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Regulation of epidermal differentiation through KDF1-mediated deubiquitination of IKKα

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Abstract

Progenitor cells at the basal layer of skin epidermis play an essential role in maintaining tissue homeostasis and enhancing wound repair in skin. The proliferation, differentiation, and cell death of epidermal progenitor cells have to be delicately regulated, as deregulation of this process can lead to many skin diseases, including skin cancers. However, the underlying molecular mechanisms involved in skin homeostasis remain poorly defined. In this study, with quantitative proteomics approach, we identified an important interaction between KDF1 (keratinocyte differentiation factor 1) and IKKα (IκB kinase α) in differentiating skin keratinocytes. Ablation of either *KDF1* or *IKKα* in mice leads to similar but striking abnormalities in skin development, particularly in skin epidermal differentiation. With biochemical and mouse genetics approach, we further demonstrate that the interaction of IKKα and KDF1 is essential for epidermal differentiation. To probe deeper into the mechanisms, we find that KDF1 associates with a deubiquitinating protease USP7 (ubiquitin-specific peptidase 7), and KDF1 can regulate skin differentiation through deubiquitination and stabilization of IKKα. Taken together, our study unravels an important molecular mechanism underlying epidermal differentiation and skin tissue homeostasis.

Keywords deubiquitination; epidermal differentiation; IKKα; KDF1; skin
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Introduction

Mammalian skin serves as an essential water-impermeable barrier that protects us from various environmental damages [1]. Epidermis of skin is a stratified epithelial tissue that consists of four major tissue layers: basal layer, spinous layer, granular layer, and stratum corneum. Tissue homeostasis of skin epidermis is sustained by the potential epidermal progenitor/stem cells that localize at the basal layer. In adult skin, these cells periodically move upward from their niche at the basement membrane and undergo terminal differentiation to replenish lost skin cells during normal tissue homeostasis or upon skin injury [1–3]. Deregulation of epidermal differentiation in skin can lead to the development of various skin diseases including skin cancers, such as SCC (squamous cell carcinoma).

Although the morphogenetic changes during epidermal differentiation have been studied [1–3], much remains unknown about the molecular mechanisms underlying this process. The role of KDF1 (keratinocyte differentiation factor 1) in epidermal differentiation was discovered before via an ENU-induced mutagenesis screen in mice [4]. It has been shown that the *KDF1* mutant fetuses developed a thick, taut, and hyperplastic epidermis with diminished barrier ability. Loss of *KDF1* also leads to aberrant cell proliferation, misexpression of basal and spinous differentiation markers, and an absence of terminal differentiation, suggesting that KDF1 is a novel player that is critically involved in epidermal differentiation. To determine the underlying molecular mechanism and dissect the signaling network involved in skin stratification, we examined KDF1's interactome by quantitative proteomics approach. Our MS (mass spectrometry) analysis identified an intriguing protein, IKKα (IκB kinase α), as a specific binding partner of KDF1 in differentiating keratinocytes.

IKKα is a well-established component within the NF-κB signaling pathway [5]. However, loss of function of *IKKα* in mice leads to striking abnormalities in skin development. The *IKKα* KO (knockout) mice developed abnormally thick and shiny skin with diminished terminal differentiation in epidermis, resembling the phenotypes in *KDF1* KO animals [2,6–8]. Interestingly, it has also been demonstrated that its function in skin differentiation is independent upon its kinase activity and its role in NF-κB-signal transduction [6,7,9]. Emerging evidence suggests that IKKα regulates skin tissue homeostasis via its own transcriptional regulatory role. A dramatic increase of IKKα level in keratinocyte nucleus precedes epidermal differentiation, which could modulate the autocrine signaling of EGF and the expression of a potential secreted keratinocyte differentiation-inducible factor (kDIF) [10]. Consistent with its role in epidermal differentiation, deregulated IKKα in skin could lead to tumorigenesis, such as cutaneous SCCs [11–15].

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To probe deeper into the molecular mechanisms, we found that *KDF1* deletion will enhance IKKα ubiquitination and abolish IKKα accumulation in differentiating keratinocytes. Protein ubiquitination is a universal mechanism for protein degradation that controls a wide variety of cellular processes [16–19]. Deubiquitination is a reverse process of ubiquitination, which is carried out by deubiquitinating enzymes (DUBs) [20,21]. Interestingly, our proteomics analysis revealed that KDF1 associates with a key DUB, USP7/HAUSP (ubiquitin-specific peptidase 7/herpesvirus-associated ubiquitin-specific protease), in keratinocytes. As a deubiquitination molecule, USP7 has been implicated in various signaling cascades, including NF-κB pathway [22,23], p53/MDM2 (mouse double minute 2 homolog), PTEN (phosphatase and tensin homolog), and FOXO4 regulation [24–27]. However, the role of USP7 in skin development and epidermal stratification remains unclear. In this study, we present compelling evidence that KDF1 regulates IKKα ubiquitination and stability through its interaction with USP7. Deletion of *USP7* in epidermal keratinocytes leads to decreased IKKα and aberrant differentiation. Taken together, our results illuminate an important molecular mechanism whereby differentiation of epidermal progenitor cells is regulated by KDF1 and IKKα proteins.

Results

KDF1 interacts with IKKα

KDF1 deficiency leads to profound abnormalities in skin development. To dissect the underlying mechanisms, we engineered expression vectors encoding HA- and His_6-tagged *KDF1*. We used tandem affinity purification to isolate the KDF1 complex from transfected cells upon calcium-induced keratinocyte differentiation and then employed SILAC (stable isotope labeling by amino acids in cell culture) coupled with LC-MS/MS (liquid chromatography and tandem mass spectrometry) to determine the KDF1 interacting proteins (Fig 1A and Dataset EV1). Our analysis identified IKKα as a KDF1 binding partner in differentiating keratinocytes. To confirm the interaction, we first conducted co-immunoprecipitation assay with ectopically expressed *KDF1* and *IKKα* in HEK293 cells (Fig 1B and C). To examine the interaction of endogenous proteins, we immunoprecipitated KDF1 in WT (wild-type) skin keratinocytes before or after high calcium-induced epidermal differentiation. Interestingly, endogenous co-immunoprecipitation can only be detected in differentiated keratinocytes, suggesting that endogenous KDF1 associates with IKKα specifically in differentiated cells (Fig 1D, quantification in Fig EV1A, and source data in Fig EV1B). The kinase activity of IKKα is dispensable for keratinocyte differentiation [6,7,9]. As expected, the kinase-dead mutant of IKKα retains strong binding affinity to KDF1 *in vitro* (Fig 1E).

In order to map the potential binding regions within KDF1 and IKKα, we generated various truncation mutants of *KDF1* and *IKKα*, based upon the known functional domains or protein characteristics (Fig EV1C and D). IKKα is a conserved helix-loop-helix kinase, which contains a serine–threonine kinase domain, a leucine zipper motif (LZ), a helix-loop-helix domain (HLH), and a NEMO-binding domain (Fig EV1C) [28]. Co-immunoprecipitation analysis suggests that the kinase domain of IKKα (mutant 1) does not bind KDF1. The IKKα truncation mutant containing LZ and HLH domain (mutant 2 and 3) can associate with KDF1, whereas the truncation mutant harboring the HLH and NEMO-binding domain (mutant 4) cannot. It strongly suggests that the LZ domain may be responsible for the interaction with KDF1. Consistent with this hypothesis, an *IKKα* truncation mutant containing LZ domain alone (mutant 5) can interact with KDF1, whereas a LZ-deletion mutant of *IKKα* (mutant 6) fails to bind KDF1 (Fig 1F).

