IKKα is a critical coregulator of a Smad4-independent TGFβ-Smad2/3 signaling pathway that controls keratinocyte differentiation

Pascal Descargues*, Alok K. Sil*†, Yuji Sano*, Olexandr Korchynskyi*‡, Gangwen Han§, Philip Owens§, Xiao-Jing Wang§, and Michael Karin*¶

*Laboratory of Gene Regulation and Signal Transduction, Department of Pharmacology, University of California at San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0723; †Department of Microbiology, University of Calcutta, Calcutta 700 073, India; ‡Department of Orthopaedic Surgery, Virginia Commonwealth University, Richmond, VA 23298; and §Department of Otolaryngology, Dermatology, and Cell and Developmental Biology, Oregon Health and Science University, Portland, OR 97239

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Cell-cycle exit and differentiation of suprabasal epidermal keratinocytes require nuclear IκB kinase α (IKKα), but not its protein kinase activity. IKKα also is a suppressor of squamous cell carcinoma (SCC), but its mode of action remains elusive. Postulating that IKKα may serve as a transcriptional regulator in keratinocytes, we searched for cell-cycle-related genes that could illuminate this function. IKKα was found to control several Myc antagonists, including *Mad1*, *Mad2*, and *Ovol1*, through the association with TGFβ-regulated Smad2/3 transcription factors and is required for Smad3 recruitment to at least one of these targets. Surprisingly, Smad2/3-dependent *Mad1* induction and keratinocyte differentiation are independent of Smad4, the almost universal coregulator of canonical TGFβ signaling. IKKα also is needed for nuclear accumulation of activated Smad2/3 in the epidermis, and Smad2/3 are required for epidermal differentiation. We suggest that a TGFβ–Smad2/3–IKKα axis is a critical Smad4-independent regulator of keratinocyte proliferation and differentiation.

epidermis | cornification | terminal differentiation

A critical mediator of NF-κB activation (1), IκB kinase (IKK) consists of two catalytic subunits, IKKα and IKKβ (2–5), and a regulatory subunit, IKKγ/NEMO (6, 7). Despite structural similarity, IKKα and IKKβ have nonredundant functions, with IKKβ being the predominant IKK (1, 8) and IKKα being a critical regulator of keratinocyte differentiation (9, 10). Without IKKα, epidermal keratinocytes exhibit enhanced proliferation and failure to differentiate. Consequently, *Ikkα*$^{-/-}$ mice are born enshrouded in a taut and thickened, nonstratified, epidermal sheet devoid of barrier function.

The mammalian epidermis is a stratified squamous epithelium in which basal keratinocytes undergo asymmetric cell divisions, giving rise to nonproliferative progeny that embark on a differentiation program as they delaminate and move upward through the spinous and granular layers before generating the cornified layer, which provides the crucial barrier function (11, 12). Without IKKα, this process is blocked, and basal keratinocytes fail to exit the cell cycle (9, 10, 13). Isolated *Ikkα*$^{-/-}$ keratinocytes proliferate uncontrollably and do not respond to differentiation-inducing signals such as high Ca^{2+} (9, 13). The reexpression of IKKα in *Ikkα*$^{-/-}$ keratinocytes induces growth arrest and allows terminal differentiation, but this function depends neither on IKKα's protein kinase activity nor on NF-κB. Instead, it requires nuclear accumulation of IKKα (14).

Recently, IKKα was identified as a tumor suppressor in squamous cell carcinoma (SCC), a type of cancer derived from squamous epithelia of the skin, oral and nasal cavities, esophagus, and other sites (15). Decreased nuclear IKKα expression was found in about one third of oral SCCs, mainly those that exhibit poorly differentiated phenotype and poor prognosis (16). These results strongly suggest that loss of nuclear IKKα contributes to malignant conversion of keratinocytes to less differentiated and hyperproliferative SCC. The mechanism by which IKKα controls the keratinocyte cell cycle is not known, but, as suggested from the above discussion, IKKα function is likely to be exerted in the nucleus.

Here, we describe IKKα target genes involved in negative regulation of keratinocyte proliferation. We also provide evidence that IKKα activates these genes through interaction with TGFβ-regulated Smad transcription factors. Although the prevailing mode of TGFβ action entails the interaction of receptor substrate Smads (R-Smads) with the co-Smad, Smad4 (17, 18), keratinocyte and epidermal differentiation, which depend on Smad2/3 and IKKα, are Smad4-independent. We suggest that IKKα serves as a nuclear cofactor for Smad2/3 in the epidermis.

