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IKKα Is Required to Maintain Skin Homeostasis and Prevent Skin Cancer

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SUMMARY

It has long been known that excessive mitotic activity due to H-Ras can block keratinocyte differentiation and cause skin cancer. It is not clear whether there are any innate surveillants that are able to ensure that keratinocytes undergo terminal differentiation, preventing the disease. IKKα induces keratinocyte terminal differentiation, and its downregulation promotes skin tumor development. However, its intrinsic function in skin cancer is unknown. Here, we found that mice with IKKα deletion in keratinocytes develop a thickened epidermis and spontaneous squamous cell-like carcinomas. Inactivation of epidermal growth factor receptor (EGFR) or reintroduction of IKKα inhibits excessive mitosis, induces terminal differentiation, and prevents skin cancer through repressing an EGFR-driven autocrine loop. Thus, IKKα serves as an innate surveillant.

INTRODUCTION

Keratinocytes constitute the stratified epidermis, the largest and most rapidly renewing organ in the body (Blanpain et al., 2007). Keratinocytes in the basal epidermis are mitotic, providing new cells to replace those that are shed. After moving to the suprabasal layers, the cells gradually differentiate and give rise to the tough, soft cornified layer at the surface of the skin that protects the internal organs. Each cell renewal cycle takes approximately 4 weeks. Stem cells in the epidermis and hair follicles also provide cells needed in case of "emergent cell loss," such as injury. Therefore, a balance between keratinocyte proliferation and differentiation is required to maintain epidermal homeostasis. Numerous extrinsic or intrinsic factors can trigger keratinocytes to produce a broad spectrum of growth factors and cytokines, influencing their differentiation and proliferation. Maintaining proper cellular responses to various stimuli is therefore a prerequisite for preventing skin disorders.

The mitogenic signaling cascade from epidermal growth factor receptor (EGFR), Ras, and extracellular signal-regulated kinase (ERK) to transcription factors, activating gene expression of growth factors including EGFR ligands, has been implicated in the regulation of keratinocyte proliferation and differentiation (Dlugosz et al., 1997). More than two decades ago, Yuspa and colleagues (1985) demonstrated that oncogenic v-H-Ras induces keratinocyte proliferation and transformation but blocks terminal differentiation. The pathology of Ras-induced keratinocyte-derived cancer resembles human squamous cell carcinomas (SCCs). The chemical carcinogen 7,12-dimethylbenz[*a*]anthracene (DMBA) activates H-Ras to initiate skin tumors (Balmain and Pragnell, 1983). The activating *Ras* mutations and elevated Ras-related pathways, as well as elevated activity of EGFR and its ligands, have also been seen in human SCCs (Leong et al., 2004; Pierceall et al., 1991). Thus, elevated activity along this cascade provides the molecular basis for the promotion of keratinocyte proliferation, dedifferentiation, and transformation. On the other hand, inactivation of EGFR has been found to inhibit Ras-mediated keratinocyte proliferation and to promote differentiation in vitro, as well as to prevent skin tumors induced by overexpression of a dominant form of son of

SIGNIFICANCE

Squamous cell carcinoma (SCC) derived from epithelial cells in many organs is one of the most common malignancies in humans. Downregulation of IKKα has been reported in SCCs of the skin, esophagus, lungs, and head and neck in humans, highlighting the importance of IKKα in human cancers. Although a reduction in IKKα expression promotes chemical carcinogen-induced SCCs, it is unknown whether IKKα deletion can cause skin SCCs. Here we show that IKKα deletion in keratinocytes induces skin SCCs in mice through upregulating transcription of EGFR ligands and ligand activators, thus activating the EGFR-driven pathway. Inactivation of EGFR prevents the development of epidermal hyperplasia and tumors. These findings shed light on therapeutic targets for preventing IKKα-related SCC development.

sevenless (SOS-F), a guanine nucleotide exchange factor that facilitates the GDP-GTP exchange of Ras protein (Sibilia et al., 2000). Thus, EGFR may serve as a switch point for keratinocyte proliferation and differentiation. However, it is unknown whether there are any surveillants that can antagonize the cascade activity to balance keratinocyte proliferation and differentiation.

Gene disruption studies have demonstrated that the epidermis of *Ikkα*$^{-/-}$ newborn mice lacks a terminally differentiated cornified layer and exhibits marked thickening; these mice die soon after birth (Hu et al., 1999; Takeda et al., 1999). Reintroduction of IKKα or kinase-inactive IKKα induces keratinocyte terminal differentiation and represses hyperproliferation in vitro and in vivo (Hu et al., 2001; Sil et al., 2004). These findings underscore a pivotal role for IKKα in regulating keratinocyte proliferation and differentiation. Furthermore, our recent studies have shown that reduced IKKα expression provides a selective growth advantage that cooperates with Ras activity to promote the formation of benign and malignant skin tumors induced by DMBA/12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Park et al., 2007). Loss of heterozygosity, a classical tumor suppressor feature, is found in most carcinomas in *Ikkα*$^{+/-}$ mice, and somatic *Ikkα* mutations are detected in *Ikkα*$^{+/+}$ carcinomas. Also, *Ikkα* genetic alterations and epigenetically downregulated IKKα expression have been reported to be associated with dedifferentiation, invasion, and progression of human SCCs (Liu et al., 2006; Maeda et al., 2007). Thus, IKKα may regulate mitogenic activity and Ras activity in keratinocyte proliferation, differentiation, and skin tumor development.

Differentiation is thought to require the withdrawal of keratinocytes from the cell cycle (Dlugosz et al., 1997; Gandarillas and Watt, 1997). Activated Ras has been suggested to increase the S phase, so that the enhanced cell progression is able to block the exit of keratinocytes from the cell cycle, thereby preventing differentiation (Dlugosz et al., 1997). We previously reported that there are more bromodeoxyuridine (BrdU)-labeled keratinocytes, an indicator of S phase, in IKKα null than in wild-type (WT) mouse epidermis (Hu et al., 1999). An elevated S phase and a reduced G2/M phase are detected in primary cultured, undifferentiated *Ikkα*$^{-/-}$ keratinocytes (Hu et al., 2001; Zhu et al., 2007). The re-expression of IKKα rescues these cell-cycle defects. Thus, IKKα-loss-enhanced cell progression may prevent keratinocyte terminal differentiation by utilizing this cell-cycle mechanism. However, the pathway that IKKα uses to regulate this switch between keratinocyte proliferation and differentiation remains to be defined.

IKKα, IKKβ, and IKKγ (NEMO) form the IKK complex that activates NF-κB by phosphorylating IκBs, the inhibitors of NF-κB (Ghosh and Karin, 2002). Although IKKα and IKKβ are highly conserved protein kinases, IKKβ shows a stronger kinase activity for IκBs than IKKα does. Mice with deletion of IKKβ or IκBα in the basal epidermis develop tumor necrosis factor receptor (TNFR)-dependent skin inflammation, and mice overexpressing IκBα in the basal epidermis develop TNFR-dependent skin tumors (Lind et al., 2004; Pasparakis et al., 2002; Rebholz et al., 2007). However, mice overexpressing IKKα in the basal or suprabasal epidermis have normal skin and develop fewer SCCs and metastases induced by DMBA/TPA than WT mice do (Liu et al., 2006; Sil et al., 2004). Elevated IKKα expression has been found to enhance terminal differentiation and antagonize chemical carcinogen-induced mitogenic and angiogenic activities. Conversely, reduced IKKα expression results in elevated mitogenic and angiogenic activities (Park et al., 2007). Thus, these physiological functions of IKKα in the skin of mice extend beyond NF-κB signaling alone.

However, a study has suggested that IKKα-mediated keratinocyte differentiation is not cell autonomous (Gareus et al., 2007), which is inconsistent with the phenotypes of IKKα-deficient mice and keratinocytes described above. Also, the function of IKKα in adult mice has not been revealed. Thus, we generated mice with IKKα deletion in keratinocytes and investigated the role of IKKα in skin homeostasis and skin cancer development.

RESULTS

IKKα Deletion in Keratinocytes Causes Thickened and Wrinkled Skin in Mice

To determine the function of IKKα in the skin of mice, we generated mice with a floxed *Ikkα* allele (*Ikkα*$^{F/F}$) (see Figures S1A–S1D available online). Genotyping confirmed that the number of *Ikkα*$^{F/F}$ homozygotes matched the expected Mendelian ratio. To study the role of IKKα in the epidermis, we crossed *Ikkα*$^{F/F}$ mice with mice expressing Cre under the control of the keratin 5 (K5) promoter, which is expressed in basal epidermal keratinocytes, to generate *Ikkα*$^{F/F}$/K5.Cre mice. It has been documented that K5.Cre or K14.Cre is expressed in oocytes of female mice, which leads to germline mutations (Hafner et al., 2004; Ramirez et al., 2004). The expression of paternally transmitted K5.Cre was reported in the mouse epidermis at embryonic day 15.5. As expected, the appearance, skins, and skeletons of *Ikkα*$^{F/F}$/K5.Cre mice generated from male *Ikkα*$^{F/F}$ and female *Ikkα*$^{F/+}$/K5.Cre mice were identical to those of *Ikkα*$^{-/-}$ mice (Figure 1A; Figures S2 and S3) (Hu et al., 1999; Takeda et al., 1999). Western blotting showed no IKKα expression in multiple organs obtained from *Ikkα*$^{F/F}$/K5.Cre mice (Figure 1B). *Ikkα*$^{F/F}$/K5.Cre newborns generated from female *Ikkα*$^{F/F}$ and male *Ikkα*$^{F/+}$/K5.Cre mice were indistinguishable from WT (*Ikkα*$^{F/F}$) mice (P1, Figure 1C). By day 5, however, the skin of *Ikkα*$^{F/F}$/K5.Cre mice was noticeably more wrinkled and thicker, and mice gradually showed retarded development (P11, Figure 1C). Most mutant mice died between 12 to 16 days after birth; a few survived longer, but no longer than 22 days. No food was found in the stomachs of the dead mice (Figure S4A), and the esophagus was smaller in mutant mice than in WT mice (Figure S4B). A similar phenotype has been described in *Ikkα*$^{-/-}$ mice, which might be relevant to the mouse deaths (Hu et al., 1999; Sil et al., 2004). Western blotting detected a low level of IKKα in the epidermis of mutant mice at day 1 after birth, and a further reduction of IKKα expression was observed later (Figure 1D), which was consistent with the loss of the WT *Ikkα* allele in DNA isolated from the epidermis of the mice (Figure S5). IKKα expression was reduced in the tongue, esophagus, and intestine of *Ikkα*$^{F/F}$/K5.Cre mice but was normal in the dermis, liver, lung, spleen (B cells), stomach, thymus (T cells), bone, brain, heart, kidney, and bone marrow and blood cells (hematopoietic cells) in *Ikkα*$^{F/F}$/K5.Cre mice compared with WT mice (Figure 1E). The level of IKKα expression depended on whether any *Ikkα* alleles remained (Figure 1E). These results indicate that K5.Cre deletes IKKα specifically in the cells that express K5 in *Ikkα*$^{F/F}$ mice. To evaluate the effect of mouse genetic backgrounds on these phenotypes, we backcrossed *Ikkα*$^{F/F}$ and K5.Cre mice with C57BL/6 or FVB mice for five generations. An

