze gene expression profiles. Data analysis was performed using Ingenuity Pathway Analysis software.

Luciferase assay

E2F and RBP-Jk activities were analyzed using the Cignal E2F and RBP-Jk Reporter (luc) Kit (Qiagen, Valencia, CA). The E2F and RBP-Jk reporter plasmids containing the control *Renilla* luciferase construct were transfected into HaCaT cells in a 48-well plate using Lipofectamine LTX with PLUS reagent. The activities of the firefly and *Renilla* luciferases were determined 48 h after transfection by using the dual luciferase reporter assay system (Promega, Madison,

WI) using a luminometer (Promega, Madison, WI). The luciferase activities were normalized for the *Renilla* luciferase activity of the internal control.

Notch1 promoter analysis and ChIP assay

A 4-kb human *Notch1* promoter sequence was analyzed with Genomatix software (Genomatix, Germany) to predict potential factor binding sites. Multiple Sp- or Klf-binding elements were predicted 3.2 kb upstream of the transcription start site and at the proximal promoter of the human *Notch1* gene. Chromatin DNA and nuclear protein complexes were extracted from the HaCaT cells transfected with His-tagged E2F1 or empty expression vectors, and ChIP assays were conducted with an anti-His antibody (Novagen, Madison, WI) and EZ ChIP kit (Millipore, Billerica, MA). ChIP primer sequences are shown in supplementary material Table S2 (Lefort et al., 2007).

Statistical analysis

The data were analyzed with Prism 4 software. Differences between groups were analyzed for statistical significance using Student's *t*-tests.

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Competing interests

The authors declare no competing interests.

Author contributions

T.N., S.F., and Y.Yamada conceived of the study. T.N. performed the majority of the experiments and analyzed the data, with some contributions from Y.Yoshitomi. and V.P. T.N., Y.Yoshitomi, V.P., K.S. and Y.Yamada interpreted the data. T.N. and Y.Yamada wrote the manuscript.

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Supplementary material

Supplementary material available online at <http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.156778/-DC1>

References

- Artavanis-Tsakonas, S., Rand, M. D. and Lake, R. J. (1999). Notch signaling: cell fate control and signal integration in development. *Science* **284**, 770–776.
- Bergh, G., Ehinger, M., Olsson, I., Jacobsen, S. E. and Gullberg, U. (1999). Involvement of the retinoblastoma protein in monocytic and neutrophilic lineage commitment of human bone marrow progenitor cells. *Blood* **94**, 1971–1978.
- Blanpain, C., Lowry, W. E., Pasolli, H. A. and Fuchs, E. (2006). Canonical notch signaling functions as a commitment switch in the epidermal lineage. *Genes Dev.* **20**, 3022–3035.
- Boukamp, P., Petrussevska, R. T., Breitkreutz, D., Hornung, J., Markham, A. and Fusenig, N. E. (1988). Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J. Cell Biol.* **106**, 761–771.
- Boulter, L., Govaere, O., Bird, T. G., Radulescu, S., Ramachandran, P., Pellicoro, A., Ridgway, R. A., Seo, S. S., Spee, B., Van Rooijen, N. et al. (2012). Macrophage-derived Wnt opposes Notch signaling to specify hepatic progenitor cell fate in chronic liver disease. *Nat. Med.* **18**, 572–579.
- Breunig, J. J., Silbereis, J., Vaccarino, F. M., Sestan, N. and Rakic, P. (2007). Notch regulates cell fate and dendrite morphology of newborn neurons in the postnatal dentate gyrus. *Proc. Natl. Acad. Sci. USA* **104**, 20558–20563.
- Burgesson, R. E. and Christiano, A. M. (1997). The dermal-epidermal junction. *Curr. Opin. Cell Biol.* **9**, 651–658.
- Cai, M., Han, L., Chen, R., Ye, F., Wang, B., Han, F., Lei, T. and Guo, D. (2009). Inhibition of LRRIG3 gene expression via RNA interference modulates the proliferation, cell cycle, cell apoptosis, adhesion and invasion of glioblastoma cell (GL15). *Cancer Lett.* **278**, 104–112.