KDF1 contains a proline- and cysteine-rich region near the N-terminus [4] (Fig EV1D), which shows no binding affinity toward IKKα (mutant 1) (Fig 1G). Therefore, we further truncated the C-terminal portion of KDF1 (mutant 2) into two halves (mutants 3 and 4). The co-immunoprecipitation analysis indicates that the binding motif of KDF1 lies within central domain of KDF1 (mutant 3). Consistently, an in-frame deletion of this region (mutant 5) abolished IKKα binding (Fig 1G). Taken together, our data show that the interaction is mediated by the LZ motif of IKKα and the central domain of KDF1.

Interaction of *IKKα* and *KDF1* is essential for epidermal stratification

Loss of *KDF1* or *IKKα* has been shown to affect skin development *in vivo* [4,6–9]. To assess the relevance of IKKα and KDF1 interaction in skin differentiation, we first systematically examined epidermal stratification in embryonic E18.5 skin of mutant animals. Histological analysis coupled with immunofluorescence staining revealed striking similarities of *KDF1* KO and *IKKα* KO in epidermal differentiation, including thickened epidermis, loss of stratum corneum (Fig 2A), expansion of basal cell marker, hyperplastic spinous layer, and loss of expression of late differentiation markers (Fig 2B, and quantification in Fig EV2A and B). To further dissect the molecular mechanisms, we isolated primary basal progenitor cells from *KDF1* KO and *IKKα* KO animals. When induced to differentiate with calcium shift, *KDF1* or *IKKα* KO cells fail to express *loricrin* or *filaggrin*, both are well-established biochemical markers for epidermal differentiation (Fig 2C). The aberrant skin stratification upon loss of *KDF1* is not due to potential changes in skin inflammation, as staining of macrophage, T-cell, and dendritic cell markers shows no significant increase of immunocytes in *KDF1* KO skin (Fig EV2C).

The phenotypic resemblance between *KDF1* and *IKKα* null cells suggests that these two proteins may act in the same pathway to regulate epidermal tissue homeostasis. To test this hypothesis, we used PiggyBac transposon system to restore expression of WT *IKKα* or *IKKα* mutant deficient in KDF1 binding (mutant 6) in *IKKα* null cells (Fig 3A). However, constitutive expression of *KDF1* in *KDF1* KO cells may lead to cell cycle arrest. To resolve this issue, we used a tetracycline-inducible system to restore expression of WT *KDF1* or *KDF1* mutant defective in IKKα interaction (mutant 5) in *KDF1* KO keratinocytes. Induction with doxycycline can lead to a dose-dependent expression of *KDF1* or its mutant in the cells (Fig 3B).

With establishment of these cell lines, we first examined the ability of these constructs to rescue epidermal differentiation upon calcium shift *in vitro*. Quantitative analysis of the expression level of *loricrin* shows that expression of WT *IKKα* or WT *KDF1* is able to significantly increase the expression of *loricrin*. By contrast, expression of *IKKα* or *KDF1* mutant failed to rescue the differentiation defect *in vitro* (Figs 3C and D, and EV3A).

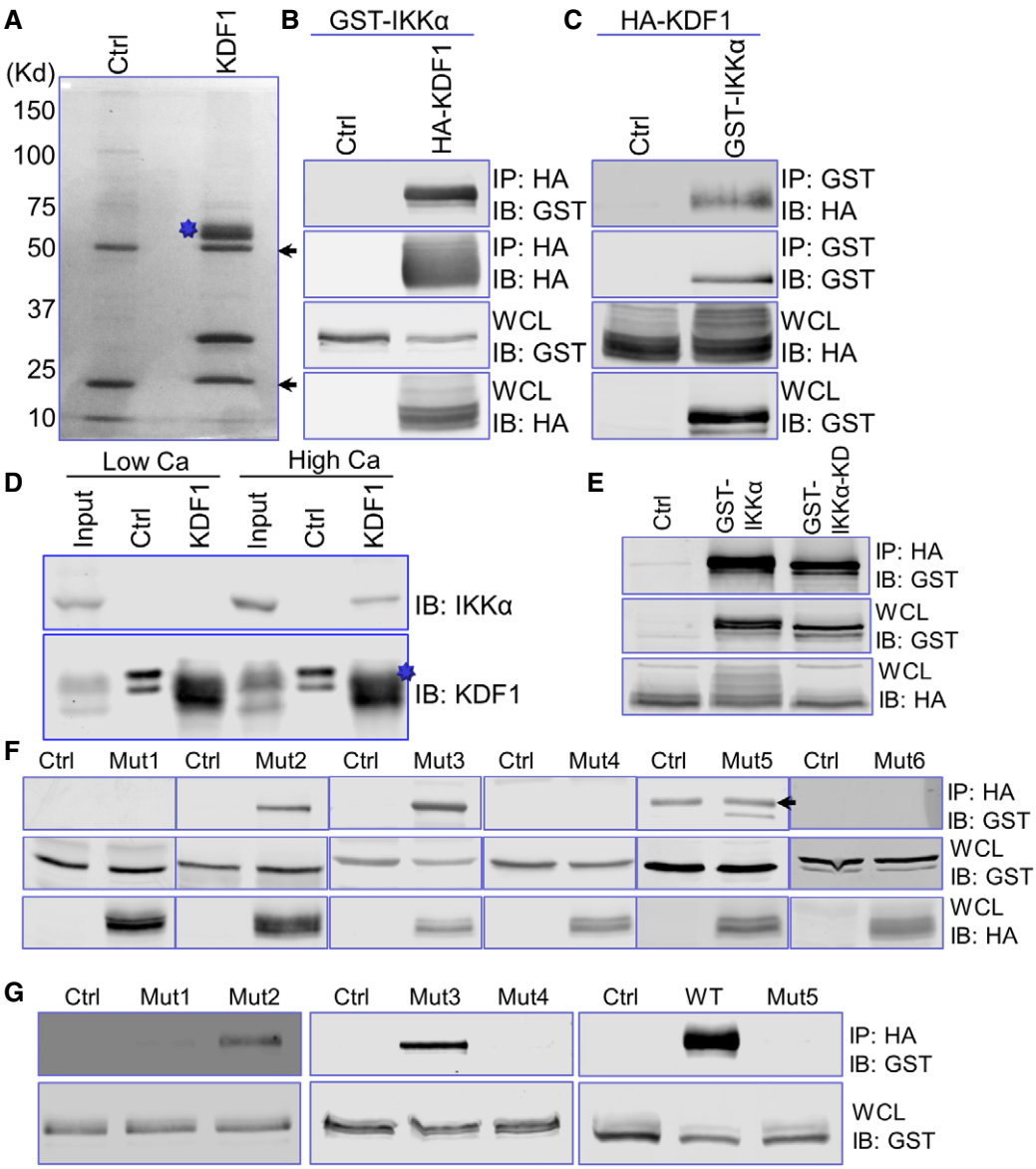

Figure 1. KDF1 associates with IKKα.

A Tandem affinity purification was used to isolate KDF1-associated proteins from SILAC-labeled keratinocytes. Precipitated proteins were resolved by SDS–PAGE and subjected to identification with LC-MS/MS. Arrows denote IgG heavy and light chains. Star denotes KDF1. Ctrl: control. Kd: kilodalton for molecular weight markers.

