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14. Cells incubated with [^{32}P]orthophosphate (2 mCi/ml) for 5 hours were TNF-stimulated, washed, and harvested in lysis buffer. IKK was immunoprecipitated from precleared lysates using anti-IKKα as described (3). Immune complexes were washed with lysis buffer containing 2 M urea. Proteins were resolved by SDS–polyacrylamide gel electrophoresis (7.5%) and transferred to Immobilon-P membrane (Millipore) for autoradiography and immunoblot analysis. IKK assays were performed as described (3).
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28. We thank D. Rothwarf, J. DiDonato, and F. Mercurio for helpful discussion and advice, J. W. Li, G. Cadwell, and J. Lewis for excellent technical assistance, and B. Thompson for help with manuscript preparation. M.D., M.H., and Y.C. were supported by postdoctoral fellowships from the D. Collen Research Foundation (Belgium), Human Frontier Science Program, and Tobacco-Related Disease Research Program, respectively. Work was supported by grants from The National Institutes of Health. (R37 ES04151 and R01 AI43477) and the U.S. Department of Energy (DE-FG03-86ER60429). M.K. is a Frank and Else Schilling–American Cancer Society Research Professor.

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Limb and Skin Abnormalities in Mice Lacking IKKα

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The gene encoding inhibitor of kappa B (IκB) kinase α (IKKα; also called IKK1) was disrupted by gene targeting. IKKα-deficient mice died perinatally. In IKKα-deficient fetuses, limb outgrowth was severely impaired despite unaffected skeletal development. The epidermal cells in IKKα-deficient fetuses were highly proliferative with dysregulated epidermal differentiation. In the basal layer, degradation of IκB and nuclear localization of nuclear factor kappa B (NF-κB) were not observed. Thus, IKKα is essential for NF-κB activation in the limb and skin during embryogenesis. In contrast, there was no impairment of NF-κB activation induced by either interleukin-1 or tumor necrosis factor–α in IKKα-deficient embryonic fibroblasts and thymocytes, indicating that IKKα is not essential for cytokine-induced activation of NF-κB.

The IκB kinase, a large protein complex, phosphorylates two serine residues of the IκB proteins. This leads to degradation of IκB and activation of NF-κB transcription factors (*1*). IKKα was identified as a subunit of the IκB kinase complex that directly phosphorylates IκB (*2, 3*). IKKβ was subsequently identified as a second subunit of the IκB kinase complex that forms a heterodimer with IKKα (*3, 4*). In vitro studies have indicated that both IKKα and IKKβ (also called IKK2) may contribute to tumor necrosis factor–α (TNF-α)– and interleukin-1 (IL-1)–induced activation of NF-κB (*2–4*).

To assess the in vivo role of IKKα, we disrupted the *IKK*α gene by homologous recombination in E14.1 embryonic stem (ES) cells (*5*). A targeting vector was constructed to replace an exon encoding subdomain VI of the kinase catalytic portion with a neomycin resistance gene. Two correctly targeted ES clones successfully transmitted the disrupted allele through the germ line (Fig. 1A). The heterozygous (IKKα$^{+/-}$) mice were phenotypically normal and healthy. To generate IKKα$^{-/-}$ mice, the heterozygotes were crossed. IKKα$^{-/-}$ progeny were born with abnormal appearance and died within 4 hours after birth. Newborn IKKα$^{-/-}$ pups had defective development of limbs and tails (Fig. 1D), and their skin was abnormally shiny. Northern (RNA) and protein immunoblot analysis of embryonic fibroblast (EF) cells confirmed that the disruption of the *IKK*α gene abolished the expression of IKKα mRNA and protein (Fig. 1, B and C). Expression of mRNA and protein for IKKβ was slightly increased in IKKα$^{-/-}$ EF cells.