Results

Identification of IKKα-Dependent Cell-Cycle Regulators. One of the earliest responses to IKKα reexpression in *Ikkα*$^{-/-}$ keratinocytes is growth arrest (13). Therefore, we examined the IKKα dependence of known cell-cycle regulators. Among the analyzed genes, the one showing the greatest IKKα dependence was *Max dimerizer 1* (*Mad1*) [Fig. 1 *A* and *B* and supporting information (SI) Fig. 7]. Mad1 is a transcriptional regulator whose immediate relatives are Mad2, Mad3, and Mad4 proteins that heterodimerize with Max to form Mad/Max dimers that antagonize Myc/Max dimers (19, 20). Mad/Max dimers were linked to the inhibition of cell proliferation and the induction of differentiation in a variety of cell types, including keratinocytes (21), in which c-Myc stimulates proliferation and inhibits differentiation (22). The expression of *Ovol1*, *Mad2*, and, to a lesser extent, *Mad3* and *Mad4* also was reduced in the absence of IKKα (SI Fig. 7*A*). Interestingly, Ovol1 acts as a c-Myc repressor in keratinocytes (23). Because of the magnitude of its IKKα dependence, we chose *Mad1* as a model gene. Mad1 expression was strikingly reduced in the *Ikkα*$^{-/-}$ epidermis, which also was deficient in the expression of Ovol1 (Fig. 1 *C* and *D*).

During differentiation triggered by Ca^{2+} (Fig. 2*A*) or confluency (Fig. 2*B*), Mad1 was induced in WT, but not in *Ikkα*$^{-/-}$, keratinocytes. The introduction of IKKα, even in a catalytically inactive form, into *Ikkα*$^{-/-}$ keratinocytes restored Mad1 expres-

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¶To whom correspondence should be addressed. E-mail: mkarin@ucsd.edu.

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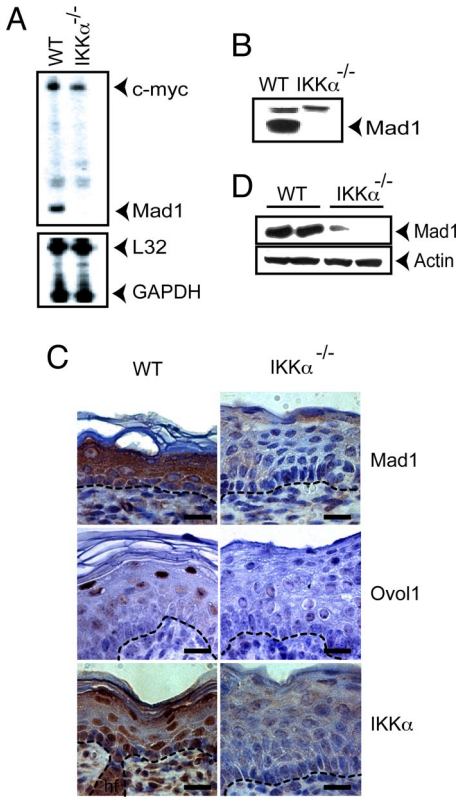

Fig. 1. Keratinocyte Mad1 expression is IKKα-dependent. (*A*) RNA from WT and *Ikkα*$^{-/-}$ keratinocytes was analyzed for the expression of the indicated genes by RNase protection. (*B*) Whole-cell lysates of WT and *Ikkα*$^{-/-}$ keratinocytes were analyzed for Mad1 expression by immunoblotting. (*C*) Cross-sections of skin from WT and *Ikkα*$^{-/-}$ E18 fetuses were stained with H&E and antibodies to Mad1, Ovol1, and IKKα. (Scale bars: 25 μm.) (*D*) Protein lysates of E18 WT and *Ikkα*$^{-/-}$ skin were analyzed for Mad1 expression by immunoblotting.

