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THE ESSENTIAL MOLECULAR MECHANISMS UNDERLYING EPIDERMAL DIFFERENTIATION AND HOMEOSTASIS

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ABBREVIATIONS

ADULT (acro-dermato-ungual-lacrimo-tooth)

AEC (ankyloblepharon, ectodermal dysplasia, clefting)

AP-1 (activator protein 1)

AP-2 (activator protein 2)

BCC (basal cell carcinoma)

BMP (bone morphogenetic protein)

CD34 (clusters of differentiation 34)

C/EBP (CCAAT/enhancer binding proteins)

CRISPR (clustered regularly interspaced short palindromic repeats)

ECM (extracellular matrix)

EEC (ectrodactyly, ectodermal dysplasia, clefting)

EGFR (epidermal growth factor receptor)

H/E (hematoxylin and eosin)

HFs (hair follicles)

IFE (interfolicular epidermis)

IKKα (IκB kinase α)

KDF1 (keratinocyte differentiation factor 1)

LADs (lamin-associated domains)

LMS (limb-mammary syndrome)

LRIG1 (Leu-rich repeats and immunoglobulin-like domains 1)

MMP (matrix metalloproteinase)

MSCP (melanoma-associated chondroitin sulphate proteoglycan)

NICD (intracellular domain of Notch)

PKC (protein kinase C)

Pkp1 (Plakophilin-1)

RIPK4 (receptor-interacting serine-threonine kinase 4)

SCC (squamous cell carcinoma)

SGs (sebaceous glands)

SHFM (split hand/foot malformation)

SNAREs (soluble NSF-attachment protein receptors)

t-SNARE (target-SNARE)

uPA (urokinase-type plasminogen activator)

v-SNARE (vesicle-associated SNARE)

CHAPTER I

INTRODUCTION

BACKGROUND AND SIGNIFICANCE

Mammalian skin structures and essential functions

Skin is the largest organ in the mammalian body, which has significant functions in protecting humans and animals from external environment (e.g. irradiation, pathogen, dehydration and physical stress), as well as elaborating the body surface with appendages [1-3].

Mammalian skin includes both of the dermis and the epidermis. The dermis locates above the subcutaneous tissue and below the epidermis. Depending on the location on human body, the thickness of skin dermis may vary from 3 mm on the back to 0.3 mm on the eyelids. Since there is no blood supply in epidermis, the dermis and its circulatory system serve as an essential support for nutrients and oxygen delivery in the organ. Besides blood vessels, the dermis is composed of about 90% collagen and 10% elastic fibers, lymph vessels, muscle fibers, pilosebaceous and sweat glands, which defines a connective tissue matrix.

The skin epidermis has a thickness varying from 1.6 to 0.04 mm, which is composed of interfolicular epidermis (IFE), hair follicles (HFs) and sebaceous glands (SGs) and eccrine sweat glands [4, 5]. The primary cell type composing epidermis is keratinocyte, with a small population of melanocytes, Langerhans cells and Merkel cells. Four layers of keratinocytes at different stages of differentiation compose skin epidermis. Listing from the innermost and least differentiated layer, the skin epidermis is consisted of stratum basale, stratum spinosum, stratum granulosum and stratum corneum. The thickness of each layer varies depending on species and the location.

The stratum basale lays on the basal lamina, which is connecting with the dermis. Keratinocyte in this layer expresses distinct biomarkers such as keratin 5 and 14. Differentiation

of keratinocyte initiates when the cells move from the basal layer to the stratum spinosum. The distinct biomarker of spinal layer includes transglutaminase-I, keratin 1 and 10. In the next layer, the stratum granulosum, keratinocyte starts to go through dramatic morphological changes as profilaggrin and loricrin are generated and packaged into keratohyalin granules. Meanwhile, lipids are largely stored into lamellar bodies, which is eventually released to establish the lipid matrix in the stratum cornuem and forms the waterproofing permeability barrier. Another essential bioprocess initiating in the granular layer is enucleation, which is a nuclear degradation process during normal cellular homeostasis. I will discuss the enucleation process in detail in a later section. At the end, the stratum cornuem provides the cornified envelop sustained with lipid matrix and enucleated structure.

Contribution of epidermal stem cells to somatic skin homeostasis

Mammalian epidermis is a dynamic and regenerating tissue. Under normal physiological conditions, human epidermis turn over about every 4-6 weeks and about 9 pounds of dead skin cells shed every year. In order to replenish the shed skin and maintain the barrier function of epidermis, skin keratinocyte continuously goes through a robust differentiation process, called stratification. On the other hand, when skin tissue encounters a cutaneous wound, an appropriate healing process is required to repair damaged epidermis and regenerate lost tissue. In both conditions, epidermal progenitor cells are essential in sustaining skin homeostasis and secure the physiological function of the skin tissue.

IFE stem cells and their progenitor cells are localized within the stratum of basale [6-11]. Based upon published literatures, human IFE stem cells express unique cellular markers, such as Leu-rich repeats and immunoglobulin-like domains 1 (LRIG1), high level of β1 and α6 integrins,

and melanoma-associated chondroitin sulphate proteoglycan (MSCP) [11-16]. There are other stem cell populations positioning at skin appendages, such as hair follicles and sweat glands [1, 17]. Human and murine HF bulge stem cells express cellular markers different from IFE stem cells, such as clusters of differentiation 34 (CD34) and keratin 15 [12, 18-20]. Although IFE stem cells are unipotent, which is different from the multipotent stem cells at HF, this stem cell population and their progenitors are extremely important in maintaining epidermal homeostasis under normal physiological conditions as well as during wound healing process [6, 9]. To supplement the pools of keratinocytes that are lost during shedding or injury, epidermal stem cells and their progenitors periodically exit from their niches at the basement membrane and initiate the program of terminal differentiation [9]. Significantly, within all four layer of skin keratinocytes in adult IFE area, only IFE stem cells and their progenitors possess the ability of self-renewal and differentiation into the suprabasal layer of epidermis. Asymmetric cell division model has been proposed to illustrate how somatic IFE stem cells maintain its equilibrium in adult mammalian body [1]. When an IFE stem cell goes through cell division parallel to the basement membrane, one daughter cell remains as a stem cell through self-renewal, while the other daughter cell becomes a progenitor, which still possesses proliferation ability but eventually commits to terminal differentiation while leaving the stratum basale. Since basal keratinocytes include IFE stem cells and all the proliferative progenitors, I will refer the whole population as basal keratinocytes. The reason why I emphasize the context of adult skin, is that there is a difference regarding the proliferation potential of suprabasal keratinocytes during embryonic skin development versus in adult epidermal stratification. During embryonic skin development, suprabasal keratinocytes temporally maintain their proliferation ability while initiating the differentiation process, in order to quickly accomplish the coverage of embryo with immature keratinocyte before the terminal differentiation occurs.

This cell population is referred as intermediate layer, which maintains self-renewal potential while expressing spinal keratinocyte marker keratin 1. However, in adult skin, intermediate layer does not exit, and the spinal layer right above basal keratinocytes completely quit mitotic cycle while expressing cellular markers like keratin 1.

The role of basal keratinocytes during wound healing process could be more complex because of the involvement of cell migration, which has been utterly discussed in a review article I published in 2017 (see reference [21]). Adult skin wound healing is commonly divided into four phases that spatiotemporally overlaps: homeostasis, inflammation, re-epithelialization and remodeling. Basal keratinocytes have striking contributions during re-epithelialization phase, when new granulated skin tissue and intact keratinocyte layer is formed [9, 22].

First of all, basal keratinocytes at the wound's edges need to be activated to initiate the migration along the upper layer of blood clot in order to fill in the gap. In general, the anchor attaching basal keratinocytes to the basal lamina, hemidesmosome, need to be dissolved before the migration can occur. Furthermore, calcium influx that occurs when wound damages the front row cells will activate AP-1 signaling and lead to the reorganization of intracellular tonofilaments in keratinocytes [23-25]. Moreover, keratinocytes at the leading edge will express new integrins, assemble actin filament networks and form dynamic focal adhesions with various extracellular matrix (ECM) components, which are all indispensable steps for keratinocyte migration [26-31]. In addition, lamellipodia crawling will direct the migration of keratinocytes, while small GTPases, such as Rho GTPases, possess significant functions in regulating these cytoskeletal mechanisms [25, 32]. Subsequently, keratinocytes facilitate the dissolving of blood clot as they passing through and migrating forward. As illustrated in the literature, migrating keratinocytes upregulate key enzymes and related proteins, such as MMP (matrix metalloproteinase) family members (e.g.

MMP-1, MMP-9 and MMP-10) [33-36], and the activators and the receptor of uPA (urokinase-type plasminogen activator), which can activate a fibrinolytic enzyme, called plasmin [37-39].

When the keratinocytes at the leading edge migrate forward, basal keratinocytes behind the front line initiate rapid proliferation and differentiation process to restore the barrier function of the epithelium [29, 40]. The EGF family of growth factors are abundantly released at the wound to promote the proliferation of basal keratinocytes [41]. Gradually, the regenerated epithelia from opposing directions fuse and close the wound gap, which forms a barrier between the eschar and the underlying tissue. Soon after the wound gap is closed, keratinocytes stop the expression of integrins and ECM-degrading molecules, and undergo robust differentiation process to restore the normal epithelium.

Skin stem cells at HFs and sweat glands may also facilitate the wound healing process, if these compartments are intact at the wound site [17, 42, 43]. Similar as the fast proliferating basal keratinocytes behind the leading edge, the dispersed HFs and sweat glands stem cells join the pools of keratinocytes to provide extra proliferating cells and fill up the wound surface. However, the contribution of stem cells from HFs and sweat glands during wound healing could be highly limited and transient. For instance, the regeneration of hair follicles and sweat glands in patients with severe wounds is challenging, because of the irreversible damage of these compartment. Moreover, during acute wound repair, keratinocytes generated from HF stem cells could disappear from the newly formed epidermis after several weeks [44].

Signaling pathways and transcription factors in epidermal stratification

The highly regulated process of keratinocyte proliferation and differentiation are the foundation for the establishment of functional skin barrier. The program of epidermal homeostasis

and keratinocyte differentiation is controlled by various signaling pathways, such as Wnt/BMP and Notch, as well as governed by multiple transcription factors like p63, AP-1/2 and Klf4. I will give a brief introduction of these important regulatory mechanisms to provide a broad view of the skin development field.

Notch signaling initiates when extracellular ligands bind to the Notch receptors on cellular membrane. Then the intracellular domain of Notch (NICD) is cut off and released to form transcriptional regulation complex with mastermind-like (MAML) protein and RBP-J, and regulate target gene expression [45]. Unlike in many other organs where Notch signaling could promote cell proliferation, it controls the transition from proliferating basal layer to post-mitotic spinal layer in mature skin keratinocytes [46, 47]. Keratin 1 is a well-known downstream target of Notch signaling within epidermis [47, 48]. Wnt and bone morphogenetic protein (BMP) signaling mainly contribute to the epidermal specification during embryonic development and the maintenance of HF stem cells whining adult skin [1, 49]. I will not talk into details here.

p63 is discovered as the first transcription factor that has critical regulatory functions specially in the epidermal lineage [50]. During embryogenesis, one of p63 isoform, TAp63, is expressed in the surface ectoderm before the commitment of epidermal stratification, and contributes to keratin 14 expression and initiation of epidermal stratification indirectly through AP-2γ [51-53]. Another p63 isoform, ΔNp63, starts to take over after the commitment of epidermal stratification, and directly induces and maintains keratin 14 expression [52, 54]. Other transcriptional targets of ΔNp63 that maintain basal keratinocyte proliferation, are thoroughly reviewed by V. Botchkarev and E. Flores [55]. These transcriptional regulations involve inhibition of cell cycle related gene (e.g. p21 and 14-3-3σ) and prevention of Notch signaling. Interestingly, ΔNp63 could also regulate the initiation of keratinocyte terminal differentiation by inducing

keratin 1 expression synergistically with Notch signaling [56] and repressing cell-cycle inhibiting proteins (e.g. cyclin B2 and cdc2) [57]. According to V. Botchkarev and E. Flores [55], the contradictory function of ΔNp63 is because of the existence of intermediate layer during embryogenesis, where ΔNp63 is required for basal keratinocyte proliferation, while in mature keratinocyte, ΔNp63 could promote terminal differentiation.

Klf4 is an essential transcription factor regulating the formation of the epidermal barrier [58, 59]. The potential downstream targets involved in this regulatory function include *Connexin26* and Sprr protein family [60]. *Connexin26* is an inhibitory target of Klf4, the overexpression of which in mature keratinocyte could cause barrier defects [61]. Sprr protein family is potentially inhibited by Klf4 as well, since their expression is upregulated in *Klf4* knock-our skin [60]. It was proposed that *Sprr* genes are responsive genes that are compensatorily activated when barrier defects occur [62-65].

AP-1 (activator protein 1) and AP-2 (activator protein 2) are two completely different transcriptional regulators. AP-1 is a transcriptional complex, consisting of two proteins, Jun and fos, which are dimerized together to regulate proliferation and differentiation of keratinocyte. AP-1 could positively regulate EGFR (epidermal growth factor receptor) and HB-EGF, which controls keratinocyte proliferation and skin tumor growth [66]. Additional downstream targets of AP-1 include loricrin, involucrin and TGase1 [67-71], which contribute to keratinocyte differentiation as well. The function of AP-2 during epidermal stratification is less understood compared to AP-1. There are indications that AP-2 works together with C/EBP (CCAAT/enhancer binding proteins), which is another family of transcription factors [72, 73].

Besides the essential transcriptional regulators and various signaling pathways, epigenetic regulations are also controlling epidermal development and keratinocyte differentiation [74-76].

Although this is beyond the scope of this thesis research, it is worth knowing that the epigenetic regulations and the transcription factor mediated mechanisms during keratinocyte differentiation are highly connected [77, 78]. This connection is reviewed by V. Botchkarev, stating that p63 could control epigenetic regulators, such as *Satb1* and *Brg1*, and further affect the expression of lineagespecific genes required for the generation of epidermal barrier [79].

Even though these signaling pathways mentioned above have been identified in the regulation of epidermal stratification, the precise molecular mechanisms and regulatory networks that orchestrate the balance between epidermal progenitor cells proliferation and differentiation remain poorly understood.

Ca^{2+} is an essential regulator of keratinocyte differentiation and epidermal stratification

In mature epidermis, there is an extracellular Ca^{2+} gradient from the basal to the cornified layers, which promotes and regulates epidermal stratification as the keratinocyte traverse the different layers of the epidermis [80, 81]. Calcium regulation of skin differentiation has been reviewed in details by many literatures [49, 82]. In general, calcium may regulate various signaling pathways including the upregulation of phosphatidylinositol metabolism and diacylglycerol levels, and the activation of the PKC (protein kinase C) [83]. Activated PKC signaling is essential in downregulation of K1 and K10 expression [84], as well as induction of loricrin, filaggrin and transglutaminase expression [84, 85]. These transitions in expression of various cellular markers correspond to the transition from stratum spinosum to stratum granulosum. Another mechanism through which calcium regulate keratinocyte differentiation is by calcium receptor protein [86-91]. As calcium receptor protein locates at both the plasma membrane and intercellular compartments like ER and trans-Golgi [92], the underlying molecular machineries involved in calcium receptor

protein are genuinely complicated. I won't review too much into details, but via various *in vitro* and *in vivo* studies, including mouse models generated using different targeting strategies, it has been illustrated that calcium receptor functions through raising intracellular calcium concentration and inducing the expression of cellular markers essential for terminal differentiation, such as loricrin and filaggrin.

Because of the essential regulatory role of Ca^{2+} gradient during keratinocyte differentiation, it has also been widely used to induce keratinocyte terminal differentiation *in vitro* to mimic the differentiation process *in vivo*. A simple increase of calcium concentration in cell culture medium (e.g. from 0.05mM to 1.5mM), will induce robust keratinocyte differentiation *in vitro*. A lot of the studies mentioned above in regarding calcium regulations of keratinocyte differentiation is based upon studies done *in vitro* cultured keratinocytes [83-85, 90, 92]. It is always important to keep in mind the limitation of such *in vitro* studies, such as the lack of comparable intra- and extra-cellular environment as well as missing the communications and regulations between dermis and epidermis. Further examination using *in vivo* system is critical to confirm and verify a proposed hypothesis or a potential mechanism illustrated from *in vitro* studies.

Other essential regulatory proteins involved in epidermal differentiation

KDF1 was discovered in 2013 by Lee *et al*. via ENU-induced mutagenesis screen in mice [93]. The homozygous mutant fetuses (E18.5) showed severe morphological defects, such as lacking of ear and mouth opening, short forelimbs, and fusions between the tail, hind limbs and the genitals. More importantly, the mutant fetuses developed a thick, taut and hyperplastic epidermis with diminished barrier ability. Several evidence indicated the abnormalities in epidermal development and differentiation of epidermal progenitor cells due to KDF1 deficiency:

1) cellular proliferation was expanded throughout mutant suprabasal keratinocytes, which was restricted to basal keratinocytes at E18.5 in wild type epidermis; 2) markers of basal keratinocytes (Keratins 14 and 5) were expressed throughout the mutant epidermis; 3) mutant suprabasal keratinocytes exhibited a mix of basal and spinous markers, while the markers of granular and cornified layers (Loricrin and Filaggrin) were strongly reduced or absent; 4) the sign of the terminal differentiation (enucleation and cornification) was absent. In addition, the ceaseless expression of KDF1 in embryonic development and adult epidermis emphasized its essential function in regulating the proliferation and differentiation process of epidermal progenitor cells in embryonic and mature skin.

KDF1 is highly conserved across mammals. The human homolog C1ORF172, in particular, falls at human Chromosome 1p36. The heterozygous deletion of 1p36 is the most common terminal deletion observed in humans, which is associated with various types of human disease [94, 95]. Furthermore, this locus has been reported to be frequently deleted in many cancer types, such as neural, epithelial and hematopoietic malignancies [96]. In the recent high-resolution analysis of somatic copy-number alterations (SCNAs) across 26 human cancer types, locus 1p36.11 (covering KDF1 and 23 different genes) was identified as one the independent regions of significant focal deletion [97]. However, the function of KDF1 remains unknown until Lee *et al*. discovered its essential role in keratinocyte differentiation [93]. Recent studies suggest that KDF1 is also involved in tooth agenesis, and mutation of *KDF1* has been identified in patients with ectodermal dysplasia, a heterogeneous group of diseases that affects the ectoderm derivative, such as skin, hair follicles, teeth, and nails [98]. Although the previous study hinted the association of KDF1 with other key regulators of epidermal stratification, such as Stratifin (14-3-3σ) and p63

[93], there is a big knowledge gap remaining in relations to the precise molecular mechanisms through which KDF1 regulates keratinocyte differentiation.

In September 1999, two publications came out on the same volume of Science Journal, where they respectively used different strategy to create IKKα (IκB kinase α) knock-out mice with the purpose of determining the biological function of IKKα [99, 100]. In both models, the IKKα knock-out fetuses (E18.5) had defective development of limbs and tails, with abnormally thick, shiny, and wrinkleless skin. Further examination confirmed the disorders in the proliferation and differentiation of epidermal keratinocytes in IKKα knock-out mice: 1) the stratum granulosum and stratum cornuem were utterly missing, indicating a complete block of keratinocyte differentiation; 2) the thickened stratum spinosum expressed both basal and spinous markers (Keratin 14 and 10); 3) the mutant epidermis was hyperplastic, with significantly higher rate of proliferation, not only in the basal keratinocytes, but also in the suprabasal keratinocytes.