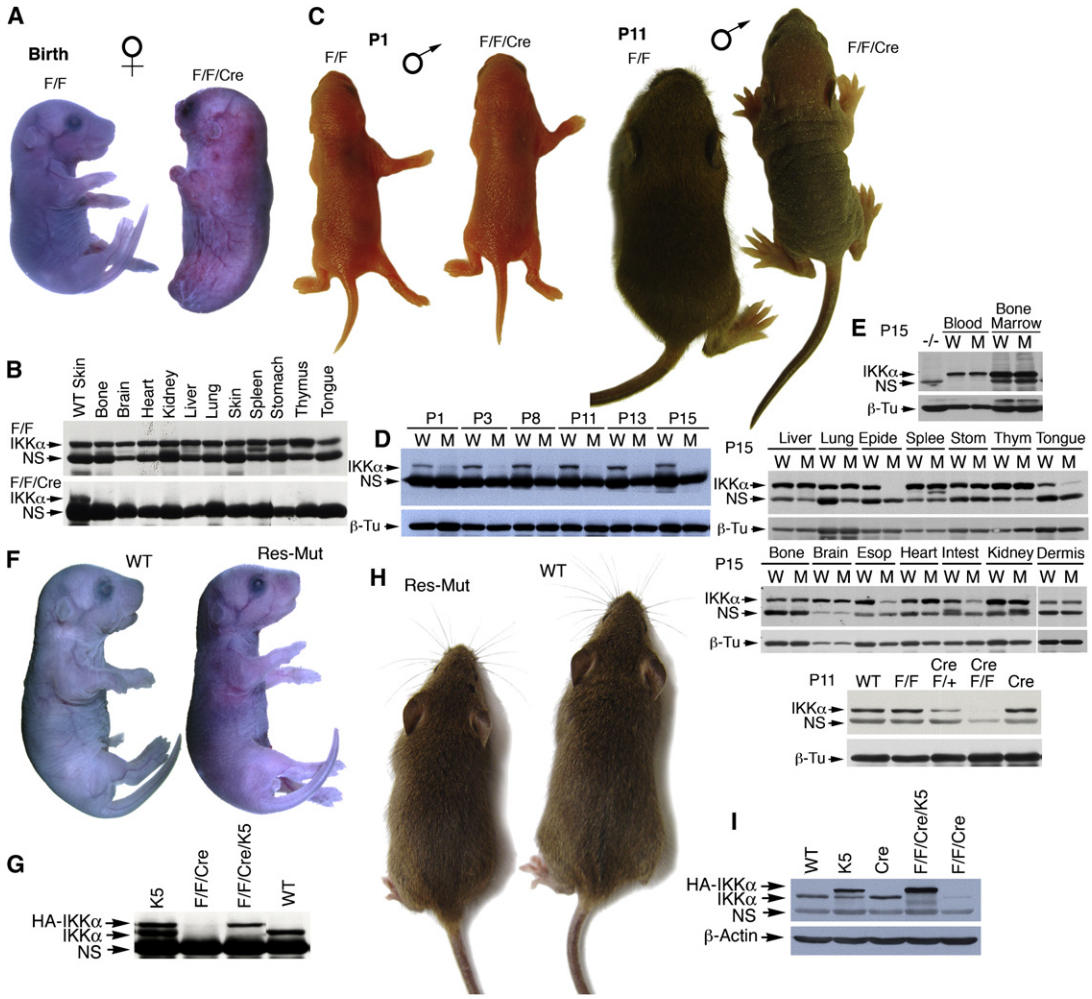

Figure 1. IKKα Deletion in Keratinocytes Causes Skin Defects in Postnatal Mice

(A) Appearance of *Ikkα*$^{F/F}$ (F/F) and *Ikkα*$^{F/F}$/K5.Cre (F/F/Cre) mice at birth. The female symbol indicates maternally transmitted K5.Cre.

(B) IKKα levels in different organs of mice in (A), as detected by western blotting. NS, nonspecific band.

(C) Appearance of *Ikkα*$^{F/F}$ (F/F) and *Ikkα*$^{F/F}$/K5.Cre (F/F/Cre) mice at postnatal days 1 and 11 (P1 and P11). The male symbol indicates paternally transmitted K5.Cre.

(D and E) IKKα levels in the epidermis of *Ikkα*$^{F/F}$ (W) and *Ikkα*$^{F/F}$/K5.Cre (M) mice in (C), as detected by western blotting. NS, nonspecific bands; β-Tu, β-tubulin as loading control; Blood, blood cells; Epide, epidermis; Splee, spleen; Stom, stomach; Thym, thymus; Esop, esophagus; Intest, intestine; WT, wild-type.

(F) Appearance of WT and maternally transmitted *Ikkα*$^{F/F}$/K5.Cre/K5 (Res-Mut) newborns.

(G) IKKα levels in the skins of mice in (F), as detected by western blotting. K5, K5.IKKα transgene; HA-IKKα, HA-tagged IKKα transgene expression.

(H) Appearance of WT and paternally transmitted *Ikkα*$^{F/F}$/K5.Cre/K5 (Res-Mut) mice at 4 weeks of age.

(I) IKKα levels in the epidermis of mice in (H), as detected by western blotting. β-actin was used as a loading control.

FVB background was found to confer a slight increase in skin thickness and slightly earlier death compared to a C57BL/6 background (data not shown). Collectively, these results suggest that a low level of IKKα in the epidermis is sufficient for normal embryonic skin development but that a further reduction causes skin lesions.

To verify that these phenotypes in *Ikkα*$^{F/F}$/K5.Cre mice were due to an IKKα-specific deletion in keratinocytes, we re-expressed a keratinocyte-specific K5.IKKα transgene in *Ikkα*$^{F/F}$/K5.Cre mice (Lomada et al., 2007). K5.IKKα expression rescued the skin phenotype of maternally transmitted *Ikkα*$^{F/F}$/K5.Cre mice (Figures 1F and 1G; Figure S6A), but the rescued mice died soon after birth, which was consistent with observations in previous reports (Lomada et al., 2007; Sil et al., 2004). K5.IKKα expression completely rescued paternally transmitted *Ikkα*$^{F/F}$/K5.Cre mice (Figures 1H and 1I; Figure S6B). No skin disorders were found in adult *Ikkα*$^{F/F}$/K5.Cre/K5.IKKα mice. Thus, IKKα deletion causes the skin phenotypes in *Ikkα*$^{F/F}$/K5.Cre mice.

IKKα Deletion Causes Epidermal Keratinocyte Hyperproliferation and Deregulates Expression of Many Genes

Histologies of the skins from WT and *Ikkα*$^{F/F}$/K5.Cre mice were almost identical on days 1 and 3 (Figure 2A), which reflected their normal appearances. From days 6 to 22, the epidermal thickness in mutants gradually increased (Figure 2A). The total

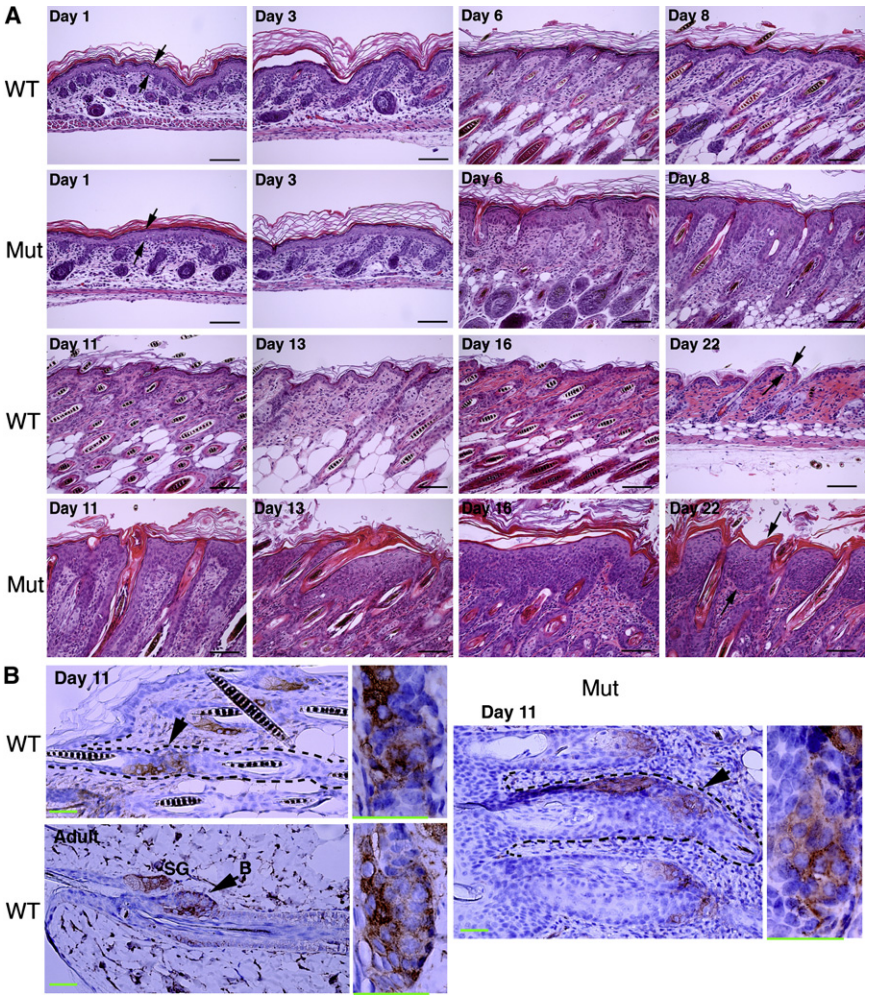

Figure 2. IKKα Deletion Causes Epidermal Hyperplasia

(A) Histology of mouse skins stained with hematoxylin and eosin (H&E). Day, postnatal day; WT, wild-type mice; Mut, *Ikkα*$^{F/F}$/K5.Cre mice. Arrows indicate epidermis. Scale bars = 30 μm.

