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IKK α 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. IKK α induces keratinocyte terminal differentiation and its reduction promotes skin tumor development. However, its intrinsic function in skin cancer is unknown. Here we found that mice with IKK α deletion in keratinocytes developed a thickened epidermis and spontaneous squamous cell-like carcinomas. Inactivation of epidermal growth factor receptor (EGFR) or reintroduction of IKK α inhibited excessive mitosis, induced terminal differentiation, and prevented skin cancer through repressing an EGFR-driven autocrine loop. Thus, IKK α 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 top 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 pre-requisite for preventing skin disorders.

The mitogenic signaling cascade from 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

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differentiation (Dlugosz et al., 1997). Two decades ago, Yuspa and colleagues demonstrated that oncogenic v-H-Ras induced keratinocyte proliferation and transformation but blocked terminal differentiation (Yuspa et al., 1985). 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, were also 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 was found to inhibit Ras-mediated keratinocyte proliferation and promote differentiation in vitro, as well as prevent skin tumors induced by overexpression of a dominant form of son of 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 demonstrated that the epidermis of *Ikkα*^{-/-} newborn mice lacked a terminally differentiated cornified layer and exhibited marked thickening; these mice died soon after birth (Hu et al., 1999; Takeda et al., 1999). Reintroduction of IKKα or kinase inactive IKKα induced keratinocyte terminal differentiation and repressed 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 showed that reduced IKKα expression provided a selective growth advantage that cooperated 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, was found in most carcinomas in *Ikkα*^{+/-} mice, and somatic *Ikkα* mutations were detected in *Ikkα*^{+/+} carcinomas. Also, *Ikkα* genetic alterations and epigenetically down-regulated IKKα expression were 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 and Ras activities 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 were more bromodeoxyuridine (BrdU)-labeled keratinocytes, an indicator for the 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 were detected in primary cultured, undifferentiated *Ikkα*^{-/-} keratinocytes (Hu et al., 2001; Zhu et al., 2007). The re-expression of IKKα rescued 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.

IKK α , IKK β , and IKK γ (NEMO) form the IKK complex that activates NF- κ B through phosphorylating I κ Bs, the inhibitors of NF- κ B (Ghosh and Karin, 2002). Although IKK α and IKK β are highly conserved protein kinases, IKK β shows a stronger kinase activity for I κ Bs than does IKK α . Mice with deletion of IKK β or I κ B α in the basal epidermis developed tumor necrosis factor receptor (TNFR)-dependent skin inflammation and mice overexpressing I κ B α in the basal epidermis developed TNFR-dependent skin tumors (Lind et al., 2004; Pasparakis et al., 2002; Rebholz et al., 2007). However, mice overexpressing IKK α in the basal or suprabasal epidermis had normal skin and developed fewer SCCs and metastases induced by DMBA/TPA than did WT mice (Liu et al., 2006; Sil et al., 2004). Elevated IKK α expression was found to enhance terminal differentiation and antagonize chemical carcinogen-induced mitogenic and angiogenic activities. Conversely, reduced IKK α expression resulted in elevated mitogenic and angiogenic activities (Park et al., 2007). Thus, these physiological functions of IKK α in the skin of mice are beyond NF- κ B signaling alone.

A study, however, suggested that IKK α -mediated keratinocyte differentiation was not cell autonomous (Gareus et al., 2007), which could not explain the phenotypes in IKK α deficient mice and keratinocytes described above. Also, the function of IKK α in adult mice has not been revealed. Thus, here we generated mice with IKK α deletion in keratinocytes and investigated the role of IKK α in skin homeostasis and skin cancer development.

RESULTS

IKK α Deletion in Keratinocytes Causes Thickened and Wrinkled Skin in Mice

To determine the function of IKK α in the skin of mice, we generated mice with a floxed *Ikka* allele (*Ikka*^{F/F}) (Figure S1A–D). Genotyping confirmed that the number of *Ikka*^{F/F} homozygotes matched the expected Mendelian ratio. To study the role of IKK α in the epidermis, we crossed *Ikka*^{F/F} mice with mice expressing Cre under the control of keratin 5 (K5) promoter, which is expressed in basal epidermal keratinocytes, to generate *Ikka*^{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 *Ikka*^{F/F}/K5.Cre mice generated from male *Ikka*^{F/F} and female *Ikka*^{F/+}/K5.Cre mice were identical to those of *Ikka*^{-/-} mice (Figures 1A, S2, S3) (Hu et al., 1999; Takeda et al., 1999). Western blotting showed no IKK α expression in multiple organs obtained from the *Ikka*^{F/F}/K5.Cre mice (Figure 1B). *Ikka*^{F/F}/K5.Cre newborns generated from female *Ikka*^{F/F} and male *Ikka*^{F/+}/K5.Cre mice were indistinguishable from wild-type (WT, *Ikka*^{F/F}) mice (P1, Figure 1C). By day 5, however, the skin of *Ikka*^{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). The esophagus was smaller in mutant mice than in WT mice (Figure S4B). A similar phenotype has been described in *Ikka*^{-/-} mice, which might be relevant to the mouse deaths (Hu et al., 1999; Sil et al., 2004). Western blotting detected a low level of IKK α in the epidermis of mutant mice at day 1 after

birth and a further reduction of IKK α expression was observed later (Figure 1D), which was consistent with the loss of the WT *Ikka* allele in DNA isolated from the epidermis of the mice (Figure S5). IKK α expression was reduced in the tongue, esophagus, and intestine of *Ikka*^{F/F}/K5.Cre mice, but was expressed normally in the dermis, liver, lung, spleen (B cells), stomach, thymus (T cells), bone, brain, heart, kidney, bone marrow (hematopoietic cells), and blood cells (hematopoietic cells) in *Ikka*^{F/F}/K5.Cre mice compared with those in WT mice (Figure 1E). The level of IKK α expression depended on whether any *Ikka* alleles remained (Figure 1E). These results indicate that K5.Cre deletes IKK α specifically in the cells that express K5 in *Ikka*^{F/F} mice. To evaluate the effect of mouse genetic backgrounds on these phenotypes, we backcrossed *Ikka*^{F/F} and K5.Cre mice with C57BL6 or FVB mice for five generations. An FVB background was found to confer a slight increase in skin thickness and slightly earlier death compared to a C57BL6 background (data not shown). Collectively, these results suggest that a low level of IKK α in the epidermis is sufficient for normal embryonic skin development, but a further reduction causes skin lesions.

To verify that these phenotypes in *Ikka*^{F/F}/K5.Cre mice were due to an IKK α -specific deletion in keratinocytes, we re-expressed a keratinocyte-specific K5.IKK α transgene in *Ikka*^{F/F}/K5.Cre mice (Lomada et al., 2007). K5.IKK α expression rescued the skin phenotype of maternally transmitted *Ikka*^{F/F}/K5.Cre mice (Figures 1F, G, 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.IKK α expression completely rescued paternally transmitted *Ikka*^{F/F}/K5.Cre mice (Figures 1H, I, S6B). No skin disorders were found in adult *Ikka*^{F/F}/K5.Cre/K5.IKK α mice. Thus, IKK α deletion did cause the skin phenotypes in *Ikka*^{F/F}/K5.Cre mice.

IKK α Deletion Causes Epidermal Keratinocyte Hyperproliferation and Deregulates Expression of Many Genes

Histologies of the skins from WT and *Ikka*^{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 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 1 to 16 days old; 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 (Figures 2B, S7), suggesting that IKK α deletion may have a stronger impact on proliferation of CD34 negative keratinocytes than on CD34 positive keratinocytes.

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

Next, we determined the mitotic activity in the epidermis using immunohistochemical staining. We found that the proliferation marker Ki67 was significantly more numerous in *Ikka*^{F/F}/K5.Cre than in WT epidermis (Figure 3A). BrdU-positive cells were consistently more numerous in the mutant than in the WT epidermis (Figure S9A, B), and expression of K6, which is highly expressed in abnormally proliferating epidermis, was elevated in the 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 the mutants 6 days old (Figure S10A). Also, TNF α expression was higher in IKK α null than in WT keratinocytes (Figure S10B).