B, C HEK293 cells were transfected with empty vector or plasmids encoding HA-tagged *KDF1* and/or GST-tagged *IKKα*. Immunoprecipitation (α-HA or α-GST) was carried out to determine their interaction. Immunoprecipitates (IP) and whole cell lysate (WCL) were immunoblotted (IB) with different antibodies as indicated.

D Immunoprecipitation was carried out with α-KDF1 antibody in keratinocyte cultured in medium with low or high concentration of calcium (Ca). IPs were blotted with different antibodies as indicated. Star denotes KDF1. The same IP was used for USP7 immunoblot as shown in Fig 5B.

E HEK293 cells were transfected to co-express HA-tagged *KDF1* with GST-tagged *IKKα* or its kinase-dead (KD) mutant. IP and WCL were blotted with different antibodies as indicated.

F HEK293 cells were transfected to co-express HA-tagged *KDF1* with GST-tagged *IKKα* or its different truncation mutants (Fig EV1B). IP and WCL were blotted with different antibodies as indicated. Arrow denotes antibody heavy chain.

G HEK293 cells were transfected to co-express GST-tagged *IKKα* with HA-tagged *KDF1* or its different truncation mutants (Fig EV1C). IP and WCL were blotted with different antibodies as indicated.

To investigate the relevance of KDF1 and IKKα interaction in an *in vivo* setting, we took advantage of the mouse epidermal organotypic culture system that has been recently developed by our laboratory. *KDF1* or *IKKα* KO cells and different rescued cells were cultured on top of acellularized dermis. Exposure to the air/liquid interphase can induce stratification of cultured cells to generate a skin-like organoid *in vitro* [29]. Transplantation of this cultured skin organoid to nude host leads to efficient skin engraftments, which are stable and can readily express exogenous genes that have been transduced to the epidermal progenitor cells. Regenerated skin from

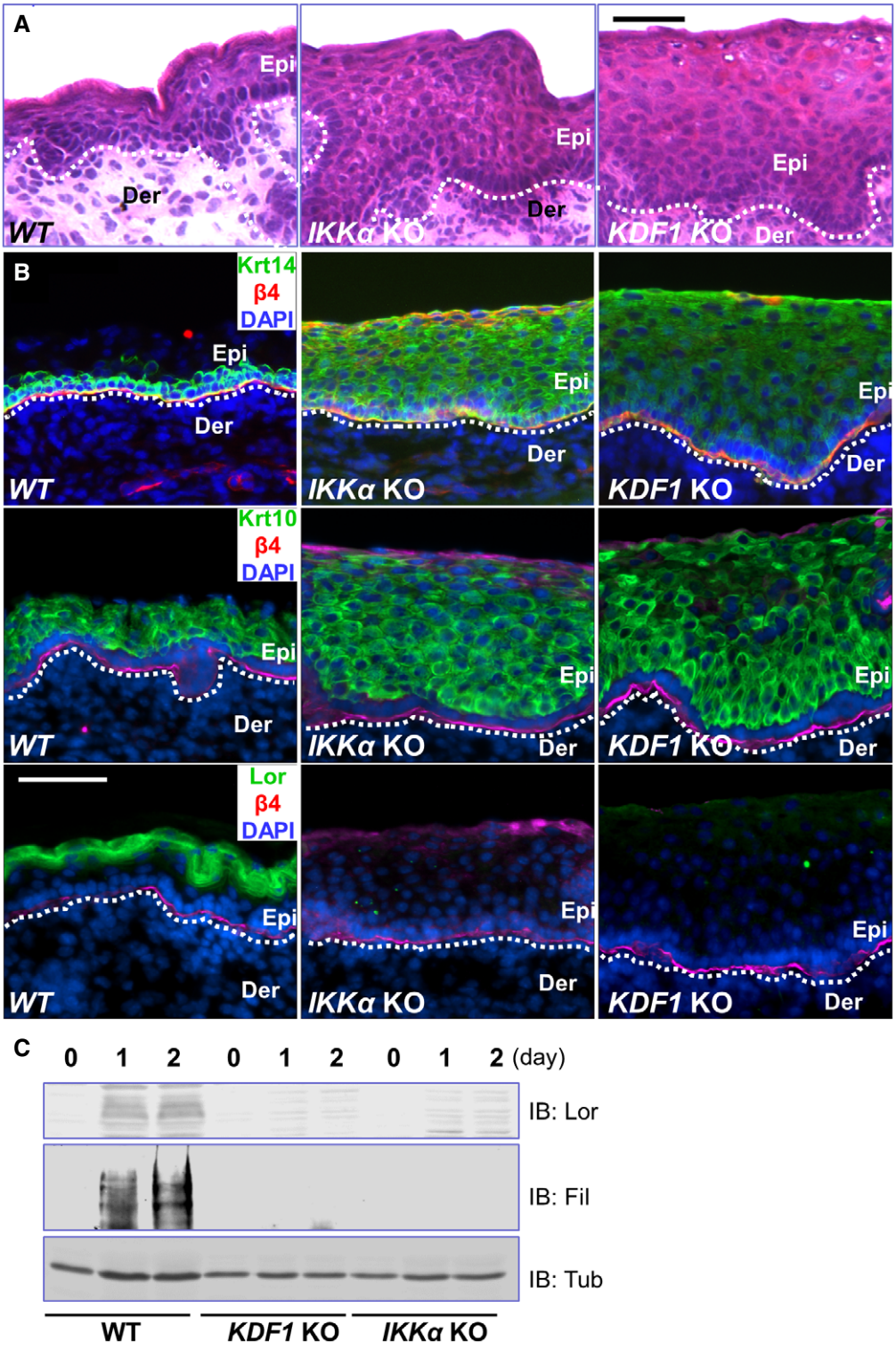

Figure 2. Loss of *KDF1* or *IKKα* leads to aberrant epidermal differentiation.

A H/E staining of E18.5 skin sections from WT, *IKKα* KO, and *KDF1* KO mice. Dotted lines denote dermal–epidermal boundaries. Epi: epidermis, Der: dermis. Scale bar = 50 μm.

B E18.5 skin sections from WT, *IKKα* KO, and *KDF1* KO mice were immunostained with different antibodies as indicated (Krt14: keratin 14, Krt10: keratin 10, Lor: loricrin, β4: β4-integrin, CD104). Dotted lines denote dermal–epidermal boundaries. Epi: epidermis, Der: dermis. Scale bar = 100 μm.

C Immunoblots of WCL collected at 0, 1, and 2 days after calcium shift with different antibodies as indicated.