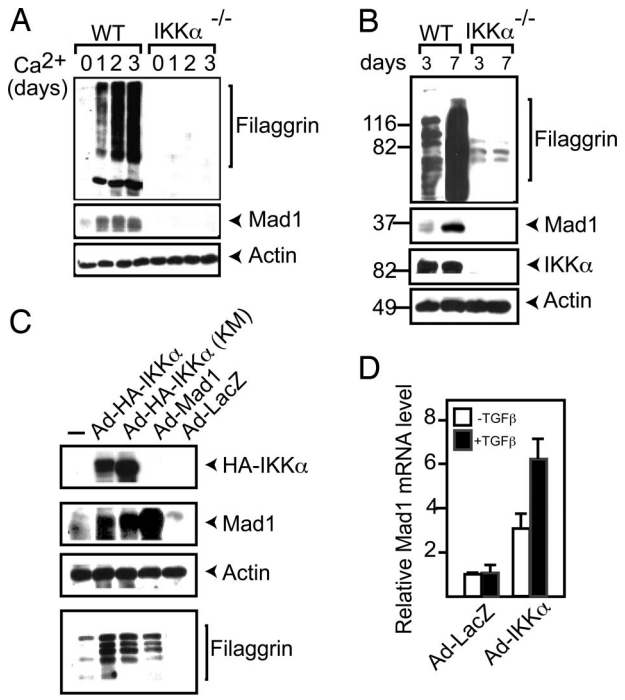

Fig. 2. Mad1 is induced during keratinocyte differentiation and by TGFβ in an IKKα-dependent manner. (*A*) WT keratinocytes were incubated with 0.2 mM Ca^{2+}. Cell lysates were prepared when indicated, and filaggrin and Mad1 expression were analyzed by immunoblotting. (*B*) WT and *Ikkα*$^{-/-}$ keratinocytes were examined after 3 or 7 days of culture for the expression of Mad1 and filaggrin by immunoblotting. (*C*) *Ikkα*$^{-/-}$ keratinocytes were infected with adenoviruses encoding the indicated proteins. After 5 days, cells were lysed, and protein expression was analyzed by immunoblotting. (*D*) RNA was extracted from IKKα- or LacZ-adenovirus-infected *Ikkα*$^{-/-}$ keratinocytes incubated with or without TGFβ1 for 2 h. *Mad1* mRNA was analyzed by real-time PCR.

sion, along with the differentiation marker filaggrin (Fig. 2*C*). Ectopic Mad1 expression resulted in a modest increase in filaggrin expression, an effect that may be due to the inhibition of *Ikkα*$^{-/-}$ keratinocyte proliferation (data not shown).

IKKα Is Needed for TGFβ-Regulated Gene Expression and Inhibition of Proliferation in Keratinocytes. In keratinocytes, Mad1 is induced by TGFβ (21), whose growth-inhibitory activity in these cells is partially dependent on Ovol1 (23). Interestingly, *Ikkα*$^{-/-}$ keratinocytes failed to arrest (SI Fig. 8) and up-regulate *Mad1* mRNA (Fig. 2*D*) also in response to TGFβ1. The reexpression of IKKα restored TGFβ responsiveness (Fig. 2*D*). The induction of $p21^{WAF1}$ showed partial IKKα dependence, but *Smad7*, encoding a TGFβ-inducible inhibitory i-Smad protein (24), was fully inducible in the absence of IKKα (SI Fig. 9). Thus, IKKα is required for many, but not all, TGFβ-elicited transcriptional responses in keratinocytes.

IKKα Interacts with TGFβ-Regulated R-Smads and Translocates to the Nucleus in Response to TGFβ. TGFβ family members signal via specific serine/threonine kinase receptors whose activation induces the association of receptor-regulated (R) Smad transcription factors with co-Smad and nuclear accumulation of the resulting complex (25). We examined whether IKKα interacts with R-Smads and co-Smads. GST-pulldown experiments using GST-Smad2–4-fusion proteins and lysates of HaCaT cells containing HA-tagged IKKα showed strong and weak binding of IKKα to Smad3 and Smad2, respectively, but no interaction with Smad4 (Fig. 3*A*). Transient transfection experiments revealed an association of IKKα with Smad3 that was potentiated by a constitutively activated form of TGFβ type 1 receptor, ALK5(T204D) (Fig. 3*B*). As predicted by the pulldown experiments, Smad4 did not interact with IKKα unless Smad3 was present (data not shown). An efficient and specific coprecipitation of endogenous IKKα with Smad3 was seen in primary mouse keratinocytes, especially after TGFβ treatment (Fig. 3*C*).

Nuclear accumulation of IKKα is critical for the induction of keratinocyte differentiation (14). TGFβ signaling caused nuclear accumulation of IKKα in primary mouse keratinocytes, whose kinetics paralleled the onset of Mad1 induction (Fig. 3*D* and SI Fig. 10).