(B) Brown indicates CD34-positive cells (cells indicated by arrows are shown enlarged in boxes at right); blue indicates nuclear counterstaining. Adult sample is shown as a control for CD34-positive cells in the bulge (B). SG, sebaceous gland. Scale bars = 30 μm.

number of keratinocytes in the epidermis was significantly higher in mutants than in WT mice. Immunohistochemical staining showed that the entire mutant epidermis expressed K5 and gradually reduced K10 with increased epidermal thickness (Figure S7). The thickened mutant epidermis did not express the terminal differentiation markers loricin and filaggrin, although the markers were weakly detected on the surface of the skin (Figure S7). These results suggest that hyperproliferation may prevent terminal differentiation in keratinocytes. We also examined CD34-positive keratinocytes, which have been identified as follicular stem cells (Blanpain et al., 2004; Morris et al., 2004). Hair follicles are still developing in mice at age 1 to 16 days; thus, the hair follicle of an adult mouse was used as a control for CD34-positive cells in the hair follicle (Figure 2B). Immunohistochemical staining showed that the number of CD34-positive keratinocytes was similar in hair follicles of WT and mutant mice, although the total number of keratinocytes was greater in mutant hair follicles than in WT hair follicles (Figure 2B; Figure S7), suggesting that IKKα deletion may have a stronger impact on proliferation of CD34-negative keratinocytes than on CD34-positive keratinocytes.

Mice with keratinocyte-specific IKKα deletion by K14.Cre generated in a previous study died at birth (Gareus et al., 2007). Although it is difficult to speculate on the observations of Gareus et al. (2007) because the authors did not describe in detail how they generated their mice, we attempted to clarify the differences by generating *Ikkα*$^{F/F}$/K14.Cre mice. Paternally transmitted *Ikkα*$^{F/F}$/K14.Cre and *Ikkα*$^{F/F}$/K5.Cre mice showed similar skin phenotypes (Figure S8A). Some of the maternally transmitted *Ikkα*$^{F/F}$/K14.Cre mice recapitulated the phenotype of *Ikkα*$^{-/-}$ mice (Figure S8B), and others developed epidermal hyperplasia after birth (data not shown). Ramirez et al. (2004) observed a partial gene deletion pattern in oocytes in some cases by Cre, which might explain these different phenotypes.

Next, we determined mitotic activity in the epidermis using immunohistochemical staining. We found that the proliferation marker Ki67 was significantly more abundant in *Ikkα*$^{F/F}$/K5.Cre epidermis than in WT epidermis (Figure 3A). BrdU-positive cells were consistently more numerous in mutant epidermis than in WT epidermis (Figures S9A and S9B), and expression of K6, which is highly elevated in abnormally proliferating epidermis, was elevated in mutant epidermis (Figure 3A). The abnormally proliferating epidermis may promote dermal cell proliferation or attract macrophages or lymphocytes. Thus, we found a slightly increase in macrophages (F4/80) but no increase in T (CD3) or B (CD45R) cells (Pasparakis et al., 2002) in the stroma of 6-day-old mutants (Figure S10A). Also, TNFα expression was higher in IKKα null than in WT keratinocytes (Figure S10B).

To determine whether the epidermal hyperplasia is intrinsic to IKKα-deficient keratinocytes, we cultured keratinocytes isolated from *Ikkα*$^{F/F}$/K5.Cre and WT newborns. Cell growth curves showed that mutant cells grew more rapidly than WT cells (Figure 3B). WT keratinocytes had terminally differentiated morphologies (Hu et al., 2001), whereas mutant cells did not (Figure 3C). Also, mutant cells formed many more and larger colonies than WT cells did (Figure 3D). Thus, IKKα deletion induces keratinocyte-autonomous hyperproliferation.

To determine which signaling pathways may promote cell proliferation, we compared the transcriptional profiles of genes in WT and mutant keratinocytes using microarray analyses

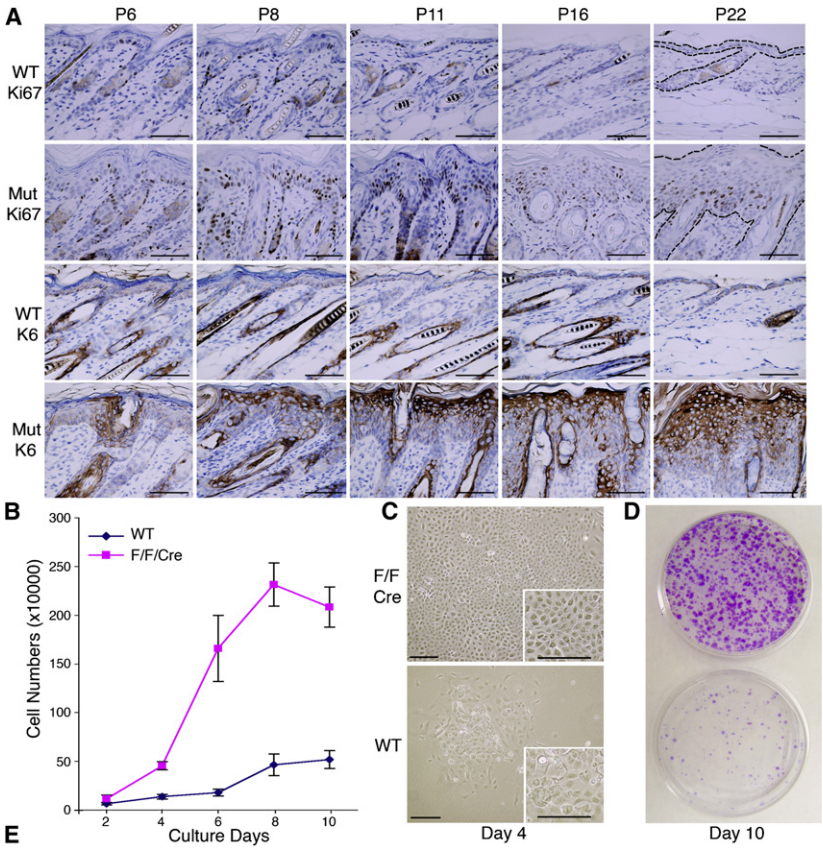

Figure 3. IKKα Deletion Causes Keratinocyte Hyperproliferation and Deregulates Expression of Many Genes

(A) Immunohistochemistry of skin sections stained for Ki67 and K6. P, postnatal day; WT, wild-type; Mut, *Ikkα*$^{F/F}$/K5.Cre. Dark brown color indicates positive cells; blue color indicates nuclear counterstaining. Scale bars = 50 μm.

(B) Keratinocyte growth curve. Equal numbers of cells from newborns were plated in cultures, and cell growth was statistically analyzed at the indicated days (n = 8). Error bars indicate ±SD.

(C) Keratinocyte morphologies in culture at day 4. Scale bars = 50 μm.

(D) Keratinocytes colony formation at day 10. Cell colonies were stained with 0.5% crystal violet solution.

(E) Summary of microarray analysis results for cultured keratinocytes. Gene upregulation and downregulation are in *Ikkα*$^{F/F}$/K5.Cre cells compared to WT cells. References for each gene's functions can be found by searching the NCBI Entrez Nucleotide database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=nuccore&itool=toolbar) using the "Systematic Name" entry in Table S1.

Genes Upregulated

Mitogenic Pathways

Egfr (AK049452/5×), Megf6 (BC039980/6×), Egf19 (NM_207666/3×), Egf17 (NM_178444/2×), Epgn (NM_053087/3×)

Braf (AK036798/2×), Fgf13 (NM_010200/6×), Igf2 (NM_01514/2×), Pdgfα (NM_008808/3×), Pdgfrβ (NM_008809/2×)

Integrin (Itg)

Itgβl1 (NM_145467/3×), Itgβ3 (NM_016780/2×), Itgα5 (NM_010577/2×), Itgα6 (AK045391/4×), Itgα7 (L23423/2×), Itgα11 (NM_176922/2×), Itgβ4 (NM_008484/4×)

A disintegrin and metalloprotease (Adam)/matrix metallopeptidase (Mmp)

Adam19 (NM_009616/3×), Mmp10 (NM_019471/2×), Mmp9 (NM_013599/2×)

Notch Pathway

Jag2 (NM_010588/4×), Jag1 (NM_013822/3×), Dll1 (NM_007865/4×)

Wnt Pathway

Wnt2 (NM_023653/7×), Wnt3α (NM_009522/8×), Wnt6 (NM_009526/3×), Wnt10β (NM_011718/3×), Fzd10 (NM_175284/2×), Dkk3 (NM_015814/3×), Dkk2 (NM_020265/2×)

Genes Downregulated

Wnt7α (NM_009528/-3×), Notch3 (NM_008716/-3×)

Claudin (Cldn)/Tight junctions/Tissue barriers

Cldn1 (NM_016674/-11×), Cldn3 (NM_009902/-17×), Cldn4 (NM_009903/-16×), Cldn6 (NM_018777/-5×), Cldn7 (NM_016887/-24×), Cldn23 (NM_027998/-23×)

(Table S1). As shown in Figure 3E, upregulated genes in mutants included those encoding EGFR, EGFR ligands, Braf, FGF13, IGF2, and PDGFα; a group of integrin genes; ADAM19, MMP10, and MMP9; Notch ligands; the Wnt inhibitors Dkk3 and Dkk2; and various Wnts. Downregulated genes included those encoding Wnt7α, Notch3, and a group of claudins (Figure 3E; Table S1). Such a broad collection of deregulated genes indicates that IKKα may have a broad impact on skin biology.