- Classon, M., Kennedy, B. K., Mulloy, R. and Harlow, E. (2000). Opposing roles of pRB and p107 in adipocyte differentiation. *Proc. Natl. Acad. Sci. USA* **97**, 10826–10831.
- de la Pompa, J. L., Wakeham, A., Correia, K. M., Samper, E., Brown, S., Aguilera, R. J., Nakano, T., Honjo, T., Mak, T. W., Rossant, J. et al. (1997). Conservation of the Notch signalling pathway in mammalian neurogenesis. *Development* **124**, 1139–1148.
- Devgan, V., Mammucari, C., Millar, S. E., Briskin, C. and Dotto, G. P. (2005). p21WAF1/Cip1 is a negative transcriptional regulator of Wnt4 expression downstream of Notch1 activation. *Genes Dev.* **19**, 1485–1495.
- Dotto, G. P. (2008). Notch tumor suppressor function. *Oncogene* **27**, 5115–5123.
- Fuchs, E. (2008). Skin stem cells: rising to the surface. *J. Cell Biol.* **180**, 273–284.
- Georgia, S., Soliz, R., Li, M., Zhang, P. and Bhushan, A. (2006). p57 and Hes1 coordinate cell cycle exit with self-renewal of pancreatic progenitors. *Dev. Biol.* **298**, 22–31.
- Guo, C. S., Degnin, C., Fiddler, T. A., Stauffer, D. and Thayer, M. J. (2003). Regulation of MyoD activity and muscle cell differentiation by MDM2, pRb, and Sp1. *J. Biol. Chem.* **278**, 22615–22622.
- Gur, G., Rubin, C., Katz, M., Amit, I., Citri, A., Nilsson, J., Amariglio, N., Henriksson, R., Rechavi, G., Hedman, H. et al. (2004). LRI1 restricts growth factor signaling by enhancing receptor ubiquitylation and degradation. *EMBO J.* **23**, 3270–3281.
- Hanahan, D. (2000). Benefits of bad telomeres. *Nature* **406**, 573–574.
- Harbour, J. W., Luo, R. X., Dei Santi, A., Postigo, A. A. and Dean, D. C. (1999). Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1. *Cell* **98**, 859–869.
- Hennings, H., Michael, D., Cheng, C., Steinert, P., Holbrook, K. and Yuspa, S. H. (1980). Calcium regulation of growth and differentiation of mouse epidermal cells in culture. *Cell* **19**, 245–254.
- Hertveldt, V., Louryan, S., van Reeth, T., Drèze, P., van Vooren, P., Szpirer, J. and Szpirer, C. (2008). The development of several organs and appendages is impaired in mice lacking Sp6. *Dev. Dyn.* **237**, 883–892.
- Ibarretxe, G., Aurrekoetxea, M., Crende, O., Badiola, I., Jimenez-Rojo, L., Nakamura, T., Yamada, Y. and Unda, F. (2012). Epiprofin/Sp6 regulates Wnt-BMP signaling and the establishment of cellular junctions during the bell stage of tooth development. *Cell Tissue Res.* **350**, 95–107.
- Jensen, K. B. and Watt, F. M. (2006). Single-cell expression profiling of human epidermal stem and transit-amplifying cells: Lrig1 is a regulator of stem cell quiescence. *Proc. Natl. Acad. Sci. USA* **103**, 11958–11963.
- Jensen, K. B., Collins, C. A., Nascimento, E., Tan, D. W., Frye, M., Itami, S. and Watt, F. M. (2009). Lrig1 expression defines a distinct multipotent stem cell population in mammalian epidermis. *Cell Stem Cell* **4**, 427–439.
- Jones, P. H. and Watt, F. M. (1993). Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression. *Cell* **73**, 713–724.
- Koster, M. I., Kim, S., Mills, A. A., DeMayo, F. J. and Roop, D. R. (2004). p63 is the molecular switch for initiation of an epithelial stratification program. *Genes Dev.* **18**, 126–131.