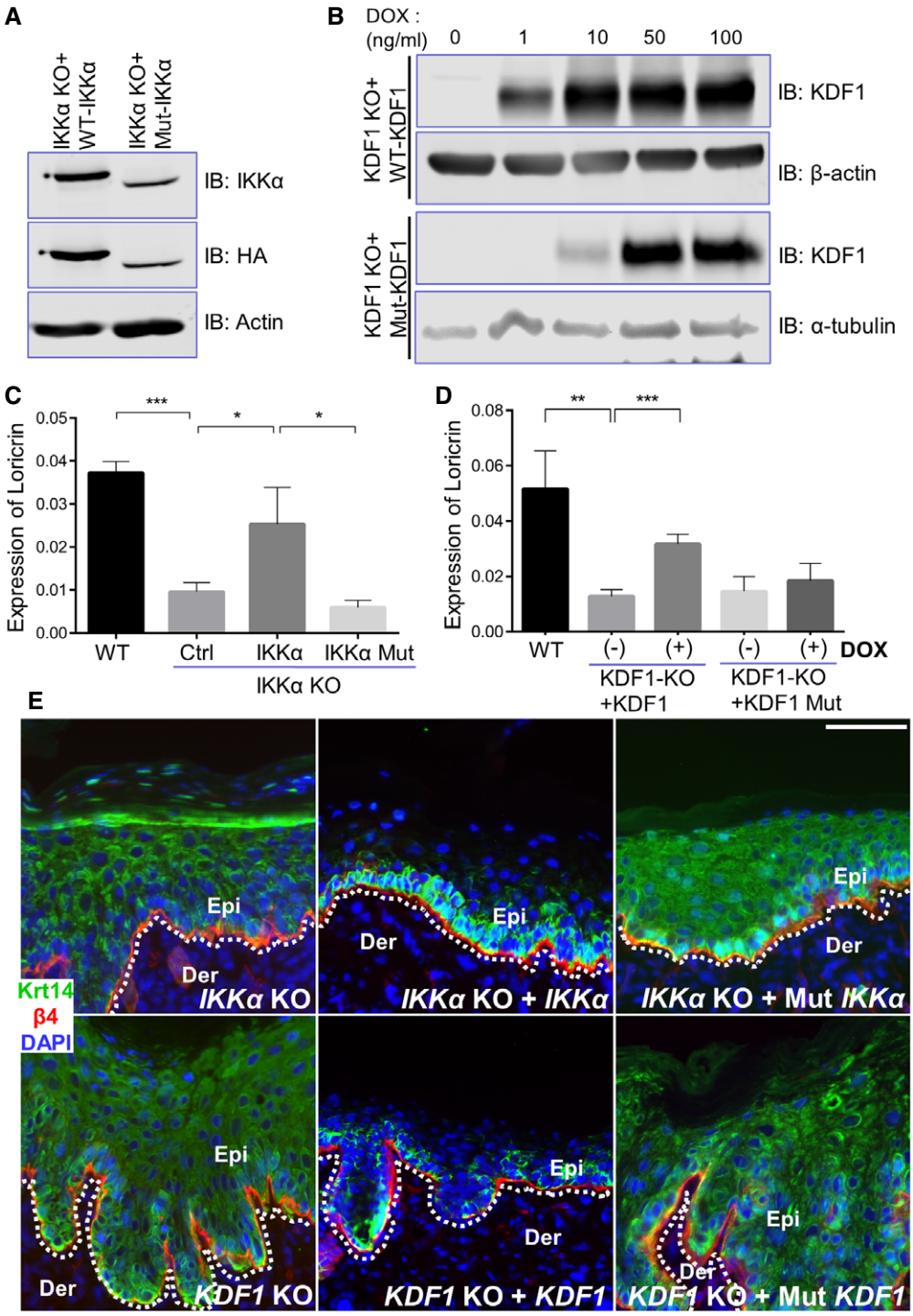

Figure 3. IKKα and KDF1 interaction is essential for skin stratification.

A Immunoblots of WCL from *IKKα* KO cells with rescued expression of HA-tagged *IKKα* or its mutant using different antibodies as indicated.

B Immunoblots of WCL from *KDF1* KO cells with rescued expression of *KDF1* or its mutant using different antibodies as indicated. Expression of exogenous *KDF1* was induced by doxycycline (DOX) at different concentration.

C, D Expression of *loricrin* is determined by immunoblots and quantified by densitometry. The relative expression level of *loricrin* upon calcium shift in different cell types was calculated and presented as bar graphs. Statistical analysis is conducted using unpaired Student's *t*-test. Error bar represents SD (standard deviation). $N = 3$ (biological replicates). $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$

E Sections of regenerated skin developed from engrafted *IKKα* KO, *KDF1* KO, and their rescued cells were immunostained with different antibodies as indicated. Dotted lines denote dermal–epidermal boundaries. Epi: epidermis, Der: dermis. Scale bar = 50 μm.

KDF1 or *IKKα* KO cells displays striking epidermal abnormalities, including thickened epidermis, loss of cornified cells, and expansion of basal cell markers, resembling the phenotypes of *KDF1* or *IKKα* KO *in vivo* (Fig 3E). Interestingly, when WT but not mutant *KDF1* or *IKKα* was re-expressed in the regenerated skin, epidermal differentiation was largely restored, including decreased epidermal thickness and restrictive expression of *Krt14* (keratin 14, a basal cell marker) in the basal layer (Fig 3E and quantification in Fig EV3B). Taken together, our results strongly suggest that epidermal differentiation requires interaction between KDF1 and IKKα.

KDF1 regulates keratinocyte differentiation via controlling the protein stability of IKKα

It has been shown that IKKα governs epidermal differentiation through its own transcriptional regulatory role in cell nucleus [10,30]. Through protein fractionation, we found that both IKKα and KDF1 show significant localization in cell nuclear fractions (Fig EV4A). Protein level of IKKα exhibits a strong increase upon keratinocyte differentiation (Fig 4A). Interestingly, although IKKα level is comparable in undifferentiated WT or *KDF1* KO epidermal progenitor cells, the level of IKKα is dramatically reduced in *KDF1* KO cells upon calcium switch-induced differentiation (Fig 4A). To investigate the potential mechanism, we found that IKKα mRNA level is not significantly changed upon loss of *KDF1* (Fig EV4B). Instead, stability of IKKα exhibits a significant decrease in *KDF1* KO cells (Fig 4B and quantification in 4C). Consistent with this notion, the ubiquitination level of endogenous or exogenously expressed *IKKα* [31] is significantly increased in *KDF1* KO cells upon differentiation (Fig 4D and quantification in 4E). Additionally, expression of WT *KDF1* but not its mutant can restore the IKKα level in *KDF1* null cells upon calcium shift (Fig 4F, and quantification in Fig EV4C). Together, these results suggest that KDF1 can regulate epidermal differentiation through controlling the protein stability of IKKα.

To further test this hypothesis, we try to rescue *KDF1* KO cells with exogenous expression of *IKKα* (Fig 4G). Interestingly, upon skin engraftment, we find that ectopic expression of *IKKα* can significantly restore skin stratification, including decreased epidermal thickness and normal expression of *Krt14* in the basal cell layer (Fig 4H, and quantification in Fig EV4D and E). Although exogenous expression of *IKKα* can reduce overall epidermal thickness, it alone cannot fully restore the normal epidermal structure, suggesting that additional factors/pathways may be involved in KDF1-regulation of epidermal differentiation as well.

KDF1 controls the ubiquitination level of IKKα via USP7

DUBs are special proteases that can recognize and specifically cleave ubiquitin or ubiquitin-like proteins from target molecules. Different DUBs have been shown to be involved in various cellular processes including protein stability, cell cycle regulation, and chromatin remodeling [20,21]. Our proteomics analysis of KDF1 interactome (Fig 1A) demonstrates another potential binding partner of KDF1, USP7, which is a DUB and can reverse both poly- and mono-ubiquitination of protein targets. The interaction between KDF1 and USP7 was confirmed *in vitro* by co-immunoprecipitation assay (Fig 5A). Interestingly, as KDF1 interaction with IKKα, endogenous KDF1 association with USP7 can only be detected in differentiated primary skin keratinocytes (Fig 5B, and source data in Fig EV5A). Consistent with previous report [24], USP7 shows strong nuclear localization in cultured epidermal keratinocytes (Fig EV4A).