IKKα Is Required for Smad3 Recruitment to *Mad1* Chromatin. We examined the transcriptional regulatory region of the *Mad1* gene and identified potential Smad-binding elements (SBEs) (18) in its 5′-flanking region at positions −534 and −21 and within intron 1 at positions +390 and +394. Mutational study revealed that both of these SBEs are required for IKKα and Smad3 responsiveness (data not shown). ChIP experiments using *Ikkα*$^{-/-}$ keratinocytes reconstituted with various HA-tagged IKKα constructs revealed TGFβ-induced IKKα binding to the SBE-containing regions of the *Mad1* promoter and intron 1, but not to the SBE-devoid exon 6 (Fig. 4*A* and SI Fig. 11). Chromatin recruitment of IKKα required its NLS, but not its kinase activity (Fig. 4*A*). Smad3 was recruited to the same regions of the *Mad1* gene after TGFβ treatment of WT keratinocytes (Fig. 4*B*). No Smad3 recruitment to the *Mad1* chromatin could be detected in

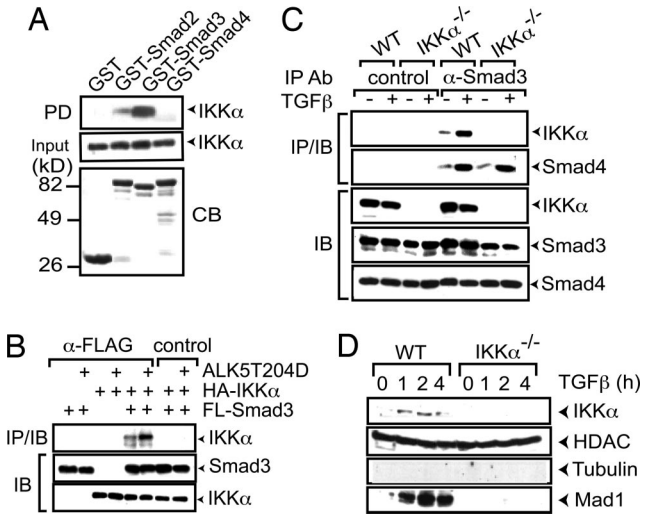

Fig. 3. TGFβ stimulates complex formation between IKKα and Smad3 and induces IKKα nuclear translocation. (*A*) GST Smad2, −3, or −4-fusion proteins on GSH agarose beads were incubated with extracts of HaCaT cells expressing HA-IKKα. After extensive washing, bound proteins were eluted, gel was separated, and the presence of HA-IKKα was examined by immunoblotting. PD, pulldown; CB, Coomassie blue-stained gel. (*B*) HaCaT cells were transfected with various combinations of HA-IKKα, Flag-Smad3, and ALK5(T204D) expression vectors. After 2 days, whole-cell lysates were immunoprecipitated (IP) with Flag antibodies, gel separated, and immunoblotted (IB) with either Flag or HA antibodies. (*C*) WT and *Ikk*$\alpha^{-/-}$ keratinocytes were prepared and treated with or without TGFβ1 for 1 h. Cell lysates were immunoprecipitated (IP) with Smad3 or isotype-matched control antibody, followed by immunoblotting (IB) with the indicated antibodies. (*D*) WT and *Ikk*$\alpha^{-/-}$ keratinocytes were incubated with TGFβ1. Nuclear extracts were prepared at the indicated times and analyzed by immunoblotting.

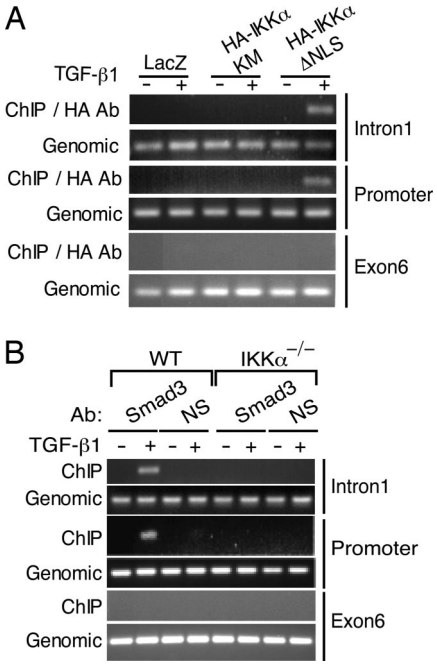

Fig. 4. IKKα is required for Smad3 recruitment to the *Mad1* regulatory region. (*A*) Primary keratinocytes were transduced with LacZ, HA-IKKα(KM), or HA-IKKα(ΔNLS) adenoviruses. After 2 days, cells were treated or not with TGFβ1 for 1 h. After fixation, nuclear extracts were prepared, sonicated, and immunoprecipitated with HA antibodies. The presence of 5′ SBE-containing region (promoter), intron 1, and exon 6 sequences was analyzed by PCR. (*B*) Primary WT and *Ikk*$\alpha^{-/-}$ keratinocytes were treated with or without TGFβ1 as indicated. After fixation, nuclear extracts were prepared, sonicated, and immunoprecipitated with Smad3 or RelA antibodies. The presence of the 5′ SBE-containing region (promoter) and exon 6 was analyzed by PCR.