IKKα Downregulates an Autocrine Loop of EGFR, ERK, EGFR Ligands, and a Group of ADAMs in the Epidermis and in Keratinocytes

To determine the signaling pathways responsible for hyperproliferation and dedifferentiation in IKKα-deficient cells (Figure 3E), we examined the EGFR-driven pathway because it has been reported to regulate keratinocyte proliferation and differentiation (Dlugosz et al., 1997). Also, the activity of ERK, a downstream target of EGFR, was previously found to be elevated in IKKα-deficient keratinocytes (Park et al., 2007; Sil et al., 2004). Western blotting showed more EGFR and ERK activity in mutant than in WT epidermis (Figures 4A and 4B). Because EGFR ligands activate EGFR and the appearance of *Ikkα*$^{F/F}$/K5.Cre mice resembles that of mice expressing increased soluble forms of heparin-binding EGF (HB-EGF) (Yamazaki et al., 2003), we examined levels of EGF and HB-EGF. Using western blotting, we found higher levels of mature EGF and HB-EGF and lower levels of EGF and HB-EGF precursors in mutant than in WT epidermis (Figure 4C). It is known that A disintegrin and metalloprotease (ADAM) sheddases cleave EGF and HB-EGF precursors to generate their active soluble forms (Huovila et al., 2005). Figure 3E shows that ADAM19 was upregulated in IKKα-deficient keratinocytes. We thus examined the mRNA levels of ADAM12, 10, 17, 19, and 9 in primary cultured keratinocytes by RT-PCR and found that their expression levels were higher in *Ikkα*$^{-/-}$ than in WT cells and that stimulation of cell growth elevated these levels (Figure 4D). Western blotting confirmed elevated levels of ADAM12, 10, and 17 in mutant epidermis (Figure 4E). The presence of elevated ADAM10 expression in mutant epidermis was confirmed by immunofluorescence staining (Figure S11A). Furthermore, zymography (Hall and Erickson, 2003) verified that ADAMs were more active in mutant than in WT epidermis, and reintroduction of IKKα repressed this activity (Figure 4F).

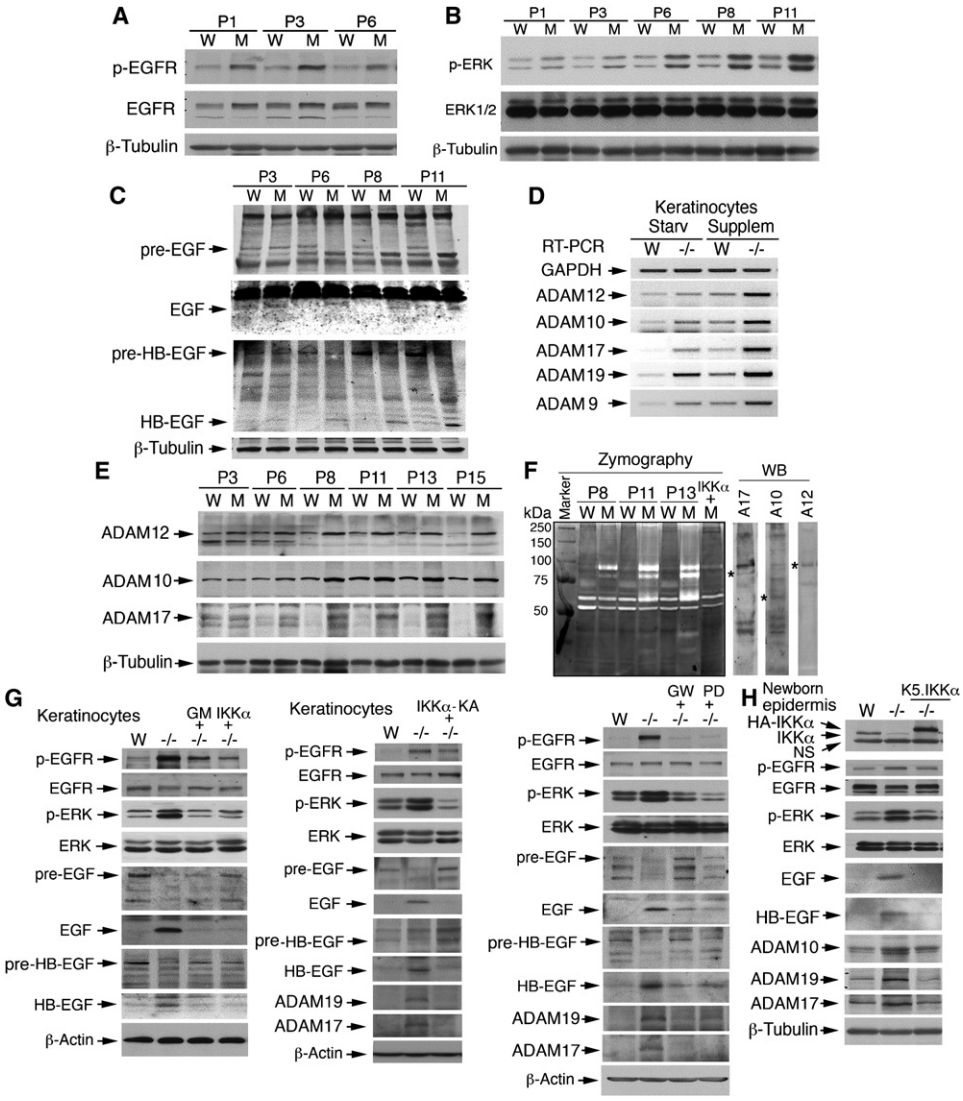

Figure 4. Elevated Autocrine Loop in IKKα-Deficient Keratinocytes

(A–C) Levels of phosphorylated EGFR (p-EGFR), p-ERK, EGF, precursor EGF (pre-EGF), HB-EGF, and pre-HB-EGF in epidermis of WT (W) and *Ikkα*$^{F/F}$/K5.Cre (M) mice, as detected by western blotting. P, postnatal day. β-tubulin was used as a loading control.

(D) mRNA levels of A disintegrin and metalloproteases (ADAMs) in WT and *Ikkα*$^{-/-}$ (−/−) primary keratinocytes cultured for 1 day with no growth supplement (Starv) or with growth supplement (Supplem), as detected by RT-PCR. GAPDH was used as a PCR control.

(E) Levels of ADAM12, 10, and 17 in epidermis of WT and *Ikkα*$^{F/F}$/K5.Cre (M) mice, as detected by western blotting.

(F) Activities of sheddases in epidermis of WT, *Ikkα*$^{F/F}$/K5.Cre (M), and *Ikkα*$^{F/F}$/K5.Cre/K5.IKKα mice (IKKα+M), as detected by gelatin zymography. WB, western blotting; * indicates proteins.

(G) Levels of p-EGFR, p-ERK, EGF, HB-EGF, and/or ADAMs in WT keratinocytes, *Ikkα*$^{-/-}$ keratinocytes (−/−), and *Ikkα*$^{-/-}$ keratinocytes treated with GM6001 (GM), GW2974 (GW), and/or PD98059 (PD) for 24 hr or with reintroduced IKKα or IKKα-KA after 48 hr, as detected by western blotting. β-actin was used as a loading control.

(H) Levels of the indicated proteins in skin of WT, *Ikkα*$^{-/-}$ (−/−), and *Ikkα*$^{-/-}$/K5.IKKα newborns, as detected by western blotting. NS, nonspecific band. β-tubulin was used as a loading control.

ERK and EGFR are believed to upregulate ADAMs (Huovila et al., 2005). We therefore hypothesized that IKKα regulates EGFR-driven mitogenic signaling and ADAM expression through the same signaling loop. To prove this hypothesis, we treated IKKα-deficient keratinocytes with inhibitors for a broad spectrum of ADAMs (GM6001), EGFR (GW2974), and/or ERK (PD98059) and re-expressed IKKα or a kinase-dead full-length IKKα (with a mutation at the ATP-binding site within the kinase domain; IKKα-KA) in the cells. Western blotting showed reduced EGFR and ERK activities and EGF, HB-EGF, and ADAM levels but elevated EGF and HB-EGF precursors in mutant keratinocytes after these treatments (Figure 4G). Similar results were obtained for transgenic IKKα in the epidermis (Figure 4H). Taken together, these results suggest that IKKα downregulates the activity of

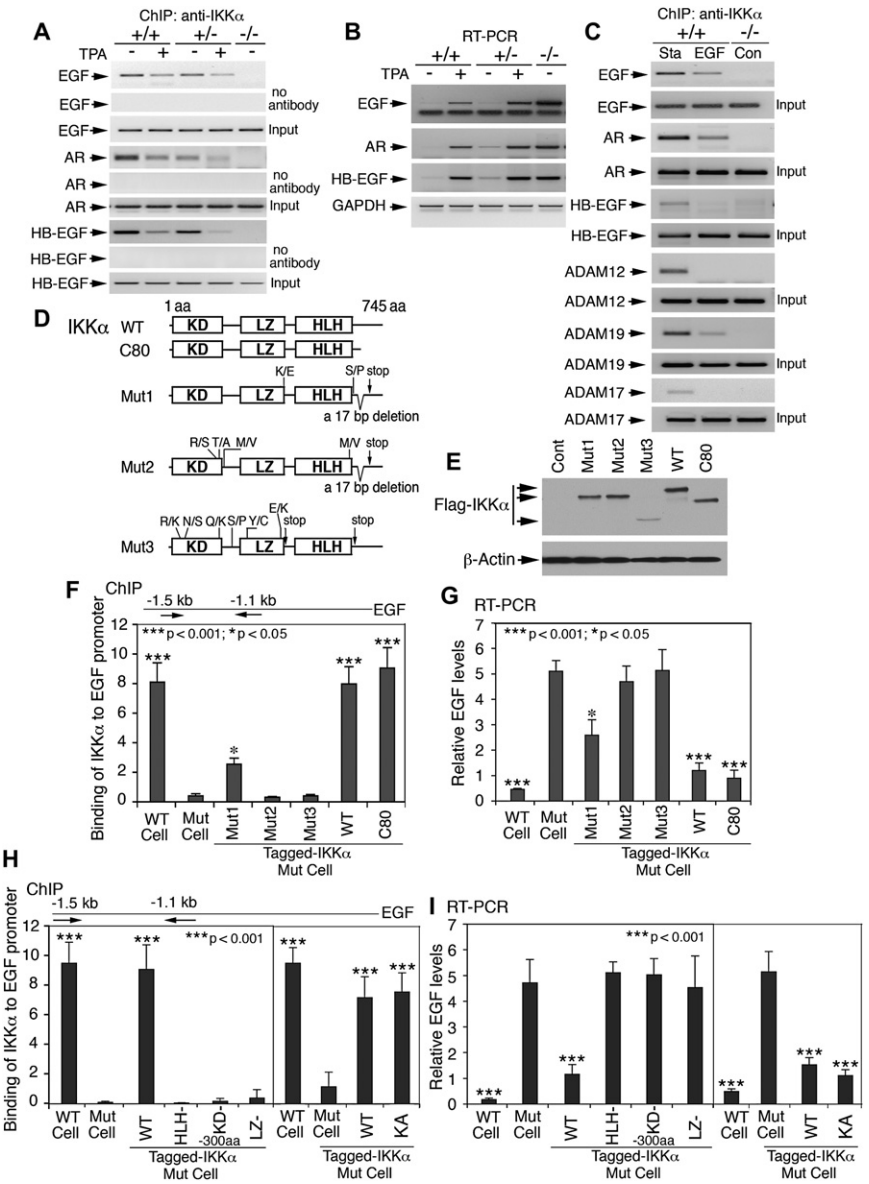

Figure 5. IKKα Regulates Promoter Activities of EGF, HB-EGF, Amphiregulin, and ADAMs

(A) Binding of IKKα to promoters of EGF, amphiregulin (AR), and HB-EGF, as detected by chromatin immunoprecipitation (ChIP) assay with an anti-IKKα antibody for immunoprecipitation. +/+, *Ikkα*$^{+/+}$ keratinocytes; +/−, *Ikkα*$^{+/-}$ keratinocytes; −/−, *Ikkα*$^{-/-}$ keratinocytes. TPA was used at 30 ng/ml. No antibody, negative control; Input, PCR control.