The potential role of USP7 in skin development and keratinocyte differentiation has not been addressed before, although it has been shown to regulate the turnover of many signaling molecules, such as p53 and PTEN [24,25]. To this end, we first employed CRISPR (clustered regularly interspaced short palindromic repeats) technology and generated *USP7* KO keratinocyte (Figs 5C and EV5B). Deletion of *USP7* in skin keratinocytes leads to decreased IKKα protein level upon differentiation (Fig 5D, and quantification in Fig EV5C). Additionally, loss of *USP7* results in enhanced IKKα ubiquitination (Fig 5E). When induced to differentiation by calcium shift, the *USP7* KO cells exhibit significantly reduced expression of *Krt10* and *loricrin* (Fig 5F and quantification in 5G). Regenerated skin from transplanted *USP7* KO cells displays striking epidermal abnormalities similar to *KDF1* and *IKKα* KO cells, including thickened epidermis and expansion of basal cells (Fig 5H and quantification in Fig EV5D and E). Together, our studies suggest that KDF1 regulates IKKα ubiquitination and protein stability by recruiting USP7, a deubiquitinating enzyme, which is essential for epidermal differentiation (Fig 5I).

Discussion

Adult tissue homeostasis and wound repair are mediated by the delicate balance between cell proliferation, cell death, and differentiation. Skin provides an essential barrier protecting us from various environmental damages. Aberrant tissue homeostasis or wound repair can lead to dire consequence for our survival. In skin, both processes are driven by the epidermal stem/progenitor cells that localize at the basal layer of the skin epidermis [1,32,33]. Differentiation of epidermal progenitor cells is a complex but fascinating process, involving the permanent withdrawal of cells from the cell cycle, the synthesis and modification of various protein and lipid components of the cornified envelop, and the controlled dissolution of cellular organelles and the nucleus [1,34]. Aberrant skin differentiation contributes to the development of various skin diseases, including psoriasis, inflammatory skin diseases, and SCC. Cutaneous SCC is the second most common human cancer, afflicting more than 250,000 patients in the United States every year [35,36]. Cutaneous SCC can be highly invasive and metastatic (3–10% rate of metastasis), and a significant number of patients with a primary SCCs develop secondary lesions within 5 years of diagnosis, leading to severe morbidity and mortality [37]. Thus, understanding epidermal differentiation and its underlying molecular mechanisms is critical for devising effective therapeutic strategies for the treatment of various skin diseases. In this study, by employing a combinatory approach encompassing mouse genetics with molecular and cell biology studies, we provided compelling evidence that KDF1 regulates epidermal differentiation by forming a regulatory complex with IKKα, and controlling IKKα protein stability via recruiting a deubiquitinating enzyme, USP7.

KDF1 was initially identified by a forward genetics screen as a key regulator of epidermal differentiation in mouse skin [4]. Recent studies suggest that KDF1 is also involved in tooth agenesis, and mutation of *KDF1* has been identified in patients with ectodermal

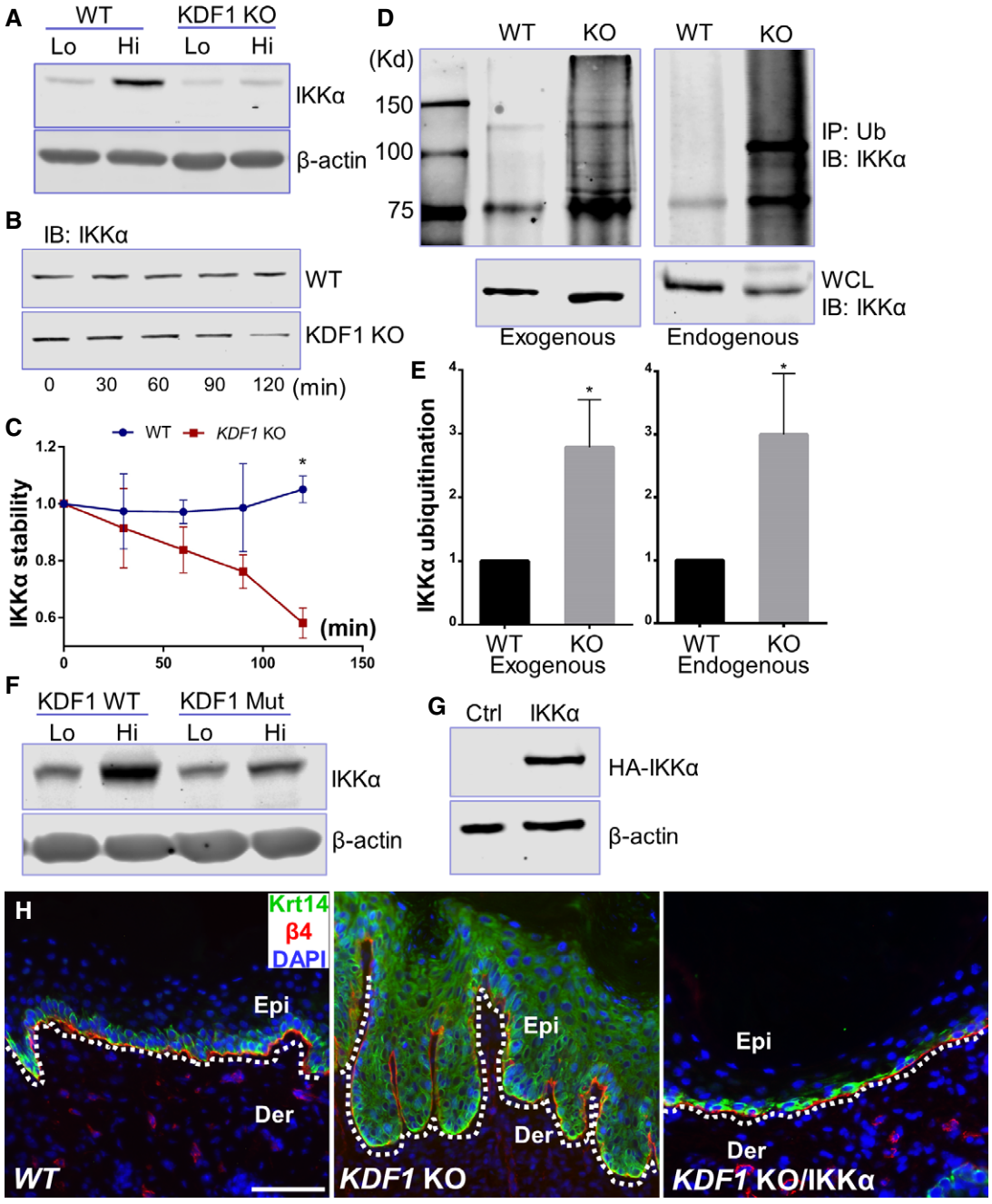

Figure 4. IKKα protein level is crucial during keratinocyte differentiation, and KDF1 regulates the ubiquitination and the protein level of IKKα.

A WCL from WT and *KDF1* KO cells before and after calcium shift were immunoblotted with different antibodies as indicated. Lo: low calcium; Hi: high calcium.

B, C WT and *KDF1* KO keratinocytes were treated with 20 nM cycloheximide (CHX). WCL was collected at 0-, 30-, 60-, 90-, and 120-min post-CHX treatment and subjected to immunoblotting with IKKα antibody (B). Band intensity is determined by densitometry, and the amount of IKKα is calculated and quantified (C). Statistical analysis is conducted using two-way ANOVA. $N = 3$ (biological replicates). $^*P < 0.05$. Error bar represents SD (standard deviation).