Ikk$\alpha^{-/-}$ keratinocytes. It indicates that IKKα and Smad3 are likely to be present within the same complex.

TGFβ Signaling Controls Mad1 Expression, IKKα Nuclear Localization, and Keratinocyte Proliferation and Differentiation. To further investigate the role of Smad transcription factors in the regulation of *Mad1* expression, we prepared primary keratinocytes from *Smad7* gene-switch-transgenic mice (26). The expression of this i-Smad in keratinocytes cultured in high Ca^{2+} inhibited Mad1, filaggrin, and loricrin expression (Fig. 5*A*). The expression of *Smad7* between embryonic day 14.5 (E14.5) and birth interfered with proper epidermal differentiation, as indicated by aberrant and reduced filaggrin and loricrin expression (SI Fig. 12). The expression of *Smad7* in adult mice prevented nuclear accumulation of IKKα, inhibited Mad1 expression, and led to marked epidermal hyperplasia (Fig. 5*B*).

Next we examined the role of Smad3 and Smad2 in epidermal differentiation. Although it is known that the disruption of either *Smad3* or *Smad2* alone does not interfere with keratinocyte differentiation (ref. 27 and data not shown), this may be due to redundancy between these two R-Smads. To assess this theory, we used siRNA-mediated knockdown of *Smad2* in *Smad3*$^{-/-}$ keratinocytes and found reduced Ca^{2+}-induced loricrin and Mad1 expression (Fig. 5*C*). In addition, we crossed *Smad2*-floxed mice (28) and CrePR1 mice (29) to the *Smad3*$^{-/-}$ background to allow for keratinocyte-specific *Smad2* deletion. Although we did not recover any mouse embryos older than E14.5 lacking both Smad3 and Smad2 in keratinocytes, *Smad3*$^{-/-}$/*Smad2*$^{F/\Delta}$ embryos that were alive at E15.5 exhibited dramatic reduction in terminal epidermal differentiation, whose specific markers are filaggrin and loricrin, relative to *Smad3*$^{-/-}$/*Smad2*$^{F/F}$ counterparts (Fig. 5*D*). Consistent with the Smad2 siRNA-silencing experiment, this result suggests that the reduction of ≈50% of Smad2 protein is sufficient to overcome the compensation for Smad3 loss and inhibit terminal epidermal differentiation.

IKKα-Dependent Smad2/3 Activation and Keratinocyte Differentiation Are Smad4-Independent. With rare exceptions, as recently demonstrated for TIF1γ-mediated erythroid differentiation (30), most R-Smad-dependent TGFβ responses require Smad4 (17). However, mice lacking Smad4 in epidermal keratinocytes do not show obvious differentiation defects (31, 32). This result prompted us to examine whether Smad4 is required for IKKα-dependent TGFβ-induced gene expression. In adult WT mouse skin, Smad4 was mainly present in basal keratinocytes, whereas nuclear phospho-Smad2/3 and IKKα were similarly distributed in nuclei of both basal and suprabasal keratinocytes (Fig. 6*A*). In the *Smad4*-deficient epidermis, IKKα and phospho-Smad2/3 remained nuclear, and their distribution appeared identical to that in WT epidermis. However, in the *Ikk*$\alpha^{-/-}$ epidermis, IKKα was no longer present, and nuclear staining with phospho-Smad2/3 antibodies was severely diminished, but Smad4 localization was not affected (Fig. 6*B*).