(B) RT-PCR for expression of the indicated genes. GAPDH was used as an mRNA loading control.

(C) Binding of IKKα to the promoters of the indicated genes. Sta, cells cultured in basic keratinocyte culture medium; Con, control. EGF was used at 10 ng/ml.

(D) Protein structures of IKKα. KD, kinase domain; LZ, leucine zipper; HLH, helix-loop-helix domain; stop, stop codon. R/S, T/A, M/V, K/E, S/P, R/K, N/S, Q/K, Y/C, and E/K indicate amino acid substitutions.

(E) Indicated IKKα protein levels in IKKα-deficient keratinocytes as detected by western blotting. Cont, no IKKα. β-actin was used as a loading control.

(F) Binding of different forms of IKKα to the EGF promoter, as detected by ChIP assay with an anti-IKKα antibody for immunoprecipitation. All samples were compared with IKKα-deficient cells (Mut). Error bars in (F)–(I) indicate ±SD.

(G) RT-PCR for expression of EGF affected by different forms of IKKα.

(H) Binding of different forms of IKKα (see Figure S11B) to the EGF promoter, as detected by ChIP assay. "−300 aa" represents a 300 aa deletion.

(I) RT-PCR for expression of EGF affected by different forms of IKKα.

the autocrine loop of EGFR, ERK, ADAMs, and EGFR ligands in keratinocytes.

IKKα Suppresses Promoter Activation of EGF, HB-EGF, Amphiregulin, and ADAMs

To determine the mechanism of how IKKα prevents EGFR autocrine loop activity in keratinocytes, we examined whether IKKα regulates the transcription of EGFR ligand genes and ADAMs, because IKKα has been shown to have transcriptional activity in the keratinocyte nucleus (Liu et al., 2006). Using a chromatin immunoprecipitation (ChIP) assay, we found that IKKα bound to the promoters of the EGF (−1511 to −1133 bp), HB-EGF (−1911 to −1534 bp), and amphiregulin (AR) (−3129 to −2785 bp) genes in *Ikkα*$^{+/+}$ and *Ikkα*$^{+/-}$ primary cultured keratinocytes, but not in *Ikkα*$^{-/-}$ keratinocytes (Figure 5A). The bonds were stronger in *Ikkα*$^{+/+}$ than in *Ikkα*$^{+/-}$ cells. Treatment with TPA (30 ng/ml) attenuated binding of IKKα to these promoters, which correlated profoundly with increased expression of the corresponding genes (Figure 5B). Treatment with EGF (10 ng/ml) also reduced binding of IKKα to the promoters of the EGF, HB-EGF, AR, ADAM12 (−2733 to −2516 bp), ADAM19 (−3402 to −3182 bp), and ADAM17 (−2389 to −2073 bp) genes in WT keratinocytes (Figure 5C), and the addition of growth factors elevated the expression of these ADAMs (Figure 4D). We found that IKKα failed to bind to the proximal promoter regions of the genes described above (data not shown). These results suggest that IKKα suppresses the induced expression of EGF, HB-EGF, AR, and ADAMs by downregulating their promoter activities.

To further elucidate how IKKα regulates gene expression, we introduced WT IKKα, an 80 aa deletion IKKα (IKKα-C80), and three IKKα mutants (Mut1, Mut2, and Mut3) isolated from poorly differentiated SCCs into IKKα-deficient keratinocytes (Figures 5D and 5E) (Hu et al., 2001; Liu et al., 2006; Zhu et al., 2007). ChIP assay and RT-PCR showed that WT IKKα and IKKα-C80 bound to the EGF promoter and suppressed EGF expression; Mut1 showed incomplete binding activity to the EGF promoter and a reduced inhibitory effect on EGF expression compared

to IKKα, and Mut2 and Mut3 failed to bind to the EGF promoter or suppress EGF expression (Figures 5F and 5G). WT keratinocytes were used as controls. Because IKKα-C80 had activity comparable to that of IKKα, the C-terminal deletion in Mut1 and Mut2 may not be a major cause of loss of IKKα function. In addition to the C-terminal deletion, a mutation occurred at the leucine zipper (LZ) motif in Mut1; Mut2 and Mut3 had multiple mutations in the helix-loop-helix (HLH) or LZ motif and in the region between the kinase and LZ domain. To further confirm whether these motifs of the IKKα protein are required to suppress the activities of the above promoters, we introduced IKKα mutants with mutations in the LZ or HLH domains (LZ- or HLH-), IKKα-KA, and/or an N-terminal 300 aa deletion (KD-) (Zhu et al., 2007) into IKKα-deficient keratinocytes (Figure S11B). ChIP assay showed that the activities of IKKα-KA in binding to the EGF promoter and repressing EGF expression were similar to those of WT IKKα; however, LZ-, HLH-, and KD- IKKα mutants were not able to bind to the EGF promoter and failed to suppress EGF expression (Figures 5H and 5I). These results indicate that the LZ and HLH motifs and protein conformation of IKKα are important for regulation of gene expression.

IKKα Regulates Keratinocyte Proliferation and Differentiation through the EGFR-Driven Pathway

To understand whether IKKα regulates keratinocyte proliferation and differentiation via the EGFR-driven loop, we treated IKKα-deficient keratinocytes with inhibitors of EGFR (AG1478 and GW2974), ADAMs (GM6001), and ERK (PD98059) and also reintroduced WT, C80, Mut1, Mut2, Mut3, LZ-, HLH-, KD-, and KA IKKα into these cells. We reported previously that reintroduced IKKα specifically induces larger terminally differentiated keratinocytes and results in the expression of the terminal differentiation markers loricrin and filaggrin in *Ikkα*$^{-/-}$ cells (Hu et al., 2001). Thus, we used cell morphologies and filaggrin induction to identify terminally differentiated keratinocytes. The results indicated that the tested inhibitors, IKKα, IKKα-KA, and IKKα-C80 inhibited proliferation and induced terminal differentiation in mutant keratinocytes; Mut1 slightly inhibited hyperproliferation and induced filaggrin to a lesser degree, and Mut2, Mut3, LZ-, HLH-, and KD- failed to inhibit cell proliferation or induce keratinocyte terminal differentiation (Figures 6A and 6B). We further compared levels of filaggrin induced by IKKα, IKKα-C80, and Mut1 in IKKα-deficient cells by diluting these cell lysates to 1/2 and 1/4 and found that, compared to IKKα and IKKα-C80, Mut1 induced lower levels of filaggrin (Figure 6C). To test whether the effect of the EGFR-driven pathway on cell proliferation is related to differentiation or apoptosis in IKKα-deficient cells, we further examined markers of apoptosis in mutant keratinocytes. Using western blotting, we found that inhibitors of EGFR, ERK, and ADAMs did not increase apoptosis in mutant keratinocytes (Figure S11C). Collectively, these results suggest that IKKα and the EGFR-driven pathway coordinately regulate keratinocyte proliferation and differentiation and that IKKα activity requires its functional domains, but not its kinase activity.

Inactivation of EGFR Reverses the Epidermal Hyperplasia in *Ikkα*$^{F/F}$/K5.Cre Mice

To further determine the physiological function of the EGFR-driven loop in *Ikkα*$^{F/F}$/K5.Cre mice, we inactivated EGFR activity in *Ikkα*$^{F/F}$/K5.Cre mice by backcrossing *Egfr*$^{wa2/wa2}$ (a kinase-inactive mutation in EGFR) (Luetteke et al., 1994) with *Ikkα*$^{F/F}$ and K5.Cre mice to generate *Ikkα*$^{F/F}$/K5.Cre/*Egfr*$^{wa2/wa2}$ mice. The epidermis of *Ikkα*$^{F/F}$/K5.Cre/*Egfr*$^{wa2/wa2}$ mice was as thin as that of WT controls, and Ki67-positive cells and EGFR and ERK activities were decreased in the epidermis (Figure 6D). Like *Ikkα*$^{F/F}$/K5.Cre mice, these mice died early. GW2974, administered orally, also inhibited epidermal thickening in *Ikkα*$^{F/F}$/K5.Cre mice (Figure S12) (Kiguchi et al., 2005). Furthermore, we generated *Ikkα*$^{F/F}$/K5.Cre/*Egfr*$^{-/-}$ mice, but these mice died within 3 days after birth (Sibilia and Wagner, 1995; Threadgill et al., 1995). Reducing the *Egfr* gene dosage by half was found to dramatically decrease epidermal thickness in *Ikkα*$^{F/F}$/K5.Cre/*Egfr*$^{+/-}$ mice, although their epidermis was still slightly thicker than that of WT mice (Figure 6E). Most of the *Ikkα*$^{F/F}$/K5.Cre/*Egfr*$^{+/-}$ mice died, but a small fraction survived. The surviving mice developed obvious defects in hair and eyes (Figure 6F). It is known that *Egfr*$^{-/-}$ mice on the same genetic background are prone to death during a wide range of ages, which underscores the importance of EGFR for individual mouse development (Sibilia and Wagner, 1995; Threadgill et al., 1995). Taken together, these results suggest that a reduction of EGFR activity can mitigate IKKα-deletion-induced excessive proliferative signals in the epidermis, thereby reversing epidermal hyperplasia.