D, E WT and *KDF1* KO keratinocyte (right panels) or cells transfected with plasmid encoding exogenous *IKKα* (left panels) were treated with MG132 at 10 μM for 6 h and then were subjected to immunoprecipitation using anti-ubiquitin (Ub) antibody. IP and WCL were analyzed by immunoblots with α-IKKα antibody (D). Overall intensity of all ubiquitinated IKKα bands was determined by densitometry. Ratio of ubiquitinated IKKα was quantified and presented as bar graphs (E). Statistical analysis is conducted using unpaired Student's *t*-test. Error bar represents SD (standard deviation). $N = 3$ (biological replicates). $^*P < 0.05$. Kd: kilodalton for molecular weight markers.

F IKKα protein level in *KDF1* KO keratinocyte expressing WT *KDF1* or *KDF1* mutant, under both low and high calcium conditions, was examined and quantified by Western blotting (quantification shown in Fig EV4C).

G PiggyBac transposon was used to ectopically express HA-tagged *IKKα* in *KDF1*-deficient cells. WCL was collected and analyzed by immunoblots with different antibodies as indicated.

H Overexpression of *IKKα* in *KDF1* KO keratinocyte can restore normal skin stratification. Skin sections from grafted tissue were immunostained with different antibodies as indicated. Dotted lines denote dermal–epidermal boundaries. Epi: epidermis, Der: dermis. Scale bars = 50 μm.

dysplasia, a heterogeneous group of diseases that affects the ectoderm derivatives, such as skin, hair follicles, teeth, and nails [38]. Despite its potentially important role in tissue development and homeostasis, little is known about the molecular role of KDF1. Previous study suggests that KDF1 can affect expression of *p63* in skin keratinocytes, and genetically interacts with stratifin (Sfn, 14-3-

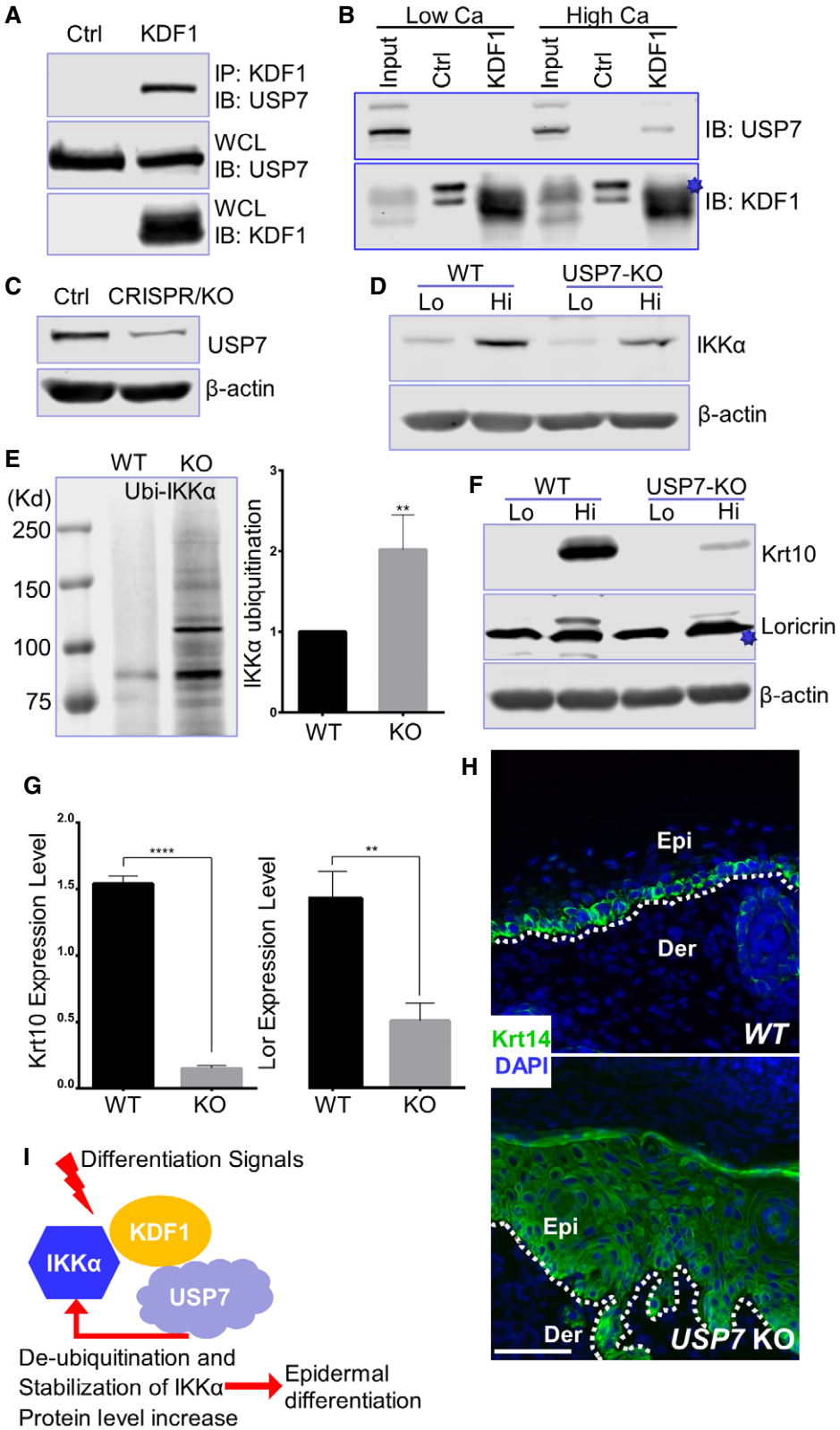

Figure 5.

Figure 5. USP7 regulates IKKα ubiquitination and skin differentiation.

A HEK293 cells were transfected to co-express *KDF1* with *USP7*. IP and WCL were blotted with different antibodies as indicated.

B Immunoprecipitation was carried out with α-KDF1 antibody in keratinocyte cultured in medium with low or high concentration of calcium (Ca), same as in Fig 1D. IPs were blotted with different antibodies as indicated. The star denotes KDF1.

C WCL from WT and *USP7* CRISPR KO cells was subjected to immunoblotting with different antibodies as indicated.

D IKKα protein level in WT and *USP7* KO keratinocyte was examined by immunoblotting before and after calcium shift.

E WT and *USP7* KO keratinocytes were treated with MG132 at 10 μM for 6 h and then subjected to immunoprecipitation using anti-ubiquitin antibody. Precipitated product was analyzed by immunoblotting with IKKα antibody. Band intensity was determined by densitometry and shown as bar graphs. Statistical analysis is conducted using unpaired Student's *t*-test. Error bar represents SD (standard deviation). $N = 3$ (biological replicates). $^{**}P < 0.01$. Kd: kilodalton for molecular weight markers.

F, G Immunoblot of Krt10 and loricrin (Lor) with WT and *USP7* KO cells before and after calcium shift (F). Star denotes an unspecific band in α-Loricrin blots. Band intensity was determined by densitometry and shown as bar graphs (G). Statistical analysis is conducted using unpaired Student's *t*-test. Error bar represents SD (standard deviation). $N = 3$ (biological replicates). $^{****}P < 0.0001$; $^{**}P < 0.01$.