Discussion

Analysis of *Ikk*α-null mice revealed that IKKα is a critical regulator of keratinocyte proliferation and differentiation (9, 13), acting in the nucleus within a kinase-independent manner (14). This view is supported by the finding that keratinocyte-restricted expression of IKKα, which, even as a "kinase-dead" form, rescues epidermal morphogenesis in *Ikk*$\alpha^{-/-}$ mice (14). However, the transplantation of *Ikk*$\alpha^{-/-}$ skin onto WT recipients also rescues differentiation (13), and a keratinocyte-

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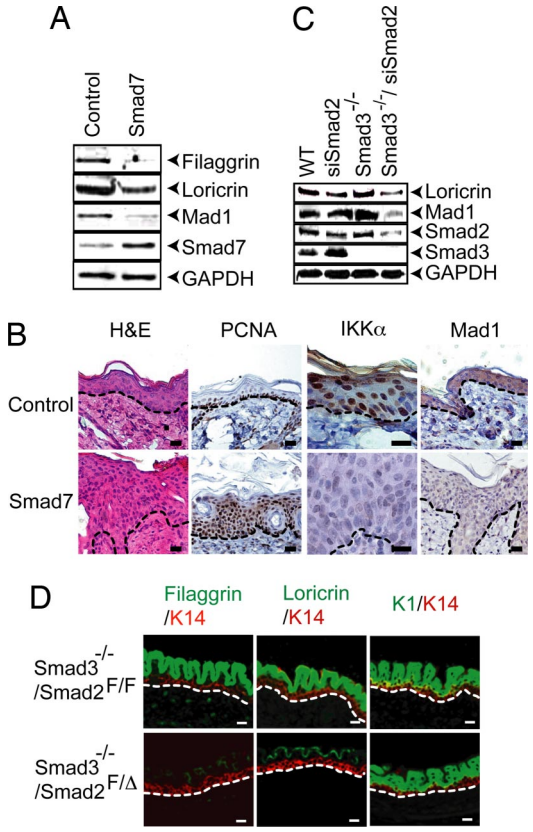

Fig. 5. Abrogation of endogenous Smad2/3 signaling inhibits epidermal differentiation, IKKα nuclear accumulation, and Mad1 expression. (*A*) Primary keratinocytes were isolated from control and bigenic Smad7 (K5.GLp65/tata.Smad7) (26) mice and cultured to confluency in high-calcium medium with RU486. After 48 h, cells were analyzed by immunoblotting. (*B*) Paraffin-embedded skin sections were prepared from adult control and Smad7 bigenic mice and were treated with RU486 for 12 days before analysis. Sections were stained with H&E and antibodies to PCNA, IKKα, and Mad1. (*C*) Primary keratinocytes from WT and *Smad3*$^{-/-}$ mice were cultured in high-Ca^{2+} medium for 48 h in the presence or absence of 100 nM Smad2-specific siRNA and analyzed by immunoblotting. (*D*) Immunofluorescence analysis of skin sections from E15.5 *Smad3*$^{-/-}$/*Smad2*$^{F/F}$ and *Smad3*$^{-/-}$/*Smad2*$^{F/\Delta}$ embryos. Dashed lines highlight the border between the epidermis and the dermis. (Scale bars: *B* and *D*, 25 μm.)

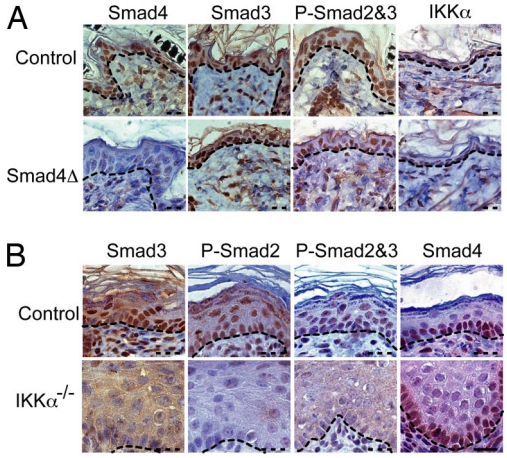

Fig. 6. IKKα, but not Smad4, is required for the nuclear accumulation of activated Smad2/3 and keratinocyte differentiation. (*A*) Paraffin-embedded skin sections were prepared from P14 control and *Smad4*$^{F/F}$*;K5-CrePR1* (*Smad4*Δ) mice and were stained with antibodies to Smad4, Smad3, phospho-Smad2,3 (P-Smad2&3), and IKKα. (*B*) Paraffin-embedded skin sections of control (WT) and *Ikk*$\alpha^{-/-}$ E18 embryos were stained with antibodies to Smad3, phospho-Smad2 (P-Smad2), P-Smad2&3, and Smad4. Dashed lines highlight the border between the epidermis and the dermis. (Scale bar: in *B* for *A* and *B*, 25 μm.)