Examination of stained skeletal preparations from the fetus at 18.5 days postcoitum (dpc) revealed that IKKα$^{-/-}$ mice had no defect in development of bone or cartilage, although the lengths of limb, tail, and craniofacial bones and cartilage were shorter than those for wild-type animals (Fig. 1E). Leg bones were compactly and tightly folded and tail cartilage was rolled up in IKKα$^{-/-}$ pups. These findings indicate that skeletal development was normal; however, limb mesenchyme outgrowth was impaired in IKKα$^{-/-}$ fetuses. Activation of NF-κB is essential for limb development in chickens (*6*). Therefore, we analyzed whether IKKα was expressed in the developing limb by whole-mount in situ hybridization (*7*). *IKK*α was expressed predominantly in the limb buds of wild-type fetuses at 12.5 dpc (Fig. 2A). In IKKα$^{-/-}$ fetuses, *IKK*α was not expressed, and the limb bud showed a slightly abnormal phenotype relative to that of wild type (Fig. 2B). *IKK*β was also expressed in the limb buds, particularly the forelimbs of wild-type as well as IKKα$^{-/-}$ fetuses at 12.5 dpc (Fig. 2, C and D). A *Drosophila melanogaster* homolog of NF-κB, Dorsal, positively and negatively regulates expression of *twist* and *decapentaplegic* (*dpp*), respectively (*8*). The murine *twist* homolog is expressed in limb bud mesenchyme (*9*), and mutations in *TWIST* lead to craniofacial and limb anomalies in humans (*10*). In the wild-type fetuses, *Twist* was expressed in the limb buds at 12.5 dpc (Fig. 2E). However, expression of *Twist* was reduced in the limb buds of IKKα$^{-/-}$ fetuses at 12.5 dpc (Fig. 2F). Expression of the bone morphogenic protein-4 gene (*BMP4*), the vertebrate *dpp* homolog, was not altered in the limb buds of IKKα$^{-/-}$ fetuses at 12.5 dpc (Fig. 2, G and H). Reduced *Twist* expression in IKKα$^{-/-}$ fetuses was also observed at 13.5 dpc (*11*). Taken together, these results indicate that IKKα regulates gene expression required for limb development, possibly through activation of NF-κB.

Tissue sections of skin at 18.5 dpc were stained with hematoxylin and eosin and examined by light microscopy. At this developmental stage, the full program of epidermal differentiation was nearly complete in wild-type mice (Fig. 3A). In contrast with the ridged and laminated normal stratum corneum of wild-type mice, IKKα$^{-/-}$ mice exhibited prominent parakeratosis without a visible stratum granulosum (Fig. 3B). The stratum spinosum of IKKα$^{-/-}$ epidermis was hyperplastic. The development

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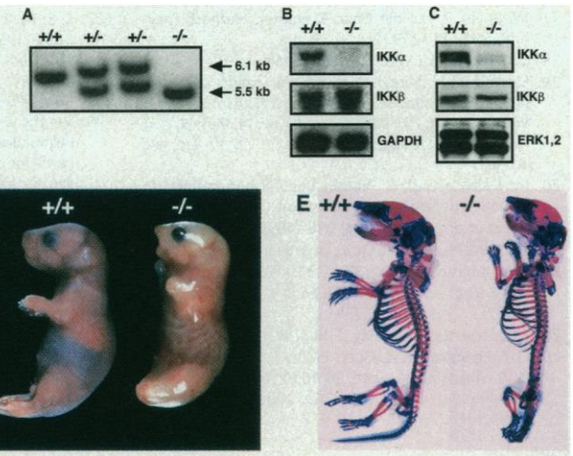


Fig. 1. Targeted disruption of the *IKK*α gene. (**A**) Southern (DNA) blot analysis of the IKKα wild-type (+/+), heterozygous (+/−), and homozygous (−/−) mutant embryos at 18.5 dpc. The 6.1-kb wild-type and the 5.5-kb targeted alleles are indicated by arrows. (**B**) Northern blot analysis of IKKα and IKKβ expression in wild-type and IKKα$^{-/-}$ EF cells. Total RNA was extracted, electrophoresed, transferred to nylon membranes, and hybridized with cDNA probes for IKKα and IKKβ. The same membrane was washed to release bound probe and rehybridized with a glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA probe. (**C**) Protein immunoblot analysis of IKKα and IKKβ expression in wild-type and IKKα$^{-/-}$ EF cells. Whole-cell lysates were immunoblotted with antibody to IKKα or IKKβ (Santa Cruz). The same membrane was blotted again with antibody to ERK1,2 (Santa Cruz). (**D**) Gross appearance of IKKα wild-type and mutant newborn mice. (**E**) The skeleton of IKKα wild-type and mutant embryos at 18.5 dpc. Embryos were double stained with Alizarin red and Alcian blue. Bone is stained red and cartilage blue.