IKKα is a subunit of IκB kinase (IKK) complex, which contains IKKα, IKKβ and NEMO. In canonical NF-κB signaling pathway [101], pro-inflammatory signals stimulate receptors on the cell membrane (e.g. tumour necrosis factor receptor or interleukin-1/Toll-like receptor), which activates the IKK complex. The activated IKK complex mediates the phosphorylation, polyubiquitination and degradation of IκB proteins, which allows the accumulating of NF-κB proteins (e.g. p50 and RelA) within the nucleus and ultimately activates the target gene (pro-inflammatory and anti-apoptotic) transcription. Although IKKα is an important regulator of NF-κB signaling, its distinct function in regulating keratinocyte differentiation adds to its novelty. Importantly, IKKα regulates keratinocyte differentiation via NF-κB-independent transcriptional regulation. Under the challenge of proinflammatory stimuli, IKK activation and IκB degradation are intact in IKKα knock-out cells (in keratinocyte, embryonic fibroblasts and liver cells) [99, 100,

102]. In contrast, IKKβ or NEMO-deficient mouse models showed impaired NF-κB signaling but possessed full differentiation ability in keratinocytes [103-105]. In the rescue experiment via adenovirus overexpression of IKKα, IKKβ or RelA, only IKKα was able to restore the differentiation ability in keratinocyte [102]. Furthermore, adenovirus infection of dominant negative IκBα in wild-type keratinocyte did not inhibit keratinocyte differentiation [102]. In addition, the ability of IKKα in inducing keratinocyte differentiation required neither the protein kinase activity, nor the direct biding to NEMO [102]. Therefore, the function of IKKα in control of keratinocyte differentiation is independent on NF-κB signaling.

In more recent studies, IKKα has been shown to be able to function within the nucleus and regulate target gene expressions. In 2004, Sil *et al*. detected increased levels of nuclear IKKα induced by keratinocyte differentiation and identified the nuclear localization sequence (NLS) within the kinase domain of IKKα [106]. The transcriptional activity of IKKα was first illustrated by Liu *et al*., that IKKα negatively regulates VEGF-A expression via binding to the distal VEGF-A promoter [107]. The transcriptional suppression targets of IKKα also include EGF, HB-EGF, and amphiregulin [108]. Remarkably, the intranuclear functions of IKKα are more than repressing target gene transcription. Zhu *et al*. demonstrated that IKKα associates with histone H3 in 14-3-3σ, which shields 14-3-3σ from histone trimethyltrasferase-mediated hypermethylation and gene silencing [109]. Although NF-κB-independent intracellular gene regulations are one of the potential functions of IKKα in regulating keratinocyte differentiation, the precise molecular mechanisms are poorly understood, and it remains unclear whether these regulatory activities of IKKα require association with other proteins.

Besides KDF1 and IKKα, in the research paper that our lab published in 2017 [110], we identified a desmosome protein, Pkp1 (Plakophilin-1), being critically involved in skin

differentiation. Upon keratinocyte differentiation, Pkp1 is significantly phosphorylated at multiple sites, among which the phosphorylation at the head domain mediated by RIPK4 (receptor-interacting serine-threonine kinase 4) is indispensable for its function in epidermal differentiation. *RIPK4* conditional knock-out mouse model (*K14-Cre-RIPK4*$^{fl/fl}$) gives rise to significantly thickened epidermis with dislocations of basal keratinocyte marker keratin 14. *Pkp1* (SE), a phosphomimetic mutant of *Pkp1*, could restore normal epidermal differentiation in *RIPK4* conditional knock-out mouse model. In contrast, WT or SA mutant of *Pkp1* could not rescue the differentiation defects saw in *K14-Cre-RIPK4*$^{fl/fl}$ mouse. We further suggested a functional model that the phosphorylation of Pkp1 by RIPK4 could block MAP kinase signaling pathway and enhance epidermal differentiation.

Enucleation process during epidermal differentiation

Although nuclei are essential organelles of eukaryotic cells to maintain normal cellular functions, some types of mammalian cells require programmed removal of the entire nuclei, called enucleation, to fulfill their functions. There are only three mammalian tissues/ cell types that have a nuclear degradation process to maintain tissue homeostasis: lens fiber cells, keratinocytes, and red blood cells (erythrocytes).

Lens need to be transparent in order to focus light on the retina, instead of scattering or refracting light as all other organelles. Therefore, lens epithelial cells need to go through a terminal differentiation process to form lens fiber cells, in which all the intracellular organelles are devoid while only crystallins proteins are left [111]. This enucleation process occurs while lens epithelial cells migrate along the lens periphery into the middle portion of the lens, which is

phenomenologically well described. However, the underlying molecular mechanisms are poorly understood.

The best studied enucleation process is in erythrocytes. Erythropoiesis initiates when hematopoietic stem cells differentiate into erythroid progenitor cells and eventually become mature erythrocytes [112]. During the maturation of erythrocytes, erythroblasts go through a process of nuclear condensation and extrusion, then temporally form a pyrenocyte containing the condensed and extruded nuclei, as well as an enucleate reticulocyte. Eventually, pyrenocyte is engulfed and degraded by macrophages, while reticulocyte becomes mature erythrocyte.

Nuclear removal in epidermal keratinocytes occurs when cells move from granular layer to the uppermost cornified layer. Granular cells remove all their organelles including the nuclei, in order to provide the cornified envelop with rigid keratin structure and water proof lipid matrix barrier [113]. The nuclei go through several morphological changes in granular layer, such as decreases in volume, more elongated and aligned to the basal membrane. Strikingly, the enucleation process occurs really rapidly, at most six hours, and continues non-stop throughout our life. In human, parakeratosis primarily features defects in keratinocyte enucleation, which causes suffering in patients with various skin diseases including cancers. Unfortunately, little is known about the pathways that lead to complete nuclear removal and about how these pathways are regulated.

A potential role of autophagy during keratinocyte enucleation

Autophagy mediated nuclear lamina proteins and nucleus degradation have been proposed to regulate nuclear removal in keratinocyte. The role of autophagy in degrading nuclear components has been accessed by Z. Dou, *et al*. in 2015 [114]. LC3/Atg8, one of the essential

autophagy proteins involved in autophagosomes trafficking, was found to localize in the nucleus. There is a directly and specific interaction between LC3 and nuclear lamina protein lamin B1, but not the other three nuclear lamin isoforms. Meanwhile, LC3-lamin B1 interaction primarily occurs at the lamin-associated domains (LADs) on chromatin, which is usually within the transcriptionally inactive heterochromatin area. Upon oncogenic stress, such as RAS activation, LC3 mediated autophagy will lead to lamin B1 and DNA component degradation, in a specifically targeted way. Therefore, autophagy mediated nuclear lamina degradation could be essential in maintaining tissue integrity and preventing tumorigenesis.

Nuclear targeted autophagy has been further illustrated in 2016 by O. Akinduro, *et al*. [115]. Various autophagy markers, such as LC3, ULK1 and WIPI1, are upregulated during embryonic skin development and are continuously active in adult mouse epidermis. shRNA knock down of *WIPI1* or *ULK1* lead to prevention of nuclear removal in differentiating keratinocytes *in vitro*. Nuclear indentation during keratinocyte differentiation was closely examined *in vitro* via immunostaining of LC3, Lamp2, p62, LMNB1 and HP1α, which further illustrated that targeted degradation of nuclear component during keratinocyte differentiation is mediated by autophagy. However, there are contradictory comments arguing if the canonical autophagy is required for keratinocyte nuclear removal. For example, *Atg7* conditional knock-out mouse model, in which *Atg7* is exclusively deleted in K14 expressing keratinocytes, gives rise to healthy skin without significant defects in epidermal cornification or skin barrier functions, although the autophagy process in mutant skin is indeed affected [116]. In addition, *Atg5* deletion in K5 expressing epithelium generate healthy and functional skin except for the premature degradation of nucleus of the cells at the preputial gland [117].

Epidermal differentiation and skin diseases, including skin cancer

Per Lee *et al*. [93], "Excessive proliferation or defective differentiation can lead to tumor formation, while the inverse scenario can result in hypoplastic organs or defects in tissue repair." Therefore, the balanced and precisely regulated proliferation and differentiation of epidermal progenitor cells are crucial in preventing cancer occurrence and ensuring the barrier function of the epidermis. Deregulation of epidermal differentiation in skin can lead to the development of various skin diseases including skin cancers. Both basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) share lineage with keratinocytes and resemble the histological characteristics of epidermal keratinocytes [118, 119]. Although BCC and SCC of the skin have a high cure rate, they are the most common cancers in the United States and causing over 1000 deaths annually [119]. Cutaneous SCC is the second most common human cancer, afflicting more than 250,000 patients in the United States every year [119-121]. 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 [122]. In addition, the oral cavity and the esophagus are also lined with keratinocytes, while the carcinomas originated from these sites represent a significantly poorer prognosis [123].

Many signaling pathways and regulatory proteins that are essential for epidermal differentiation are also related to skin diseases, especially in skin cancers. Therefore, there is an urgent need to expand our knowledge of molecular mechanisms and signaling networks involved in skin differentiation. It is dramatically important to reveal the important molecular mechanisms 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. In below, I will give a brief introduction of the molecules and signaling pathways involved in keratinocyte differentiation and skin tumorigenesis.

p63 gene mutation in human could lead to five disease syndromes, which share abnormalities in skin, teeth or nails structures, limb or craniofacial skeleton development [124]: EEC (ectrodactyly, ectodermal dysplasia, clefting), AEC (ankyloblepharon, ectodermal dysplasia, clefting), LMS (limb-mammary syndrome), ADULT (acro-dermato-ungual-lacrimo-tooth) and SHFM (split hand/foot malformation). *p63* mutation is rarely discovered in many cancer types. However, *p63* is frequently upregulated in SCC of the skin and in up to 80% of primary HNSCCs [125, 126]. To dissect the function and molecular mechanisms of p63 in tumorigenesis could be challenging, because of the presence of multiple *p63* isoforms. However, *ΔNp63*, more specifically *ΔNp63α*, is the predominant isoform that is upregulated in skin SCCs and contribute to tumorigenesis based upon many studies up to date (see review articles in reference [125, 126]). Moreover, *ΔNp63α*, has been shown to cooperate with constitutively active Ras/ MAPK signaling and promote a proliferative advantage for tumorigenesis [127].

The function of Notch signaling in skin tumorigenesis is different from many other tissue types, where traditionally Notch signaling can promote cell proliferation and act as proto-oncogene [128]. According to K. Sakamoto [129], inactivating mutations of Notch1 are present in about 10% SCC of the skin, oral cavity, esophagus and lung. As I mentioned earlier, Notch signaling controls the transition from proliferating basal layer to post-mitotic spinal layer in mature skin keratinocytes [46, 47]. In another word, it is required for blocking basal keratinocyte proliferation and initiating terminal differentiation. Inactivating Notch1 in mature mouse skin leads to spontaneous skin tumors and the mouse model becomes more prompt to carcinogen mediated skin tumorigenesis [130]. In addition, ectopic-expression of a dominant-negative form of MAML1, the coactivator

required for activating canonical Notch signaling, could cause spontaneous development of skin SCC in mouse model [131]. Therefore, Notch signaling guards the skin tissue from hyperplasia and acts as a tumor suppressor.

In 2006, Liu *et al*. examined the function of IKKα in human SCCs, where they identified significant reduction in IKKα expression in poorly differentiated human SCCs via tissue arrays of 114 SCC patients [107]. Soon after that, downregulation of IKKα in human oral carcinomas was validated [132]. Besides alterations in protein expression level, eight out of nine SCC patients possessed somatic mutations within exon 15 of IKKα [107]. Further functional studies using Lori• IKKα-Tg mice (IKKα overexpressed in suprabasal keratinocyte) revealed that overexpression of IKKα in the epidermis could repress chemical carcinogen induced tumor progression and metastases with decreased mitogenic and angiogenic activities [107]. In contrast, reduction in IKKα expression or conditional knock out of IKKα in basal keratinocytes, both promoted the development of SCCs in genetic mouse models [132, 133]. Therefore, IKKα may serve as a novel target in the development of therapeutic approaches in the treatment of human SCCs, based upon its characteristic of tumor suppressor. It is worth to notice that, there were a few research studies indicating that IKKα could also promote tumor growth [134, 135], although these research were mainly based upon *in vitro* studies and xenograft mouse models, which remains questionable and requires much more solid evidence. Therefore, without a fully understanding of IKKα's role in the regulation of keratinocyte differentiation at the molecular level, it is difficult to apply IKKα into clinical use. A better understanding of IKKα's intrinsic functions may lead to the development of effective novel targets for the treatment of human cancers originated from epidermal keratinocyte.

Loss-of-function mutations of *Pkp1* could cause ectodermal dysplasia/skin fragility (EDSF) syndrome in human [136, 137]. Patients could suffer from many symptoms, such as hyperkeratosis

of the skin at soles and palms, alopecia or hair loss, and blistering with erosions when skin encounters mechanical stress. Our recent study demonstrated that loss of *Pkp1* in skin progenitor cells could cause abnormal epidermal differentiation and promote skin carcinogenesis and progression [110]. Since phosphorylation of Pkp1 by RIPK4 is required during normal keratinocyte differentiation, we further demonstrated that loss of *RIPK4* in mice leads to cutaneous SCCs. The same gene has been identified as a frequently mutated gene in human head and neck SCCs [138].

In regarding KDF1, recent studies suggest that KDF1 is not only involved in regulating epidermal differentiation, but also involved in tooth agenesis [98]. Mutation of *KDF1* has been identified in patients with ectodermal dysplasia, a heterogeneous group of diseases that affects the ectoderm derivative, such as skin, hair follicles, teeth, and nails [98]. However, the role of KDF1 in skin tumorigenesis has never been explored, which is essential to be tested for deeply understanding the function of KDF1 in regulating epidermal differentiation.

Primary keratinocyte culturing and technical limitations

A lot of functional studies are based upon genetic mouse models. However, many of the knock-out mouse model are embryonic or perinatal lethal, such as IKKα and KDF1 knock-out mouse. Therefore, it is difficult to further identify the protein functions in adult skin. In addition, as I mentioned previously, there is a big difference between embryonic skin development and adult epidermal stratification, in terms of the existence of intermediate layer during embryonic development. Therefore, the protein functions and mechanisms illustrated from these mouse models may not reflect the real case as in adult skin. For example, two *p63* knock-out mouse models using different targeting constructs are presenting distinct phenotypes, which makes a

confliction in determining the real function of p63 during keratinocyte differentiation [139, 140]. Isolating primary keratinocyte and culturing *in vitro* makes it possible to bypass the lethality issue of the original mouse models. Since the 1940's, many studies have been focusing on using cultured human epidermal cells [141-143]. Similarly, isolating and culture mouse epidermal cells are also well-developed. However, as I mentioned earlier, *in vitro* studies cannot fully recapitulate cell behaviors *in vivo*, as the environment and tissue context are dramatically different. It is extraordinarily important to develop a procedure to graft cultured keratinocyte back into an *in vivo* environment.

In 1975, James and Howard published a report stating that human keratinocytes can be cultured to form stratified squamous epithelium in a serial culture [144]. Later, another study stated that multiple stratified human keratinocyte colonies could ultimately fuse and form a skin-like epithelium [145]. Although currently established protocol for culturing skin epidermal stem cells makes it possible to generate functional skin tissue from cultured stem cells, engraftment of passaged epidermal stem cells via traditional skin stem cell technique is technically challenging. To resolve this issue, our lab has recently developed the organotypic culture of epidermal keratinocytes *in vitro* by culturing the cells 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* [146]. 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 stem cells. Via this novel technique, we can closely mimic the *in vivo* conditions of skin development while allowing various manipulations in the donor cells. Up to now, we have been using this technique to access the role of Pkp1 and RIPK4 in keratinocyte differentiation and skin tumorigenesis, retrieving the mechanisms underlying keratinocyte mobility and wound

healing, as well as utilizing grafted keratinocyte as a tool of gene delivery for treating disease [110, 147-149].

In clinical settings, skin grafts generated form cultured epidermal stem cells have been popularly developed to treat patients with severe burns or other chronic conditions. In my review paper [21], I have introduced several medical devices for disease treatment, which are designed based on cultured human epidermal keratinocytes. For example, ReCell (Avita Medical) is a unique method to harvest autogenous skin through simple biopsy from patients, and apply cell suspension to facilitate wound healing. Upon applying, the keratinocyte autografts could provide treatment similar to standard skin grafting, but with benefits of smaller harvesting wound and accelerated healing process with improved scar formation. SkinGun^{TM} is another medical device similar to ReCell that has an optimized spraying device. In addition to keratinocyte autografts, allograft can also facilitate wound healing and be used in a clinical setting. Apligraf® (Organogenesis, MA) is a bi-layered skin substitute, with one bottom layer of neonatal foreskin-derived fibroblasts and an upper layer of stratified cultured epidermal keratinocytes. It has been approved by FDA to treat chronic venous leg ulcers (VLU) and diabetic foot ulcers (DFU).

CHAPTER II

REGULATION OF EPIDERMAL DIFFERENTIATION THROUGH KDF1-MEDIATED DEUBIQUITINATION OF IKK1

Introduction

As part of the largest organ in the adult mammalian body, the skin epidermis has significant functions in protecting humans and animals from external environment [1-3]. Four layers of keratinocytes at different stages of differentiation compose the inter-follicular epidermis (IFE). Only basal keratinocytes possess the ability of proliferation and giving rise to the other compartments in IFE [21, 49]. Therefore, basal keratinocytes, also called epidermal progenitor cells, are crucial in fueling the skin epidermal turnover (desquamation) and facilitating the replacement of damaged or lost tissue [1, 3, 21, 49, 150]. Abnormal epidermal differentiation is significantly involved with the development of various skin diseases including skin cancers, such as basal cell carcinomas (BCCs) and squamous cell carcinomas (SCCs).

Although the morphogenetic and genetic changes during epidermal differentiation were largely studied [2, 76, 151], much is unknown about the molecular mechanisms and regulatory networks underlying this process. The function of *KDF1* in epidermal differentiation was discovered in 2013 via an ENU-induced mutagenesis screen in mice [93]. The homozygous mutant *KDF1* fetuses (E18.5) developed a thick, taut and hyperplastic epidermis with diminished barrier ability. Crucial evidence, such as an uncontrolled cellular proliferation, a mixture of basal and spinous differentiation markers, and an absence of terminal differentiation, indicated the abnormalities in epidermal development and differentiation of epidermal progenitor cells due to *KDF1* deficiency. However, the precise molecular mechanisms through which KDF1 regulates keratinocyte differentiation is poorly understood. In order to shed light to this process, our lab took

advantage of stable isotope labeling by amino acids in cell culture (SILAC) technology paired with tandem mass spectrometry (MS/MS), and conducted a screening assay. We were able to identify a list of targets that are interacting with KDF1, which provides an important guideline to study the function of KDF1.

Within the long list of KDF1 interacting proteins, IKKα caught our eyes since it has been reported to have essential functions during epidermal differentiation [99, 100, 151]. *IKKα* knockout fetuses developed abnormally thick, shiny, and wrinkleless skin [99, 100]. We carefully examined the epidermal differentiation process in both IKKα and KDF1 deficient mouse models, and confirmed their similarities in the disorders of epidermal stratifications, such as uncontrolled proliferation and abolished terminal differentiation. Although IKKα has been known as a key component within NF-κB signaling pathway [101], its distinct function in regulating keratinocyte differentiation does not require its kinase activity and is NF-κB-independent [99, 100, 102]. It remains unclear whether the regulatory activity of IKKα in keratinocyte differentiation requires association with other proteins, and little is known about how it functions to regulate epidermal differentiation at the molecular level. In addition, aberrant IKKα function could lead to skin cancers, such as human SCCs [107, 132, 133, 152, 153]. Substantial reduction of IKKα protein level was significantly associated with poorer prognosis of human SCCs. Further functional studies using transgenic mouse models provided compelling evidence that IKKα is a suppressor of skin cancer. Therefore, understanding how IKKα fulfills its regulatory functions during epidermal differentiation is not only essential for skin development, but is also critical for cancer biology.

In this study, we discovered that KDF1 and IKKα form a complex and regulate similar process of keratinocyte differentiation. The importance of their interaction during epidermal differentiation is confirmed in our *in vitro* and *in vivo* studies. Moreover, *KDF1* deficiency

contributes to skin tumorigenesis due to aberrant keratinocyte differentiation. However, we wanted to further investigate the novelties of this interaction. Increase of IKKα expression level during embryonic skin development has been reported in the past [154]. Although there were indications of changes at the transcriptional level [154, 155], we found that transcriptional regulation cannot subsidize IKKα protein level change during primary keratinocyte differentiation. Moreover, loss of *KDF1* would diminish the elevation of IKKα protein level but not reflected at the transcription level. For the first time, we proposed a potential mechanism of controlling IKKα protein level during epidermal stratification through ubiquitination mediated regulatory pathway.