To determine if the hyperplastic epidermis is intrinsic to IKK α -deficient keratinocytes, we cultured keratinocytes isolated from *Ikka*^{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 did WT cells (Figure 3D). Thus, IKK α deletion induced keratinocyte-autonomous hyperproliferation.

To determine which signaling pathways may promote cell proliferation, we compared the transcriptional profiles of genes in WT and mutant keratinocytes by using microarray analyses (Table S1). As shown in Figure 3E, up-regulated genes in mutants include EGFR, EGFR ligands, Braf, FGF13, IGF2, and PDGF α ; a group of integrin genes; ADAM19, MMP10 and MMP9; Notch ligands; Wnt inhibitors Dkk3 and Dkk2; and various Wnts. Down-regulated genes include Wnt7 α , Notch3, and a group of Claudins (Figure 3E and Table S1). Such a broad collection of deregulated genes indicates that IKK α may have a broad impact on skin biology.

IKK α 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 IKK α -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 IKK α deficient keratinocytes (Park et al., 2007; Sil et al., 2004). Western blotting showed more EGFR and ERK activity in mutant than in WT epidermis (Figure 4A, B). Because EGFR ligands activate EGFR and the appearance of *Ikka*^{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 the mutant than in the WT epidermis (Figure 4C). It is known that ADAMs shedases cleave EGF and HB-EGF precursors to generate their active soluble forms (Huovila et al., 2005). Figure 3E shows that ADAM19 was up-regulated in IKK α -deficient keratinocytes. We thus examined the mRNA levels of ADAM12, 10, 17, 19, and 9 in primary cultured keratinocytes by using reverse transcription polymerase chain reaction (RT-PCR) and found that their expression levels were higher in *Ikka*^{-/-} than in WT cells and that the stimulation of cell growth elevated these levels (Figure 4D). Western blotting confirmed elevated levels of ADAM12, 10, and 17 in the 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 the mutant than in the WT epidermis, and reintroduction of IKK α repressed this activity (Figure 4F).

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

IKK α Suppresses Promoter Activation of EGF, HB-EGF, Amphiregulin (AR), and ADAMs

To determine the mechanism of how IKK α prevents EGFR autocrine loop activity in keratinocytes, we examined whether IKK α regulated the transcription of EGFR ligand genes and ADAMs because IKK α has been shown to have transcriptional activity in the keratinocyte nucleus (Liu et al., 2006). Using the ChIP assay, we found that IKK α bound to the promoters of the EGF (-1511 to -1133 bp), HB-EGF (-1911 to -1534 bp), and AR (-3129 to -2785 bp) genes in *Ikka*^{+/+} and *Ikka*^{+/-} primary cultured keratinocytes but not in *Ikka*^{-/-} keratinocytes (Figure 5A). The bonds were stronger in *Ikka*^{+/+} than in *Ikka*^{+/-} cells. Treatment with TPA (30 ng/ml) attenuated binding of IKK α to these promoters, which profoundly correlated with increased expression of these corresponding genes (Figure 5B). Treatment with EGF (10 ng/ml) also reduced binding of IKK α 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 IKK α failed to bind to the proximate promoter regions of the genes described above (data not shown). These results suggest that IKK α suppresses the induced expression of EGF, HB-EGF, AR, and ADAMs by down-regulating their promoter activities.

To further elucidate how IKK α regulates gene expression, we introduced WT IKK α , an 80-amino-acid-deletion IKK α (IKK α -C80) and three IKK α mutants (Mut1, Mut2, and Mut3) isolated from poorly differentiated SCC into IKK α -deficient keratinocytes (Figure 5D, E) (Hu et al., 2001; Liu et al., 2006; Zhu et al., 2007). ChIP assay and RT-PCR showed that IKK α and IKK α -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 IKK α ; Mut2 and Mut3 failed to bind to the EGF promoter or suppress EGF expression (Figure 5F, G). WT keratinocytes were used as controls. Because IKK α -C80 had activity comparable to that of IKK α , the C-terminal deletion in Mut1 and Mut2 may not be a major cause of loss of IKK α function. In addition to the C-terminal deletion, a mutation occurred at the LZ motif in Mut1; Mut2 and Mut3 had multiple mutations in the HLH or LZ motif and in the region between the kinase and LZ domain. To further confirm whether these motifs of IKK α protein are required to suppress the activities of the above promoters, we introduced IKK α mutants with mutations in its LZ or HLH domains (LZ- or HLH-), IKK α -KA, and/or an N-terminal 300-amino-acid (aa) deletion (KD-) (Zhu et al., 2007) into IKK α -deficient keratinocytes (Figure S11B). ChIP assay showed that the activities of IKK α -KA in binding to the EGF promoter and repressing EGF expression were similar to those of WT IKK α , however, LZ-, HLH-, and KD- IKK α mutants were not able to bind to the EGF promoter and failed to suppress EGF expression (Figure 5H, I). These results indicate that the LZ and HLH motifs and protein conformation of IKK α are important for regulation of gene expression.

IKK α Regulates Keratinocyte Proliferation and Differentiation through the EGFR-led Pathway

To understand whether IKK α regulates keratinocyte proliferation and differentiation via the EGFR-driven loop, we treated IKK α -deficient keratinocytes with inhibitors of EGFR (AG1478, GW2974), ADAMs (GM6001), and ERK (PD98059) and also reintroduced IKK α , IKK α -C80, Mut1, Mut2, Mut3, LZ-, HLH-, KD-, and KA into these cells. We previously reported that reintroduced IKK α specifically induced larger terminally differentiated keratinocytes and resulted in the expression of the terminal differentiation markers loricrin and filaggrin in *Ikka*^{-/-} cells (Hu et al., 2001). Thus, we used the cell morphologies and filaggrin induction to identify terminally differentiated keratinocytes. The results showed that those tested inhibitors, IKK α , IKK α -KA, and IKK α -C80 inhibited proliferation and induced terminal differentiation in mutant keratinocytes; Mut1 slightly inhibited hyperproliferation and induced filaggrin to a lesser degree, but Mut2, Mut3, LZ-, HLH-, and KD-failed to inhibit cell proliferation or induce keratinocyte terminal differentiation (Figure 6A, B). We further compared levels of filaggrin induced by IKK α , IKK α -C80, and Mut1 in IKK α -deficient cells by diluting these cell lysates to 1/2 and 1/4 and found that, compared to IKK α and IKK α -C80, Mut1 induced lower levels of filaggrin (Figure 6C). To test whether the effect of the EGFR-driven pathway on cell proliferation was related to differentiation or apoptosis in IKK α deficient cells, we further examined the 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 IKK α and the EGFR-driven pathway

coordinately regulate keratinocyte proliferation and differentiation and that the IKK α activity requires its functional domains but not its kinase activity.

Inactivation of EGFR Reverses the Hyperplastic Epidermis in *Ikka*^{F/F}/K5.Cre Mice

To further determine the physiological function of the EGFR-driven loop in *Ikka*^{F/F}/K5.Cre mice, we inactivated the EGFR activity in *Ikka*^{F/F}/K5.Cre mice by backcrossing *Egfr*^{wa2/wa2} (a kinase inactive mutation in EGFR) (Lueteteke et al., 1994) with *Ikka*^{F/F} and K5.Cre mice to generate *Ikka*^{F/F}/K5.Cre/*Egfr*^{wa2/wa2} mice. The epidermis of *Ikka*^{F/F}/K5.Cre/*Egfr*^{wa2/wa2} mice was as thin as that of the WT control, and Ki67-positive cells and EGFR and ERK activities were decreased in the epidermis (Figure 6D). Like the *Ikka*^{F/F}/K5.Cre mice, these mice died early. GW2974, given orally, also inhibited epidermal thickening in *Ikka*^{F/F}/K5.Cre mice (Figure S12) (Kiguchi et al., 2005). Furthermore, we generated *Ikka*^{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 the epidermal thickness in *Ikka*^{F/F}/K5.Cre/*Egfr*^{+/-} mice although their epidermis was still slightly thicker than that of WT mice (Figure 6E). Most of the *Ikka*^{F/F}/K5.Cre/*Egfr*^{+/-} mice still died but a small fraction survived (Figure 6F). The survived mice developed obvious defects in hair and eyes. It is known that *Egfr*^{-/-} mice on the same genetic background are prone to die during a large 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 IKK α -deletion-induced excessive proliferative signals in the epidermis, thereby reversing epidermal hyperplasia.