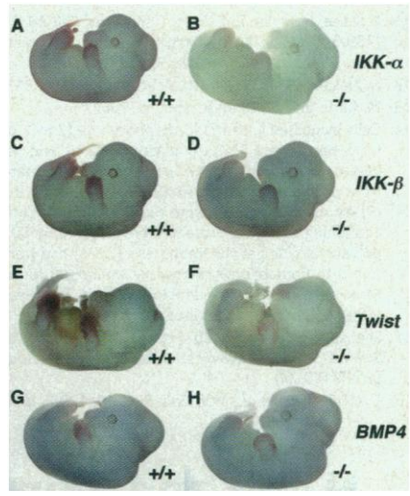


Fig. 2. Defective limb development in IKKα$^{-/-}$ mice. Expression of *IKK*α in the limb buds of wild-type embryos at 12.5 dpc (**A**), but not in IKKα$^{-/-}$ embryos (**B**). *IKK*β expression in the limb buds of wild-type (**C**) and IKKα$^{-/-}$ embryos (**D**) at 12.5 dpc. Reduced expression of *Twist* in the limb buds of IKKα$^{-/-}$ embryos (**E** and **F**). *BMP4* expression in wild-type embryos (**G**) and IKKα$^{-/-}$ embryos (**H**) at 12.5 dpc.

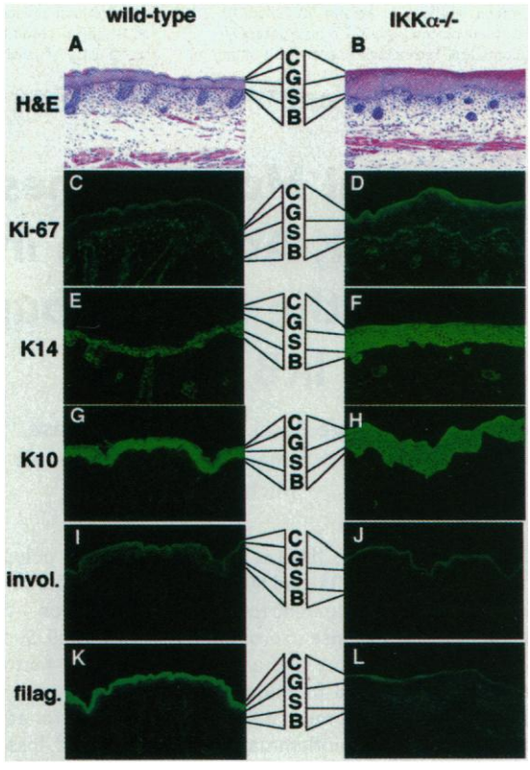
of hair follicles was retarded, and only small premature hair follicles were seen in IKKα$^{-/-}$ mice at this stage. The presence of hyperplastic epidermis prompted us to evaluate proliferative activity in the epidermis of IKKα$^{-/-}$ mice. We stained the skin sections with antibody to Ki-67 (anti–Ki-67), which is expressed in proliferating cells. Relatively few Ki-67–positive cells were observed in the basal cell layer of the epidermis of wild-type mice (Fig. 3C). In contrast, almost all of the basal cells and a few suprabasal cells expressed Ki-67 in IKKα$^{-/-}$ mice, indicating abnormal proliferation of the IKKα$^{-/-}$ cells in the basal layer (Fig. 3D).

A panel of antibodies for proteins expressed at defined stages of epidermal differentiation were used to examine whether IKKα deficiency affects keratinocyte maturation. Keratin K14 was expressed in one to two layers of basal cells in wild-type mice, whereas it was strongly expressed in the whole thickened epidermis in IKKα$^{-/-}$ mice (Fig. 3, E and F). Although keratin K10, one of the markers for terminal differentiation of stratified epithelia, was expressed in all keratinocytes except for the basal layer in both wild-type and IKKα$^{-/-}$ epidermis (Fig. 3, G and H), expression of several differentiation markers was impaired in IKKα$^{-/-}$ mice. These included involucrin and filaggrin, which are early and late differentiation markers of keratinocytes, respectively. Membranous expression of involucrin was observed in the upper stratum spinosum and stratum corneum from wild-type animals. In contrast, weak cytoplasmic expression was observed in the upper layer of epidermis from IKKα$^{-/-}$ mice (Fig. 3, I and J). Filaggrin was expressed in the stratum corneum and granulosum in wild-type epidermis; however, its expression was reduced in IKKα$^{-/-}$ epidermis (Fig. 3, K and L). Thus, immunohistological analysis of epidermis revealed that epidermal terminal differentiation was dysregulated in IKKα$^{-/-}$ mice. IKKα$^{-/-}$ mice might die shortly after birth as a result of the impaired skin barrier function, as demonstrated in mice lacking transglutaminase-1 or expressing dominant negative retinoic acid receptor (*12*).