Ubiquitination, is a universal mechanism for protein degradation that controls a wide variety of cellular processes [156-159]. Deubiquitination is a reverse process of ubiquitination which is performed by deubiquitinating enzymes (DUBs) [160, 161]. USP7 is an essential DUB which was caught up during our SILAC-MS screening for KDF1 interacting proteins. Although USP7 has be implicated in controlling NF-κB pathway [162, 163], and regulating proteins related to tumorigenesis such as p53/MDM2 (mouse double minute 2 homolog), PTEN and FOXO4 [164-167], the function of USP7 in skin development and epidermal stratification is unknown. We found that KDF1 has an interaction with USP7 during keratinocyte differentiation and loss of *USP7* has effects on IKKα protein level as well as ubiquitination level. Keratinocyte differentiation process is also hindered *in vitro* upon loss of *USP7*. Taken together, our results strongly supported our hypothesis that USP7 is recruited by KDF1 to the regulatory complex involving IKKα, which significantly contributes to the upregulation of IKKα protein level during epidermal differentiation. Our studies illuminated a novel molecular mechanism whereby differentiation of epidermal progenitor cells is regulated by ubiquitination.

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, Rabbit Krt 5 and Krt 10 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^{TM} 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, 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. Ki-67 antibody (KI505) was obtained from Nordic MUbio (Susteren, The Netherlands). 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; 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 Proteomic 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 hours 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 were 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 [110]. Epidermis of E18.5 or newborn mice 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 keratinocytes can healthily and independently grow. 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 60mM. HEK293-T cells were cultured in DMEM medium supplemented with 10% FBS.

Animals

KDF1 mutant strain was a generous gift from Dr. Scott D. Weatherbee (Yale University). The *IKKα* KO strain was a generous gift from Dr. Anning Lin (the University of Chicago). 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.

Protein Biochemical Analysis

Western blot was conducted as previously described [168]. 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) for 1 hour at room temperature, followed by an overnight incubation with desired primary antibody at 4 degree. Immunoblot was washed three times with 1 X Tween 20/Phosphate Buffered Saline (PBST) and incubated with secondary antibody (1:10000 dilution) at room temperature for 1 hour. Blot was washed with 1 X 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.

Skin Organotypic Culture and Grafting

Skin organotypic culture and grafting were performed as previously described [110]. Decellularized dermis (1cm X 1cm square shape) was prepared from newborn CD1 mice skin via EDTA treatment [146]. 2 X 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 6-8 weeks were anaesthetized. Two 1cm X 1cm 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 one week after surgery [110, 148, 149]. All the experiments were repeated more than three times (three biological replicates). For phenotypic analysis using immunostaining, at least 3 sections were taken from each graft for quantifications.

Histology and Immunofluorescence

Skin or tumor 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 were conducted as previously described [169]. 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, a Student's *t* test was used to evaluate the statistical significance (P value) of the difference. For results in **Figure 2.8 D**, two-way ANOVA (analysis of variance) was used for statistical analysis.

Results

IKKα interacts with KDF1

KDF1 deficiency leads to profound abnormality in epidermal development. To dissect the underlying mechanisms, we engineered expression vectors encoding HA and His_6-tagged *KDF1*. We then used tandem affinity purification to isolate the KDF1 complex from transfected cells upon calcium-induced keratinocyte differentiation, and then employed SILAC (stable isotope labelling by amino acids in cell culture) coupled with LC-MS/MS (liquid chromatograph and tandem mass spectrometry) **(Figure 2.1 A)**. KDF1 associated proteins can be readily differentiated from the background signals via amino acid labeling. Our analysis suggests that KDF1 associates with IKKα in skin keratinocytes. To confirm the binding affinity between KDF1 and IKKα, we conducted co-immunoprecipitation (co-IP) assay in HEK293T cells **(Figure 2.1 B and C)**. HA-KDF1 showed strong binding ability with GST-IKKα, while the empty vector without KDF1 did not. Furthermore, GST-IKKα could co-IP with HA-KDF1 as well. The interaction between endogenous IKKα and KDF1 was confirmed via immunoprecipitation, using anti-KDF1 antibody in WT keratinocyte **(Figure 2.1 D)**.

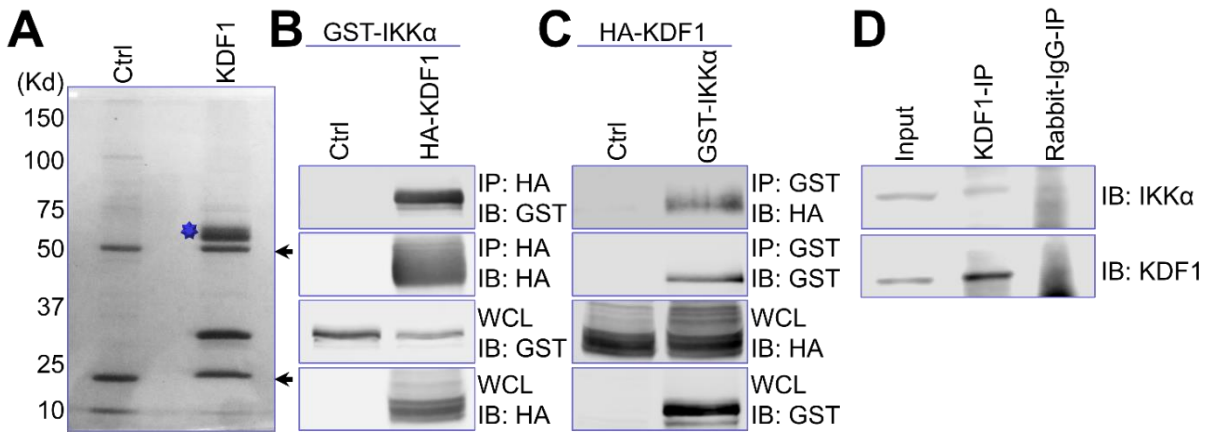

Figure 2.1: Exogenous expression of KDF1 and examination of IKKα KDF1 interaction via Co-IP in HEK293T cells and immunoprecipitation within keratinocyte. A. Tandem affinity purification was used to isolate KDF1 associated protein after transfection. Precipitated proteins were resolved in SDS-PAGE and subjected to identification with LC-MS/MS. Arrows denote IgG heavy and light chains. Star denotes KDF1 band. **B.** Using HA-KDF1 as the bait to pull down GST-IKKα. Control is pKH3 (empty vector used to construct HA-KDF1). **C.** Using GST-IKKα as the bait to pull down HA-KDF1. Control is pDH-GST-puro (empty vector used to construct GST-IKKα). **D.** Immunoprecipitation using anti-KDF1 antibody in WT keratinocyte. Normal rabbit IgG was used as a negative control.

Loss of *IKKα* or *KDF1* causes similar alterations in epidermal stratification

Consistent with previous reports [99, 100, 102], our examination indicates similar alterations in epidermal stratification upon loss of *KDF1* or *IKKα* **(Figure 2.2 A and B)**. Both models have striking phenotypes including: a hyperplastic spinous layer indicated by immunostaining of keratin 10, a lack of cornified layer suggested by the loss of loricrin expression, and a failure of enucleation process. In addition, the expression of keratin 14, which is a biochemical marker of basal keratinocyte, was abnormally expanded throughout the whole epidermis. With isolated basal stem cells, we found that ablation of *KDF1* or *IKKα* leads to altered cell morphology upon calcium shift compared to WT keratinocyte **(Figure 2.2 C)**. Additionally, under calcium shift *in vitro*, *KDF1* or *IKKα* knock-out (KO) keratinocyte have reduced expression of cross-linked loricrin and modified filaggrin, both are biochemical markers for epidermal differentiation in keratinocytes **(Figure 2.2 D)**. Together, our results provide compelling evidence that epidermal differentiation is regulated by both KDF1 and IKKα.

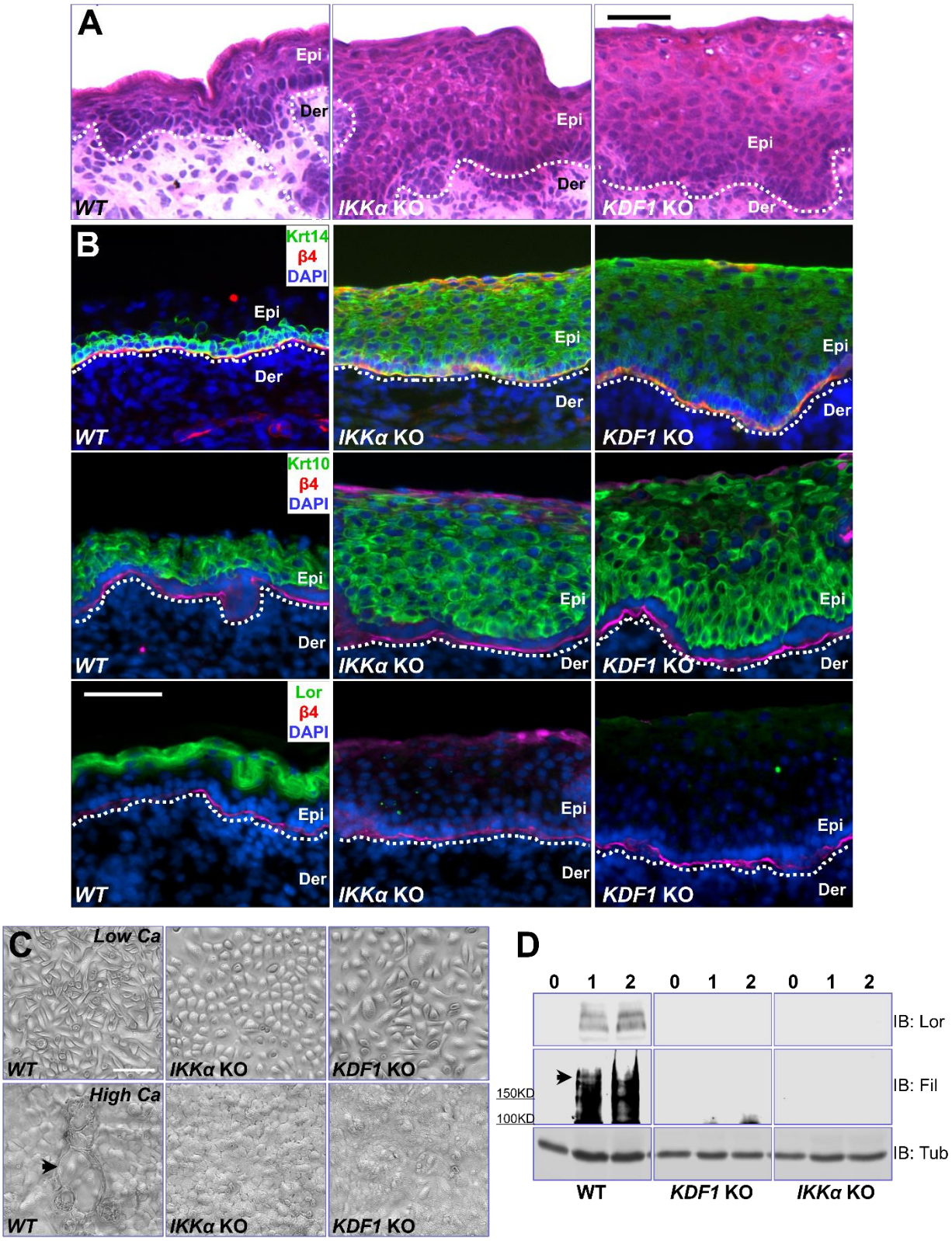

Figure 2.2: Comparison of the alterations in epidermal stratification upon loss of *KDF1* or *IKKα*. Continued on next page.

Figure [2.2], continued. Analysis of E18.5 WT, *KDF1* mutant and *IKKα* KO epidermis, via H/E staining **(A)** and immunofluorescence staining of different antibodies **(B)** as indicated (Krt14: keratin 14, Krt10: keratin 10, Lor: Loricrin, β4: β4-integrin, CD104). In H/E staining, scale bar=50 μm. In immunofluorescence staining, scale bar=100 μm. Dotted lines denote dermal–epidermal boundaries. Epi: epidermis, Der: dermis. **C.** Cell morphology is evaluated at 0h and 48h using light microscope. Black arrow denotes cell differentiation in culture. Scale bar=100 μm. **D.** Western blot of loricrin and filaggrin using whole cell lysate collected at 0, 1 and 2 days after calcium shift. Tubulin is used as loading control. Arrow denotes modified Filaggrin.

Mapping of the interacting domains within IKKα and KDF1

In order to address the mechanisms underlying IKKα and KDF1 regulated keratinocyte differentiation, we first mapped the binding motif within KDF1 and IKKα **(Figure 2.3 and 2.4)**. We generated various truncation mutants of KDF1 and IKKα, based upon the already-known function domains or protein characteristics. IKKα is a conserved helix-loop-helix ubiquitous kinase, which contains a serine-theronine kinase domain, a leucine zipper motif [170], a helix-loop-helix domain (HLH) and a NEMO binding domain **(Figure 2.3 A)** [171]. KDF1 contains proline and cystein-rich regions near the N-terminus, although it lacks any recognizable functional domains **(Figure 2.4 A)** [93]. All the KDF1 constructs are HA-tagged, while all the IKKα mutants are tagged with GST. Then, different combination of mutant constructs were transiently transfected into HEK293T cells at 1:1 ratio, and the binding was tested by co-IP **(Figure 2.3 B and 2.4 B)**. Since co-IP using HA as the bait has much higher pull-down efficiency than GST, we performed co-IP using anti-HA affinity beads as our primary approach. The empty vectors were used as a negative control for co-IP.

As shown in **Figure 2.3 B**, the serine-theronine kinase domain of IKKα does not have the binding ability with KDF1 and the interaction may happen in the other three domains. The IKKα truncation mutant involving LZ and HLH domain can interact with KDF1, while the mutant including HLH and NEMO binding domain cannot. Therefore, LZ domain may be responsible for the interaction with KDF1. To confirm this hypothesis, we generated two more mutant IKKα, either solely containing LZ domain or involving all the functional domains but without LZ domain. Upon co-IP assay result, IKKα does interact with KDF1 at its LZ domain. Although the binding between IKKα and KDF1 does not occur at the kinase domain of IKKα, we further examined whether the kinase activity is required for binding, using kinase-dead mutant K44M. The amino

acid replacement of Lysine 44 with Methionine in this mutant destroys IKKα kinase activity [172]. Since the kinase activity of IKKα is not required for keratinocyte differentiation [99, 100, 102], it is unlikely that the kinase-dead mutant will impair its interaction with KDF1. As expected, the kinase-dead mutant of IKKα can still interact with KDF1 upon co-IP assay result.

For KDF1 **(Figure 2.4 B)**, the proline and cystein-rich regions near the N-terminus cannot bind to IKKα. Therefore, we further truncated the C-terminal portion of KDF1 into two halves. The co-IP assay indicates that the binding motif of KDF1 lies within 149-276aa, and the loss of binding ability with IKKα upon in-frame deletion of this region further confirmed this result. Together, we were able to identify the crucial regions in KDF1 and IKKα that mediate their mutual binding, which lead to the generation of non-binding mutant KDF1 and IKKα for functional studies.

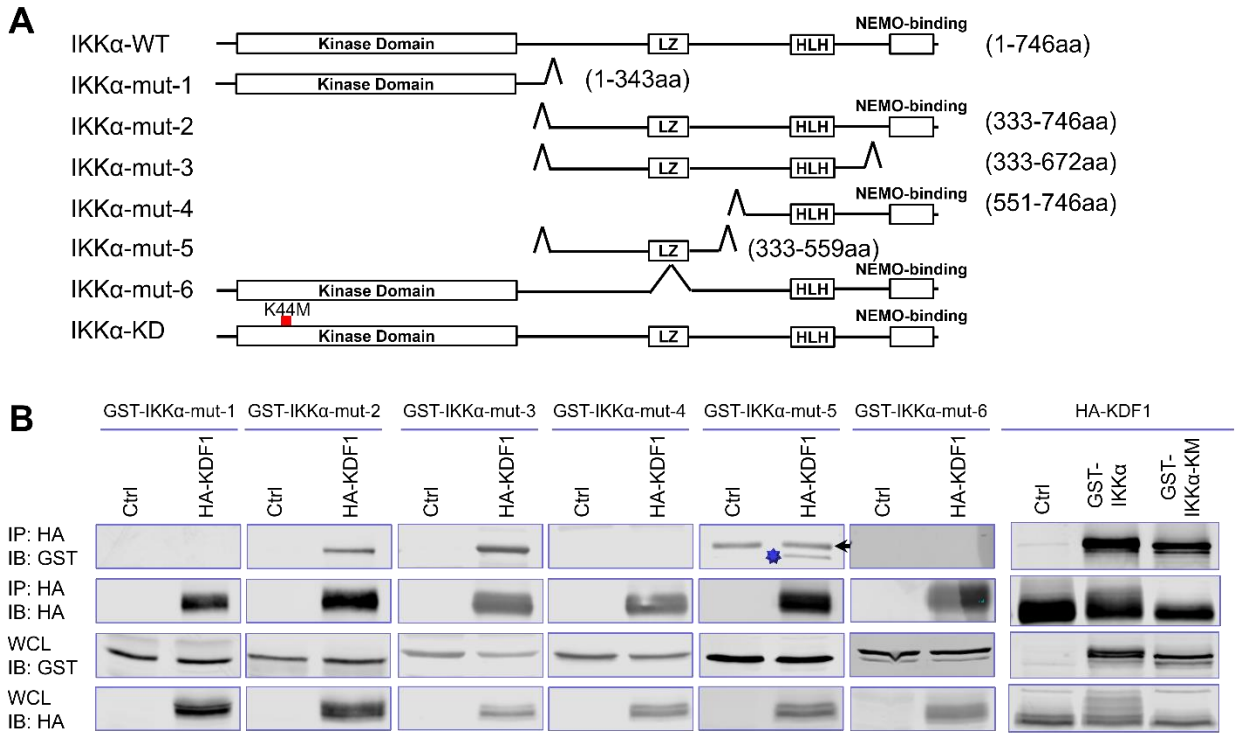

Figure 2.3: Diagram of *IKKα* mutants generating strategies and investigation of the interacting domains within IKKα via Co-IP in HEK293T cells. All the IKKα mutants are tagged with GST. Different combination of mutant constructs with HA tagged KDF1 are transiently transfected into HEK293T cells at 1:1 ratio, and the binding is tested by co-IP. **A.** IKKα is a conserved helix-loop-helix ubiquitous kinase, which contains a serine-theronine kinase domain, a leucine zipper motif [170], a helix-loop-helix domain (HLH) and a NEMO binding domain [171]. **B.** IKKα-mut-1 contains the N-terminal fraction with kinase activities. Since the binding motif falls inside the C-terminal fraction (IKKα-mut-2), we further truncate it into IKKα-mut-3 and 4. Via IKKα-mut-5 (contains LZ motif) and IKKα-mut-6 (in-frame deletion of LZ motif), the binding motif is narrowed down to LZ motif. In order to examine whether the kinase activity is required for binding, co-IP is conducted using kinase-dead IKKα mutant (IKKα-KD) K44M. Control is pDH-GST-puro (empty vector used to construct IKKα mutants).