Inactivation of IKK α kinase in *Ikka*^{K44A/K44A} mice was found not to cause hyperproliferation in the epidermis (Figure S13) (Zhu et al., 2007). Thus, the elevated epidermal thickness in *Ikka*^{F/F}/K5.Cre mice is IKK α kinase independent. IKK α loss also elevated TNF α expression (Figure S10B). Overexpression of TNF α can cause severe inflammation-dependent skin lesions in mice (Lind et al., 2004). To determine whether the epidermal phenotype was TNF α dependent, we generate *Ikka*^{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 (Figure S14A, B). No significant alterations in the levels of IKK β , IKK γ , p65, p100, p52 and I κ B α , and in IKK kinase activity were found in the epidermis of WT and mutant mice (Figure S15A, B). Collectively, these results show that excessive EGFR-driven loop activity, but not TNFR, is a primary trigger responsible for inducing the epidermal hyperplasia in *Ikka*^{F/F}/K5.Cre mice.

Mice with IKK α Deletion in Keratinocytes Develop Spontaneous Skin Cancer

To determine if IKK α deletion in keratinocytes induces skin tumors, we used mouse mammary tumor virus (MMTV).Cre mice to delete IKK α . *Ikka*^{F/F}/MMTV.Cre mice were distinguished from WT mice by their sparse body hair 3 weeks after birth. We found that 8 of 9 *Ikka*^{F/F}/MMTV.Cre mice began to develop spontaneous tumors on their face at 1 to 5 months of age (Figure 7A, B). 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 *Ikka*^{F/F}

or MMTV.Cre mice developed any spontaneous tumors. The dorsal epidermis of *Ikka^{F/F}/MMTV.Cre* mice was hyperplastic, with elevated EGFR and ERK activities compared with the WT epidermis (Figure S16).

Because MMTV.Cre is also expressed in other types of cells (Wagner et al., 1997), we investigated whether inducible IKK α 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 IKK α in mice by using RU846. We treated 7 *Ikka^{F/F}/K15.Cre* mice with RU846. Four months after treatment, the 7 mice began to develop skin tumors (Figure 7D, E). The appearance of these tumors was similar to that of DMBA/TPA-induced skin papillomas and malignant carcinomas (Figure 7D, F). The mouse in Figure 7D developed more than 10 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 *Ikka^{F/F}/K15.Cre* skins. Western blotting showed that filaggrin expression was lower in *Ikka^{F/F}/K15.Cre* than in WT skin and that treatment with GW2974 elevated filaggrin expression (Figure 7G). In *Ikka^{F/F}/K15.Cre* skin, increased keratinocyte numbers in the hair follicles and the epidermis surrounding the hair follicles and elevated EGFR and ERK activities were detected, but CD34-positive cells were restricted in the hair follicle (Figures 7H, S17). GW2974 inhibited hyperproliferation and EGFR and ERK activities in the mutant epidermis (Figures 7H, S17). Also, 7 GW2974-treated *Ikka^{F/F}/K15.Cre* mice had not developed any tumors at 6 months after treatment with RU846, suggesting that inactivation of EGFR induced terminal differentiation, inhibited proliferation, and prevented tumor development.

The *Ikka* gene was deleted in all the tumors found in *Ikka^{F/F}/MMTV.Cre* and *Ikka^{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 *Ikka^{F/F}/MMTV.Cre* mice (Figure 7I). Taken together, these results indicate that IKK α deletion in keratinocytes can cause skin carcinomas, which are accompanied by elevated EGFR and ERK activities (Figures S16A, S17).

In addition, we generated tamoxifen-inducible *Ikka^{F/F}/K5.Cre* mice. The dorsal epidermis of the mice exhibited hyperplasia (Figure S18A). Four of the 8 inducible *Ikka^{F/F}/K5.Cre* mice developed skin tumors 10 to 12 months after tamoxifen treatment (Figure S18B). Only 1–3 tumors per mouse were observed and the tumors lost IKK α . Thus, the number and incidence of skin tumors were lower in inducible *Ikka^{F/F}/K5.Cre* mice than in inducible *Ikka^{F/F}/K15.Cre* mice. The K5 and K15 promoters are expressed in different subtypes of keratinocytes. Keratinocytes expressing K5 are physiologically 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 IKK α -null than in WT keratinocytes (Figure 8A). Reintroduced

IKK α , EGFR inhibitor, and dominant negative RasN17 suppressed Ras and ERK activities and cell proliferation but induced terminal differentiation in IKK α -null keratinocytes (Figure 8A, B, C). Increased Ras activity was found to suppress terminal differentiation in WT keratinocytes (Figure 8D). Thus, IKK α deletion may activate Ras via the EGFR-driven loop in keratinocytes (Figure 8E).

DISCUSSION

IKK α Functions as a Switch for Keratinocyte Differentiation and Proliferation via the EGFR-led Pathway and Is Required for Skin Homeostasis

Our results, here, demonstrated that a low level of IKK α in keratinocytes was sufficient to maintain embryonic skin development in mice. However, a further IKK α 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 S7, 6A, C). Inactivation of EGFR by genetic and pharmacological inhibitor approaches as well as transgenic IKK α were able to suppress keratinocyte hyperproliferation and direct the cells to terminal differentiation, reversing the epidermal phenotype in mice with IKK α deletion in keratinocytes. Furthermore, IKK α and IKK α functional domains were required to suppress the transcription of EGFR's ligands and these ligands' activators and to suppress EGFR and ERK activities in mutant keratinocytes. Taken together, these results provide evidence that IKK α 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 *Ikka* (Mut1) impaired the capacity of IKK α to induce terminal differentiation and suppress hyperproliferation at the same time in IKK α -deficient keratinocytes (Figure 6A, C), providing further, direct evidence that IKK α is a switch for keratinocyte proliferation and differentiation; the integrity of *Ikka*, but not its kinase activity, is required for this switch. Previously, we showed that the culture medium obtained from WT keratinocytes was able to temporarily induce terminal differentiation in *Ikka*^{-/-} keratinocytes (Hu et al., 2001), which was inactivated by treatment with trypsin or heat (90°C). The proteinaceous factor(s) was named keratinocyte differentiation inducible factor (KDIF). We also suggested that the grafted *Ikka*^{-/-} 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 previously placed *Ikka*^{-/-} 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 *Ikka*^{-/-} keratinocytes to proliferate, causing them instead to undergo terminal differentiation. Thus, in addition to secreted KDIF, which may include inhibitors to components in the EGFR-driven loop, intrinsic conditions also regulate the status of IKK α -deficient keratinocytes.

Although here we found that depleting TNFR was not able to prevent the development of hyperplastic epidermis in *Ikka*^{F/F}/K5.Cre mice (Figure S14), the elevated TNF α may affect late phenotypes in these mice. Also inflammatory cells in the dermis (Figure S10A) may

contribute to epidermal cell proliferation in mutant mice. IKK α loss was previously found to upregulate vascular endothelial growth factor expression (Liu et al., 2006). Indeed, an increase in microblood vessels was observed in the skin of *Ikka*^{F/F}/K5.Cre mice (data not shown). In addition, we observed very short hair in survived *Ikka*^{F/F}/K5.Cre/*Egfr*^{+/-} mice and sparse hair in *Ikka*^{F/F}/MMTV.Cre mice, suggesting that IKK α 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, MMPs, Notch receptor ligands, Wnts, and Wnt inhibitors in IKK α -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 an IKK α deletion in keratinocytes. Thus, IKK α plays a broad role in maintaining normal skin.

IKK α Is an Innate Surveillant to Prevent Skin Cancer

In mouse models of skin carcinogenesis, DMBA-activated Ras or over-expressed Ras in basal or suprabasal keratinocytes can induce skin (squamous cell-like) 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 developed into malignant tumors because skin stem cells located there were thought to be targeted by the tumor initiator (Brown et al., 1998; Kangsamaksin et al., 2007).