The stratum spinosum is also thickened in transgenic mice with skin-specific expression of dominant negative IκB proteins (*13*). The expression of several keratin genes is transcriptionally controlled by NF-κB (*14*). Therefore, we immunohistologically analyzed expression of IκBs and NF-κB in the epidermis (*15*). In wild-type mice, IκBα and IκBβ were expressed in the stratum spinosum at 18.5 dpc; however, expression of IκBα and IκBβ was reduced in the basal epithelial layer (Fig. 4, A and C). In contrast, no reduction of expression of IκBα and IκBβ was observed in the basal layer of IKKα$^{-/-}$ mice (Fig. 4, B and D). We further analyzed the subcellular localization of RelA, a p65 protein of the NF-κB family. In wild-type mice, cytoplasmic expression of RelA was reduced in the basal layer as compared with that in the stratum spinosum (Fig. 4E). In addition, RelA was expressed in the nucleus in some basal layer cells (Fig. 4, E, G, and I). These observations indicate that NF-κB was activated in the basal cell layer of wild-type epidermis. In contrast, RelA was expressed in the cytoplasm but not in the nucleus of all cells in the basal layer in IKKα$^{-/-}$ epidermis, indicating that NF-κB activation did not occur in the basal layer of IKKα$^{-/-}$ epidermis (Fig. 4, F, H, and J). These results indicate that IKKα-induced NF-κB activation in the basal layer cells may be required for terminal differentiation of the epidermis at this developmental stage.

IKKα was originally identified as a kinase responsible for IL-1– and TNF-α–induced activation of NF-κB (*2–4*). Therefore, we analyzed the response to IL-1 and TNF-α in EF cells. Wild-type EF cells produced IL-6 in response to IL-1 and TNF-α. However, IKKα$^{-/-}$ EF cells produced almost the same amount of IL-6 in response to IL-1 and TNF-α (Fig. 5A). We next examined whether these cytokines induced degradation of IκBs and activation of NF-κB (*16*). IL-1 and TNF-α stimulation induced rapid degradation of IκBα and IκBβ in both wild-type and IKKα$^{-/-}$ EF cells (Fig. 5B). IL-1 and TNF-α stimulation also induced similar NF-κB DNA binding activity in wild-type and IKKα$^{-/-}$ EF cells and thymocytes (Fig. 5, C and D). This inconsistency with studies in which IKKα was required for IL-1- and TNF-α–induced NF-κB activation reflect compensative action of IKKβ in IKKα$^{-/-}$ mice. Indeed, IKKβ can act as a homodimer (*3, 4*).

Knockout mice have revealed a direct functional role of each member of the NF-κB family in immune regulation (*17*). However, these knockout mice do not show any developmental abnormalities despite a strong similarity between the NF-κB family members and the *D. melanogaster* protein Dorsal, which is activated early during embryogenesis and essential for development. However, there is the possibility that multiple NF-κB subunit gene knockouts may cause developmental abnormalities. Our present finding that IKKα activity is involved in mouse limb development is consistent with previous reports in chickens that inhibition of NF-κB activity by overexpression of dominant