H Sections of engrafted skin developed from *USP7* KO or control WT cells were immunostained with different antibodies as indicated. Dotted lines denote dermal–epidermal boundaries. Epi: epidermis, Der: dermis. Scale bar = 100 μm.

I A working model of epidermal differentiation regulated by KDF1. Upon differentiation signals, KDF1 associates with IKKα and recruits deubiquitination enzyme USP7 to the protein complex. USP7 can deubiquitinate IKKα and promote its protein stability, which will in turn promote epidermal differentiation.

3σ). Our current study shows that KDF1 association with IKKα is essential for epidermal differentiation, and ectopic expression of *IKKα* can rescue skin stratification in *KDF1* null cells, strongly suggesting that IKKα acts as an important downstream partner of KDF1 in epidermal differentiation.

It has been demonstrated in multiple independent studies that loss of *IKKα* leads to striking skin abnormalities, including defective epidermal differentiation [6,7,39]. Studies by Hu, *et al* [9] and by Gareus *et al* [40] also indicate that ablation of *IKKα* in cultured skin keratinocytes can directly inhibit epidermal differentiation, strongly suggesting that IKKα regulates skin differentiation via a keratinocyte cell-autonomous manner. Conditional KO (knockout) of *IKKα* with a Krt14 promoter-driven *Cre* transgenic line leads to perinatal lethality. Mutant mice have rather normal epidermal stratification but aberrant skin barrier function, suggesting impaired terminal differentiation capability of *IKKα*-deficient keratinocytes [40]. By contrast, mice with conditional deletion of *IKKα* by a Krt5 promoter-driven *Cre* can survive to adulthood but exhibit epidermal hyperproliferation and skin carcinogenesis phenotypes [10]. It is possible that this discrepancy results from potential difference from different *Cre* transgenic lines used [41]. Additionally, it is noteworthy that IKKα may act in a paracrine or autocrine manner by controlling the secretion of a hitherto unidentified kDIF (keratinocyte differentiation-inducing factor) to regulate epidermal differentiation [9]. Thus, an incomplete deletion of *IKKα* may lead to mild phenotypes *in vivo*.

It remains incompletely understood how IKKα regulates epidermal stratification. IKKα has been shown to be able to translocate to the cell nucleus and directly regulate target gene expressions. In 2004, Sil *et al* [42] detected increased levels of nuclear IKKα induced by keratinocyte differentiation and identified the putative NLS (nuclear localization sequence) within the kinase domain of IKKα. The transcriptional activity of IKKα was further illustrated by Liu *et al*, [11] that IKKα negatively regulates VEGF-A expression via binding to the distal *VEGF-A* (*vascular endothelial growth factor-A*) promoter. Besides *VEGF-A*, a number of other transcriptional targets of IKKα have been identified, including *EGF* (*epidermal growth factor*), *HB-EGF* (*heparin-binding EGF-like growth factor*), and *amphiregulin* [10]. Interestingly, it is noteworthy that IKKα can also regulate transcription of *Sfn* by preventing its hypermethylation and silencing [43]. *Sfn* has been shown to genetically interact with *KDF1* in epidermal development pathways [4]. In this study, we illustrated a critical interaction of KDF1 with IKKα, which is mediated by the LZ domain of IKKα. Deletion of LZ domain in *IKKα* will abolish this interaction and suppress epidermal differentiation. However, LZ domain is also involved in heterodimerization or homodimerization of IKKα and IKKβ [44], and deletion of LZ domain can block kinase activity of IKKα [45,46]. Future analysis will be required to delineate the precise molecular pathways whereby LZ domain of IKKα is involved in skin differentiation.

Protein ubiquitination and deubiquitination are highly dynamic but tightly controlled processes, regulating not only proteostasis but also function of the target proteins. The DUBs are special proteases that can reverse the modification of target proteins by single ubiquitin and polyubiquitin. The human genome encodes nearly one hundred DUBs, and their substrate specificity can be modulated by different mechanisms [20]. Ubiquitin-specific DUBs usually contain multiple domains with insertions and/or extensions that can control their substrate specificity. Additionally, the substrate specificity and subcellular localization of DUBs can be regulated by their protein binding partners. USP7 was first discovered as a binding protein for a herpes virus regulatory protein [47,48]. However, accumulating evidence suggests that USP7 plays critical but diverse roles in many different cellular processes including host–virus interaction, DNA repair, transcription, epigenetic regulation, and tumorigenesis, potentially through its many identified downstream targets, including p53, PTEN, FOXO4, and NFκB pathway proteins [22–27]. Null mutation of *USP7* in mice leads to early embryonic lethality, suggesting its important role in development [27]. Our data showed that it is also an essential gene involved in differentiation of skin epidermal cells. Although our results demonstrated regulation of IKKα deubiquitination by USP7, we cannot rule out the possibility that loss of *USP7* in skin keratinocytes may lead to changes of other signaling proteins. Characterization of potential *IKKα* mutant deficient for ubiquitination together with proteomics analysis of *USP7* KO epidermal cells and functional studies *in vitro* and *in vivo* will be essential to resolve this issue in the future.

In closing, our findings reveal an important molecular mechanism underlying epidermal differentiation and provide an important basis for the development of rationally based, molecularly targeted drugs for the treatment of various skin diseases, including skin cancers.

Materials and Methods

Antibodies, reagents, and plasmid DNA constructions

Loricrin and filaggrin antibodies were generous gifts from Dr. Elaine Fuchs at the Rockefeller University. Chicken Krt14, and rabbit Krt5 and Krt10 antibodies were obtained from Covance (Princeton, NJ). Rat monoclonal β4-integrin (CD104) was obtained from BD Pharmingen (Franklin lakes, NJ). KDF1 antibody (HPA028639), α-Flag antibody, and EZview™ Red anti-HA affinity beads were obtained from Sigma (St. Louis, MO). IKKα antibody (#2682) and normal rabbit IgG were obtained from Cell Signaling Technology (Danvers, MA). Loricrin (55439-1-AP), α/β-tubulin, and β-actin were obtained from Proteintech® (Rosemont, IL). Rabbit polyclonal antibodies against HA and GST, and protein A/G beads were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mono- and polyubiquitinylated conjugates monoclonal antibody (FK2) was obtained from Enzo (Farmingdale, NY). Other chemicals or reagents were obtained from Sigma (St. Louis, MO), unless otherwise indicated.