Inactivation of IKKα kinase in *Ikkα*$^{K44A/K44A}$ mice was found not to cause hyperproliferation in the epidermis (Figure S13) (Zhu et al., 2007). Thus, the increased epidermal thickness in *Ikkα*$^{F/F}$/K5.Cre mice is IKKα kinase independent. IKKα loss also elevated TNFα expression (Figure S10B). Overexpression of TNFα can cause severe inflammation-dependent skin lesions in mice (Lind et al., 2004). To determine whether the epidermal phenotype was TNFα dependent, we generated *Ikkα*$^{F/F}$/K5.Cre/*Tnfr1*$^{-/-}$ mice and found that depleting TNFR1 did not attenuate the epidermal phenotype but did lead to the slightly earlier death of the mice (Figures S14A and S14B). No significant alterations in the levels of IKKβ, IKKγ, p65, p100, p52, and IκBα or in IKK kinase activity were found in the epidermis of WT or mutant mice (Figures S15A and S15B). Collectively, these results show that excessive EGFR-driven loop activity, but not TNFR, is a primary trigger responsible for inducing the epidermal hyperplasia in *Ikkα*$^{F/F}$/K5.Cre mice.

Mice with IKKα Deletion in Keratinocytes Develop Spontaneous Skin Cancer

To determine whether IKKα deletion in keratinocytes induces skin tumors, we used mouse mammary tumor virus (MMTV).Cre mice to delete IKKα. *Ikkα*$^{F/F}$/MMTV.Cre mice were distinguishable from WT mice by their sparse body hair 3 weeks after birth. We found that 8 of 9 *Ikkα*$^{F/F}$/MMTV.Cre mice began to develop spontaneous tumors on their face at 1 to 5 months of age (Figures 7A and 7B). Because the oldest mice that we observed were 8 months old, it is possible that the tumor incidence may reach 100% as the mice get older. These tumors expressed pan-keratin and resembled SCCs pathologically (Figures 7C). None of the *Ikkα*$^{F/F}$ or MMTV.Cre mice developed spontaneous tumors. The dorsal epidermis of *Ikkα*$^{F/F}$/MMTV.Cre mice was hyperplastic, with elevated EGFR and ERK activities compared to WT epidermis (Figure S16).

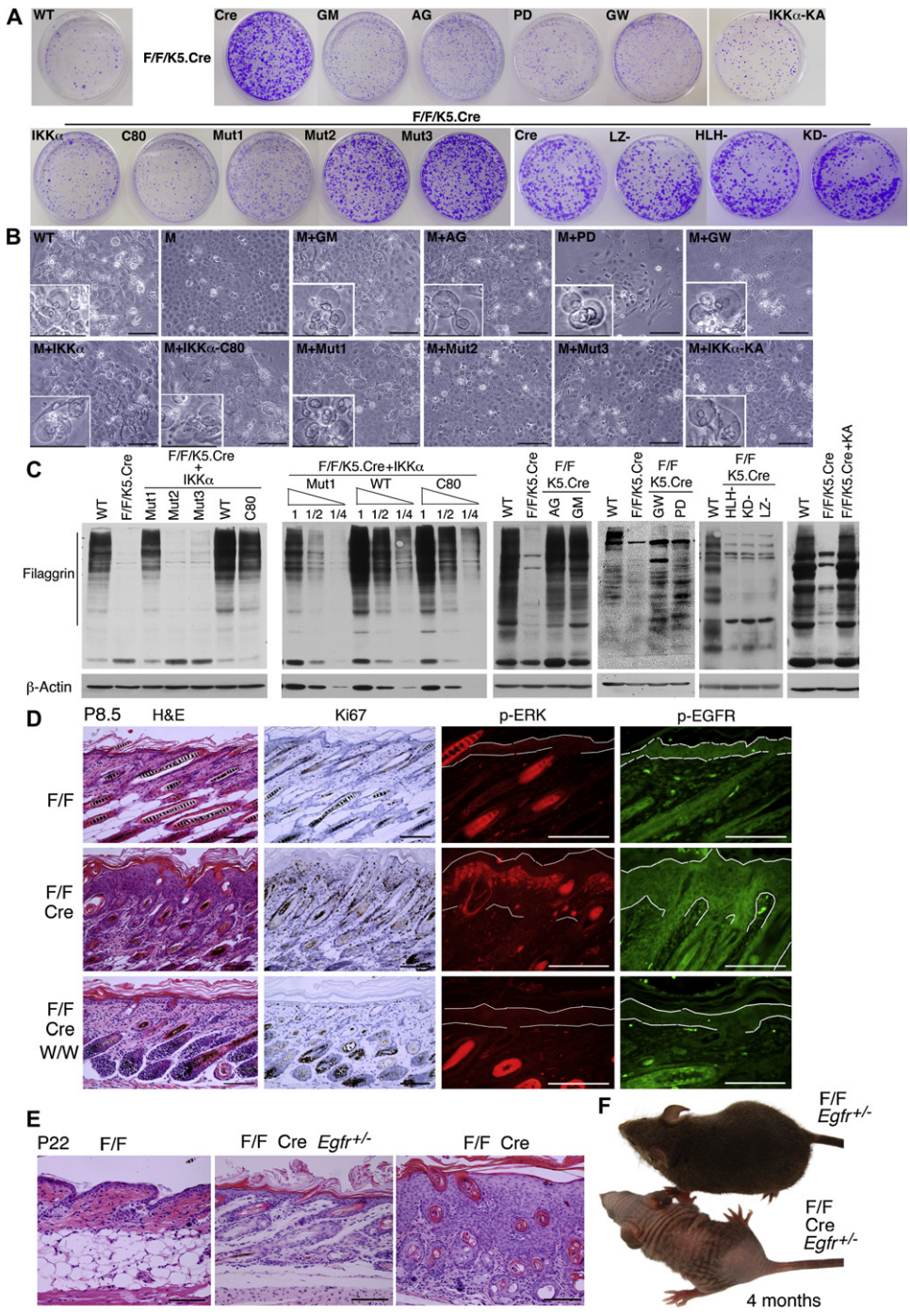

Figure 6. IKKα Switches Keratinocyte Proliferation and Differentiation via the EGFR Pathway

(A) Effects of different forms of IKKα and the indicated inhibitors on keratinocyte colony formation. WT, *Ikkα*$^{F/F}$ keratinocytes; F/F/K5.Cre, *Ikkα*$^{F/F}$/K5.Cre keratinocytes. GM, GM6001; AG, AG1478; PD, PD98059; GW, GW2974. Keratinocyte colonies were stained with 0.5% crystal violet.

(B) Keratinocyte morphologies in culture. Cells in small boxes represent terminally differentiated keratinocytes. M, *Ikkα*$^{F/F}$/K5.Cre keratinocytes. Scale bars = 40 μm.

(C) Filaggrin levels in the indicated keratinocytes, as detected by western blotting. 1, 1/2, and 1/4 represent protein dilutions. β-actin was used as a loading control.

(D) Effect of inactivation of EGFR on the epidermis in mice at P8.5. Ki67 is shown by dark brown staining with immunohistochemistry, p-ERK by red staining with immunofluorescence, and p-EGFR by green staining with immunofluorescence. Lines in p-ERK and p-EGFR indicate the limits of the epidermis. Cre, K5.Cre; W/W, *Egfr*$^{wa2/wa2}$. Scale bars = 50 μm.

(E) Skin histology of the indicated mouse genotypes, stained with H&E. Scale bars = 60 μm.

(F) Appearance of the indicated mouse genotypes at 4 months of age.

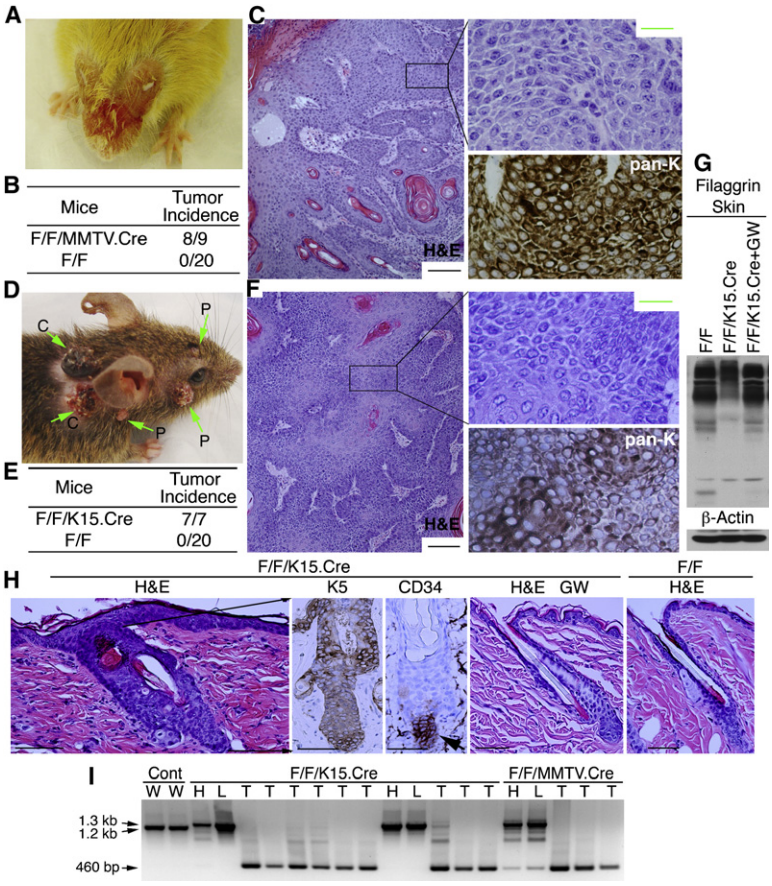

Figure 7. IKKα Deletion in Keratinocytes Causes Spontaneous Skin Cancer

(A) Tumors in an 8-month-old *Ikkα*$^{F/F}$/MMTV.Cre mouse.

(B) Tumor incidence in *Ikkα*$^{F/F}$/MMTV.Cre (F/F/MMTV.Cre) and WT (F/F) mice.

(C) Pathologies of H&E-stained tumor sections from *Ikkα*$^{F/F}$/MMTV.Cre mice. Panels at right show a magnification of the boxed area at left and pan-keratin staining (brown). Black scale bar = 25 μm; green scale bar = 10 μm.

(D) Tumors in an *Ikkα*$^{F/F}$/K15.Cre mouse 6 months after RU486 treatment to induce IKKα deletion. P, papillomas; C, carcinomas.