- Kurata, S., Okuyama, T., Osada, M., Watanabe, T., Tomimori, Y., Sato, S., Iwai, A., Tsuji, T., Ikawa, Y. and Katoh, I. (2004). p51/p63 Controls subunit alpha3 of the major epidermis integrin anchoring the stem cells to the niche. *J. Biol. Chem.* **279**, 50069–50077.
- Laederich, M. B., Funes-Duran, M., Yen, L., Ingalla, E., Wu, X., Carraway, K. L., 3rd and Sweeney, C. (2004). The leucine-rich repeat protein LRI1 is a negative regulator of ErbB family receptor tyrosine kinases. *J. Biol. Chem.* **279**, 47050–47056.
- Lefort, K., Mandinova, A., Ostano, P., Kolev, V., Calpini, V., Kolfschoten, I., Devgan, V., Lieb, J., Raffoul, W., Hohl, D. et al. (2007). Notch1 is a p53 target gene involved in human keratinocyte tumor suppression through negative regulation of ROCK1/2 and MRCKalpha kinases. *Genes Dev.* **21**, 562–577.
- Li, F. Q., Coonrod, A. and Horwitz, M. (2000). Selection of a dominant negative retinoblastoma protein (RB) inhibiting satellite myoblast differentiation implies an indirect interaction between MyoD and RB. *Mol. Cell. Biol.* **20**, 5129–5139.
- Lin, S. Y., Black, A. R., Kostic, D., Pajovic, S., Hoover, C. N. and Azizkhan, J. C. (1996). Cell cycle-regulated association of E2F1 and Sp1 is related to their functional interaction. *Mol. Cell. Biol.* **16**, 1668–1675.
- Lowell, S., Jones, P., Le Roux, I., Dunne, J. and Watt, F. M. (2000). Stimulation of human epidermal differentiation by delta-notch signalling at the boundaries of stem-cell clusters. *Curr. Biol.* **10**, 491–500.
- Mills, A. A., Zheng, B., Wang, X. J., Vogel, H., Roop, D. R. and Bradley, A. (1999). p63 is a p53 homologue required for limb and epidermal morphogenesis. *Nature* **398**, 708–713.
- Moriyama, M., Durham, A. D., Moriyama, H., Hasegawa, K., Nishikawa, S., Radtke, F. and Osawa, M. (2008). Multiple roles of Notch signaling in the regulation of epidermal development. *Dev. Cell* **14**, 594–604.
- Muller, S., Lindquist, D., Kanter, L., Flores-Staino, C., Henriksson, R., Hedman, H. and Andersson, S. (2013). Expression of LRI1 and LRI3 correlates with human papillomavirus status and patient survival in cervical adenocarcinoma. *Int. J. Oncol.* **42**, 247–252.
- Nakamura, T., Unda, F., de-Vega, S., Vilaxa, A., Fukumoto, S., Yamada, K. M. and Yamada, Y. (2004). The Krüppel-like factor epiprofin is expressed by epithelium of developing teeth, hair follicles, and limb buds and promotes cell proliferation. *J. Biol. Chem.* **279**, 626–634.
- Nakamura, T., de Vega, S., Fukumoto, S., Jimenez, L., Unda, F. and Yamada, Y. (2008). Transcription factor epiprofin is essential for tooth morphogenesis by regulating epithelial cell fate and tooth number. *J. Biol. Chem.* **283**, 4825–4833.
- Nguyen, B. C., Lefort, K., Mandinova, A., Antonini, D., Devgan, V., Della Gatta, G., Koster, M. I., Zhang, Z., Wang, J., Tommasi di Vignano, A. et al. (2006). Cross-regulation between Notch and p63 in keratinocyte commitment to differentiation. *Genes Dev.* **20**, 1028–1042.
- Nicolas, M., Wolfer, A., Raj, K., Kummer, J. A., Mill, P., van Noort, M., Hui, C. C., Clevers, H., Dotto, G. P. and Radtke, F. (2003). Notch1 functions as a tumor suppressor in mouse skin. *Nat. Genet.* **33**, 416–421.
- Papadimou, E., Ménard, C., Grey, C. and Pucéat, M. (2005). Interplay between the retinoblastoma protein and LEK1 specifies stem cells toward the cardiac lineage. *EMBO J.* **24**, 1750–1761.