specific ablation of *Ikk*α results in only a partial differentiation defect, preventing formation of the epidermal barrier (33). These results suggest that, in addition to acting autonomously in isolated keratinocytes, IKKα also may act in dermal fibroblasts to produce factors that allow IKKα-deficient keratinocytes to partially differentiate when in contact with a normal dermis. Nonetheless, studies of SCC, cancers derived from squamous epithelial keratinocytes, underscore the importance of nuclear IKKα in negative control of keratinocyte proliferation and demonstrate that its loss results in an aggressive phenotype with poor prognosis (15, 16). To understand how IKKα controls keratinocyte proliferation, we identified cell-cycle genes whose expression is IKKα-dependent. Intriguingly, several of these genes encode negative regulators of Myc action or expression, *Mad1*, *Mad2*, and *Ovol1* that are induced by TGFβ. These results are consistent with the finding that Myc is a positive regulator of keratinocyte proliferation and an inhibitor of terminal differentiation (22). Importantly, the expression of these genes in TGFβ-treated or differentiating keratinocytes depends on IKKα, but not on Smad4, the canonical coregulator of TGFβ-induced transcription (17, 18).

Investigation of the mechanism by which IKKα controls *Mad1* transcription revealed that IKKα interacts with Smad3-containing transcription-activating complexes in a TGFβ-dependent manner and is needed for their recruitment to the *Mad1* control region. IKKα is recruited to the same region of the *Mad1* control region as Smad3, and our unpublished results reveal that IKKα also interacts with the control region of the *Ovol1* gene (data not shown). Most likely, IKKα is recruited to chromatin via its interaction with Smad2/3 because IKKα lacks a recognizable DNA-binding domain. Although IKKα was proposed to act as a histone H3 kinase (34, 35), its function in TGFβ signaling does not depend on its protein kinase activity, which is not TGFβ-responsive (SI Fig. 11). The effect of IKKα on Mad1 expression and keratinocyte differentiation is unique to this particular IKK subunit, and the deletion of IKKβ has no effect on either parameter.

Although the physiological cues that control cell-cycle exit in keratinocytes, a prerequisite for differentiation (11, 12), are ill defined, TGFβ production is elevated during the differentiation of human oral keratinocytes, which can be induced by exogenous TGFβ (36). Furthermore, TGFβ inhibits keratinocyte proliferation and that is partially dependent on the IKKα target *Ovol1* (23). We suggest that the antiproliferative effect of TGFβ is exerted via several IKKα-dependent genes, including *Mad1*, *Mad2*, and *Ovol1*. This explains why disruption of any single *Mad* gene does not perturb keratinocyte differentiation (37–39) and disruption of *Ovol1* leads to keratinocyte hyperproliferation, but its effect on differentiation is relatively modest (23).

Until now, however, the role of TGFβ in epidermal differentiation has been obscured by the previous observations that the absence of Smad4, the ubiquitous coregulator of R-Smad-dependent transcription (17, 18), does not interfere with keratinocyte differentiation (31, 32). Until recently (30), Smad4 was thought to be obligatory for all R-Smad-mediated transcriptional responses, therefore the presence of normal epidermis in the absence of Smad4 suggested that TGFβ family members do not have a critical role in keratinocyte differentiation. However, we found that IKKα and activated Smad2/3 accumulate in the nuclei of differentiating keratinocytes independently of Smad4. In addition, IKKα is required for the nuclear accumulation of

activated Smad2/3 in epidermis. These results indicate that IKKα is required in mouse keratinocytes for both TGFβ-induced growth arrest and differentiation. In conclusion, IKKα is a critical component of the TGFβ-signaling pathway in keratinocytes, where Smad4 plays a very minor role, if any, in the activation of the differentiation program.

By controlling keratinocyte proliferation, TGFβ and its relatives also may influence keratinocyte differentiation. The ectopic expression of Smad7 in epidermis, at a level sufficient to block Smad2/3 activation (26), prevents nuclear IKKα and Smad2/3 accumulation in epidermal keratinocytes and is associated with abnormal epidermal differentiation. Likewise, silencing of *Smad2* in *Smad3*$^{-/-}$ keratinocytes reduced Mad1 expression and epidermal differentiation, and *in vivo* reduction of the *Smad2* gene dosage in *Smad3*$^{-/-}$ epidermis resulted in a significant reduction of the expression of epidermal terminal differentiation markers. Therefore, it would be interesting to investigate the exact roles of individual TGFβ family members, which include activins, nodal, and BMPs in keratinocyte differentiation. In addition, the TGFβ–Smad2/3–IKKα axis may not control all aspects of epidermal differentiation, and the expression of barrier-related genes may depend on an interaction between IKKα and the retinoic acid receptor (33).