(E) Tumor incidence in *Ikkα*$^{F/F}$/K15.Cre (F/F/K15.Cre) and WT (F/F) mice.

(F) Pathologies of H&E-stained tumor sections from *Ikkα*$^{F/F}$/K15.Cre mice. Panels at right show a magnification of the boxed area at left and pan-keratin staining (brown). Black scale bar =25 μm; green scale bar = 10 μm.

(G) Filaggrin levels in skin, as detected by western blotting. β-actin was used as a loading control.

(H) Skin sections stained with H&E and antibodies against K5 or CD34. Brown color indicates K5 or CD34 staining. The two arrows in the leftmost H&E panel indicate hair follicles seen in the K5 and CD34 panels to the right. The arrow in the CD34 panel indicates CD34 staining. GW, GW2974. Scale bar = 40 μm.

(I) Genotyping. Cont, WT skin; H, heart; L, liver; T, tumor. 1.3 kb, allele containing loxP sites; 1.2 kb, WT allele; 460 bp, *Ikkα* gene deletion.

Because MMTV.Cre is also expressed in other types of cells (Wagner et al., 1997), we investigated whether inducible IKKα deletion specifically in keratinocytes causes spontaneous skin tumors. We used inducible K15.Cre, which is specifically expressed in keratinocytes of hair follicles (Morris et al., 2004), to delete IKKα in mice using RU846. We treated seven *Ikkα*$^{F/F}$/K15.Cre mice with RU846. Four months after treatment, all of the mice began to develop skin tumors (Figures 7D and 7E). The appearance of these tumors was similar to that of DMBA/TPA-induced skin papillomas and malignant carcinomas (Figures 7D and 7F). The mouse in Figure 7D developed more than ten tumors. The tumors resembled SCCs pathologically and expressed pan-keratin (Figure 7F).

We further examined the status of keratinocyte differentiation and proliferation in WT and *Ikkα*$^{F/F}$/K15.Cre skin. Western blotting showed that filaggrin expression was lower in *Ikkα*$^{F/F}$/K15.Cre than in WT skin and that treatment with GW2974 elevated filaggrin expression (Figure 7G). In *Ikkα*$^{F/F}$/K15.Cre skin, increased keratinocyte numbers in hair follicles and epidermis surrounding the hair follicles and elevated EGFR and ERK activities were detected, but CD34-positive cells were restricted in the hair follicle (Figure 7H; Figure S17). GW2974 inhibited hyperproliferation and EGFR and ERK activities in mutant epidermis (Figure 7H; Figure S17). Also, seven GW2974-treated *Ikkα*$^{F/F}$/K15.Cre mice had not developed any tumors at 6 months after treatment with RU846, suggesting that inactivation of EGFR induces terminal differentiation, inhibits proliferation, and prevents tumor development.

The *Ikkα* gene was deleted in all of the tumors found in *Ikkα*$^{F/F}$/MMTV.Cre and *Ikkα*$^{F/F}$/K15.Cre mice (Figure 7I). Because MMTV.Cre is expressed in various types of cells, weak deletion bands were detected in the heart and liver of *Ikkα*$^{F/F}$/MMTV.Cre mice (Figure 7I). Taken together, these results indicate that IKKα deletion in keratinocytes can cause skin carcinomas, which are accompanied by elevated EGFR and ERK activities (Figures S16A and S17).

In addition, we generated tamoxifen-inducible *Ikkα*$^{F/F}$/K5.Cre mice. The dorsal epidermis of these mice exhibited hyperplasia (Figure S18A). Four of the eight inducible *Ikkα*$^{F/F}$/K5.Cre mice developed skin tumors 10 to 12 months after tamoxifen treatment (Figure S18B). Only one to three tumors per mouse were observed, and IKKα was deleted in these tumors. Thus, the number and incidence of skin tumors were lower in inducible *Ikkα*$^{F/F}$/K5.Cre mice than in inducible *Ikkα*$^{F/F}$/K15.Cre mice. The K5 and K15 promoters are expressed in different subtypes of keratinocytes. Keratinocytes expressing K5 are physically closer to suprabasal differentiating cells than hair follicle keratinocytes expressing K15. The difference may have an impact on the susceptibility to tumorigenesis in mice.

Activated Ras, a downstream target of EGFR and upstream target of ERK, is involved in keratinocyte transformation (Yuspa et al., 1985). We further found that Ras activity was significantly higher in IKKα null than in WT keratinocytes (Figure 8A). Reintroduced IKKα, EGFR inhibitor, or dominant-negative RasN17 suppressed Ras and ERK activities and cell proliferation but induced terminal differentiation in IKKα null keratinocytes (Figures 8A–8C). Increased Ras activity was found to suppress terminal differentiation in WT keratinocytes (Figure 8D). Thus, IKKα deletion may activate Ras via the EGFR-driven loop in keratinocytes (Figure 8E).

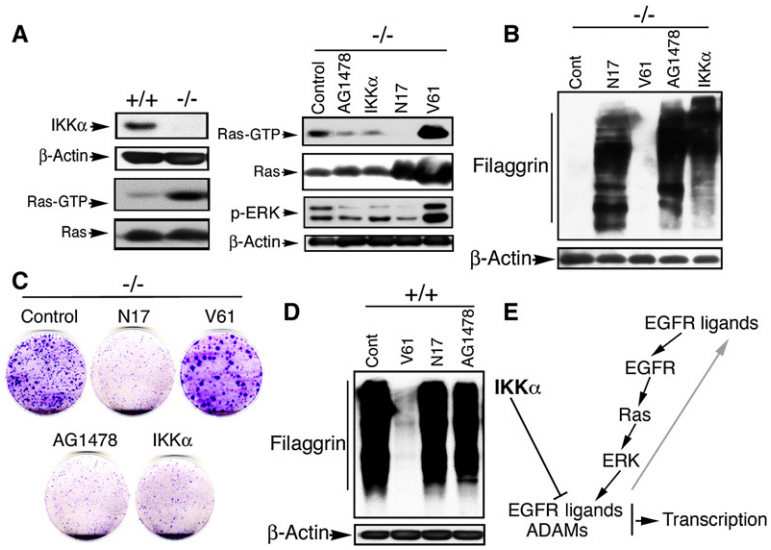

Figure 8. Elevated Ras Activity Incorporates with the EGFR Pathway in IKKα Null Keratinocytes

(A) Ras activity in keratinocytes as detected by GST pull-down and western blotting. Elevated Ras and ERK activities were downregulated by IKKα, inactivation of Ras, and an EGFR inhibitor. +/+, $Ikk\alpha^{+/+}$ keratinocytes; −/−, $Ikk\alpha^{-/-}$ keratinocytes. Ras-GTP, active form of Ras; AG1478, EGFR inhibitor; N17, dominant-negative Ras mutant; V61, active Ras mutant.

(B) Effects on filaggrin expression in $Ikk\alpha^{-/-}$ keratinocytes by the indicated proteins, as detected by western blotting. β-actin was used as a loading control.

(C) Effects on colony formation of $Ikk\alpha^{-/-}$ keratinocytes by the indicated proteins. Colonies were stained with 0.5% crystal violet.

(D) Effects on filaggrin expression in $Ikk\alpha^{+/+}$ keratinocytes by the indicated proteins, as detected by western blotting. β-actin was used as a loading control.

(E) A working hypothesis of IKKα function in keratinocytes for maintaining skin homeostasis and preventing skin cancer. Arrows indicate promotion; blunted line indicates inhibition.

DISCUSSION

IKKα Functions as a Switch for Keratinocyte Differentiation and Proliferation via the EGFR-Driven Pathway and Is Required for Skin Homeostasis

Our results here have demonstrated that a low level of IKKα in keratinocytes is sufficient to maintain embryonic skin development in mice. However, further IKKα deletion induced epidermal hyperplasia in postnatal and adult mice. Epidermal thickness in mutant mice was found to gradually increase after birth, which was accompanied by gradually elevated EGFR and ERK activities and levels of EGFR ligands and ADAMs; this also occurred in cultured mutant keratinocytes. The hyperproliferative keratinocytes in the epidermis or in cultures lacked terminal differentiation (Figures 6A and 6C; Figure S7). Inactivation of EGFR by genetic and pharmacological inhibitor approaches as well as transgenic IKKα was able to suppress keratinocyte hyperproliferation and direct the cells to terminal differentiation, reversing the epidermal phenotype in mice with IKKα deletion in keratinocytes. Furthermore, IKKα and IKKα functional domains were required to suppress the transcription of EGFR ligands and these ligands' activators and to suppress EGFR and ERK activities in mutant keratinocytes. Taken together, these results provide evidence that IKKα regulates keratinocyte proliferation and differentiation through the EGFR-driven loop, which is required for maintaining skin homeostasis in mice.

Quantitation assays showed that mutations in *Ikkα* (Mut1) impaired the capacity of IKKα to induce terminal differentiation and suppress hyperproliferation at the same time in IKKα-deficient keratinocytes (Figures 6A and 6C), providing further, direct evidence that IKKα is a switch for keratinocyte proliferation and differentiation; the integrity of *Ikkα*, but not its kinase activity, is required for this switch. Previously, we showed that culture medium obtained from WT keratinocytes was able to temporarily induce terminal differentiation in $Ikk\alpha^{-/-}$ keratinocytes (Hu et al., 2001), which was inactivated by treatment with trypsin or heat (90°C). The proteinaceous factor was named keratinocyte differentiation-inducing factor (kDIF). We also suggested that the grafted $Ikk\alpha^{-/-}$ skin was induced to undergo terminal differentiation by kDIF from surrounding WT keratinocytes in mice (Hu et al., 2001). Our findings here further explain this transplant result. We had previously placed $Ikk\alpha^{-/-}$ skin in basic keratinocyte culture medium at 4°C for longer than overnight before grafting (Hu et al., 2001). Such conditions might affect the ability of the $Ikk\alpha^{-/-}$ keratinocytes to proliferate, causing them to instead undergo terminal differentiation. Thus, in addition to secreted kDIF, which may include inhibitors of components of the EGFR-driven loop, intrinsic conditions also regulate the status of IKKα-deficient keratinocytes.