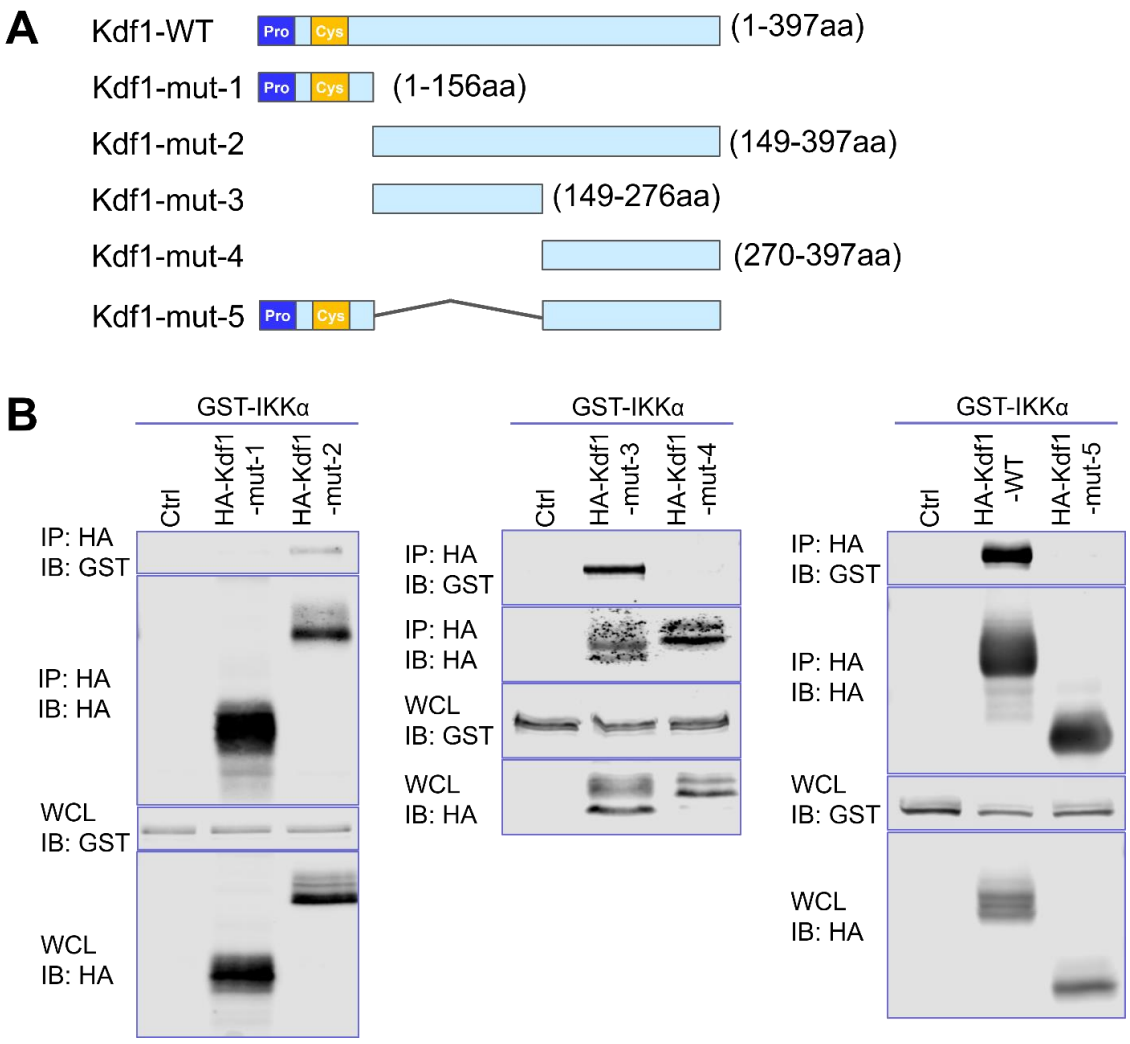

Figure 2.4: Diagram of *KDF1* mutants generating strategies and investigation of the interacting domains within KDF1 via Co-IP in HEK293T cells. All the KDF1 constructs are HA-tagged. Different combination of mutant constructs and GST tagged IKKα are transiently transfected into HEK293T cells at 1:1 ratio, and the binding is tested by co-IP. **A.** KDF1 contains proline and cysteine-rich regions near the N-terminus, and it lacks any recognizable functional domains. **B.** KDF1-mut-1 contains the proline and cysteine-rich regions and KDF1-mut-2 does not. Since the binding motif falls inside KDF1-mut-2, we further truncate it into two parts (KDF1-mut-3 and 4) and narrowed down the binding motif to 149-276aa via in-frame deletion (KDF1-mut-5). Control is pKH3 (empty vector used to construct KDF1 mutants).

IKKα and KDF1 interaction is crucial for epidermal differentiation *in vitro*

We hypothesize that IKKα and KDF1 regulate epidermal differentiation together as a regulatory complex in epidermal keratinocytes. Loss of either protein leads to aberrant epidermal differentiation and skin stratification. Understanding the underlying molecular mechanisms will be essential to delineate the signaling cascades involved in epidermal development and homeostasis. To determine the significance of KDF1 and IKKα interaction in skin differentiation, we used the mutants of *KDF1* and *IKKα* deficient for this interaction, which was identified in the last section. Using PiggyBac transposon system, we generated *IKKα* KO lines rescued with WT or mutant *IKKα*, tagged with triple-HA **(Figure 2.5 A)**. Since our pilot study indicated that cell growth may be hindered by overexpression of KDF1, we chose Tet-On inducible system to express non-binding mutant *KDF1* or WT *KDF1* in *KDF1* deficient keratinocytes **(Figure 2.5 B)**.

The ability of these constructs to rescue defects in epidermal differentiation were determined upon calcium shift *in vitro*. The differentiation of basal keratinocytes were assessed by morphological analysis of cells after calcium shift **(Figure 2.5 E)**. The expression level of loricrin, a biochemical marker for late stage epidermal differentiation, were examined via western blotting and quantified using housekeeping genes as loading controls **(Figure 2.5 C and D)**. Our results indicate that, expression of WT *IKKα* or WT *KDF1* were able to restore the morphological changes in differentiation deficient cells during calcium shift and partially restore the expression of loricrin, a late-stage differentiation marker. In contrast, non-binding mutant *IKKα* or *KDF1* were unable to rescue differentiation defect in *IKKα* and *KDF1* knockout cells.

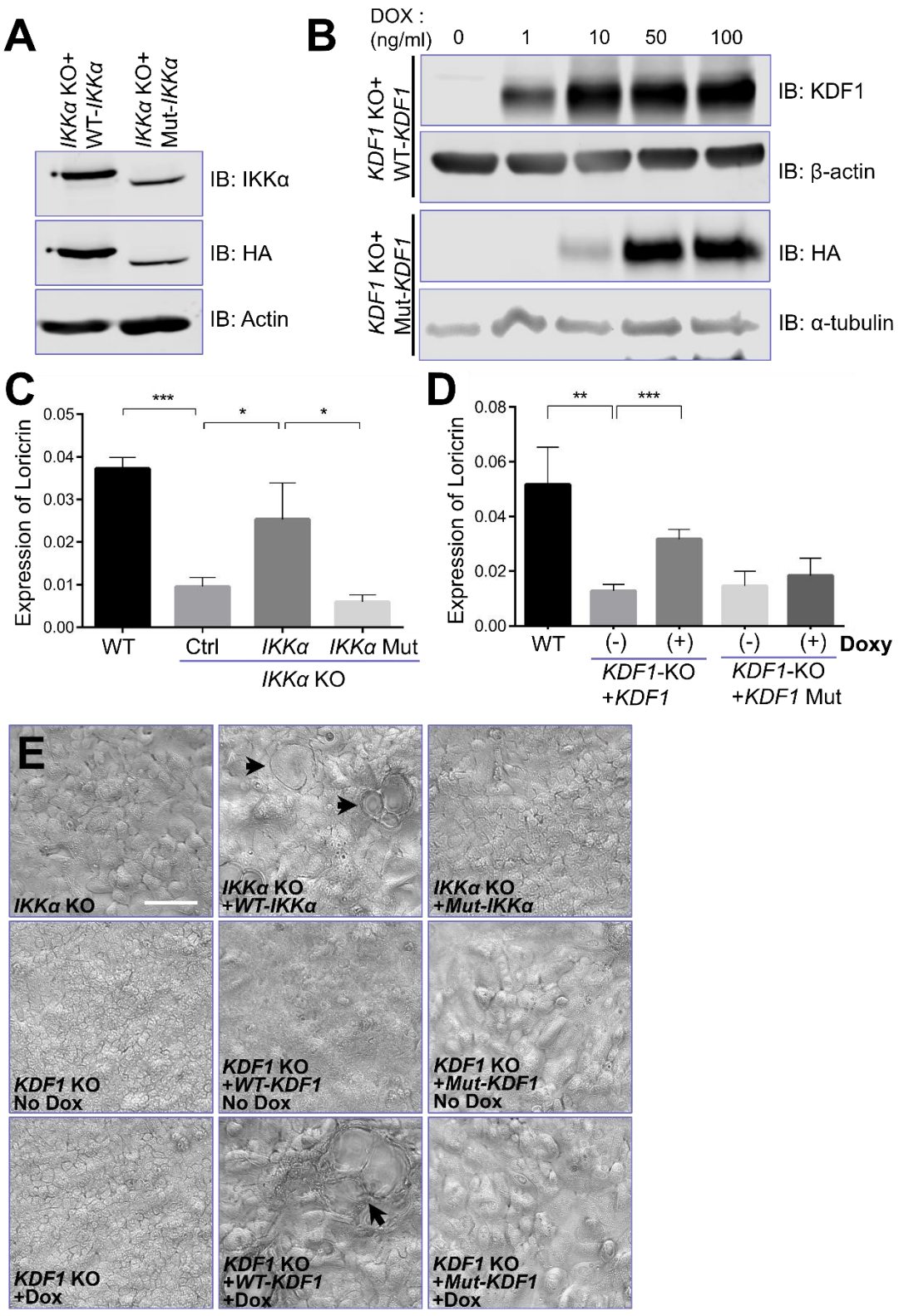

Figure 2.5: IKKα and KDF1 interaction is essential for skin stratification *in vitro*. Continued next page.

Figure [2.5], continued. A. Using PiggyBac transposon system, we generated *IKKα* KO lines rescued with WT or mutant *IKKα*, tagged with triple-HA. Expression of exogenous protein was tested via western blotting. β-actin was used as a loading control. **B.** Tet-On inducible system was used to express non-binding mutant *KDF1* or WT *KDF1* in *KDF1* deficient keratinocytes. Serious concentration of doxycycline (Dox) was added to the culture medium and incubated overnight. The expression of exogenous protein was tested via western blotting. α-tubulin or β-actin was used as a loading control. 50ng/ml Dox was settled to be used in the following studies. **C and D.** Western blot of loricrin in conducted using whole cell lysate collected at 2 days after calcium shift. β-actin is used as a loading control. Band intensity is determined by densitometry and the relative expression of loricrin is calculated and quantified. Statistical analysis is conducted using unpaired Student's *t*-test. Error bar represents S.D. (standard deviation). ***, $p<0.0001$; **, $p<0.01$; *, $p<0.05$ **C.** Exogenous expression of WT-*IKKα* significantly increases loricrin expression in *IKKα* KO cells. *IKKα* KO keratinocyte rescued with non-binding mutant *IKKα* has similar loricrin expression as *IKKα* KO cells. **D.** Expression of WT-*KDF1* with Dox induction significantly increases loricrin expression in *KDF1* KO cells. Non-binding mutant *KDF1* cannot rescue loricrin expression. **E.** Cell morphology is evaluated at 0h and 48h using light microscope. Black arrow denotes cell differentiation in culture. Scale bar=100 μm.

IKKα and KDF1 interaction is crucial for skin stratification *in vivo*

The established protocol for culturing skin epidermal stem cells makes it possible to generate functional skin tissue from engineered stem cells, which can carry defined genetic alterations to investigate the consequence in epidermal stratification *in vivo.* However, engraftment of passaged epidermal stem cells via traditional skin stem cell technique is technically challenging. To resolve this issue, our lab has recently developed the organotypic culture of epidermal keratinocytes *in vitro* by culturing the cells 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* [146]. 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 stem cells.

With this novel platform, we have successfully engrafted WT, *KDF1* KO and *IKKα* KO keratinocytes. All the grafted cells were infected with lentivirus encoding H2B-RFP, and could be readily detected and distinguished after skin regeneration. Regenerated skin from *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* **(Figure 2.2)**. 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 *Krt-14* (Keratin 14, a basal cell marker) in the basal layer **(Figure 2.6 A)**. The thickness of *Krt-14* plus layer and the whole epidermis are measured and quantified **(Figure 2.6 B and C)**. The thickness of epidermis is an important feature of skin development and an indicator of skin differentiation. Expression of WT *IKKα* or WT *KDF1* were able to significantly decrease the thickness of epidermis to a level indistinguishable from WT

regenerated skin **(Figure 2.6 C)**. In comparison, non-binding mutant *IKKα* or *KDF1* had limited ability to affect the thickness of epidermis **(Figure 2.6 C)**. Taken together, our results strongly suggest that epidermal differentiation requires interaction between KDF1 and IKKα.

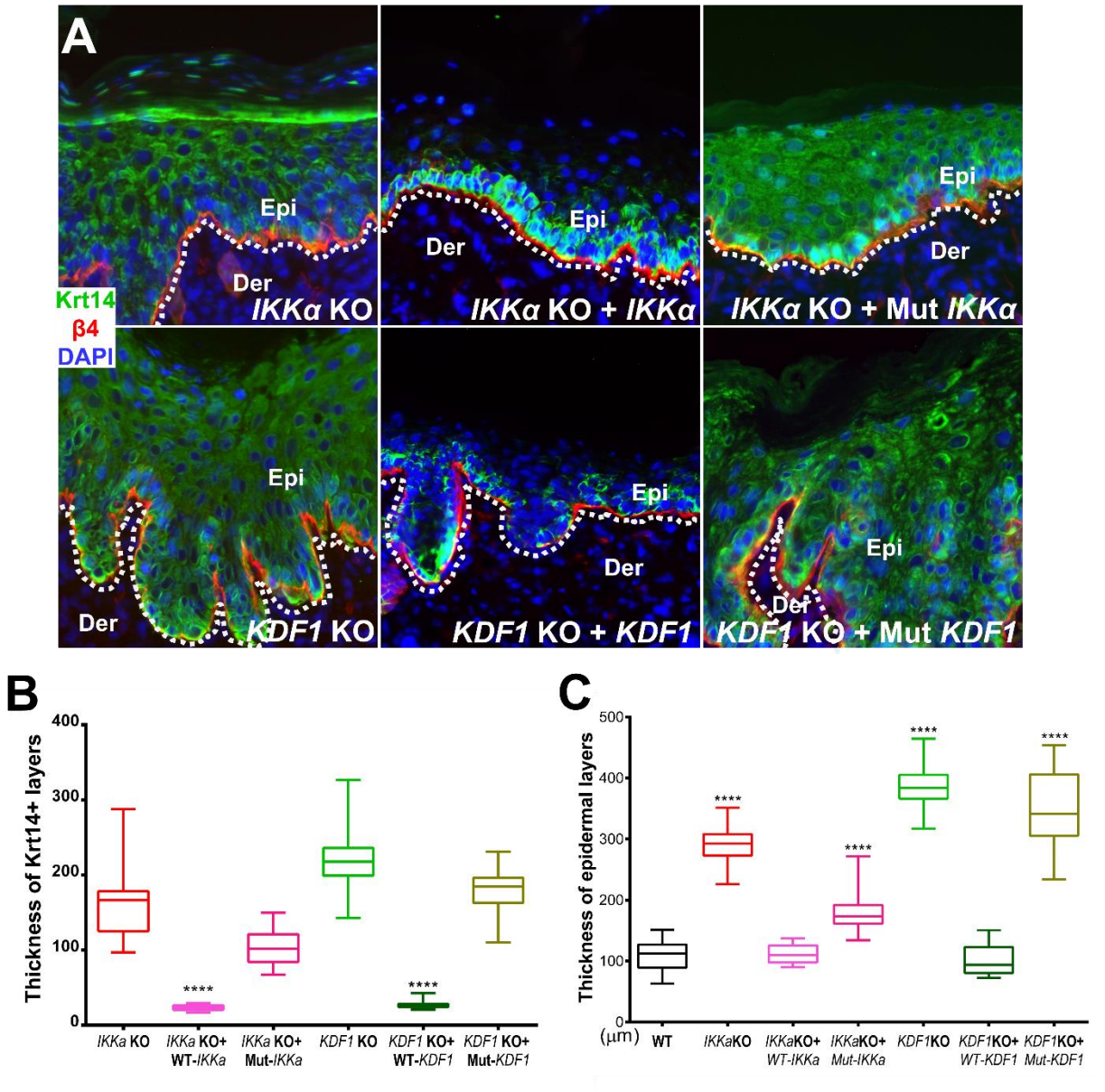

Figure 2.6: IKKα and KDF1 interaction is essential for skin stratification *in vivo*. A. Sections of regenerated skin developed from engrafted *IKKα* KO, *KDF1* KO, and their rescued cells were immunostained with different antibodies as indicated. Dotted lines denote dermal–epidermal boundaries. Scale bar=50 μm. **B and C.** Thickness of Krt14 positive layer and the whole epidermis were quantified and showed as box and whisker plots. The plot indicates first quartile (bottom line of the box), median (middle line of the box), third quartile (top line of the box), minimum and maximum measurements (whiskers). **(B)** The difference between each WT rescuing line and their original knock-out line is statistically significant. **(C)** The difference between WT and two knock-out lines and their non-binding mutant rescuing lines is statistically significant. (****, P<0.00001, unpaired Student's *t*-test)

KDF1 deficiency promotes skin tumor development

IKKα has been reported in the literatures to be a tumor suppressor of skin cancers [107, 132, 133, 153]. However, the function of KDF1 in skin tumorigenesis has not been illustrated. Since KDF1 and IKKα are both key regulators of epidermal stratification and their interaction has an essential role during keratinocyte differentiation, it is possible that KDF1 has similar function in skin cancers as IKKα. To test this hypothesis, we infected WT and *KDF1* KO cells with lentivirus encoding *Ha-Ras*, which has a G12V mutant. Upon engraftment to nude mice, the WT cells usually give rise to visible growth of benign papilloma. By contrast, engraftment of *KDF1* KO with *G12V-Ras* expression leads to significantly larger and more aggressive tumors **(Figure 2.7 A)**. Tumor sizes were measured weekly post engraftment. The tumors generated from mutant *Ha-Ras* infected *KDF1* KO cells grew significantly faster than control **(Figure 2.7 B)**. At the end, tumor samples from WT or *KDF1* KO cells were collected and subjected to H/E staining as well as immunostaining **(Figure 2.7 C)**. Histologically, the tumors derived from *KDF1* KO cells displayed more advanced progression toward SCC, including the characteristic squamous pearls in the H/E (hematoxylin and eosin) staining and islands of dysplastic squamous epithelial cells lying in the dermis, which distinctly indicated an invasive form of SCC. By contrast, tumors from the WT cells usually have intact basement membrane with hyperplasia of the overlying epidermis. Moreover, there is dramatic decrease of expression of differentiation-associated markers, including Krt10 and Loricrin, further demonstrates that the differentiation process is hindered. In addition, Ki-67 staining revealed that cell proliferation is more abundantly distributed in KO tumors, whereas proliferating cells in WT tumors primarily locate at the basal epidermis. Together, these results strongly suggest that enhanced tumorigenesis and progression in *KDF1* KO skin is

related to aberrant epidermal differentiation. KDF1 is essentially involved in epidermal differentiation and loss of *KDF1* promotes tumor growth *in vivo.*

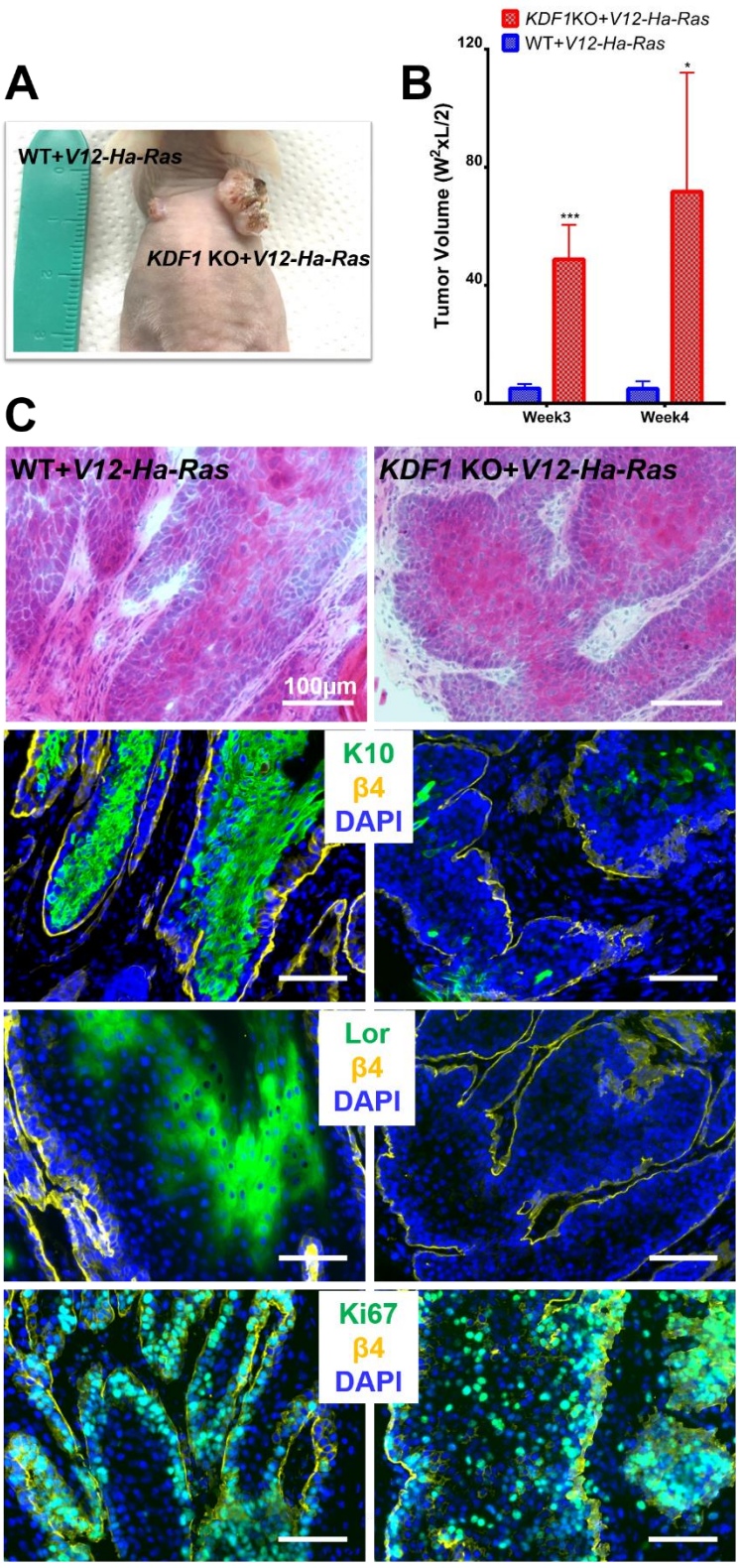

Figure 2.7: *KDF1* deficiency promotes tumor growth *in vivo*. Continued next page.