Primers used to generate *IKKα* and *KDF1* mutants are listed as follows (core sequence only): IKKα-mut-1: forward-ATG GAG CGG CCC, reverse-ACG CTC AAT ACG AGA CTG TAG TGA ATG A; IKKα-mut-2: forward-AGT CTT CAT TCA CTA CAG TCT CGT AT, reverse-TCA TTC TGT TAA CCA ACT CCA ATC A; IKKα-mut-3: forward-GTG CAC TAT GTG TCT GGA CTA A, reverse-TTC TAG ACT GGA TCC TAC AAG GG; IKKα-mut-4: forward-AGA CGT CAG GGA GAC TTG AT, reverse-TCA TTC TGT TAA CCA ACT CCA ATC A; IKKα-mut-5: forward-GTG CAC TAT GTG TCT GGA CTA A, reverse-AGA TTC CAT CAA GTC TCC CTG AC; IKKα-mut-6 (bridge gap): forward-ACA AAG GGC AGC AAT TCA GCT TGA CT, reverse-AGT CAA GCT GAA TTG CTG CCC TTT GT; KDF1-mut-1: forward-ATG CCC AGG CCG GGA CAG CCC CG, reverse-GCC CAT GCT TGT CTT GAG CCT C; KDF1-mut-2: forward-CAG AGG CTC AAG ACA AGC AT, reverse-GCA GTA CAC CTG CAG CAG GGG TG; KDF1-mut-3: forward-CAG AGG CTC AAG ACA AGC AT, reverse-TGA GAT CTT GCT GGT CTT CTC; KDF1-mut-4: forward-GAG AAG ACC AGC AAG ATC TCA G, reverse-GCA GTA CAC CTG CAG CAG GGG TG; and KDF1-mut-5 (bridge gap): forward-AGG CTC AAG ACA AGC GAG AAG ACC AGC AAG, reverse-CTT GCT GGT CTT CTC GCT TGT CTT GAG CCT. The mRNA level of IKKα was examined via RT–qPCR using primers: GAC TGT ATA TGA AGG ACC ATT TGC; GTC TTC CTT TAG CCC AGA TAC G.

SILAC-MS and proteomics analysis

Undifferentiated WT keratinocytes were subjected to SILAC label. L-lysine-2HCl (4, 4, 5, 5-D4) and L-arginine-HCl (μ-^{13}C6) (Cambridge Isotope Laboratories Inc, Andover, MA) were used to replace the regular lysine and arginine in the medium for heavy isotope labeling. Cells with light isotope labeling were used as a control. Heavy isotope-labeled cells were transfected with construct expressing HA- and His_6-tagged *KDF1*. The cells were subjected to calcium shift for 24 h before lysis with RIPA (radioimmunoprecipiation assay) buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 10% Glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitors. We mixed the same amount of heavy labeled proteins and light labeled proteins, and conducted sequential purification with Ni-NTA column and immunoprecipitation with anti-HA affinity agarose (Sigma, St. Louis, MO). The product from tandem affinity purification was resolved in SDS–PAGE and subjected to identification with LC-MS/MS. Fractionation was conducted using trypsin digestion.

Cell culture

Primary mouse keratinocytes were isolated using previously reported methods [49]. Epidermis of newborn mice or E18.5 was separated from dermis by an overnight treatment with dispase. Then, the primary keratinocytes were dispersed from the epidermis using trypsin. Keratinocytes were co-cultured with mitomycin C-treated 3T3 fibroblast feeder cells until the third passage. Cells were maintained in E-media supplemented with 15% FBS. The final concentration of Ca^{2+} is 0.05 mM. High calcium shift was performed using E-media supplemented with 15% FBS, with Ca^{2+} at a final concentration of 1.5 mM. HEK293 cells were cultured in DMEM supplemented with 10% FBS.

Cell transfection was carried out with Lipofectamine™ 3000 reagent (Invitrogen, CA), following manufacturer suggested protocol. A Tet-On 3G tetracycline-inducible expression system (Takara/Clontech) was used for inducible transgene expression. 50 ng/ml of doxycycline was used to induce gene expression *in vitro*.

Animals

KDF1 mutant strain was a generous gift from Dr. Scott D. Weatherbee (Yale University) [4]. The *IKKα* KO strain was a generous gift from Dr. Anning Lin (the University of Chicago) [6]. Nude mice for skin transplantation were purchased from the Jackson Laboratory (002019-NU/J). Around 8-week-old female nude mice were used for skin grafting. All mice used in this study were bred and maintained at the ARC (animal resource center) of the University of Chicago in accordance with institutional guidelines. For skin inflammation analysis, adult CD1 WT mice (8–11 weeks old) were treated topically with commercial Aldara cream (5% imiquimod cream) for 7 days.

Protein biochemical analysis

Western blot was conducted as previously described [50]. Cell lysates were prepared with RIPA buffer containing protease inhibitors. After the concentration of total protein is assessed, equal amounts of the cell lysates were resolved in sodium dodecyl sulfate–polyacrylamide gel and electroblotted onto a nitrocellulose membrane. The membrane was incubated with Odyssey blocking buffer (Li-COR biosciences, Lincoln, NE) for 1 h at room temperature, followed by an overnight incubation with desired primary antibody at 4°C. Immunoblots were washed three times with 1× Tween 20/phosphate-buffered saline (PBST) and incubated with secondary antibody (1:10,000 dilution) at room temperature for 1 h. Blot was washed with 1× PBST for another three times. LI-COR Odyssey scanner was used to visualize the blotting signals, and LI-COR Biosciences Software was used to conduct the quantification.

For immunoprecipitation, cell lysates were prepared with RIPA buffer containing protease inhibitors. After the concentration of total protein is assessed, equal amounts of the cell lysates (200–400 μg) were pre-cleaned using 30 μl protein A/G beads (Santa Cruz, CA).

5–10% samples were kept as input, and the remaining samples were incubated with desired antibody overnight at 4°C. 30 μl protein A/G beads (Santa Cruz, CA) were added on the following day and kept rotating for another 2 h at 4°C. The antibody and associated proteins were precipitated with beads via centrifuge, followed by five times washing step using lysis buffer. The precipitated proteins were analyzed by Western blot as described above.

Skin organotypic culture and grafting

Skin organotypic culture and grafting were performed as previously described [49]. Decellularized dermis (1 × 1 cm square shape) was prepared from newborn CD1 mice skin via EDTA treatment [29]. 2×10^6 cultured keratinocytes with desired genomic modifications were seeded onto the dermis in cell culture insert. Then, the skin culture was exposed to air/liquid interphase after an overnight attachment to form skin organoids. For grafting with skin organoids, nude mice aged ~ 8 weeks were anaesthetized. Two 1 × 1 cm square shape wounds were introduced to the back skin of the nude mice. After transplantation of skin organoids to the fresh wounds, the wound edge was sealed with surgical glue. The animals with skin graft were housed separately, and the bandages over the wound could be removed 1 week after surgery [49,51,52]. To induce exogenous gene expression in Tet regulated system, doxycycline food (TD. 120658, Envigo, Huntington, UK) was given after bandages removed until the end of the study. All the experiments were repeated more than three times (three biological replicates). For phenotypic analysis using immunostaining, at least three sections were taken from each graft for quantifications.

Histology and immunofluorescence

Skin samples were embedded in optimal cutting temperature (OTC) compound, sectioned, and fixed in 4% paraformaldehyde. Hematoxylin and eosin (HE) staining or immunofluorescence staining of desired sections was conducted as previously described [53]. Antibodies were diluted following the manufacturer's instructions unless indicated. Images were taken using EVOS FL imaging system. Evaluation of epidermal differentiation markers and measurement of epidermal thickness were carried out using ImageJ.

Statistical analysis

Statistical analysis was performed using Excel or GraphPad Prism software. Box plots were used to represent the entire population without assumptions on the statistical distribution. In most experiments, Student's *t*-test was used to evaluate the statistical significance of the difference (*P* value). For results in Fig 4C, two-way ANOVA (analysis of variance) was used to assess the statistical significance.

Data availability

The datasets produced in this study are available in the following databases: Proteomics data: PRIDE (accession: PXD015673; http://www.ebi.ac.uk/pride/archive/projects/PXD015673).

Expanded View for this article is available online.

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Author contributions
YL, LT, AL, SDW, and XW designed the experiments. YL, JY, and XG performed the experiments. YL and XW analyzed the data. XW wrote the manuscript. All authors edited the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

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