Although here we found that depleting TNFR was not able to prevent the development of epidermal hyperplasia in $Ikk\alpha^{F/F}$/K5.Cre mice (Figure S14), the elevated TNFα may affect late phenotypes in these mice. Also, inflammatory cells in the dermis (Figure S10A) may contribute to epidermal cell proliferation in mutant mice. IKKα loss was previously found to upregulate vascular endothelial growth factor expression (Liu et al., 2006). Indeed, an increase in blood microvessels was observed in the skin of $Ikk\alpha^{F/F}$/K5.Cre mice (data not shown). In addition, we observed very short hair in surviving $Ikk\alpha^{F/F}$/K5.Cre/$Egfr^{+/-}$ mice and sparse hair in $Ikk\alpha^{F/F}$/MMTV.Cre mice, suggesting that IKKα plays a role in hair development, although the mechanism remains to be elucidated. Moreover, our microarray analyses revealed the elevated expression of multiple integrins, various growth factors, matrix metalloproteases (MMPs), Notch receptor ligands, Wnts, and Wnt inhibitors in IKKα-deficient keratinocytes (Figure 3E). A group of tight-junction proteins, claudins, which affect barrier formation and hair development, were dramatically downregulated in mutant keratinocytes (Figure 3E). Taken together, these molecular alterations may contribute to the skin phenotypes in mice with IKKα deletion in keratinocytes. Thus, IKKα plays a broad role in maintaining normal skin.

IKKα Is an Innate Surveillant to Prevent Skin Cancer

In mouse models of skin carcinogenesis, DMBA-activated Ras or overexpressed Ras in basal or suprabasal keratinocytes can induce squamous cell-like skin tumors (Brown et al., 1998;

Greenhalgh et al., 1993; Wang et al., 2000), indicating that uncontrolled proliferation is able to block terminal differentiation at different stages, allowing a single cell to grow into a mass. Additional alterations cause the mass to irreversibly invade and become malignant. Hair follicular keratinocytes targeted by Ras efficiently develop into malignant tumors because skin stem cells located there are thought to be targeted by the tumor initiator (Brown et al., 1998; Kangsamaksin et al., 2007).

Here we showed that IKKα deletion in keratinocytes results in skin carcinomas that resemble DMBA/TPA-induced tumors (Figures 7D and 7F). In IKKα-deficient keratinocytes, elevated Ras activity, which is incorporated into the EGFR-driven loop, and elevated expression of multiple growth factors, integrins, cytokines, and MMPs provide the molecular bases for promotion of keratinocyte proliferation and transformation in vivo (Figure 3E; Figures 8A–8D). Inactivation of EGFR or reintroduction of IKKα induces terminal differentiation, inhibits hyperproliferation in IKKα-deficient keratinocytes (as described above), and prevents tumor development. These results underscore the importance of IKKα-mediated terminal differentiation as an innate, primary mechanism for preventing skin carcinogenesis. In addition to the effect of EGFR on keratinocyte differentiation, Sibilia et al. (2000) reported that inactivation of EGFR prevents overexpressed SOS-F-induced skin tumors through a survival pathway but does not affect ERK activity, which suggests that overexpressed SOS-F and IKKα loss may affect different internal cellular pathways.

Treatment with TPA or EGF was found to attenuate binding of IKKα to the promoters of growth factors, which was accompanied by the elevated expression of their corresponding genes (Figures 5A–5I). Previously, TPA, ultraviolet radiation, and/or ETS-1, a proto-oncogenic protein, were found to upregulate IKKα expression (Gu et al., 2004; Li and Karin, 1998; Park et al., 2007). Elevated expression of IKKα may suppress the activities of its targets to ensure that excessive numbers of keratinocytes undergo terminal differentiation, thereby preventing cell proliferation and tumor formation. Moreover, p63 regulates IKKα expression, and impaired *Ikkα* may affect p63-related tumor development (Koster et al., 2007). DNA methylation has been shown to downregulate IKKα expression in human oral SCCs (Maeda et al., 2007). IKKα prevents DNA methylation of the *14-3-3σ* gene (Zhu et al., 2007). It will be interesting to see whether IKKα can be self-regulated in an epigenetic manner. Collectively, these results indicate that IKKα executes its role as a surveillant in preventing skin cancer through multiple avenues.

We observed that $Ikk\alpha^{F/F}$/MMTV.Cre mice developed carcinomas only on their faces, and most of the tumors in $Ikk\alpha^{F/F}$/K15.Cre mice were close to the neck and face. It is possible that microenvironmental conditions, such as the presence of more blood microvessels in the face than in dorsal skin, may have contributed to these phenotypes. Finally, we would like to emphasize that western blotting using commercial antibodies against IKKα detects a strong, nonspecific band closely underneath the IKKα protein in tissue lysates, but not in cultured cell lysates. Therefore, proper techniques can avoid misleading findings and help us to understand the physiological functions of IKKα.

Perspectives

Here we have identified the pathway that IKKα utilizes in its role as a keratinocyte proliferation and differentiation switch, which is required for maintaining skin homeostasis and preventing skin cancer. Although many deregulated genes were identified in IKKα-deficient keratinocytes, it remains to be elucidated how IKKα crosstalks with other pathways. It will also be important to develop new tools to identify the target cells of IKKα-deletion-induced skin tumors, which will facilitate the design of efficient therapeutic drugs to battle cancer. Stem cells are thought to be targets for most skin tumors (Kangsamaksin et al., 2007). It remains to be determined whether suprabasal keratinocytes are resistant to dedifferentiation and whether hair follicle keratinocytes or daughters of stem cells are resistant to differentiation when IKKα is absent during carcinogenesis. Also, how microenvironmental inflammation interacts with cancer-initiating cells remains to be defined. In addition, it will be important to determine whether IKKα plays a role in stem cell maintenance, renewal, and division. Overall, these animal models provide good opportunities for understanding many fundamental biological questions and identifying new therapeutic targets for preventing cancer.

EXPERIMENTAL PROCEDURES

Mice

$Tnfr1^{-/-}$ mice (stock number 002818) on a C57BL/6 background and $Egfr^{wa2/wa2}$ mice (stock number 000317) on a C57BL/6 background were purchased from The Jackson Laboratory. $Egfr^{+/-}$ mice on a CD-1 background (stock number 002857) were purchased from The Jackson Laboratory 3.5 years ago. These mice were backcrossed with our $Ikk\alpha^{+/-}$ mice on a 129/C57BL/6 background for over six generations. The $Egfr^{+/-}$ mice were then crossed with $Ikk\alpha^{F/F}$ mice on a 129/C57BL/6 background. MMTV.Cre mice (stock number 003553) were purchased from The Jackson Laboratory. These mice were on a B6/129 background, but the strain originated on an FVB background. GW2974 (GlaxoSmithKline), an inhibitor of EGFR and ErbB2, was used in the diet at 200 ppm as described previously (Kiguchi et al., 2005). Pregnant mothers of $Ikk\alpha^{F/F}$/K5.Cre embryos (Brakebusch et al., 2000; Lopez-Rovira et al., 2005) received the diet containing GW2974 at 13 days postcoitum. $Ikk\alpha^{F/F}$/K15.Cre mice (stock number 005249; strain name B6;SJL-Tg(Krt1-15-cre/PGR)22Cot/J) at 4 to 6 weeks of age (Morris et al., 2004) received the diet containing GW2974 and were treated 1 week later with RU486 (M8046, Sigma-Aldrich). RU486 (40 μg) in 200 μl of acetone was applied topically to the dorsal mouse skin every day for 10 days. Tamoxifen-inducible K5.Cre mice were obtained from P. Chambon (Indra et al., 1999). The dorsal skin of $Ikk\alpha^{F/F}$/K5.Cre mice at age 6–8 weeks was shaved and treated topically with tamoxifen (1 mg per mouse in 200 μl ethanol; catalog no. T5648, Sigma) every day for 14 days.

$Ikk\alpha^{F/F}$ Mice

To create the mouse lines used in this study, an 8.86 kb genomic DNA fragment containing exons 3–9 of the *Ikkα* gene was isolated from R1 ES cells on a 129 background, cloned into the pBluescript II SK+ vector, and sequenced. A neomycin cassette fragment with loxP sites at both ends was inserted into intron 6 (Cao et al., 2001). One loxP site was created in intron 9. The construct plasmid DNA was linearized with ClaI and electroporated to R1 ES cells, and the ES cells were selected using G418 in the Genetically Engineered Mouse Facility at the M.D. Anderson Cancer Center. Using Southern blotting, 166 clones were screened, 11 of which were found to be positive for homologous recombination. The neomycin cassette was deleted by introducing a Cre vector (Cao et al., 2001). Cre recombination can thus induce a deletion from exon 6 to 8, resulting in a frameshift for IKKα. Three ES cell clones with the floxed *Ikkα* allele were identified by Southern blot analysis and used to generate chimeras. The chimeras were backcrossed with WT C57BL/6 mice to generate $Ikk\alpha^{F/+}$ mice. The $Ikk\alpha^{F/+}$ mice obtained from the three ES cell colonies were identical. The $Ikk\alpha^{F/+}$ mice were intercrossed to generate $Ikk\alpha^{F/F}$ mice on a 129/C57BL/6 background, which were normal.

Animal Experiments

All mice used in this study were cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee of The University of Texas M.D. Anderson Cancer Center (animal protocol 04-01-05732).

Keratinocyte Culture

Mouse primary keratinocytes were isolated and cultured as described previously (Hu et al., 2001). Briefly, skin specimens isolated from newborn mice or E19 embryos were treated with 0.25% trypsin (15050-065; Invitrogen) for 8 to 10 hr at 4°C, and the epidermis was separated from the dermis. The isolated keratinocytes were plated on 60 cm cell dishes containing keratinocyte serum-free medium (10785; GIBCO). To determine cell growth curves, equal numbers of different types of cells were plated and then counted on the days indicated in the figures. The cells were treated with 0.02 μM AG1478 (658552; Calbiochem), 20 μM GM6001 (364205; Calbiochem), 5 μM GW2974, and/or 30 μM PD98059 (B59034; Calbiochem). Reintroduction of different IKKα forms in cultured keratinocytes was performed as described previously (Hu et al., 2001). To analyze cell proliferation, 2×10^5 cells were plated on each dish. Cell colonies were then fixed in 100% ethanol for 20 min at −20°C, stained with 0.5% crystal violet solution (HT901-8F0Z; Sigma) for 15 min, and washed with H_2O.

ACCESSION NUMBERS

Original microarray data have been deposited at the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE12111.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, Supplemental References, eighteen figures, and one table and can be found with this article online at http://www.cancercell.org/cgi/content/full/14/3/212/DC1/.

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