Figure [2.7], continued. WT and *KDF1* KO cells were infected with lentivirus encoding *Ha-Ras* G12V mutant. Infected cells were transplanted to nude mice for tumorigenesis analysis. **A.** Representative picture of WT and *KDF1* KO tumors appearing on nude mice. **B.** Measurement of WT and *KDF1* KO tumor size were plotted at week 3 and week 4 post engraftment. Tumor size was calculated using equation: $V=W^2xL/2$. Statistical analysis is conducted using unpaired Student's *t*-test. Error bar represents S.D. ***, $p<0.0001$; *, $p<0.05$ **C.** WT and *KDF1* KO tumors were collected and subjected to H/E staining and immunofluorescence staining with different antibodies as indicated. K10: keratin 10; Lor: Loricrin. Loss of *KDF1* leads to enhanced tumorigenesis in skin. The KO tumors display reduced level of epidermal differentiation markers, and more abundant and disorganized proliferating cell marker (Ki-67). Scale bars = 100 μm.

KDF1 regulates keratinocyte differentiation via controlling the ubiquitination level of IKKα

As we mentioned, IKKα and KDF1 may regulate epidermal differentiation together in epidermal keratinocytes, possibly via the same signaling cascades. In order to illustrate the mechanisms underlying IKKα and KDF1 mediated keratinocyte differentiation, we first examined their protein levels under calcium shift *in vitro*. Interestingly, KDF1 level remained stable after calcium shift (data not shown), while IKKα protein level dramatically increased during differentiation **(Figure 2.8 A and B)**. In contrast, IKKα protein level was significantly lower in *KDF1* KO cells, especially under the high calcium condition **(Figure 2.8 A and B)**. However, we discovered that the changes in IKKα protein levels were not due to its mRNA level **(Figure 2.8 E)**. We further examined the protein stability of IKKα in WT and *KDF1* KO keratinocytes, and we found that IKKα was less stable in *KDF1* KO cells compared to WT even without the differentiation cue **(Figure 2.8 C and D)**. Therefore, we hypothesized that the ubiquitination level of IKKα may contribute to its protein level change during keratinocyte differentiation, which serves as an essential regulatory mechanism in epidermal development and homeostasis.

To test this hypothesis, we examined and quantified the ubiquitination level of IKKα in WT and *KDF1* KO cells **(Figure 2.8 F and G)**. Immunoprecipitation using anti-ubiquitin antibody was conducted in WT and *KDF1* KO whole cell lysate under calcium-shift. Level of ubiquitination was quantified by adjusting to IKKα level among the input. Our results revealed that the ubiquitination level of IKKα is significantly increased in *KDF1* KO cells upon differentiation **(Figure 2.8 F and G)**. We further investigated this hypothesis via exogenously expressing IKKα in WT and *KDF1* KO keratinocyte and examining the level of ubiquitination **(Figure 2.8 H and I)**. Then, we conducted immunoprecipitation using anti-HA affinity beads and quantified the level of ubiquitinated HA-IKKα via adjusting to the exogenous protein expression in input. As expected,

HA-IKKα was more ubiquitinated in *KDF1* KO keratinocyte than in WT cells **(Figure 2.8 F and G)**. Our study results provide compelling evidence that increased ubiquitination of IKKα significantly correlates with decreased IKKα protein level, which could lead to the abnormal function of IKKα during differentiation in *KDF1* KO keratinocyte.

Our result indicate that IKKα protein level is essential for its regulatory function during epidermal stratification. We examined IKKα protein level in *KDF1* KO keratinocyte expressing WT *KDF1* or non-binding mutant *KDF1* **(Figure 2.9 A and B)**. *KDF1* KO cells rescued with WT *KDF1* can partially restore the increased IKKα protein level under calcium-shift, while the non-binding mutant *KDF1* cannot. In addition, we tested if overexpression of IKKα in *KDF1* KO keratinocyte could rescue the differentiation defect **(Figure 2.9 C-E)**. *KDF1* KO cells rescued with HA-IKKα were engrafted to nude mice and the regenerated skin was subjected to immunofluorescence staining of various differentiation markers. As shown in **Figure 2.9 D and E**, overexpression of IKKα in *KDF1* KO keratinocyte can restore the normal expression pattern of keratin 14, which is a biochemical marker of basal epidermis. Together, we provide strong evidence that IKKα protein level is essential in regulating keratinocyte differentiation. KDF1 and IKKα regulate epidermal stratification together via controlling the ubiquitination level of IKKα, which contributes to the regulation of IKKα protein level during differentiation process.

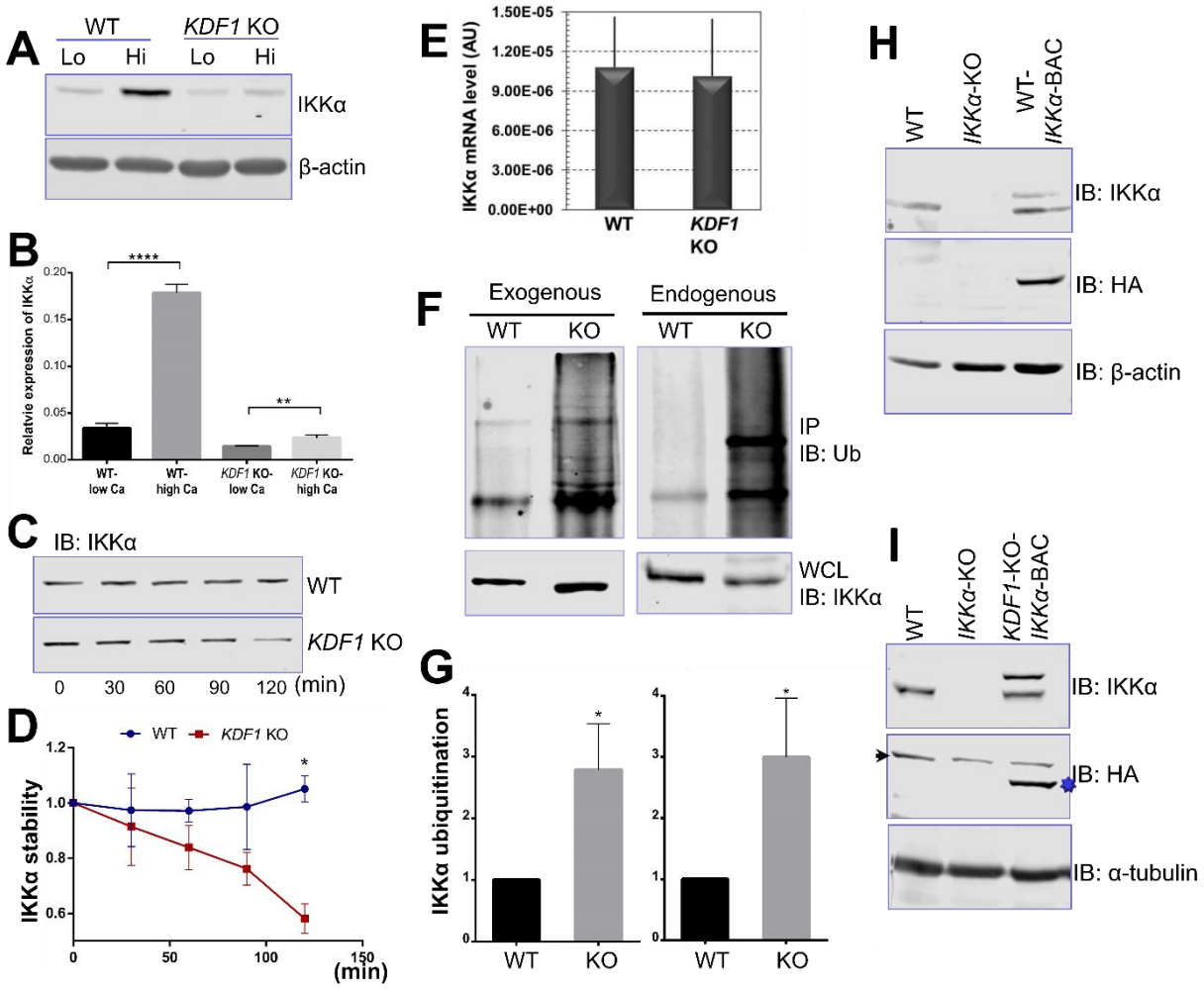

Figure 2.8: IKKα protein level is crucial during keratinocyte differentiation and KDF1 regulates the ubiquitination and the protein level of IKKα, part 1. A. WCL from WT and *KDF1* KO cells before and after calcium shift were immunoblotted with different antibodies as indicated. Lo: low calcium; Hi: high calcium. **B.** Band intensity in **(A)** is determined by densitometry and the relative expression of IKKα is calculated and quantified. Statistical analysis is conducted using unpaired Student's *t*-test. Error bar represents S.D. (standard deviation). ****, $p<0.00001$; **, $p<0.01$ **C and D.** WT and *KDF1* KO keratinocyte were treated with 20nM cycloheximide (CHX). WCL was collected at 0, 30, 60, 90, 120 min post CHX treatment and subjected to immunoblotting with IKKα antibody. Band intensity is determined by densitometry and the amount of IKKα is calculated and quantified. Statistical analysis is conducted using 2-way ANOVA. *, $p<0.05$. Error bar represents S.D. (standard deviation). **E.** mRNA level of IKKα was investigated using absolute RT-qPCR. PCR product containing IKKα coding sequence is used as a template to generate standard curve. No significant difference was detected. **Continued next page.**

Figure [2.8], continued. F and G. WT and *KDF1* KO keratinocyte (right panels) or cells transfected with plasmid encoding exogenous *IKKα* (left panels) were treated with MG132 at 10μM for 6 hours, then were subjected to immunoprecipitation using anti-ubiquitin (Ub) antibody. IP and WCL were analyzed by immunoblots with α-IKKα antibody. Band intensity in (F) was determined by densitometry. Ratio of ubiquitinated IKKα was quantified and presented as bar grafts (G). Statistical analysis is conducted using unpaired Student's *t*-test. Error bar represents S.D. (standard deviation). *, $p<0.05$. **H.** WT skin stem cells with BAC-transgenic *IKKα* are tested for IKKα and HA expression. WT keratinocytes are used to identify endogenous IKKα level. *IKKα* KO cells are used as negative control. **I.** *KDF1* mutant skin stem cells with BAC-transgenic *IKKα* are tested for IKKα and HA expression. WT keratinocytes are used to identify endogenous IKKα level. *IKKα* KO cells are used as negative control. Black arrow denotes non-specific bands. Blue star denotes HA band.

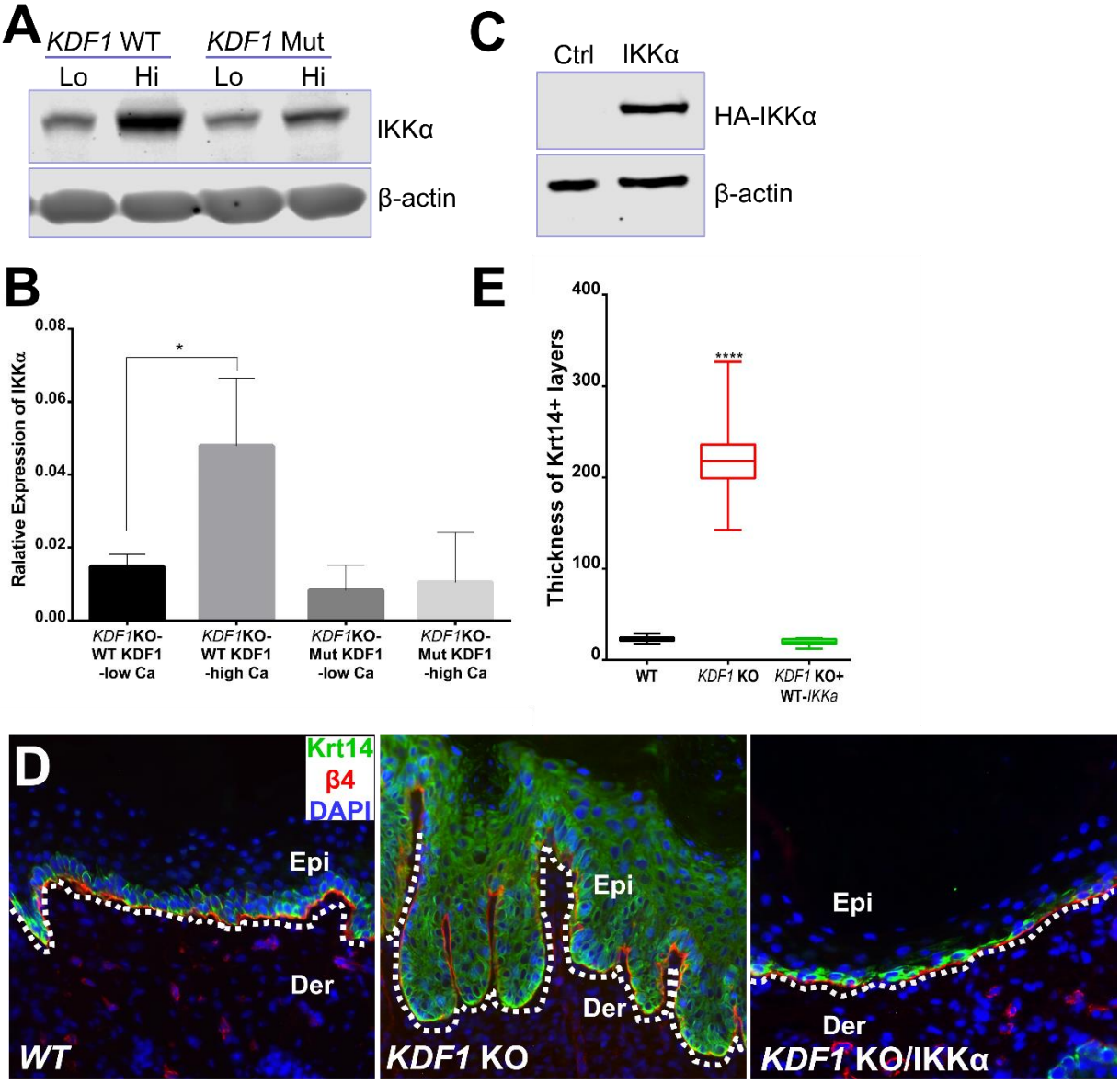

Figure 2.9: IKKα protein level is crucial during keratinocyte differentiation and KDF1 regulates the ubiquitination and the protein level of IKKα, part 2. A and B. IKKα protein level in *KDF1* KO keratinocyte expressing WT *KDF1* or *KDF1* mutant, under both low and high calcium conditions, was examined and quantified by western blotting. β-actin is used as a loading control. Band intensity is determined by densitometry and the relative expression of IKKα is calculated and quantified. Statistical analysis is conducted using unpaired Student's *t*-test. Error bar represents S.D. (standard deviation). *, $p<0.05$ **C.** PiggyBac transposon was used to ectopically express HA-tagged *IKKα* in *KDF1* deficient cells. WCL were collected and analyzed by immunoblots with different antibodies as indicated. **D.** Overexpression of *IKKα* in *KDF1* KO keratinocyte can restore normal skin stratification. Skin sections from grafted tissue were immunostained with different antibodies as indicated. Scale bars = 50 μm **Continued next page.**

Figure [2.9], continued. E. Thickness of keratin 14 positive layer in **(D)** was quantified and showed as box and whisker plots. The plot indicates first quartile (bottom line of the box), median (middle line of the box), third quartile (top line of the box), minimum and maximum measurements (whiskers) ($P<0.00001$, unpaired Student's *t*-test).

KDF1 controls the ubiquitination level of IKKα via USP7

Our studies have shown that KDF1 facilitates the stabilization of IKKα via regulating its ubiquitination level. However, KDF1 does not possess functional domains related to this function. We re-examined the list of the potential KDF1-interacting proteins obtained from our SILAC/MS screening. Excitingly, USP7 is one of the top hit on our list, which is a deubiquitinating enzyme (DUB) and could reverse both poly- and mono-ubiquitination of a specific protein.

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 [160, 161]. Our proteomics analysis of KDF1 interactome 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 **(Figure 2.10 A)**.

The function of USP7 in skin development and keratinocyte differentiation has not been discovered, although it has been shown to regulate the turnover of many signaling molecules, such as p53, PTEN [164, 165]. To this end, we first employed CRISPR (clustered regularly interspaced short palindromic repeats) technology and generated *USP7* KO keratinocyte **(Figure 2.10 B)**. IKKα protein level was assessed and quantified in both WT and *USP7* knock-out cells upon calcium shift *in vitro* **(Figure 2.10 C)**. Our results indicate that under differentiation signals, IKKα protein level is significantly lower in *USP7* knock-out cells than in WT. We further examined and quantified the ubiquitination level of IKKα in both WT and *USP7* knock-out keratinocyte **(Figure 2.10 D and E)**. Immunoprecipitation using anti-ubiquitin antibody was conducted in WT and *USP7* knock-out whole cell lysate under calcium-shift. Level of ubiquitination was quantified by

adjusting to IKKα level among the input. Our results revealed that IKKα is significantly more ubiquitinated in *USP7* knock-out keratinocyte compared to WT. In addition, differentiation process was evaluated by assessing the expression of differentiation markers upon calcium shift *in vitro* **(Figure 2.10 F and G)**. As shown in the result, when induced to differentiation by calcium shift, the *USP7* KO cells exhibits significantly reduced expression of *Krt10*. Together, our studies suggest that KDF1 regulates IKKα ubiquitination and protein stability by recruiting USP7, a deubiquitinating enzyme, which is essential for epidermal differentiation **(Figure 2.10 H)**.