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

Treatment with TPA or EGF was found to attenuate binding of IKK α to the promoters of growth factors, which was accompanied by the elevated expression of their corresponding genes (Figure 5A–I). Previously, TPA, ultraviolet light radiation, and/or ETS-1, a proto-oncogenic protein, were found to up-regulate IKK α expression (Gu et al., 2004; Li and Karin, 1998; Park et al., 2007). Elevated expression of IKK α 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 IKK α

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 also 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 *Ikkα^{FF}/MMTV.Cre* mice developed the carcinomas only on their faces, and most of the tumors in *Ikkα^{FF}/K15.Cre* mice were close to neck and face. It is possible that microenvironmental conditions, such as the presence of more micro-blood vessels in the faces than in the 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 cross-talks with other pathways. It also will 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 the hair follicle keratinocytes or daughters of stem cells are resistant to differentiation when IKKα is absent during carcinogenesis. Also, how the microenvironmental inflammation interacts with cancer initiation 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, the animal models provide good opportunities for understanding many fundamental biological questions and identifying new therapeutic targets for preventing cancer.

EXPERIMENTAL PROCEDURES

Animal Experiments

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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SIGNIFICANCE

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

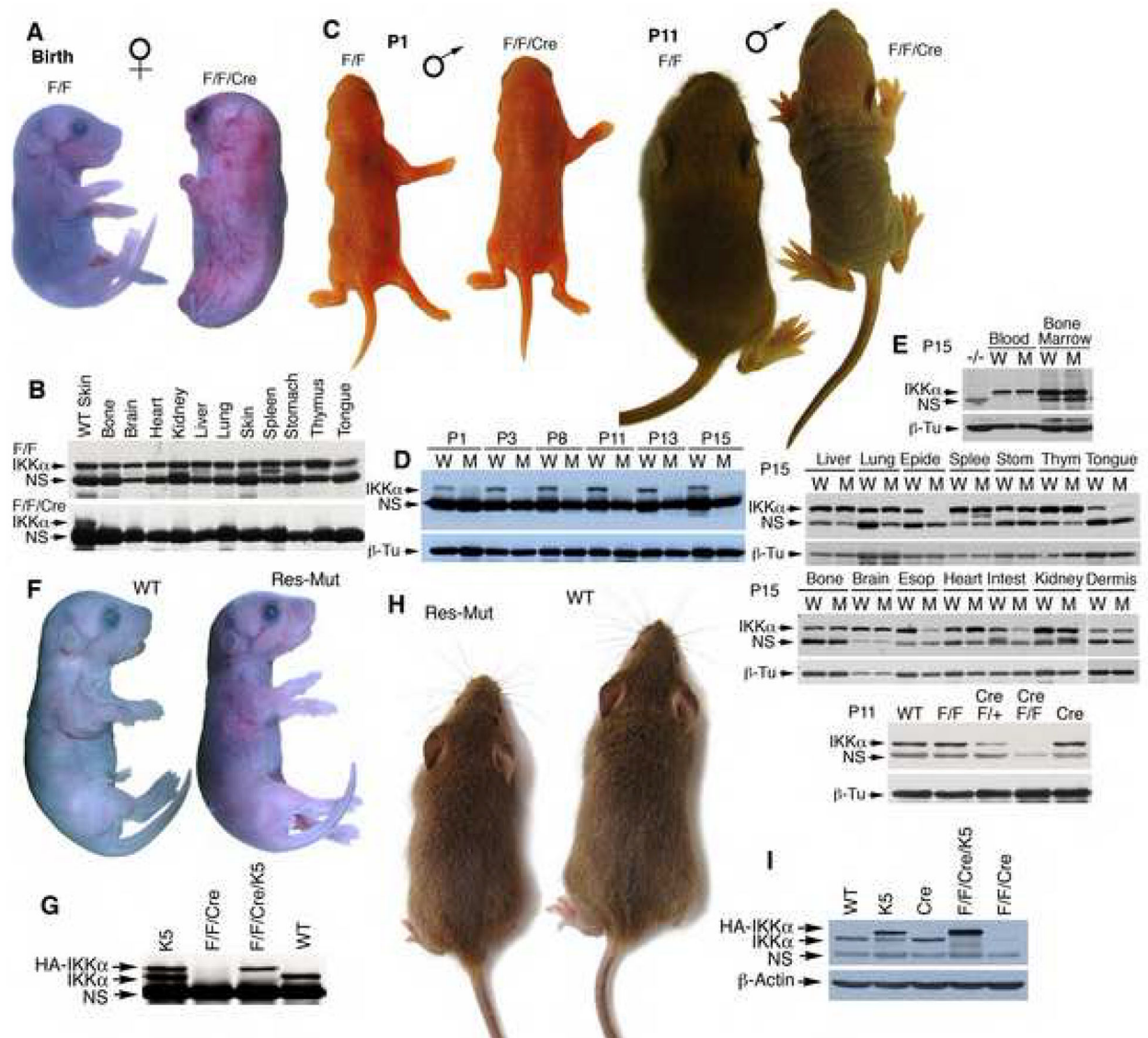


Figure 1. IKK α Deletion in Keratinocytes Causes Skin Defects in Postnatal Mice

(A) Appearances of *Ikka*^{F/F} (F/F) and *Ikka*^{F/F}/K5.Cre (F/F/Cre) mice at birth. Female symbol, maternally transmitted K5.Cre.

(B) IKK α levels in different organs of mice (A), as detected by Western blotting. NS, non-specific band.

(C) Appearances of *Ikka*^{F/F} (F/F) and *Ikka*^{F/F}/K5.Cre (F/F/Cre) mice. Male symbol, paternally transmitted K5.Cre; P, postnatal day.

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

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

- (G) IKK α levels in the skin of indicated mice (F), as detected by Western blotting. K5, K5.IKK α transgene; HA-IKK α , HA-tagged IKK α transgene expression.
- (H) Appearances of WT and paternally transmitted *Ikk α ^{F/F}/K5.Cre/K5* (Res-Mut) mice at 4 weeks old.
- (I) IKK α levels in the epidermis of indicated mice (H), as detected by Western blotting. β -Actin, loading control.