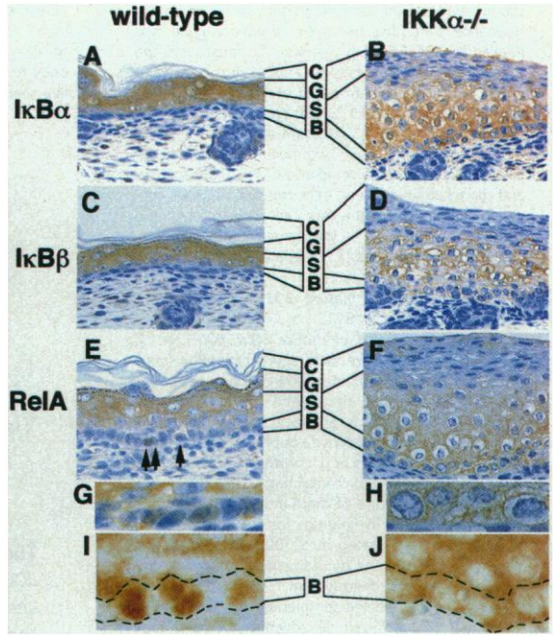


Fig. 3 (left). Defective epidermal development in IKKα$^{-/-}$ mice. Dorsal skins from wild-type (**A**) and IKKα$^{-/-}$ (**B**) fetuses at 18.5 dpc were sectioned and stained with hematoxylin and eosin (H&E). Dorsal skin sections from wild-type (C, E, G, I, and K) and IKKα$^{-/-}$ (D, F, H, J, and L) fetuses at 18.5 dpc were immunostained with anti-Ki67 (**C** and **D**), anti-keratin K14 (**E** and **F**), anti-keratin K10 (**G** and **H**), anti-involucrin (**I** and **J**), and anti-filaggrin (**K** and **L**) (*19*). C, stratum corneum; G, stratum granulosum; S, stratum spinosum; B, stratum basale. Image width for (A) to (L), 71 μm. **Fig. 4 (right).** Impaired NF-κB activation in IKKα$^{-/-}$ epidermis. Dorsal skin sections from wild-type (A, C, E, G, and I) and IKKα$^{-/-}$ (B, D, F, H, and J) fetuses at 18.5 dpc were immunostained with anti-IkBα (**A** and **B**), anti-IκBβ (**C** and **D**), and anti-RelA (**E** to **J**) (*15*). Arrows in (E) indicate the nuclear-localized RelA stainings. Higher magnification of the basal cell layer shows the nuclear-localized RelA in wild-type (G and I), and the cytoplasmic RelA stainings in IKKα$^{-/-}$ epidermis (H and J). Immunoreacted sections were counterstained with hematoxylin (A to H) or not (I and J). C, stratum corneum; G, stratum granulosum; S, stratum spinosum; B, stratum basale. Image width for (A) to (F), 32 μm; for (G) to (J), 14 μm.

negative IκB proteins resulted in limb deformity (*6*). The skin abnormality seen in IKKα knockout mice is pathologically similar to that in transgenic mice expressing dominant negative IκB proteins (*13*). Taken together, these results indicate that NF-κB activation mediated by IKKα-dependent IκB phosphorylation is essential for outgrowth in vertebrate limb development and the terminal differentiation of skin.

IL-1– and TNF-α–induced NF-κB activation and biological responses were not impaired in IKKα$^{-/-}$ cells. Thus, IKKα does not seem to be essential for cytokine-induced NF-κB activation, and IKKβ or other kinases may compensate for IKKα deficiency in IKKα$^{-/-}$ cells. RelA-deficient mice died around 14.5 dpc as a result of apoptosis of fetal hepatocytes (*18*). Despite the developmental defects, IKKα$^{-/-}$ embryos showed no apparent abnormality in the liver and were alive until birth. These results indicate that the contributions of IKKα and IKKβ to NF-κB activation may depend on the cell type and extracellular stimuli.