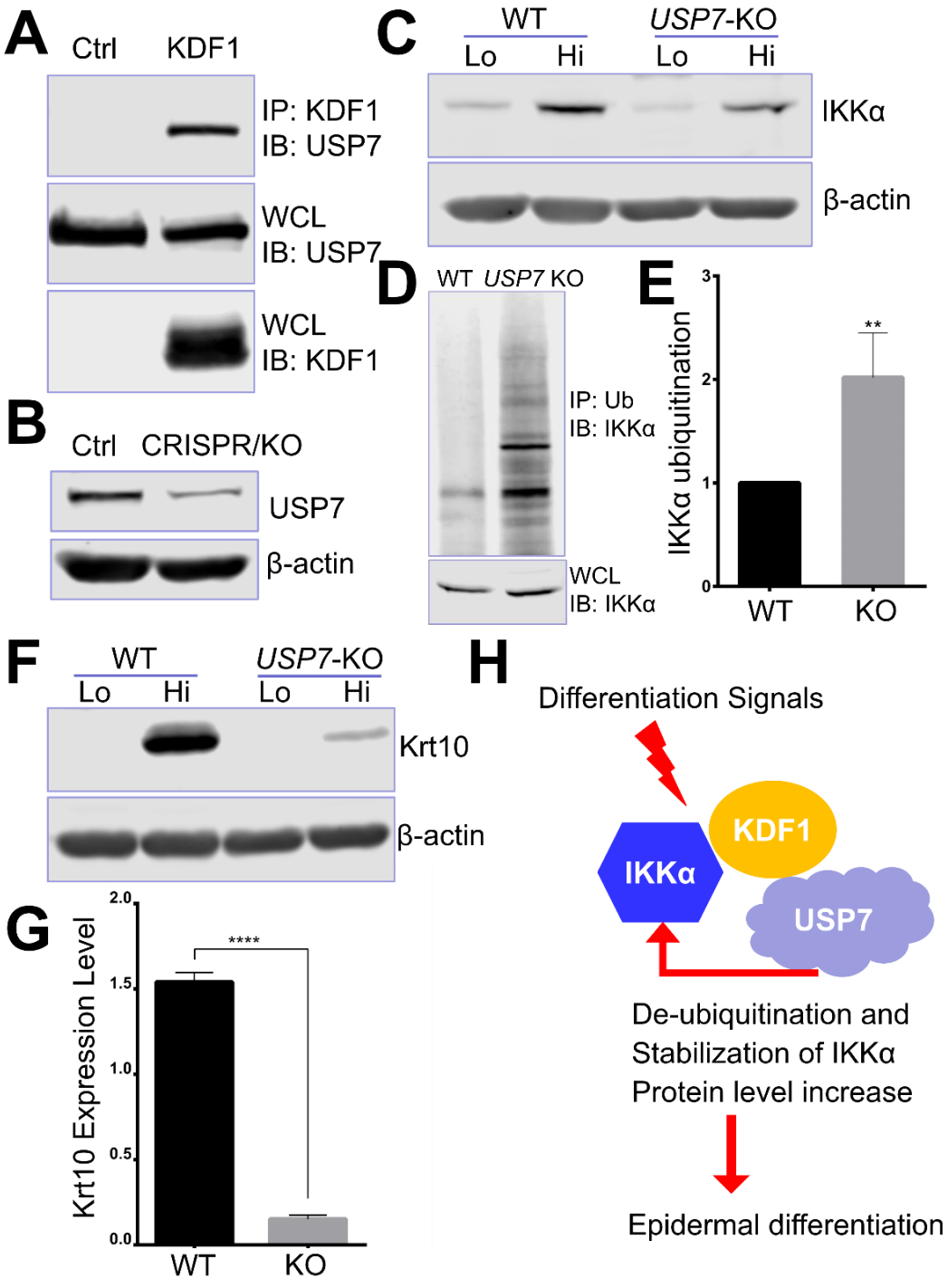

Figure 2.10: USP7 regulates IKKα ubiquitination and skin differentiation. A. HEK293 cells were transfected to co-express *KDF1* with *USP7*. IP and WCL were blotted with different antibodies as indicated. **B.** WCL from WT and *USP7* CRISPR KO cells were subjected to immunoblotting with different antibodies as indicated. **C.** IKKα protein level in WT and *USP7* KO keratinocyte were examined by immunoblotting before and after calcium shift. **Continued next page.**

Figure [2.10], continued. D-E. WT and *USP7* KO keratinocyte were treated with MG132 at 10μM for 6 hours, then subjected to immunoprecipitation using anti-ubiquitin antibody. Precipitated product was analyzed by immunoblotting with IKKα antibody. Band intensity in **(D)** was determined by densitometry and shown as bar grafts **(E)**. Statistical analysis is conducted using unpaired Student's *t*-test. Error bar represents S.D. (standard deviation). **, $p<0.01$. **F.** Immunoblot of Krt10 with WT and *USP7* KO cells before and after calcium shift. Band intensity in **(F)** was determined by densitometry and shown as bar grafts **(G)**. Statistical analysis is conducted using unpaired Student's *t*-test. Error bar represents S.D. (standard deviation). ****, $p<0.00001$. **H.** 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 promotes its protein stability, which will in turn translocate to cell nucleus and promote epidermal differentiation.

Discussion

The mammalian skin epidermis provides essential protections against the environmental challenges [1-3]. Skin keratinocytes carry out the barrier function and maintain the tissue homeostasis via a complex and choreographed program of epidermal stratification. Although the morphological and genetic alterations during skin differentiation have been largely studied [2, 76, 151], little is known about the molecular mechanisms that govern this process. In this study, by employing a combinatory approach encompassing TAP and SILAC-MS/MS screening, molecular and cell biology studies, we provided compelling evidence that KDF1 regulates keratinocyte differentiation by forming a regulatory complex with IKKα, and controlling IKKα protein stability via recruiting a deubiquitinating enzyme USP7.

KDF1 has been indicated as an essential regulator of epidermal differentiation via an ENU mutagenesis screening [93]. In our report, we examined the phenotypes of *KDF1* mutant mice that are consistent with previous studies. *KDF1* deficient animals are perinatal lethal with severe morphological defects. More importantly, the mutant fetuses possess striking defects in skin development and keratinocyte differentiation: 1) cellular proliferation was expanded throughout the mutant suprabasal keratinocytes, which was restricted to basal keratinocytes in wild type epidermis; 2) markers of basal keratinocytes (Keratin 14 and 5) were expressed throughout the mutant epidermis; 3) mutant suprabasal keratinocytes exhibited a mix of basal and spinous markers, while the markers of granular and cornified layers (Loricrin and Filaggrin) were strongly reduced or absent; 4) the sign of the terminal differentiation (enucleation and cornification) was absent. In addition, the ceaseless expression of KDF1 in adult epidermis emphasized its essential functions in regulating the differentiation of somatic epidermal progenitor cells [93]. Therefore, it is important to investigate the regulatory function of KDF1 at the molecular level, which is poorly

understood. Although other molecules or transcriptional factors (e.g. p63, Notch and IKKα) have been reported to be involved in keratinocyte differentiation [1-3, 49, 150], it is difficult to uncover the actual connections underlying and the role of KDF1 within the network without a guideline. Our screening approach using TAP and SILAC-MS/MS serves as a powerful tool to identify potential KDF1 interacting proteins, which opens up a new path to explore KDF1 mediated epidermal differentiation and the underlying regulatory networks.

With the list of screening, we were able to set our focus at IKKα. The role of IKKα in controlling keratinocyte differentiation was revealed by *IKKα* knockout mouse models [99, 100]. We examined the phenotypes of *IKKα* knockout mice (E18.5, perinatal lethal) and confirmed the disorders in epidermal differentiation as previously indicated. Interestingly, both morphological and skin developmental defects in *KDF1* and *IKKα* deficient mice are highly alike. However, similar phenotypes upon loss of a certain molecule are not sufficient to support their connected functions during epidermal differentiation. Excitingly, we discovered that there is a binding between KDF1 and IKKα at the molecular level, in addition to their phenotypic similarity. Via *in vitro* molecular study approaches, we were able to map the binding motifs within KDF1 and IKKα, which facilitated us to further examine the indispensability of this interaction during keratinocyte differentiation using knock-out and rescue strategies.

Besides the classic *in vitro* method to assess differentiation properties via calcium shift, we took advantage of skin grafting with high-passage keratinocyte to perform *in vivo* functional validations. Engraftment of cultured keratinocytes is an innovative method to best mimics the *in vivo* conditions of skin development and allows various manipulations in the donor cells. Our previous studies have revealed the effective use of this technique in basic scientific research and genetic engineering as well as therapeutic gene deliveries [110, 147-149]. In this report, we

discovered that expression of WT *IKKα* or *KDF1* in differentiation deficient cells have significant rescuing effects, especially *in vivo*. However, non-binding mutant *IKKα* or *KDF1* were unable to completely restore normal differentiation in knock-out cells. It is worth noting that KDF1 and IKKα may also have independent functions in regulating keratinocyte differentiation, given that non-binding mutant constructs seemed to slightly facilitate partial expression of loricrin *in vivo* (data not shown). However, other indicators, especially the aberrant expression of basal keratinocyte marker and the abnormally thickened epidermis, emphasized the indispensability of KDF1 IKKα interaction during epidermal differentiation. Taken together, we were able to present strong evidence that KDF1 and IKKα regulates epidermal stratification by forming a regulatory complex, and loss of the interaction between KDF1 and IKKα would significantly impair the differentiation process.

The precisely regulated epidermal progenitor cells are essential in preventing skin related diseases, particularly skin cancer. Skin cancer originated from keratinocyte, such as BCC and SCC of the skin, accounts for the most common cancers in the U.S. [119]. Furthermore, patients diagnosed with head and neck squamous cells carcinoma or carcinoma of esophagus, which are originated from the keratinocyte lining up those cavities, may have much worse prognosis and poor survival rate [123]. Therefore, there is an urgent need to expand our knowledge of molecular mechanisms and signaling networks involved in skin tumorigenesis. Many genes that have been reported to be involved in epidermal differentiation, such as *IRF6*, *Notch*, and *P63*, have aberrant functions in SCCs [125, 173, 174]. IKKα has also been reported to be a tumor suppressor in skin cancers and reduction in IKKα protein level was significantly associated with poorer prognosis of human SCCs [107, 132, 133, 152, 153]. Since KDF1 regulates epidermal stratification along with IKKα, it is essential to test the function of KDF1 during skin tumorigenesis. We explored KDF1

expression level and mutations in TCGA SCC patient datasets, although no significant correlation was found largely due to lack of valid controls (data not shown). We further explored the effect of *KDF1* deficiency on skin tumor growth via skin grafting of primary keratinocytes with an extra genetic hit of *Ha-Ras* mutation and increased potency of benign papilloma [175]. Our results strongly indicated that loss of *KDF1* promotes tumor growth *in vivo* and the enhanced tumor progression in *KDF1* KO skin is due to aberrant epidermal differentiation.

Another significant and novel finding in our study is that, ubiquitination serves as an essential regulatory mechanism facilitating the stability and protein level of IKKα during keratinocyte differentiation. We detected increased IKKα protein level in WT keratinocyte during differentiation, while the rising was significantly abolished in *KDF1* deficient cells **(Figure 2.8A)**. There is no correlation between protein and transcriptional level changes, although transcriptional regulation of *IKKα* has been indicated [154, 155]. Through cellular and molecular approaches, we obtained strong evidence that the regulation of IKKα ubiquitination and protein levels is dependent upon KDF1. Moreover, overexpression of exogenous *IKKα* in *KDF1* deficient cells could significantly rescue the differentiation defects **(Figure 2.9)**. Although we don't believe it is the only mechanism underlying KDF1 mediated keratinocyte differentiation, it is the first time we disclosed the significance of ubiquitination in regulating molecular functions during epidermal stratification. However, it is unlikely that KDF1 directly regulate ubiquitination of IKKα since there is no related functional domains detected within KDF1. Luckily, our screening of KDF1 interacting proteins helped us narrowing down the potential target of controlling IKKα ubiquitination level within the regulatory complex. USP7, an essential deubiquitinating enzyme, has essential regulatory functions within NF-κB pathway [162, 163] and controlling tumorigenesis related proteins (e.g. p53/MDM2, PTEN and FOXO4) [164-167]. In this report, we revealed

decreased IKKα protein level accompanied with increased IKKα ubiquitination upon *USP7* knock-out. Moreover, keratinocyte differentiation is impaired *in vitro* in *USP7* knock-out cells. It is noticeable that the knock-out is incomplete. Thus, the differentiation defect could be more striking if we could obtain a more complete knock-out line. However, generating *USP7* KO cells may not be easy to improve since *USP7* KO mice suffer lethality at a very early stage of embryonic development [167], suggesting the indispensable function of USP7 within multiple developmental signaling. Taken together, our results strongly supported our hypothesis that USP7 is recruited by KDF1 to the regulatory complex involving IKKα, which significantly contributes to the upregulation of IKKα protein level during epidermal differentiation.

In closing, our findings provide critical insights into the molecular machineries orchestrating the epidermal differentiation as well as skin tumorigenesis, which has important implications in the fields of skin development and epidermal progenitor cells research.

CHAPTER III

VAMP2 MEDIATED KERATINOCYTE ENUCLEATION

Introduction

Mammalian skin serves as an essential water-impermeable barrier that protects us from various environmental damages [1-3]. 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, 21, 49]. Deregulation of epidermal differentiation in skin can lead to the development of various skin diseases including skin cancers. Although the morphogenetic changes during epidermal differentiation have been studied [2], much remains unknown about the molecular mechanisms underlying this process. We conducted a shRNA library screening and uncovered many essential genes associated with the phenotype of resisting high calcium induced differentiation. Interestingly, *Vamp2* appeared on our top hit list, and his function in keratinocyte has never been identified.

Vamp2, also known as synaptobrevin 2, belongs to the large family of membrane proteins who share a common sequence called the SNARE motif [176]. SNAREs (soluble NSF-attachment protein receptors) mainly mediate intracellular vesicle fusions in eukaryotes, such as synaptic vesicle fusion in forebrain synapses [176]. There are two groups of SNAREs: vesicle-associated SNARE (v-SNARE), also known as R-SNARE, locates on the vesicle membrane; target-SNARE (t-SNARE), also called Q-SNARE, locates on cellular plasma membrane. When vesicle fusion occurs, t- and v-SNAREs form a complex which undergoes structural rearrangement from *trans* to *cis*, and facilitate the fusion process partially via catalyzing the fusion and ensuring the specificity of the fusion. Vamp2 is a v-SNARE and the systematic knock-out of *Vamp2* in mouse

lead to perinatal lethality and abnormal body shape [176]. Although striking decrease in spontaneous and triggered synaptic vesicle fusion has been caught in *Vamp2* knock-out mouse model, no phenotypical and histological examination of the skin has been conducted or mentioned. Through a closely examination, we found that the enucleation process in *Vamp2* knock-out epidermis is hindered.

Keratinocyte enucleation is a process of nuclear degradation occurring when keratinocyte undergo terminal differentiation from stratum granulosum to stratum corneum. At stratum granulosum, keratinocytes undergo various morphological changes including nuclear shape alterations such as indentation [113]. The enucleation process starts and happens very quickly when the stratum corneum forms, in at most 6 hours [113, 177]. In human, defects in keratinocyte enucleation is associated with various skin diseases including skin cancers, such as parakeratosis seen in patients with eczema and dermatitis. Unfortunately, little is known about the molecular mechanisms underlying nuclear degradation in keratinocytes. Interestingly, autophagy, another membrane trafficking system in mammalian cells, has been shown to be involved in keratinocyte enucleation [114, 115]. People have shown that autophagy could mediate nuclear lamina proteins degradation and target nuclear component for degradation during keratinocyte differentiation [114, 115]. Although there are contradictory comments arguing if the canonical autophagy is required for keratinocyte nuclear removal [116, 117], the potential mechanism that membrane targeting and trafficking is involved in keratinocyte enucleation is well worth to be further tested.

In this study, we are able to detect and visualize keratinocyte nuclear degradation both *in vitro* and *in vivo*. The subcellular localization and aggregation of Vamp2 around the nuclear membrane illustrated the potential role of Vamp2 in nuclear degradation. Via conducting SILAC-MS/MS screening, we identified various proteins associated with Vamp2 coated intracellular

vesicles, including the ones involved in autophagosomes trafficking. Further functional studies are still undergoing.

Materials and Methods

Antibodies and Reagents

Loricrin and Filaggrin antibodies were generous gifts from Dr. Elaine Fuchs at the Rockefeller University. Chicken Krt14, Rabbit Krt 10 antibodies were obtained from Covance (Princeton, NJ). Rat monoclonal β4-integrin (CD104) was obtained from BD Pharmingen (Franklin lakes, NJ). Loricrin (55439-1-AP), α/β-tubulin, β-actin and rabbit Vamp2 antibodies were obtained from Proteintech® (Rosemont, IL). EZview^{TM} Red anti-HA affinity beads were obtained from Sigma (St. Louis, MO). Other chemicals or reagents were obtained from Sigma (St. Louis, MO), unless otherwise indicated.

Cell Culture

Primary mouse keratinocytes were isolated using previously reported methods [110]. Epidermis of E18.5 or newborn mice 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 keratinocytes can healthily and independently grow. 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 60mM. HEK293-T cells were cultured in DMEM medium supplemented with 10% FBS.

shRNA library screening

Cellecta pooled bar-coded lentiviral shRNA libraries (mouse modules) were purchased from Cellecta, Inc. (Mountain View, CA). There are two modules: one includes essential genes involved in signaling pathways, and another one includes disease-associated genes. In total, 59,000 bar-coded shRNA constructs are targeting 9,175 mRNA/genes. Virus packaging follows commercial protocols and laboratory safety regulations. To ensure transfection efficiency and coverage, two modules of virus libraries are used to transfect two dishes of keratinocytes that are cultured with E-low medium, respectively. Meanwhile, the two modules of virus libraries are used to transfect two dishes of keratinocytes that are cultured with E-high medium, respectively. After several passaging, keratinocytes that can overcome the inhibition of high calcium condition and keep proliferating will be subjected to genomic DNA extraction. Both dishes of cells, transfected with two modules of virus libraries respectively, are combined together for DNA extraction. Meanwhile, cells that are cultured with E-low medium were collected as controls.

shRNA specific bar-codes were amplified from extracted genomic DNA obeying commercial protocol, and were submitted to sequencing using Illumina HT sequencer. Afterwards, decipher of the bar-coded shRNA sequence and the corresponding gene were conducted using Decipher Bar Code Deconvoluter software provided by Cellecta, Inc. (Mountain View, CA).

Animals

Vamp2 knock-out mouse model was obtained from The Jackson Laboratory. 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.

Protein Biochemical Analysis

Western blot was conducted as previously described [168]. 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) for 1 hour at room temperature, followed by an overnight incubation with desired primary antibody at 4 degree. Immunoblot was washed three times with 1 X Tween 20/Phosphate Buffered Saline (PBST) and incubated with secondary antibody (1:10000 dilution) at room temperature for 1 hour. Blot was washed with 1 X 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.

Skin Organotypic Culture and Grafting

Skin organotypic culture and grafting were performed as previously described [110]. Decellularized dermis (1cm X 1cm square shape) was prepared from newborn CD1 mice skin via EDTA treatment [146]. 2 X 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 6-8 weeks were anaesthetized. Two 1cm X 1cm 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 one week after surgery [110, 148, 149]. All the experiments were repeated more than three times (three biological replicates). For

phenotypic analysis using immunostaining, at least 3 sections were taken from each graft for quantifications.

Histology and Immunofluorescence

Skin or tumor samples were embedded in optimal cutting temperature (OTC) compound, sectioned, and fixed in 4% paraformaldehyde. Hematoxylin and eosin (H/E) staining or immunofluorescence staining of desired sections were conducted as previously described [169]. Antibodies were diluted following the manufacturer's instructions unless indicated. Images were taken using EVOS FL imaging system.