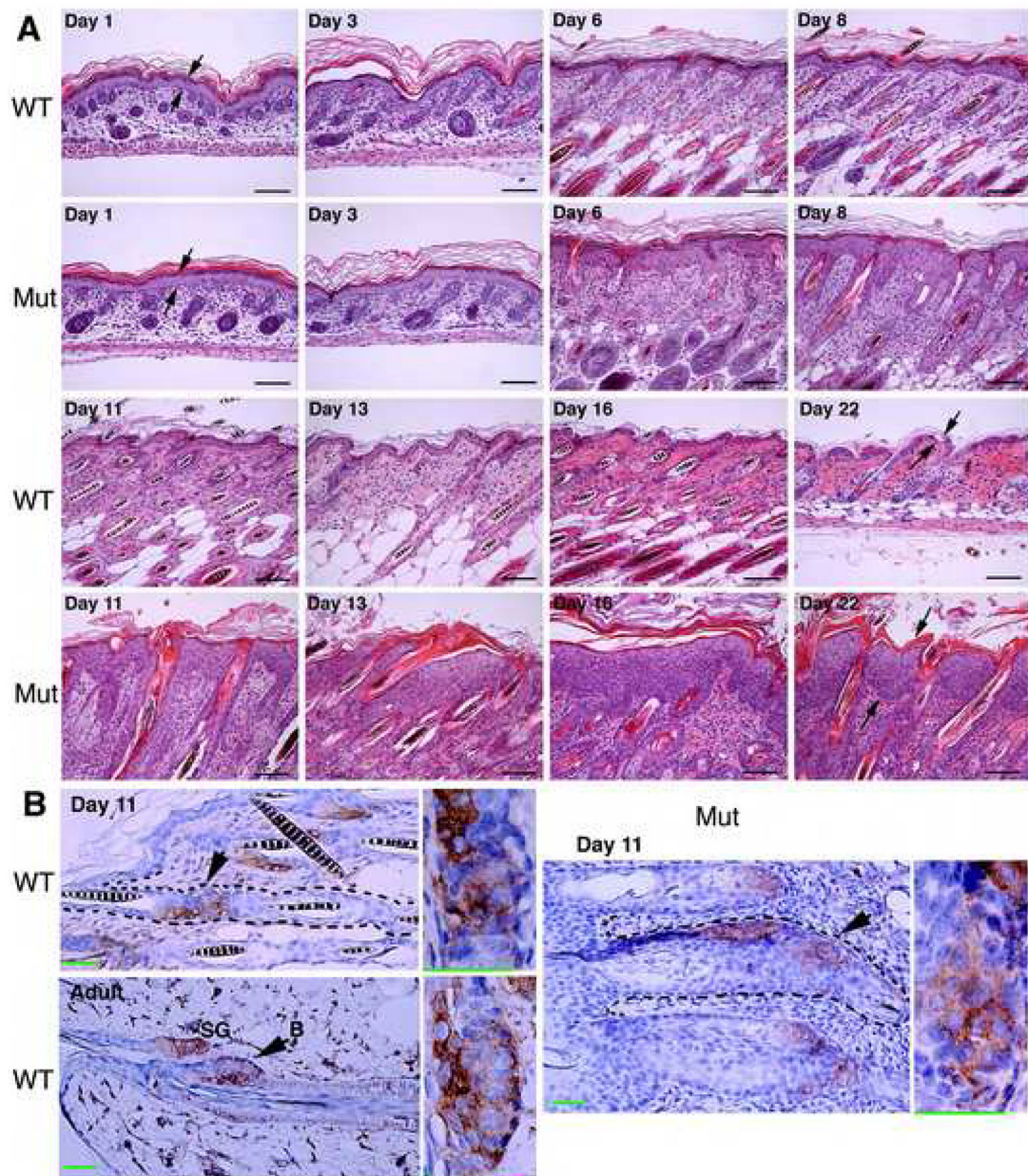


Figure 2. *IKKα* Deletion Causes Epidermal Hyperplasia

(A) Histology of the skin of indicated mice, stained with hematoxylin and eosin (H&E). WT, wild-type mice; Mut, *Ikka^{F/F}/K5.Cre* mice; Day, postnatal day; arrows, indicating epidermis. Scale bars, 30 μ m.

(B) Brown, CD34-positive cells indicated by arrows, enlarged in boxes; blue, nuclear counter-staining; SG, sebaceous gland; adult, control for CD34-positive cells in bulge. Scale bars, 30 μ m.

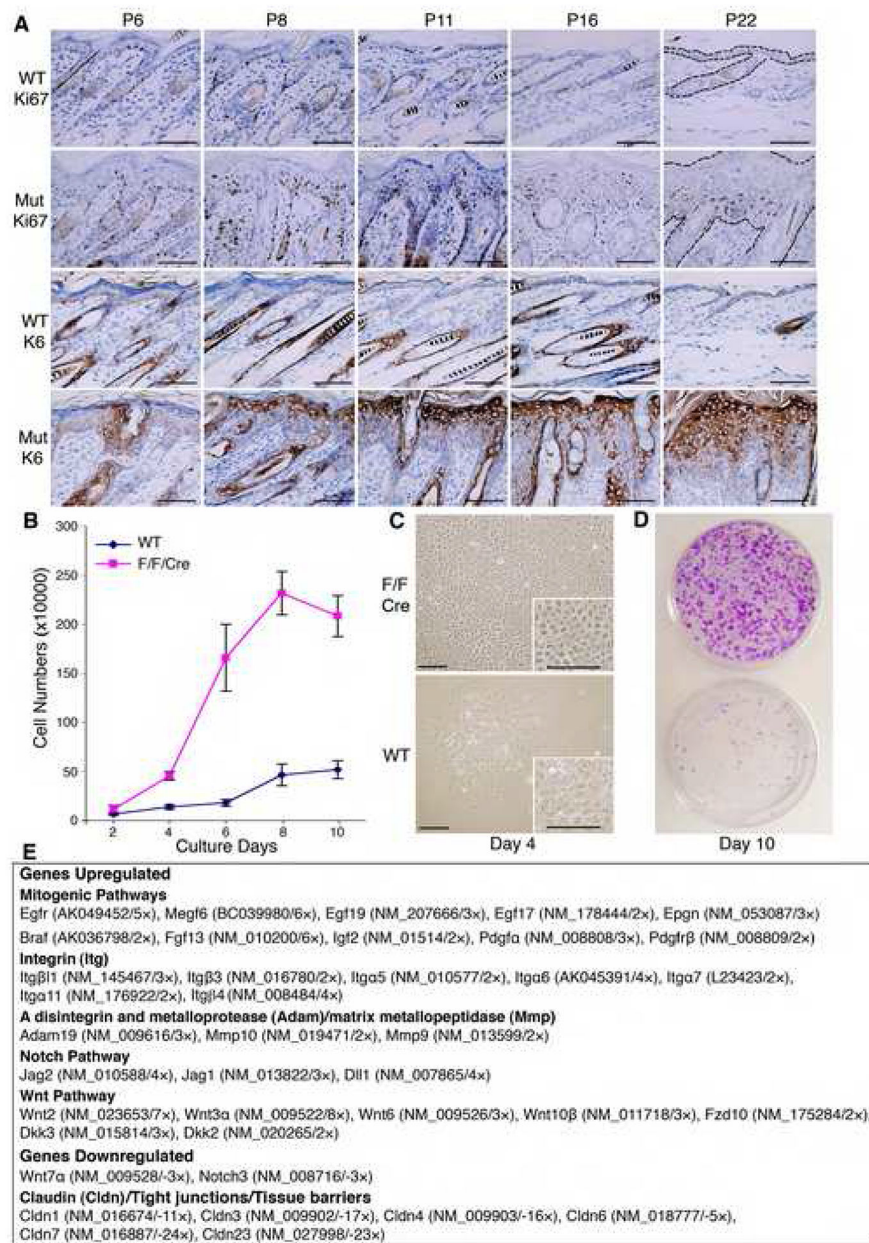


Figure 3. IKK α Deletion Causes Keratinocyte Hyperproliferation and Deregulates Expression of Many Genes

(A) Immunohistochemical skin sections stained for Ki67 and K6. Mut, *Ikka*^{F/F}/K5.Cre; dark brown color, positive cells; blue color, nuclear counter-staining; P, postnatal day. Scale bars, 50 μ m.

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

(C) Keratinocyte morphologies in culture at day 4. Scale bars, 50 μ m.

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

(E) A summary of microarray analysis results for cultured keratinocytes. Genes were up-regulated and down-regulated in *Ikka*^{F/F}/K5.Cre compared to WT cells. References for each gene's functions can be found in the Systematic Name database via NCBI. Detailed results are shown in Table S1.