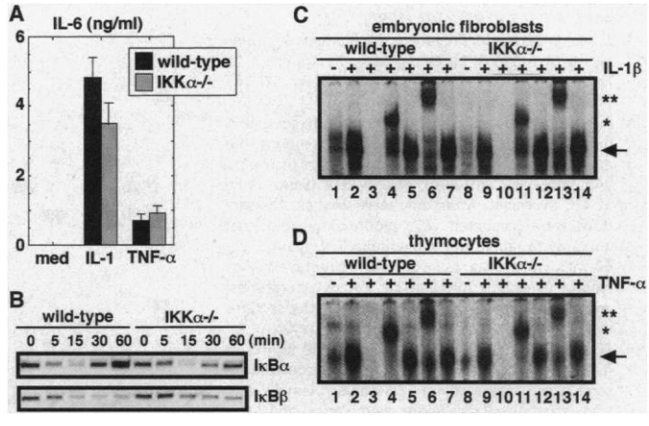


Fig. 5. Normal responsiveness to TNF-α and IL-1 in IKKα$^{-/-}$ mice. (**A**) Wild-type and IKKα$^{-/-}$ EF cells were treated with TNF-α (10 ng/ml) or IL-1β (100 U/ml) or left untreated in the culture medium (med) for 24 hours. Concentrations of IL-6 in the culture supernatants were measured by enzyme-linked immunosolvent assay (Genzyme). (**B**) Wild-type and IKKα$^{-/-}$ EF cells were deprived of serum by incubation in culture medium containing 0.1% fetal calf serum for 6 hours, then stimulated with TNF-α (50 ng/ml) for the indicated periods. Cells were lysed, and proteins were separated on SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and immunoblotted with anti-IκBα and anti-IκBβ. IL-1β stimulation resulted in similar results. Wild-type and IKKα$^{-/-}$ EF cells (**C**) or thymocytes from RAG2-deficient mice reconstituted with fetal liver cells (**D**) were stimulated for 20 min with IL-1β (1000 U/ml) or TNF-α (50 ng/m), respectively. Nuclear extracts were then prepared and incubated with a specific probe containing NF-κB–binding sites. NF-κB activity was determined by a gel mobility shift assay. Specificity was determined by adding nothing (lanes 2 and 9), specific competitor (lanes 3 and 10), or anti–NF-κB (lanes 4 and 11, anti-p50; lanes 5 and 12, anti-RelB; lanes 6 and 13, anti-RelA; lanes 7 and 14, anti-cRel). Inducible NF-κB complex is indicated by the arrow. The single and double asterisks indicate the supershifts induced by antibody to p50 and RelA, respectively.

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5. Genomic fragments corresponding to the murine *IKKα* locus were isolated from 129/SvJ genomic DNA library (Stratagene) with a murine *IKKα* cDNA fragment as a probe. A targeting vector was constructed to replace the 0.5-kb genomic fragment containing an exon encoding

subdomain VI of the catalytic portion with a neomycin resistance gene. The neomycin resistance gene was flanked by the 5.6-kb 5′ genomic fragment and the 1.1-kb 3′ fragment. A herpes simplex virus–thymidine kinase cassette was introduced at the 5′ end of the genomic fragment. E14.1 ES cells were transfected with the linearized targeting vector and selected by addition of G418 and gancyclovir. Two independently targeted ES clones were microinjected into C57BL/6 blastocysts, and the resulting chimeric mice from both clones successfully transmitted the targeted allele through the germ line. Generation of the homozygotes was done essentially as described [K. Takeda *et al.*, *Nature* **380**, 627 (1996)].

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7. The desired fragments of mouse *IKKα*, *IKKβ*, *BMP4*, and *Twist* cDNAs were subcloned into pBluescript (Stratagene). Complementary RNA probes were then prepared by using a digoxigenin RNA labeling kit (Boehringer Mannheim). Embryos at 12.5 dpc were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 5 hours at 4°C with rocking. Embryos were washed with PBS containing 0.1% Tween 20, treated with a mixture of methanol and 30% hydrogen peroxide (5:1 in volume) for 5 hours at room temperature, dehydrated into 100% methanol, and stored at −20°C. For hybridization, embryos were incubated at 65°C overnight in hybridization buffer with digoxigenin-labeled complementary RNA probes (0.4 μg/ml). Hybridized digoxigenin-labeled probes were detected with a nucleic acid detection kit (Boehringer Mannheim).
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15. Pieces of the dorsal skin were removed, fixed in 10% buffered formalin, and used for paraffin sections. The deparaffin was removed and sections were incubated with anti-IκBα, anti-IκBβ, or anti-RelA (Santa Cruz) at 4°C overnight. After they were washed, the sections were incubated with biotin-conjugated goat antibody to rabbit immunoglobulin G (Dako A/S) for 60 min, which was visualized with streptavidin-peroxidase (Vecstatin ABC Elite kit; Vector Laboratories) and diaminobenzene (Sigma). Some of the sections were lightly counterstained with hematoxylin.
16. EF cells were stimulated with TNF-α (50 ng/ml) or IL-1β (1000 U/ml) and lysed. Proteins from cell lysates were separated on SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and incubated with antibodies to IκBα, IκBβ, IKKα, or IKKβ (Santa Cruz). Bound antibody was visualized with an enhanced chemiluminescence system (DuPont). Preparation of nuclear extracts and electrophoretic mobility shift assays were done as described [O. Adachi *et al.*, *Immunity* **9**, 143 (1998)].
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19. Pieces of the dorsal skin were removed and used for frozen sections. After fixation with cold acetone, frozen sections (5 μm) were incubated with primary antibodies for 1 hour at room temperature, washed in PBS, and incubated with fluorescein isothiocyanate–conjugated second antibodies. Slides were then analyzed by fluorescence microscopy. Primary antibodies used in this study were as follows: mouse antibody to K14 (LL002, Novocastra), rabbit antibody to keratin 10 (BAbCO), rabbit antibody to involucrin (BAbCO), mouse antibody to filaggrin (Biomedical Technologies), and rabbit antibody to Ki67 (Novocastra).
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Abnormal Morphogenesis But Intact IKK Activation in Mice Lacking the IKKα Subunit of IκB Kinase