Isolation of Vamp2 coated subcellular vesicles and SILAC-MS proteomic analysis

Mouse primary keratinocyte ectopically expression HA-tagged *Vamp2* were subjected to SILAC labeling with light isotope. WT keratinocytes were subjected to SILAC labeling using high isotope: L-Lysine-2HCl (4, 4, 5, 5-D4) and L-Arginine-HCl (µ-^{13}C6) (Cambridge Isotope Laboratories Inc, Andover, MA). Upon calcium shift, all labeled cells were collected using homogenization buffer (10nM Hepes, 100mM KCl, 1mM EDTA, 25mM sucrose, PH 7.2-7.4) with proteinase inhibitors. A complete cell homogenization is obtained by passing the samples through 27-gauge needle for a couple times. Unwanted cellular compartments were removed via centrifuge the lysates at 500g for 5min for two repeated times. The supernatant is now considered as a pool of cellular vesicles. To conduct immunoprecipitation, 30ul of anti-HA affinity beads (Sigma, St. Louis, MO) were added into the processed lysate and rotated at 4 degree overnight. The beads were then washed with homogenization buffer for two times (no direct centrifuge is allowed, only with natural settling). Finally, the conjugated proteins and vesicles were resolved in SDS-PAGE gel and subjected to LC-MS/MS identification. Fractionation was conducted using trypsin digestion.

Statistical Analysis

Statistical analysis was performed using Excel or GraphPad Prism software. In most experiments, a Student's *t* test was used to evaluate the statistical significance (P value) of the difference.

Results

shRNA library screening to identify genes essential for keratinocyte differentiation

Although some signaling pathways and regulatory proteins have been identified to regulate epidermal differentiation, the precise molecular mechanisms and regulatory networks are poorly understood. In order to expand our knowledge and identify more genes that are essential for keratinocyte differentiation, we conducted a shRNA library screening using commercially designed shRNA virus libraries. Keratinocytes are extremely sensitive to calcium concentration and exposure to high-calcium medium *in vitro* will lead to cell cycle exit and terminal differentiation. However, if some essential genes that are required for calcium induced cellular differentiation are knocked down by the shRNA library, those cell populations will continue to grow when passaging in high calcium medium. Therefore, through HT sequencing and deciphering the bar-codded shRNA, we could find potential targeted genes that facilitate cells to escape calcium mediated mitotic inhibition.

After statistical analysis of the sequencing result, we obtained a long list of targeted genes **(Table 3.1)**. The relevance of these targeted genes are ranked by the ratio of Reads obtained from E-High medium versus E-Low medium, through which way we can identify the most enriched shRNA in the wanted condition (E-High) versus background (E-low).

It is expected to identify genes related to cell proliferation and mitotic regulations in our list, such as TGFβ. Interestingly, *Vamp2* is one of the top heat within our list **(Table 3.1)**. Vamp2 is a SNARE protein that has been illustrated to mediate synaptic vesicle fusions in forebrain synapses [176]. The function of Vamp2 in keratinocyte has never been accessed before. Luckily, the systematic *Vamp2* knock-out mouse model is available, although no phenotypical and histological examination of the skin has been conducted or mentioned [176].

Table 3.1: Top list of shRNA library screen to identify genes essential in regulating keratinocyte differentiation. Table represents the top 20 candidates with the highest ratio of Reads-E High/Reads- E Low. Red font color indicates gene of interested.

Barcode Sequence	Gene Symbol	Reads-E High	Read Ratio-E High	Reads-E Low	Read Ratio-E Low	Ratio Change
GTTGTGACCAGTACTGGT	PIR	996372	0.006894883	859	6.19198E-06	1113.517652
ACTGGTACGTTGACACCA	TGFBR1	8034321	0.055597408	9783	7.05194E-05	788.3986669
CAGTGTCATGCACAACTG	DAPP1	6356898	0.04398966	7890	5.6874E-05	773.458462
GTTGTGACTGGTACACGT	PIK3CD	773912	0.005355462	1094	7.88595E-06	679.1145169
ACCACAACTGCACAACTG	PLSCR1	781543	0.005408268	1429	1.03007E-05	525.0363889
TGTGCAACACACACTGGT	VAMP2	7533400	0.05213104	16877	0.000121656	428.5135238
ACGTCATGCAGTGTACAC	INPPL1	7922181	0.0548214	18885	0.00013613	402.7138137
GTGTTGGTACGTCAGTTG	PCTK1	260067	0.001799661	627	4.51964E-06	398.1864365
GTTGTGCAACGTTGACGT	DMD	6083914	0.042100614	14729	0.000106172	396.5322914
GTACGTTGACACGTTGTG	NPR3	7961399	0.055092788	20854	0.000150323	366.4956093
CAGTGTGTACCACAGTAC	OLFR706	81917	0.000566865	243	1.75163E-06	323.6208698
TGCAGTACCAGTTGACTG	THBS2	734700	0.005084115	2294	1.6536E-05	307.4577054
GTGTCACACAACACTGTG	REG1	856613	0.005927752	2834	2.04285E-05	290.1707087
TGTGCAGTCATGTGACGT	CRK	6363319	0.044034093	21881	0.000157726	279.1806311
CATGACGTGTCACAGTAC	DGCR8	180916	0.001251937	630	4.54127E-06	275.6799511
CACATGTGGTCAACCACA	FBXL18	89422	0.000618799	317	2.28505E-06	270.80325
TGGTCACATGGTTGTGCA	EFTUD2	2216	1.53347E-05	8	5.76669E-08	265.9184829
GTACTGTGCACAGTTGCA	AXL	198979	0.001376932	743	5.35581E-06	257.0911847
CACATGTGACGTCATGTG	4933401F05RIK	309669	0.002142906	1170	8.43378E-06	254.0859351
TGTGACTGACACCAGTCA	BC055324	160956	0.001113814	722	5.20444E-06	214.0122971

Defects of enucleation process in *Vamp2* KO mouse epidermis

Since *Vamp2* KO mouse is perinatal lethal, we obtained fetus at E18.5 to access the epidermal phenotypes. *Vamp2* KO fetus can be distinguished from its wild type or heterozygous littermates based upon its abnormal spinal curvature **(Figure 3.1 A)** [176]. Keratinocytes isolated from *Vamp2* KO fetus and WT littermates were subjected to calcium shift *in vitro*. Readily increased expression of Vamp2 protein is visualized using western blot, while Vamp2's expression is completely wiped off in *Vamp2* KO keratinocyte **(Figure 3.1 A)**. The histological examination of *Vamp2* KO epidermis did not show prominently abnormal differentiation defects, except for the sustention of nucleus at the topmost epidermis **(Figure 3.1 B)**. Consistently, immunofluorescence staining of various differentiation markers did not suggest striking phenotypes like we seen in *KDF1* or *IKKα* deficient mouse models, except for the potential defects in the enucleation process associated with *Vamp2* KO **(Figure 3.1 C)**.

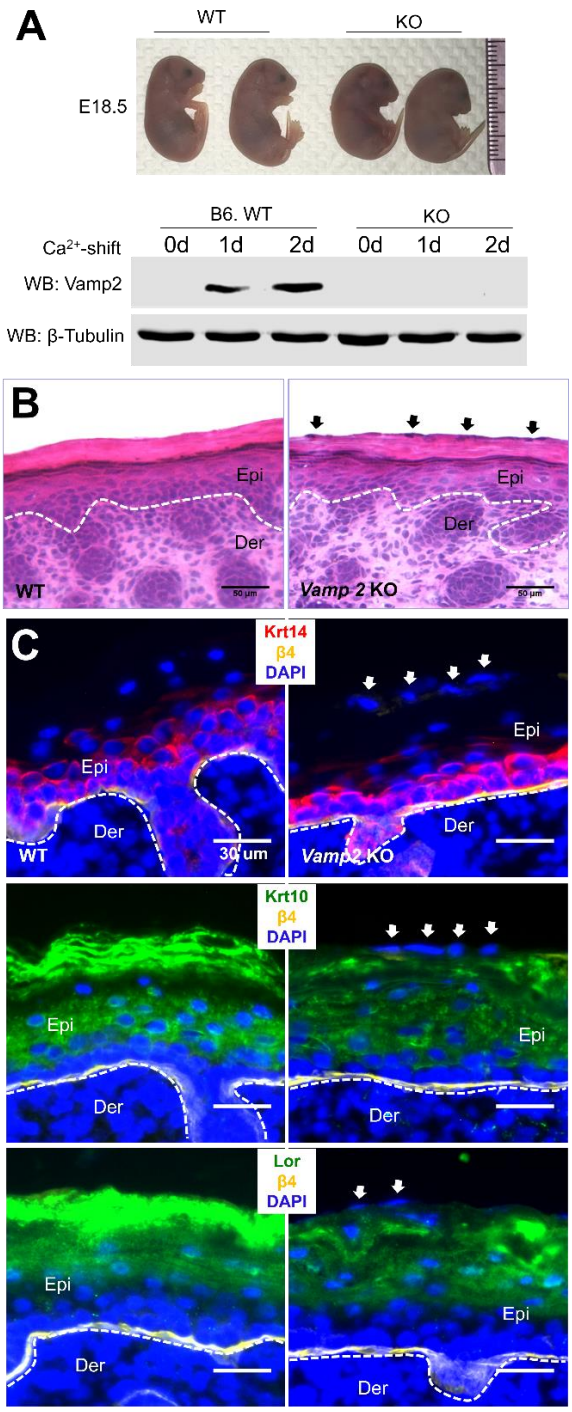

Figure 3.1: Phenotypical analysis of *Vamp2* KO mouse epidermis. A. Primary keratinocytes were isolated from WT and *Vamp2* KO E18.5 embryonic skin. Expression of Vamp2 during calcium shift is examined via western blotting. **B and C.** Analysis of E18.5 WT and *Vamp2* KO epidermis, via H/E staining **(B)** and immunofluorescence staining of different antibodies **(C)** as indicated (Krt14: keratin 14, Krt10: keratin 10, Lor: Loricrin, β4: β4-integrin, CD104). In H/E staining, scale bar=50 μm. In immunofluorescence staining, scale bar=30 μm. Dotted lines denote dermal–epidermal boundaries. Epi: epidermis, Der: dermis.

Vamp2 KO hinders enucleation process during epidermal stratification

In order to evaluate the enucleation process more closely, we examined the nuclear morphology changes upon calcium shift under the microscope **(Figure 3.2 A and B)**. Nuclear invagination indicated by DAPI staining of the nucleus was robustly evident as the days passed **(Figure 3.2 A and B)**. The invagination of nucleus can also be detected if ectopically expressed H2B-RFP is used to visualize and trace the nucleus under the microscope in real time **(Figure 3.2 C and E)**. In contrast, the shape of *Vamp2* KO nucleus remains unchanged within both of the DAPI and H2B-RFP systems.

Since *in vitro* differentiation induced by calcium shift may not fully recapitulate the regulations *in vivo*, we engrafted WT and *Vamp2* KO keratinocytes ectopically expressing H2B-RFP. Using confocal microscopy to follow the nuclear morphology changes in real time mode with living animals, we could precisely catch and trace the degradation of nucleus *in vivo* and analyze the imaging quantitatively **(Figure 3.2 D, F and G)**. As shown in the results, nucleus of WT keratinocyte was degraded much faster than *Vamp2* KO cells, and more WT keratinocytes were undergoing nuclear degradation than *Vamp2* KO cells **(Figure 3.2 F and G)**. Taken together, our data strongly suggest that missing of *Vamp2* hinders enucleation process during keratinocyte differentiation both *in vitro* and *in vivo*.

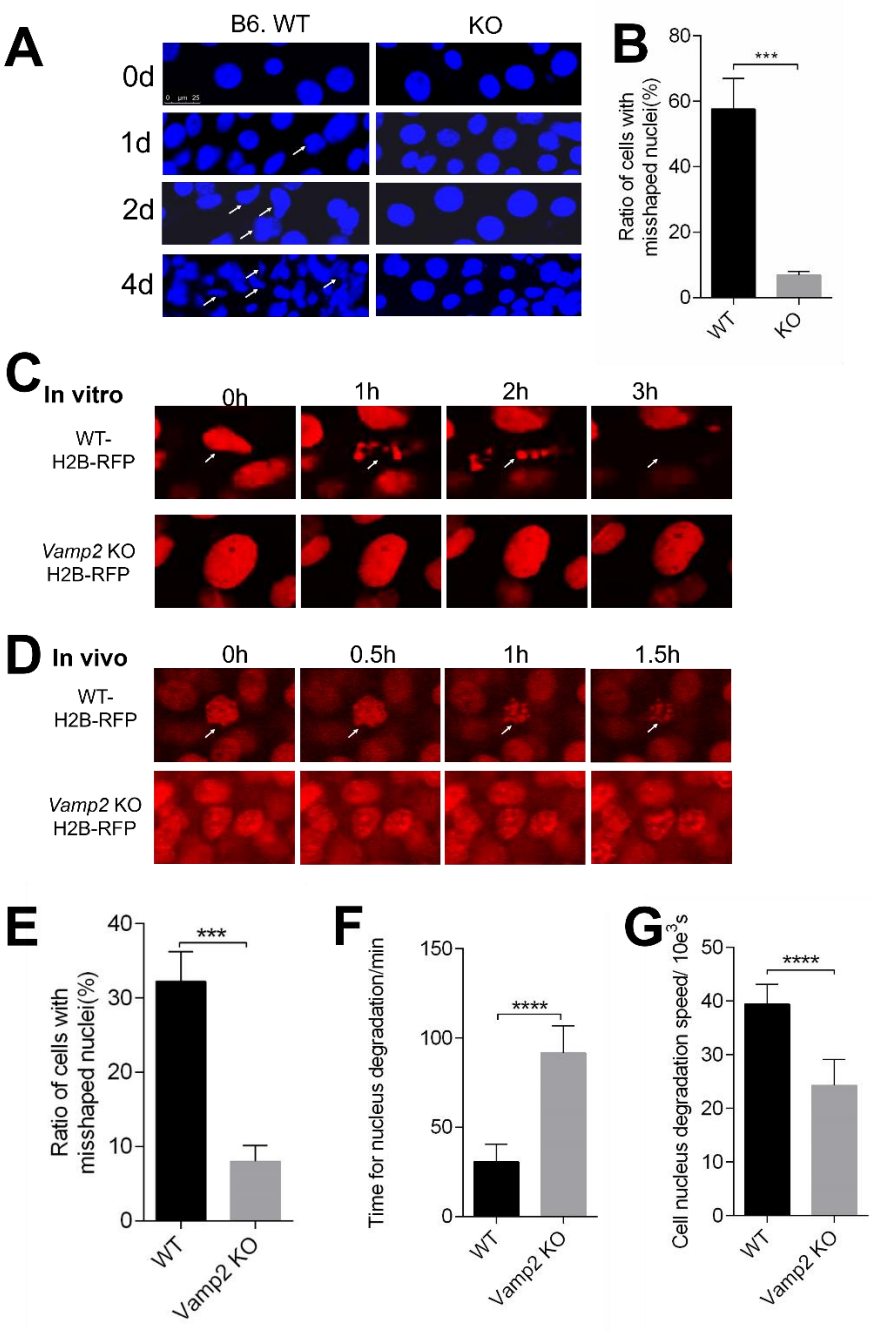

Figure 3.2: Evaluation the effect of *Vamp2* KO on nuclear degradation *in vitro* and *in vivo*. **A.** WT and *Vamp2* KO cells were undergoing calcium shift induced differentiation. Cells were fixed and stained for DAPI at different time point as illustrated. **B.** Quantification of cells undergoing nuclear degradation in **(A)**. **C.** WT and *Vamp2* KO cells were ectopically expressing H2B-RFP. Real time imaging was taken using confocal microscope to trace nuclear changes under calcium shift. Quantification of cells undergoing nuclear degradation in quantified in **(E)**. **D.** WT and *Vamp2* KO cells expressing H2B-RFP were engrafted to nude mice. Regenerated skin was subjected to real time imaging using confocal microscope to trace nuclear changes. The speed of nuclear degradation in engrafted skin was quantified in **(F and G)**. ****, P<0.00001; ***, p<0.0001, unpaired Student's *t*-test

Loss of *Vamp2* promotes skin tumorigenesis *in vivo*

Vamp2 KO mouse model performs defects in nuclear degradation process. In human, defects in keratinocyte enucleation is associated with various skin disease including skin cancers, such as parakeratosis seen in patients suffering from eczema and dermatitis. However, the function of Vamp2 in skin tumorigenesis has not been illustrated. Therefore, we infected WT and *Vamp2* KO cells with lentivirus encoding *Ha-Ras,* which has a G12V mutant. Upon engraftment to nude mice, the WT cells usually give rise to visible growth of benign papilloma. By contrast, engraftment of *Vamp2* KO with *G12V-Ras* expression leads to significantly larger and more aggressive tumors **(Figure 3.3 A)**. Tumor sizes were measured weekly post engraftment. The tumors generated from mutant *Ha-Ras* infected *Vamp2* KO cells grew significantly faster than control **(Figure 3.3 B)**. At the end, tumor samples were collected and subjected to H/E staining as well as immunostaining **(Figure 3.3 C)**. Histologically, the tumors derived from *Vamp2* KO cells displayed more advanced progression toward SCC, including the characteristic squamous pearls in the H/E staining and islands of dysplastic squamous epithelial cells lying in the dermis, which distinctly indicated an invasive form of SCC. Moreover, there is dramatic decrease of expression of differentiation-associated markers, including Krt10 and Loricrin, further demonstrates that the differentiation process is hindered. Together, these results strongly suggest that enhanced tumorigenesis and progression in *Vamp2* KO skin is related to aberrant epidermal differentiation.

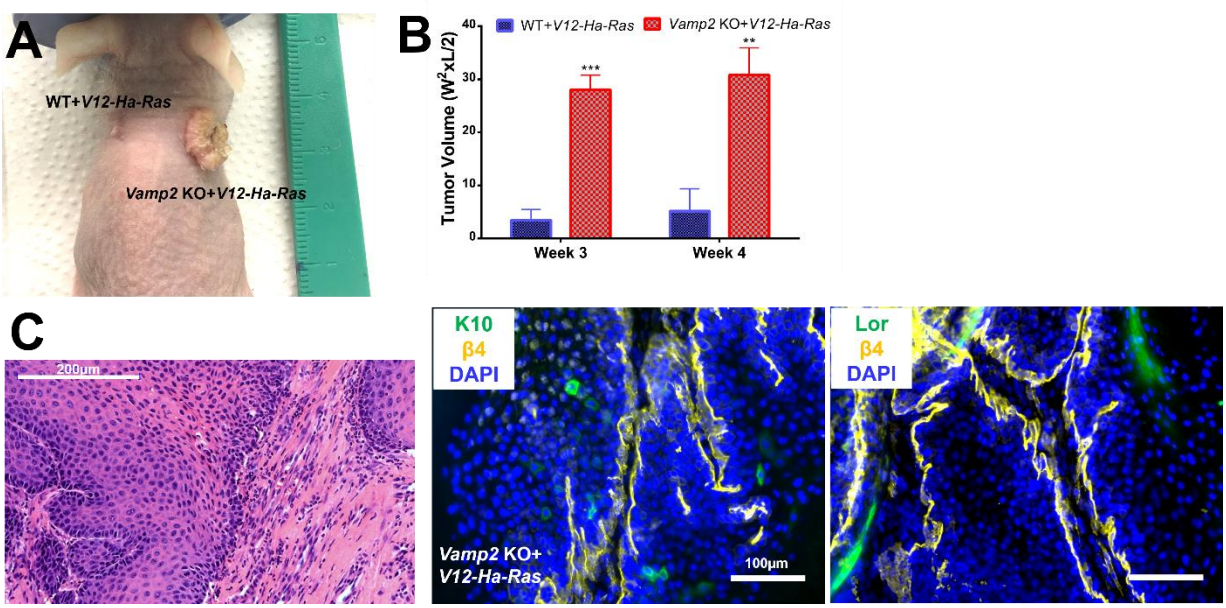

Figure 3.3: *Vamp2* deficiency promotes tumor growth *in vivo*. WT and *Vamp2* KO cells were infected with lentivirus encoding *Ha-Ras* G12V mutant. Infected cells were transplanted to nude mice for tumorigenesis analysis. **A.** Representative picture of WT and *Vamp2* KO tumors appearing on nude mice. **B.** Measurement of WT and *Vamp2* KO tumor size were plotted at week 3 and week 4 post engraftment. Tumor size was calculated using equation: $V=W^2xL/2$. Statistical analysis is conducted using unpaired Student's *t*-test. Error bar represents S.D. ***, $p<0.0001$; **, $p<0.01$ **C.** *Vamp2* KO tumors were collected and subjected to H/E staining and immunofluorescence staining with different antibodies as indicated. K10: keratin 10; Lor: Loricrin. Loss of *Vamp2* leads to enhanced tumorigenesis in skin and the KO tumors display reduced level of epidermal differentiation markers. In H/E staining, scale bars = 200 μm. In IF staining, scale bars = 100 μm.