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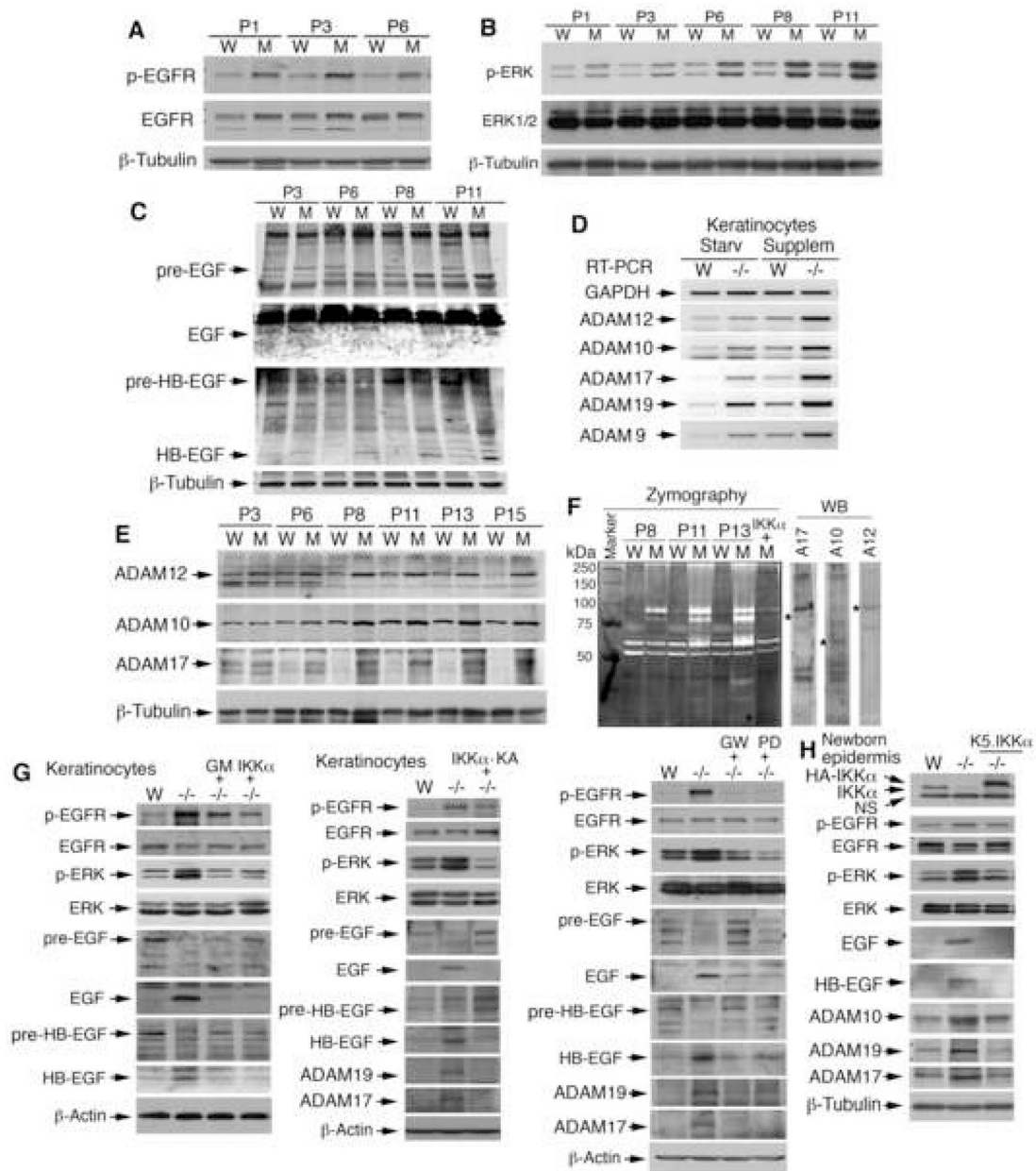


Figure 4. Elevated Autocrine Loop in *IKKα* Deficient Keratinocytes

(A-C) Levels of phosphorylated (p)-EGFR and p-ERK, HB-EGF, EGF, precursor (pre)-EGF, and pre-HB-EGF in the epidermis of WT (W) and *Ikka*^{F/F}K5.Cre (M) mice, as detected by Western blotting. P, postnatal day; β-Tubulin, loading control.

(D) mRNA levels of ADAMs in WT and *Ikka*^{-/-} (-/-) primary keratinocytes cultured with no growth supplement for 1 day (Starv) or with growth supplement (Supplem), as detected by RT-PCR. GAPDH, PCR control.

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

(F) Activities of sheddases in the epidermis of WT, *Ikkα^{F/F}/K5.Cre* (M) and *Ikkα^{F/F}/K5.Cre/K5.IKKα* mice (M+IKKα), 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, *Ikkα^{-/-}* (-/-), and *Ikkα^{-/-}* keratinocytes treated with GM6001 (GM), GW2974 (GW), and/or PD98059 (PD) for 24h or with reintroduced IKKα or IKKα-KA after 48 h, as detected by Western blotting. β-Actin, loading control.

(H) Levels of indicated proteins in the skin of WT, *Ikkα^{-/-}* (-/-), *Ikkα^{-/-}/K5.IKKα* newborns, as detected by Western blotting. NS, non-specific band.

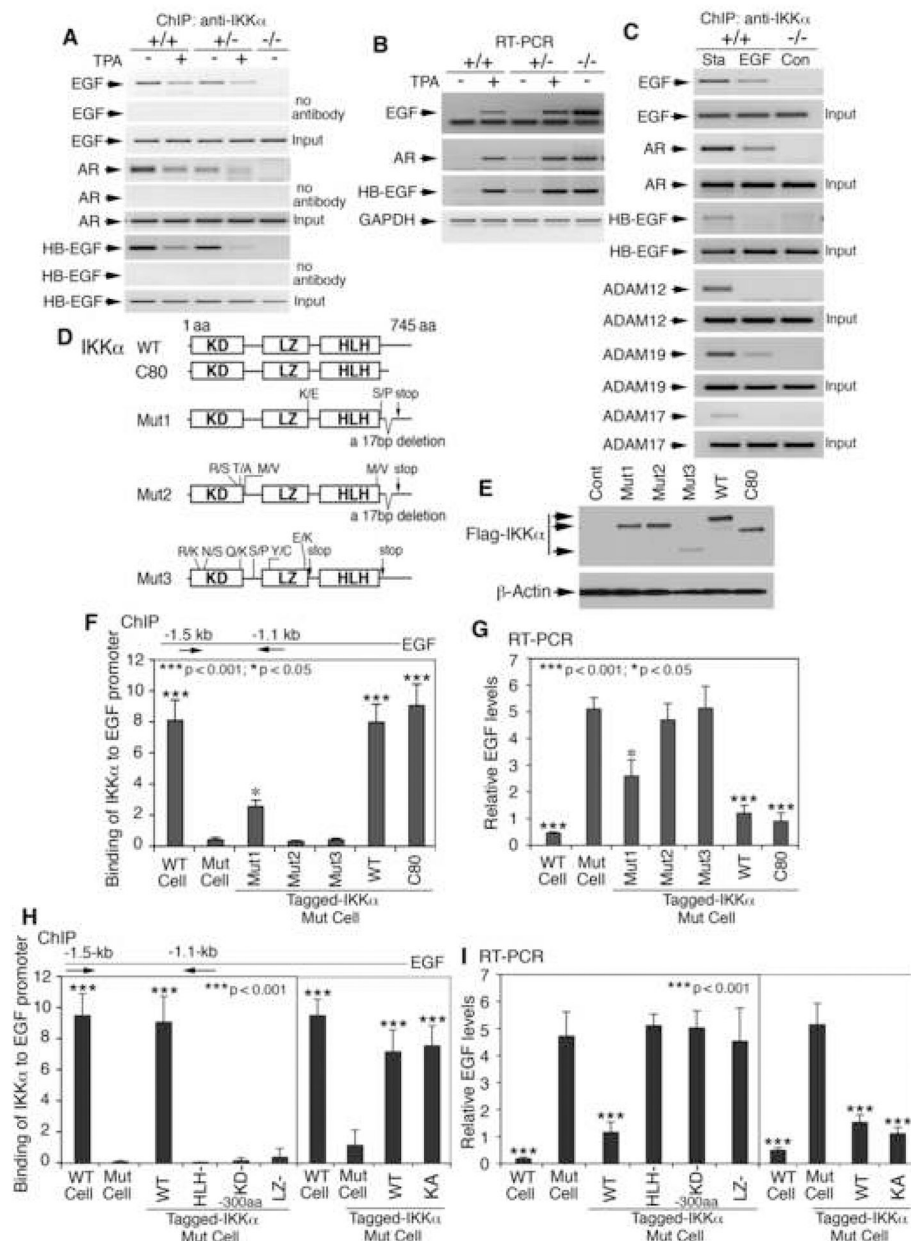


Figure 5. IKK α Regulates Promoter Activities of EGF, HB-EGF, AR, and ADAMs

(A) Binding of IKK α to promoters of EGF, HB-EGF, and AR, detected by ChIP assay with an anti-IKK α antibody for immunoprecipitation. No antibody, negative control; Input, PCR control; TPA, 30 ng/ml; $+/+$, *Ikka*^{+/+}; $+/-$, *Ikka*^{+/-}; $-/-$, *Ikka*^{-/-} keratinocytes.