- Pellegrini, G., Dellambra, E., Golisano, O., Martinelli, E., Fantozzi, I., Bondanza, S., Ponzin, D., McKeon, F. and De Luca, M. (2001). p63 identifies keratinocyte stem cells. *Proc. Natl. Acad. Sci. USA* **98**, 3156–3161.
- Powell, A. E., Wang, Y., Li, Y., Poulin, E. J., Means, A. L., Washington, M. K., Higginbotham, J. N., Juchheim, A., Prasad, N., Levy, S. E. et al. (2012). The pan-ErbB negative regulator Lrig1 is an intestinal stem cell marker that functions as a tumor suppressor. *Cell* **149**, 146–158.
- Rangarajan, A., Talora, C., Okuyama, R., Nicolas, M., Mammucari, C., Oh, H., Aster, J. C., Krishna, S., Metzger, D., Chambon, P. et al. (2001). Notch signaling is a direct determinant of keratinocyte growth arrest and entry into differentiation. *EMBO J.* **20**, 3427–3436.
- Romano, R. A., Smalley, K., Magraw, C., Serna, V. A., Kurita, T., Raghavan, S. and Sinha, S. (2012). ΔNp63 knockout mice reveal its indispensable role as a master regulator of epithelial development and differentiation. *Development* **139**, 772–782.
- Rondahl, V., Holmlund, C., Karlsson, T., Wang, B., Faraz, M., Henriksson, R. and Hedman, H. (2013). Lrig2-deficient mice are protected against PDGFB-induced glioma. *PLoS ONE* **8**, e73635.
- Rotheder, H., Geymayer, S. and Haidweiger, E. (1999). Transcription factors of the Sp1 family: interaction with E2F and regulation of the murine thymidine kinase promoter. *J. Mol. Biol.* **293**, 1005–1015.
- Ruiz, S., Santos, M., Segrelles, C., Leis, H., Jorcano, J. L., Berns, A., Paramio, J. M. and Vooijs, M. (2004). Unique and overlapping functions of pRb and p107 in the control of proliferation and differentiation in epidermis. *Development* **131**, 2737–2748.
- Suske, G., Bruford, E. and Philipson, S. (2005). Mammalian SP/KLF transcription factors: bring in the family. *Genomics* **85**, 551–556.
- Suzuki, Y., Miura, H., Tanemura, A., Kobayashi, K., Kondoh, G., Sano, S., Ozawa, K., Inui, S., Nakata, A., Takagi, T. et al. (2002). Targeted disruption of LIG-1 gene results in psoriasisiform epidermal hyperplasia. *FEBS Lett.* **521**, 67–71.
- Talamillo, A., Delgado, I., Nakamura, T., de-Vega, S., Yoshitomi, Y., Unda, F., Birchsmeier, W., Yamada, Y. and Ros, M. A. (2010). Role of Epiprofin, a zinc-finger transcription factor, in limb development. *Dev. Biol.* **337**, 363–374.
- Truong, A. B., Kretz, M., Ridky, T. W., Kimmel, R. and Khavari, P. A. (2006). p63 regulates proliferation and differentiation of developmentally mature keratinocytes. *Genes Dev.* **20**, 3185–3197.
- Watt, F. M. and Jensen, K. B. (2009). Epidermal stem cell diversity and quiescence. *EMBO Mol. Med.* **1**, 260–267.
- Wong, V. W., Stange, D. E., Page, M. E., Buczacki, S., Wabik, A., Itami, S., van de Wetering, M., Poulosom, R., Wright, N. A., Trotter, M. W. et al. (2012). Lrig1 controls intestinal stem-cell homeostasis by negative regulation of ErbB signalling. *Nat. Cell Biol.* **14**, 401–408.
- Xiong, W., Morillo, S. A. and Rebay, I. (2013). The Abelson tyrosine kinase regulates Notch endocytosis and signaling to maintain neuronal cell fate in *Drosophila* photoreceptors. *Development* **140**, 176–184.
- Yang, A., Schweitzer, R., Sun, D., Kaghad, M., Walker, N., Bronson, R. T., Tabin, C., Sharpe, A., Caput, D., Crum, C. et al. (1999). p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature* **398**, 714–718.