Vamp2 aggregates around nucleus during keratinocyte differentiation

Subcellular localization of a protein is usually associated with its molecular functions. We ectopically expressed both H2B-RFP and GFP-Vamp2 in WT keratinocytes (**Figure 3.4 A)**. Thus, we can trace the nuclear degradation process with simultaneous visualization of Vamp2 subcellular localization upon calcium shift *in vitro*. Interestingly, Vamp2 start aggregation around nucleus when indentation initiated and the intensive signals diffused away once nuclear degradation is completed. We further examined Vamp2 localization in mouse epidermis via immunostaining using anti-Vamp2 antibody **(Figure 3.4 B)**. In WT new born skin, Vamp2 is mainly expressed in suprabasal layer of epidermis and the signals are primarily around nuclei. In comparison, Vamp2 signal is completely missing in the knock-out cells. To visualize subcellular localization of Vamp2 via a better resolution, fresh skin from new born WT mouse was subjected to gold staining using anti-Vamp2 antibody **(Figure 3.4 C)**. Via electronmicroscope, each Vamp2 molecule appears as a dark dot and it is located both inside the nucleus as well as near the nuclear membrane where chromatin condensation occurs. The aggregation of Vamp2 around nucleus in differentiating keratinocyte suggests that Vamp2 may facilitate nuclear degradation via its function of vesicle trafficking, which is similar to the autophagy mediated nuclear degradation.

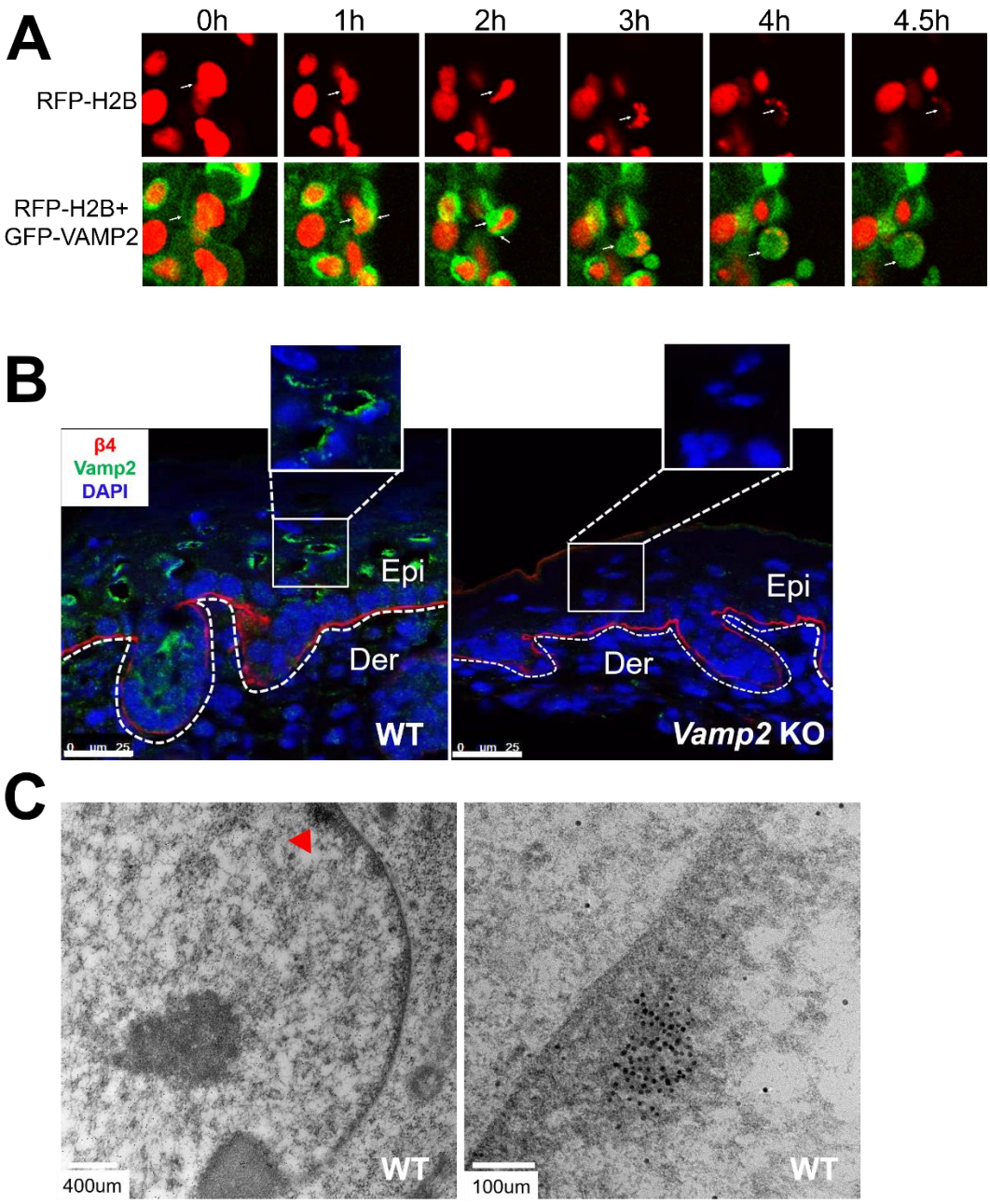

Figure 3.4: Vamp2 aggregates around nucleus during keratinocyte differentiation. A. WT keratinocytes were ectopically expressing H2B-RFP and GFP-Vamp2. Real time imaging was taken using confocal microscope to trace nuclear changes under calcium shift. The upper panel displays RFP channel only, and the lower panel displays both RFP and GFP channels. White arrows denote cells undergoing nuclear degradation. **B.** WT and *Vamp2* KO fetus skin (E.18.5) were subjected to immunostaining of different antibodies as indicated (β4: β4-integrin, CD104). Scale bar = 25 μm. Dotted lines denote dermal–epidermal boundaries. Epi: epidermis, Der: dermis. **C.** WT skin of new born mouse was subjected to gold staining using anti-Vamp2 antibody and images were taken via electronmicroscope. The right panel is an enlarged area shown in the left panel, indicated by the red arrow.

SILAC-MS/MS screening of proteins involved in Vamp2 coated intracellular vesicles

The best known function of Vamp2 is participating intracellular vesicle fusion as a SNARE family protein. If Vamp2 mediated nuclear degradation in keratinocyte is also related to vesicle trafficking and fusion, it is important to identify the proteins involved in this process, which could provide a full picture of the functional machinery. Via isolating Vamp2 coated subcellular vesicles combinatory of SILAC-MS/MS screening, we identified many proteins associated with Vamp2 (data not published). Excitingly, some potential targets involved in autophagosomes trafficking are also caught by our screening. We are continuing with further functional studies and are researching on the molecular mechanisms underlying Vamp2 mediated keratinocyte enucleation.

Discussion

Epidermal differentiation and epidermal stratification is a precisely regulated and well-orchestrated process involving many signaling pathways and regulatory proteins. Disruption of the balance between proliferation and differentiation could cause many skin diseases including skin cancers. However, the underlying mechanism are still poorly understood. Our shRNA library screening is aiming to expand our knowledge and provide new candidates that regulate keratinocyte differentiation. The screening design is straightforward with good controls for background. The phenotype we selected was the ability of cells overcoming high calcium induced proliferation inhibition. It is worth to notice that, some population of cells may not present phenotype of interests because they died upon depletion of essential genes. In addition, cells undergoing proliferation does not mean the differentiation process is completely stopped. The regulation of proliferation and differentiation process within keratinocyte are related but also independent, which need to be precisely orchestrated. Therefore, a well-designed validation and examination when study the target of interests during keratinocyte differentiation is essential.

Our study, for the first time, illustrated a potential regulatory function of Vamp2 in keratinocyte enucleation. Based upon *in vitro* calcium shift and examination of Vamp2 expression, Vamp2 level is undetectable at basal condition, which is consistent with immunostaining of Vamp2 in WT mouse skin **(Figure 3.1 A and 3.4 B)**. This indicates that during epidermal stratification, Vamp2 is induced when terminal differentiation is initiated and potentially functions at a later stage of differentiation. However, it may also raise a concern that ectopically expressing Vamp2 at basal keratinocyte may interfere with normal cellular functions, although we have not seen any sign or proof of this concern. From a methodological point of view, we provide a sufficient way to visualize nuclear degradation in real time using living mouse. The engraftment of *in vitro* cultured primary keratinocyte can give rise to fully functional epidermis with normal tissue textures. Since the donor cells can be genetically modified before undergone engrafting, we can label the protein of interests with various tags that can be readily traced under confocal microscope **(Figure 3.2 D)**. Further testing using WT keratinocyte expression H2B-RFP and GFP-Vamp2 to visualize Vamp2 localization during nuclear degradation upon engraftment is needed.

Although this project is still undergoing, via isolating the Vamp2 coated subcellular vesicles and identify the proteins associated with it, we could be more confident in pursuing the molecular mechanism underlying Vamp2 mediated keratinocyte enucleation. These associated proteins may or may not be directly interacting with Vamp2 itself. They could just adjacently locate on the same membrane. We need confirm and validate the target proteins via multiple cell and molecular methods and functional studies both *in vitro* and *in vivo*. In together, this research will provide valuable knowledge of the nuclear degradation process in keratinocyte at the molecular level.

CHAPTER IV

DISCUSSION, CONCLUSIONS AND FUTURE DIRECTIONS

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 challenges, such as irradiation, pathogen, dehydration and physical stress. 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 [2, 48, 178]. These cells can periodically migrate upward from their niche at the basement membrane and undergo terminal differentiation to replenish skin epithelial cells that are continuously lost in a process known as epidermal stratification. During wound repair, the epidermal stem cells and their progenitors play essential roles in filling up the wound field and regenerating lost tissue.

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, as well as the controlled dissolution of cellular organelles and the nucleus [2, 49]. Aberrant skin differentiation contributes to the development of various skin diseases, such as psoriasis, inflammatory skin diseases and skin cancers. Defects in keratinocyte enucleation are also associated with skin diseases like eczema and dermatitis, in which patients can suffer from severe parakeratosis and ulcer. Cutaneous SCC is the second most common human cancer in the U.S., afflicting more than 250,000 patients every year [119-121]. Even with a relatively higher cure rate, cutaneous SCC still can be highly metastatic and lethal (3-10% rate of metastasis), and a significant number of patients with a primary SCCs relapse with second lesions within 5 years of diagnosis, leading to severe morbidity and mortality

[122]. Patients with HNSCCs, which is originated from keratinocytes lining at internal surface areas, have a much worth prognosis [123]. Thus, understanding epidermal differentiation and its underlying molecular mechanisms is critical for devising effective therapeutic strategies for the treatment of various skin diseases. In the first part of this thesis 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. In the second part, by conducting a shRNA library screening, followed with examinations using mouse model as well as cell and molecular studies, we identified *Vamp2* as an essential gene regulating keratinocyte enucleation during epidermal stratification. Overall, my contributions as described in this thesis research have provided key insights into the molecular mechanism regulating keratinocyte differentiation and epidermal homeostasis, and have strong impact on the research of skin development and skin diseases.

KDF1 was initially identified by a forward genetics screen as a key regulator of epidermal differentiation in mouse skin [93]. Recent studies suggest that KDF1 is also involved in tooth agenesis, and mutation of *KDF1* has been identified in patients with ectodermal dysplasia, a heterogeneous group of diseases that affects the ectoderm derivative, such as skin, hair follicles, teeth, and nails [98]. Despite its potentially important role in tissue development and homeostasis, little is known about the role of KDF1 at the molecular level. Previous study suggests that KDF1 can affect expression of *p63* in skin keratinocytes, and genetically interacts with Stratifin (14-3-3σ). Our current study shows that KDF1 association with IKKα is essential for epidermal differentiation, and ectopic expression of *IKKα* can restore skin stratification in *KDF1* null cells. It will thus be interesting to determine whether the p63 regulation and interaction with Stratifin by

KDF1 are also mediated by IKKα in the future. There were studies indicating that p63 has a regulatory role on IKKα expression at the transcriptional level [154, 155]. However, our current study confirmed the increase of IKKα protein level during keratinocyte differentiation is not related with its transcriptional activity, which seems to be contradictory conclusions. The possible explanations are: previous studies were focusing on IKKα expression regulations during embryonic development. As I mentioned in the introduction, the existence of intermediate layer during embryonic skin development involves complicated and even conflicting functions of p63 and its various isoforms [49]. However, in adult mammalian skin, intermediate layer does not exist. Therefore, the regulatory mechanisms discovered from previous studies may not apply to our system.

Protein ubiquitination and deubiquitination are highly dynamic but precisely 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 removing single ubiquitin and polyubiquitin. The human genome encodes nearly one hundred DUBs, and their substrate specificity can be modulated by different mechanisms [160]. 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 [179, 180]. However, accumulating evidence suggest 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 [162-167]. Null mutation of *USP7* in mice leads to early embryonic lethality, suggesting its important

role in embryonic development [167]. Our data show that it is also an essential gene involved in differentiation of skin epidermal cells by regulating IKKα ubiquitination level and its protein stability. CRISPR knock-out of *USP7* cause defects in keratinocyte differentiation *in vitro*. However, we cannot rule out the possibility that loss of *USP7* in skin keratinocytes may lead to changes of other signaling proteins. Proteomics analysis coupled with functional studies *in vitro* an *in vivo* will be essential to address this hypothesis in the future.

Moreover, we may need to improve knock-out strategy to further confirm the phenotype in *USP7* KO keratinocytes. Since the current knock-out is incomplete, some of the striking consequence lead by *USP7* KO may not be able to be identified. Designing a different knock-out locus and isolating single colony during selection may help us to improve the efficiency. It is also beneficial to generate skin tissue specific conditional *USP7* knock-out. However, it is important to keep in mind that, complete *USP7* depletion may lead to cell or tissue death since USP7 is such an essential gene for early embryonic development and is involved in too many types of cellular processes. In addition, the interaction between USP7 and IKKα need to be further examined. Although we were able to confirm the binding between USP7 and KDF1, the connection between USP7 and IKKα is unclear. Together, we can ask several questions for our further studies: What is the signaling molecule or acting machinery that trigger or reinforce the interaction between KDF1 and IKKα as well as the recruitment of USP7? Is IKKα a direct substrate of USP7? Is there any other protein involved in the regulatory function of this KDF1/IKKα complex?

The tissue homeostasis of epidermis has to be precisely maintained, and deregulation of this process will potentially lead to skin tumorigenesis. Skin carcinogenesis, including both BCC (basal cell carcinoma) and SCC, is the most common human cancer type [119]. In addition to cutaneous carcinogenesis, head and neck SCC or tumors of esophagus can originate from the

epithelial cells lining up those cavities. These tumors usually have much worse prognosis and poor survival rate [123], but may share very similar molecular mechanisms underlying carcinogenesis. For instance, our recent study demonstrated that loss of *RIPK4* in mice leads to cutaneous SCCs. The same gene has been identified as a frequently mutated gene in human head and neck SCCs. Therefore, there is an urgent need to expand our knowledge of molecular mechanisms and signaling networks involved in skin differentiation. IKKα has been reported to be a tumor suppressor in skin cancers and reduction in IKKα protein level was significantly associated with poorer prognosis of human SCCs [107, 132, 133, 152, 153]. The role of USP7 has been well established in many different types of cancers, including prostate cancer, lung cancer, colon cancer, brain cancer, ovarian carcinoma, and leukemia [181]. As USP7 acts on a large variety of viral substrates, it has also been implicated in virus-associated tumors, such as nasopharyngeal cancer, lymphomas, and Kaposi's sarcoma. Beyond USP7 and IKKα, many other genes that have also been reported to be involved in epidermal differentiation, such as *IRF6*, *Notch*, and *p63*, have aberrant functions in SCCs [125, 173, 174]. It will be interesting to determine whether the KDF1/IKKα pathway may interact or affect these molecules and contribute to skin differentiation and tumorigenesis.

In the second part of this thesis study, we conducted a shRNA library screening to identify unfamiliar genes that are essential in regulating keratinocyte differentiation. Since we were selecting living cells that can stay proliferating through serial passaging in high calcium medium, the shRNA enriched in these survived cells are proposed to silence the genes essential in inhibiting cellular proliferation and promote differentiation. One technical limitation is that silence in some critical genes can cause cell death and we were unable to recover this population. However, as an initial large scale screening process, it is sufficient enough that we can catch some targets of

interest. More importantly, we were able to identify an essential regulatory protein, Vamp2, in controlling nuclear degradation process during keratinocyte terminal differentiation.

Enucleation, the degradation of nuclei, is one of the final steps during epidermal stratification that is essential in generating rigid and waterproof cornified skin barrier. Defect in enucleation process will cause retention of the nuclei and immature epidermis with aberrant barrier function. Patients with defect in keratinocyte enucleation will suffer from parakeratosis, such as in eczema and dermatitis disease. However, it is still unclear about the molecular regulatory mechanisms underlying this process. The involvement of Vamp2 in enucleation is unknown. Even its general function in skin keratinocyte is not clear. We went ahead to examine the skin phenotype of the *Vamp2* KO mouse model, and we observed intact nuclei on the topmost *Vamp2* KO mouse epidermis **(Figure 3.1)**. We further caught prohibited nuclear degradation in *Vamp2* KO keratinocyte using both *in vitro* and *in vivo* analysis **(Figure 3.2)**. We also tested the effect of *Vamp2* depletion during skin tumorigenesis **(Figure 3.3)**. However, decipher the mechanism through which Vamp2 functions during nuclear degradation is the hardest part. We need to go back to its general and already known functions illustrated in other cell types.

Vamp2 is a v-SNARE protein primarily mediating intracellular vesicle fusions such as the neurotransmitter trafficking in forebrain synapse [176]. By the meaning of its name, v as vesicle, Vamp2 locates at the membrane of trafficking vesicles. Interestingly, when we analyze the subcellular localization of Vamp2 within keratinocytes, we identified an around-nucleus pattern of Vamp2 during cell differentiation, not only *in vitro*, but also in mouse skin tissue (Figure 3.4). If the behavior of Vamp2 during nuclear degradation is related to its vesicle delivery function, is it possible that Vamp2 is mediating engulfment of nucleus, a process similar to a type of autophagy, nucleophagy? The involvement of autophagy in keratinocyte nuclear removal has been proposed

and examined in the literatures [114, 115]. Autophagy mediated nuclear lamina and DNA component degradation could be essential in maintaining tissue integrity and preventing tumorigenesis. A colocalization of LC3, Lamp2 and p62 at the nucleus undergoing indentation has also been caught during keratinocyte differentiation *in vitro*, indicating a targeted degradation of the nuclei. In addition, shRNA knock down of *WIPI1* or *ULK1* lead to prevention of nuclear removal in differentiating keratinocytes *in vitro*. Although there are studies arguing about the role of classical autophagy within keratinocyte enucleation, maybe we are working with a unique and non-canonical vesicle engulfment and trafficking system. It is interesting to conduct following tests: we can test the colocalization and potential bindings between Vamp2 and LC3, Lamp2 or p62; we can create keratinocyte ectopically expressing tagged LC3 and Vamp2, and visualize their behaviors during nuclear degradation *in vivo* using our engraftment and life-imaging platform.

Another essential test is to identify the proteome associated with Vamp2 coated intercellular vesicles, which we have already completed and currently undergoing analysis and further functional studies. Since this project is still in progress, and the molecular mechanism are not been tested yet, a lot of work can be proposed at this point and a lot of questions can be asked. First of all, we need to test if WT keratinocyte expression H2B-RFP and GFP-Vamp2 can successfully provide real-time life imaging of nuclear degradation with the proposed behavior of Vamp2 protein during this process. Second of all, is Vamp2 mediating early initiation of nuclear engulfment or the vesicle trafficking? If vesicle trafficking is involved, can we recover the Vamp2 coated vesicles in the suprabasal layer of epidermis? Can we validate these models and hypotheses through our list of proteins associated with Vamp2? We need to confirm and validate the target proteins via multiple cell and molecular methods and functional studies both *in vitro* and *in vivo*.

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