(B) RT-PCR for expression of indicated genes; GAPDH, mRNA loading control.

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

(D) Protein structures of IKK α . aa, amino acid; R/S, T/A, M/V, K/E, S/P, R/K, N/S, Q/K, Y/C, and E/K: amino acid substitutions; R, arginine; S, serine; T, threonine; A, alanine; M, methionine; V, valine; C, cysteine; K, lysine; E, glutamic acid; P, proline; N, asparagine; Q,

glutamine; Y, tyrosine; stop, stop codon; KD, kinase domain; LZ, leucine zipper; HLH, helix-loop-helix domain.

(E) Indicated IKK α protein levels in IKK α -deficient keratinocytes detected by Western blotting. Cont, no IKK α ; β -Actin, loading control.

(F) Binding of different forms of IKK α to the EGF promoter detected by ChIP assay with an anti-IKK α antibody for immunoprecipitation. All the samples were compared with IKK α -deficient cells (Mut). Error bars indicate \pm SD.

(G) RT-PCR for expression of EGF affected by different forms of IKK α . Error bars indicate \pm SD.

(H) Binding of different forms of IKK α (see Figure S11B) to the EGF promoter detected by ChIP assay. -300aa, a 300aa-deletion. Error bars indicate \pm SD.

(I) RT-PCR for expression of EGF affected by different forms of IKK α . Error bars indicate \pm SD.

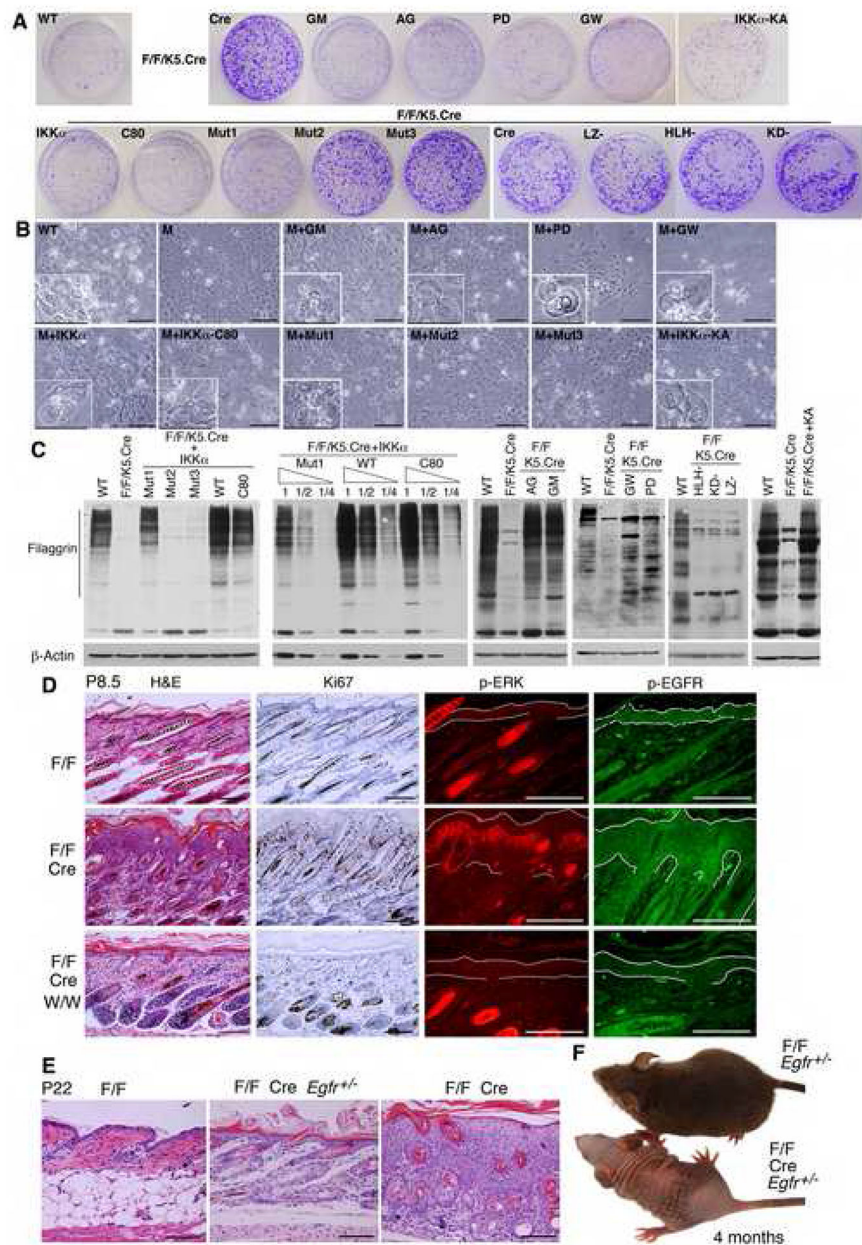


Figure 6. IKK α Switches Keratinocyte Proliferation and Differentiation via the EGFR Pathway

(A) Effects of different forms of IKK α and inhibitors on colony formation of indicated keratinocytes. Different IKK α forms include IKK α , C80, Mut1, Mut2, Mut3, LZ-, HLH-, KD-, and KA. AG, AG1478; GM, GM6001; GW, GW2974; PD, PD98059; keratinocyte colonies, stained with 0.5% crystal violet; WT, *Ikka*^{F/F} keratinocytes; F/F/K5.Cre, *Ikka*^{F/F}/K5.Cre keratinocytes.

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

(C) Filaggrin levels in indicated keratinocytes, as detected by Western blotting. 1, 1/2, 1/4, protein dilutions, β -Actin, loading controls.

(D) The effect of inactivation of EGFR on the epidermis in the indicated mice. P, postnatal day; W/W, *Egfr^{wa2/wa2}*; Cre, K5.Cre; H&E, hematoxylin and eosin; Ki67, dark brown color stained by immunohistochemistry; p-ERK, red color stained by immunofluorescence; p-EGFR, green stained by immunofluorescence; lines in p-ERK and p-EGFR, limits of the epidermis. Scale bars, 50 μ m.

(E) Skin histology of indicated mice, stained with H&E. Scale bars, 60 μ m.

(F) Appearances of indicated mice at 4 months of age.

