ARTICLE OPEN

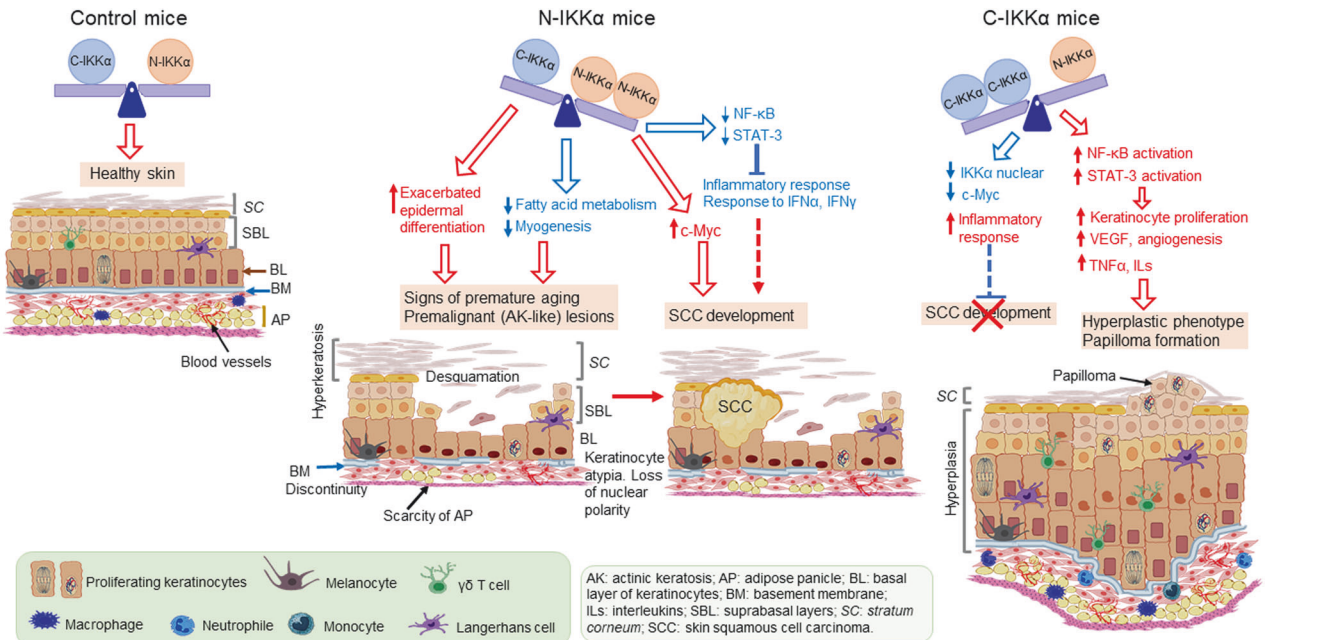
Nuclear versus cytoplasmic IKKα signaling in keratinocytes leads to opposite skin phenotypes and inflammatory responses, and a different predisposition to cancer

Verónica A. García-García[1,2,7], Josefa P. Alameda[1,2,3,7], M. Jesús Fernández-Aceñero[4], Manuel Navarro[1,2,3], Ramón García-Escudero[1,2,3], Angustias Page[1,2,3], Raúl Mateo-Gallego[1,2], Jesús M. Paramio[1,2,3], Ángel Ramírez[1,2,3], Rosa A. García-Fernández[5], Ana Bravo[6] and M. Llanos Casanova[1,2,3]✉

IKKα is known as an essential protein for skin homeostasis. However, the lack of suitable models to investigate its functions in the skin has led to IKKα being mistakenly considered as a suppressor of non-melanoma skin cancer (NMSC) development. In this study, using our previously generated transgenic mouse models expressing exogenous IKKα in the cytoplasm (C-IKKα mice) or in the nucleus (N-IKKα mice) of basal keratinocytes, we demonstrate that at each subcellular localization, IKKα differently regulates signaling pathways important for maintaining the balance between keratinocyte proliferation and differentiation, and for the cutaneous inflammatory response. In addition, each type of IKKα-transgenic mice shows different predisposition to the development of spontaneous NMSC. Specifically, N-IKKα mice display an atrophic epidermis with exacerbated terminal differentiation, signs of premature skin aging, premalignant lesions, and develop squamous cell carcinomas (SCCs). Conversely, C-IKKα mice, whose keratinocytes are nearly devoid of endogenous nuclear IKKα, do not develop skin SCCs, although they exhibit hyperplastic skin with deficiencies in terminal epidermal differentiation, chronic cutaneous inflammation, and constitutive activation of STAT-3 and NF-κB signaling pathways. Altogether, our data demonstrate that alterations in the localization of IKKα in the nucleus or cytoplasm of keratinocytes cause opposite skin changes and differentially predispose to the growth of skin SCCs.