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The oligomeric IκB kinase (IKK) is composed of three polypeptides: IKKα and IKKβ, the catalytic subunits, and IKKγ, a regulatory subunit. IKKα and IKKβ are similar in structure and thought to have similar function—phosphorylation of the IκB inhibitors in response to proinflammatory stimuli. Such phosphorylation leads to degradation of IκB and activation of nuclear factor κB transcription factors. The physiological function of these protein kinases was explored by analysis of IKKα-deficient mice. IKKα was not required for activation of IKK and degradation of IκB by proinflammatory stimuli. Instead, loss of IKKα interfered with multiple morphogenetic events, including limb and skeletal patterning and proliferation and differentiation of epidermal keratinocytes.

NF-κB/Rel proteins are dimeric transcription factors whose activity is regulated by interaction with IκB inhibitors (*1*). In nonstimulated cells NF-κB proteins are retained in the cytoplasm because IκBs mask their nuclear localization sequence. Exposure to proinflammatory stimuli results in rapid phosphorylation, ubiquitilation, and degradation of the IκBs (*1*). Freed

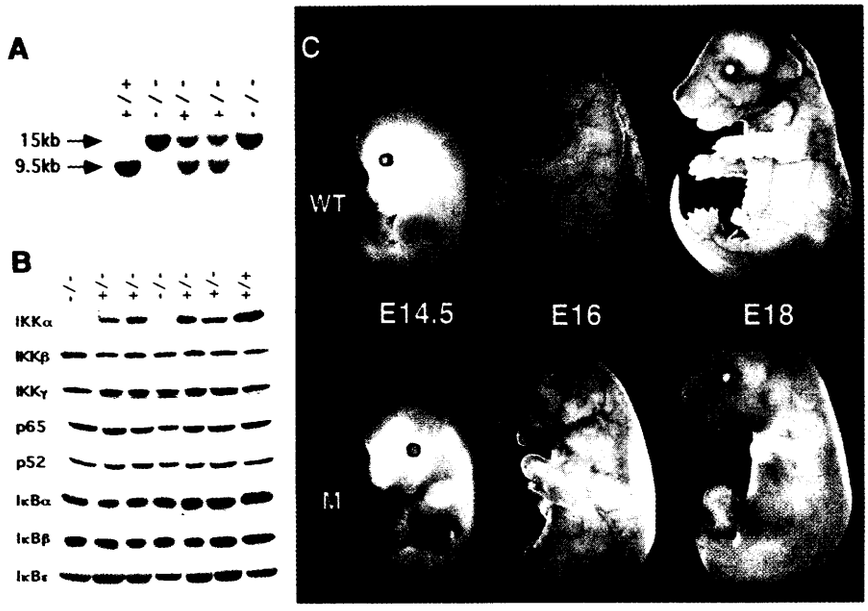

Fig. 1. Phenotypic and genotypic analysis of IKKα-deficient mice. (**A**) Southern blot analysis of Sac I–digested genomic DNA derived from E18 fetuses of different genotypes. (**B**) Protein immunoblot analysis of protein extracts prepared from muscle tissue of E18 fetuses of the indicated genotypes. Extracts were separated by SDS-PAGE, transferred to a nylon membrane, and probed with antibodies against the indicated proteins. (**C**) Appearance of wild-type (WT) and *Ikkα*$^{-/-}$ (M) fetuses collected at E14.5, E16, and E18. The tight and smooth appearance of mutant skin is apparent.