Experimental Procedures

***In Vivo* Mouse Models.** Gene-switch-*Smad7* mice were generated as described previously (26). Smad7 expression was induced *in vivo* by topical application of 20 μg per mouse RU486 as described (26). To generate mice with reduced *Smad2* gene dosage in the *Smad3*$^{-/-}$ background, *Smad3*$^{-/-}$ mice were bred with K14.CrePR1/Smad2^{F}/Smad3$^{+/-}$ mice. Daily RU486 (100 μg per mouse) was injected into pregnant mice to induce Cre-mediated *Smad2* deletion in embryonic epidermis, starting at day 12 of gestation. Then Progesterone (0.5 mg per mouse) was coinjected to protect pregnancy as described (29). Epidermal Smad4 KO mice (*Smad4*Δ) were generated by cross-breeding Smad4-floxed mice (no. 119) (31) with K5.CrePR1 mice, and *Smad4* gene deletion in keratinocytes was achieved by topical application of RU486 (20 μg per mouse daily for 5 days, beginning on postnatal day 1 on bigenic mouse skin.

Cell Culture and Transfection. Primary mouse keratinocytes from WT, *Smad3*$^{-/-}$, and *Ikk*$\alpha^{-/-}$ were cultured as described (13). For Smad7 overexpression in keratinocytes, primary keratinocytes obtained from K5.GLp65/tata.Smad7 were treated with 10^{-7} M RU486. Keratinocytes differentiation was induced by using 0.35 mM Ca^{2+} containing medium. HaCaT and HeLa cells were maintained in DMEM supplemented with 10% FBS, penicillin, and streptomycin. HaCaT and HeLa cells were transfected by using Lipofectamine Plus (Invitrogen). Primary keratinocytes were infected with adenoviral vectors as described below For TGFβ treatment, we normally used 100 pM TGFβ1 (Sigma).

Adenoviral Constructs and Infections. Adenoviruses encoding WT and mutant IKKα were described (13). Adenovirus-expressing Mad1 was constructed by using BD-Adeno-X Expression System1 (BD Bioscience/Clontech) according to the manufacturer's instructions. Adenoviruses encoding Smad3, Smad4, or Smad7 were kindly provided by A. Moustakas (Ludwig Institute for Cancer Research, Uppsala, Sweden). Keratinocytes were infected at a moi of 50. Virus-containing medium was replaced with fresh medium after 1 day.

RNA and Real-Time PCR Analyses. Total RNA was prepared by using an RNeasy Mini Kit (Qiagen), and cDNA was synthesized with a SuperScript II reverse transcription system (Invitrogen). Real-time quantitative PCR was performed by using cyclophilin mRNA for normalization (14). RNase protection assays were performed as described (40).

GST Pulldown and Coimmunoprecipitations. GST Smad2–4 were expressed in *Escherichia coli* and purified on GSH agarose beads. HaCaT cells expressing HA-IKKα were lysed in buffer [10 mM Tris·HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1% Nonidet P-40 with proteinase and phosphatase inhibitors]. Then $\approx$20 μg of purified recombinant GST-fusion protein was mixed with cell lysates at 4°C overnight with rotation, followed by five washes in lysis buffer. The washed beads were boiled in SDS/PAGE sample buffer and, after gel separation, were analyzed by immunoblotting with anti-HA antibody (Santa Cruz Biotechnology).

HaCaT cells transfected with Smad and IKKα expression vectors were lysed. After centrifugation, cleared lysates were incubated with anti-HA or anti-FLAG antibodies (Sigma) on ice. Protein G beads were added at 4°C for 2 h on a rotating platform. After three washes in lysis buffer, immune complexes were isolated, separated by SDS/PAGE, and analyzed by immunoblotting.

ChIP. ChIP assays were performed as described (41).

Histological, Immunohistochemical, and Immunofluorescence Analysis. Skin from E18 embryos were fixed in 10% neutral-buffered formalin. Then 5-μm paraffin-embedded skin sections were stained with H&E and specific antibodies. For immunohistochemistry, skin sections were kept at 60°C for 1 h and then incubated either overnight at 80°C in citrate buffer (pH 6) or at 94°C for 40 min in Dako's antigen retrieval solution S2368. A specific signal was detected by using the appropriate Dako EnVision System, HPR (DAB) kit. Immunofluorescence studies were performed as described (42).

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