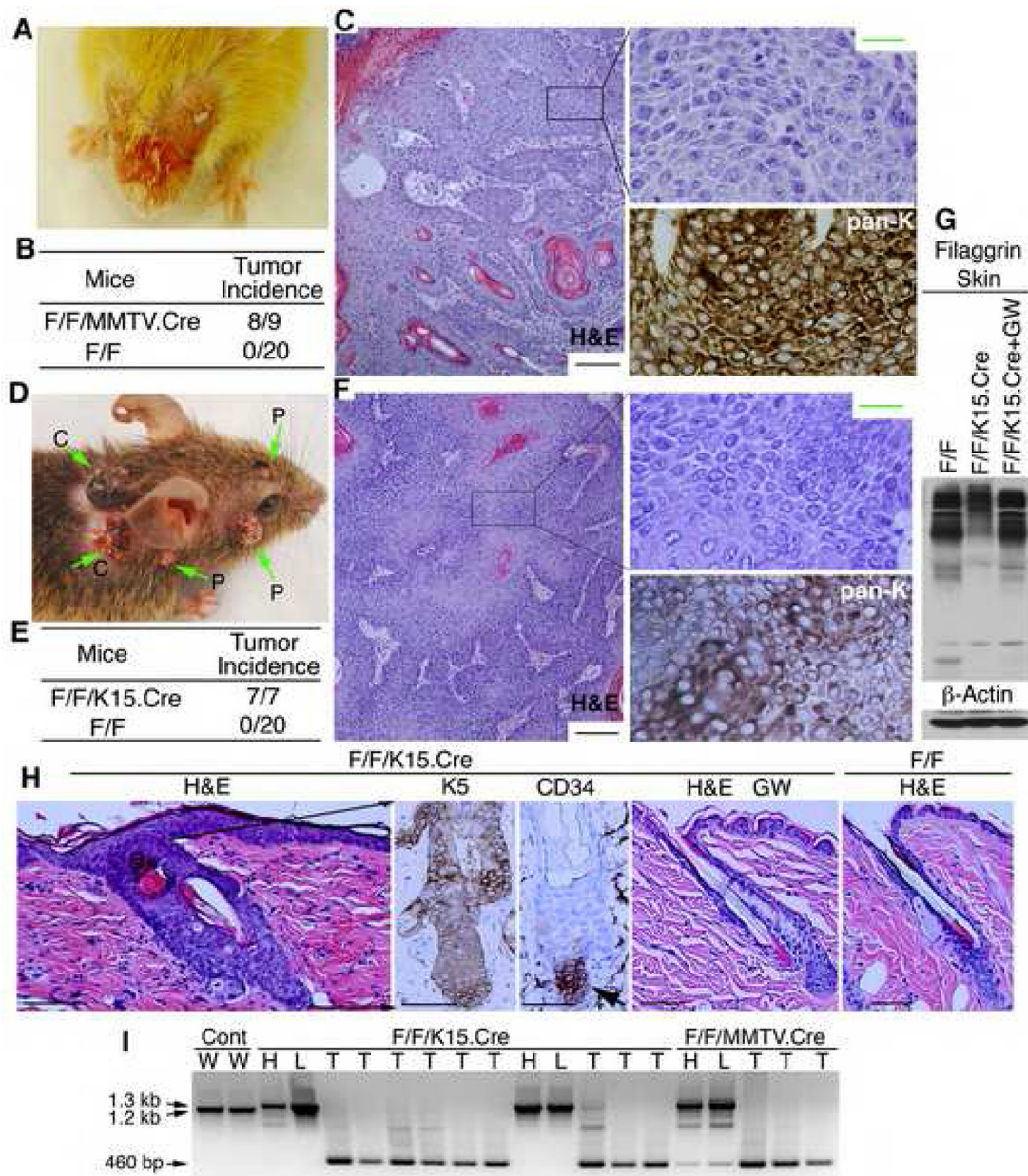


Figure 7. IKK α Deletion in Keratinocytes Causes Spontaneous Skin Cancer

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

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

(C) Pathologies of H&E stained tumor sections of *Ikka*^{F/F}/MMTV.Cre mice. Boxes, amplified (indicated by lines) and pan-keratin staining (brown). Black scale bar, 25 μ m; green scale bar, 10 μ m.

(D) Tumors in an *Ikka*^{F/F}/K15.Cre mouse 6 months after IKK α deletion. P, papillomas; C, carcinomas.

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

(F) Pathologies of H&E stained tumor sections of *Ikka^{F/F}/K15.Cre* mice. Boxes, amplified (indicated by lines) and pan-keratin staining (brown). Black scale bar, 25 μm ; green scale bar, 10 μm .

(G) Filaggrin levels in indicated skins, as detected by Western blotting. β -Actin, loading control.

(H) Skin sections from indicated mice stained with H&E and antibodies against K5 or CD34 (indicated by arrow). Brown color, K5 or CD34; lines, indicate hair follicle; GW, GW2974. Scale bar, 40 μm .

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

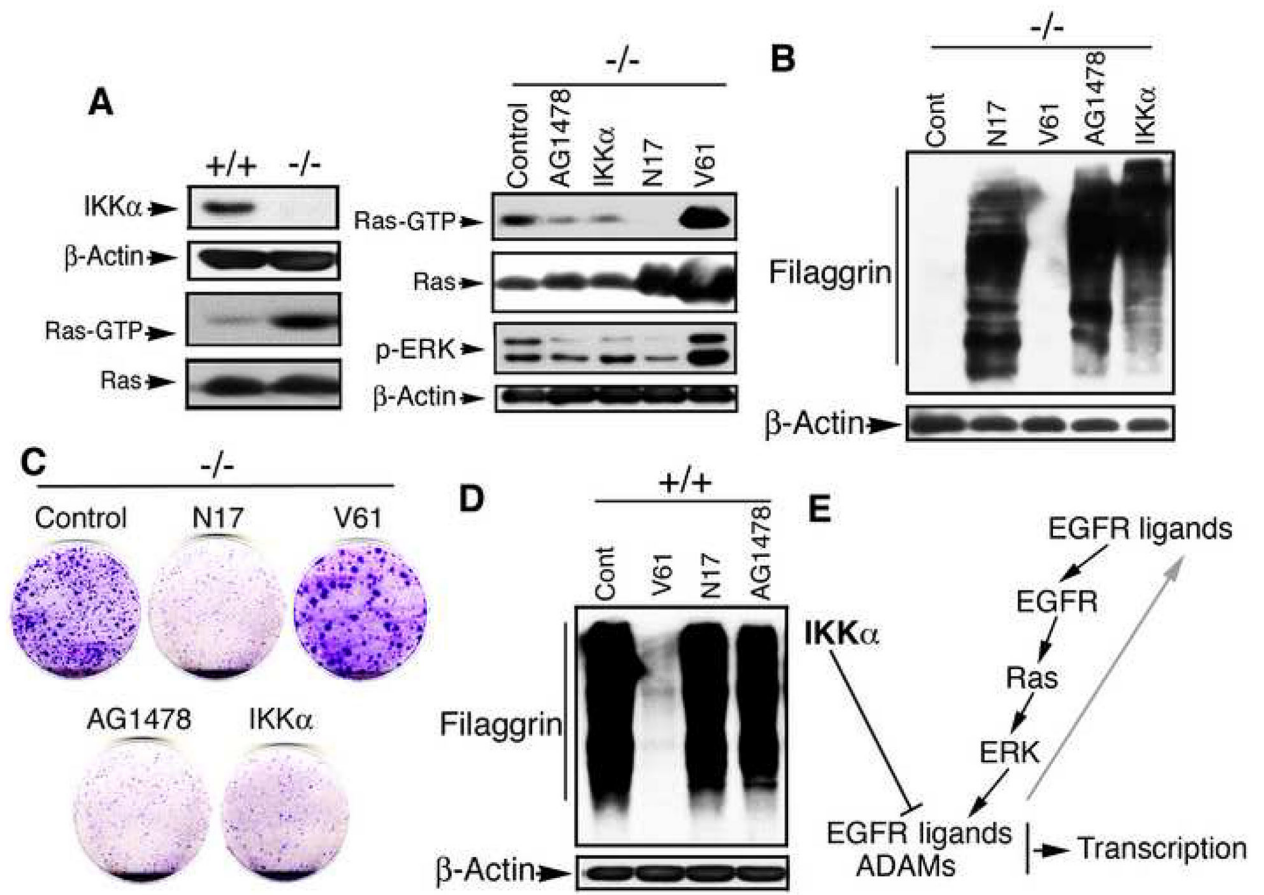


Figure 8. Elevated Ras Activity Incorporated with the EGFR Pathway in *IKKα*-null Keratinocytes

(A) Ras (GTP) activity in keratinocytes, as detected by GST-pull down and Western blotting; elevated Ras and ERK activities were down-regulated by *IKKα*, inactivation of Ras, and EGFR inhibitor. *+/+*, *Ikka*^{+/+}; *-/-*, *Ikka*^{-/-} keratinocytes; Ras-GTP, active form of Ras; Ras, p-ERK, and β -Actin, detected by Western blotting; AG1478, EGFR inhibitor; N17, domain negative form of Ras; V61, active form of Ras.

(B) Filaggrin expression in *Ikka*^{-/-} keratinocytes affected by indicated proteins, as detected by Western blotting. β -Actin, loading control.

(C) Colony formation of *Ikka*^{-/-} keratinocytes affected by indicated proteins, stained with 0.5% crystal violet blue.

(D) Filaggrin expression in *Ikka*^{+/+} keratinocytes affected by indicated proteins, as detected by Western blotting.

(E) A work hypothesis of *IKKα* function in keratinocytes for maintaining skin homeostasis and preventing skin cancer. Arrows, promotion; line, inhibition.