Oncogene; https://doi.org/10.1038/s41388-024-03203-0

Graphical Abstract



A full list of author affiliations appears at the end of the paper.

Received: 20 November 2023 Revised: 15 October 2024 Accepted: 17 October 2024
Published online: 07 November 2024

INTRODUCTION

IKKα is a member of the IKK complex that mediates NF-κB activation through both the canonical and non-canonical (or alternative) pathways [1]. In the canonical pathway, upon activation by different stimulus the IKK complex consisting of IKKα, IKKβ and IKKγ proteins phosphorylates the inhibitory IκB proteins. This leads to the release of the p50/65 NF-κB dimer, which enters the nucleus activating the expression of genes involved in cellular functions such as inflammatory and immune responses, cell survival and apoptosis [1]. The noncanonical pathway primarily relies on IKKα signaling and is essential for the maturation of secondary lymphoid organs. Knockout mouse models targeting the different IKK subunits have revealed that mice deficient in IKKβ or IKKγ die from liver cell apoptosis during embryonic development [2, 3]. However, IKKα$^{-/-}$ mice develop to term, although they exhibit multiple morphological defects and die shortly after birth, presumably due to dehydration resulting from impaired terminal epidermal differentiation and skin barrier function [4–6]. These observations indicate that IKKα plays different roles than the other proteins in the IKK complex and highlight its unique and essential function in the control of epidermal proliferation and differentiation.

The epidermis is a stratified tissue composed mostly (>90%) by keratinocytes [7]. Those of the basal layer express cytokeratins 5 and 14 (K5/K14) and have high proliferative potential. Suprabasal layers include the *stratum spinosum*, formed by differentiating keratinocytes that migrate upwards and express keratins K1/K10 and Involucrin (markers of early epidermal differentiation), the *stratum granulosum*, with keratinocytes that express Loricrin and Filaggrin (markers of late epidermal differentiation), and the *stratum corneum*, consisting of flat, enucleated lipid-rich keratinocytes (corneocytes). Maintaining homeostasis of the epidermis is crucial, as imbalances may lead to the development of different skin conditions, among them, non-melanoma skin cancer (NMSC), which is the most commonly diagnosed neoplasm, accounting for 90% of cancers in the Caucasian population [8]. Although the incidence of NMSC is much higher in older adults [9], it is steadily increasing in patients younger than 40 years [10], making understanding the molecular pathways underlying NMSC development an urgent issue. NMSC includes two major entities: basal cell carcinomas (BCCs) and squamous cell carcinomas (SCCs) which represent approximately 80% and 20% of NMSC, respectively. BCCs rarely metastasize. In contrast, SCCs can be very aggressive, metastasizing in ~5% of cases; therefore, given the high incidence of NMSC, it results in significant concomitant mortality [11]. Although the development of NMSC is strongly associated to exposure to ultraviolet radiations [12], there are other risk factors, both genetic and environmental, that also influence their development and progression. Some groups have proposed that *CHUK*, the gene encoding the human IKKα protein, functions as a tumor suppressor of skin SCC development [13–17]. However, our group and others have reported that overexpression of IKKα in keratinocytes increases the susceptibility to the development and progression of murine and human cutaneous SCCs [18–22]. The different localization of IKKα in the cytoplasm or nucleus of keratinocytes has been suggested as a possible explanation for these apparently contradictory results, since in addition to its involvement in NF-κB signaling, IKKα also exerts relevant functions in the nucleus [23]. Among others, a key role of nuclear IKKα is the regulation of keratinocyte proliferation and terminal differentiation, which occurs independently of its kinase and NF-κB activities [24–26]. Notably, our group and others have found a direct correlation between elevated levels of nuclear IKKα and increased tumor progression of skin SCCs in mice and humans [20, 22].

In this work, through the analysis of the skin of our transgenic mouse models that express exogenous IKKα only in the nucleus (N-IKKα mice) or in the cytoplasm (C-IKKα mice) of basal keratinocytes, we have revealed that the increase in nuclear IKKα levels – but not cytoplasmic levels- induces the spontaneous development of skin SCCs. These findings are very relevant since other studies hypothesized that when localized in the nucleus of keratinocytes IKKα acted as a tumor suppressor of skin SCC development without actually studying the effect that the nuclear expression of IKKα had on the keratinocytes [14, 27]. Additionally, we describe the downregulation of endogenous IKKα expression, and thus the almost complete absence of nuclear IKKα in C-IKKα keratinocytes, further reinforcing that nuclear IKKα is not a tumor suppressor of cutaneous SCC development, since although C-IKKα mice do not express nuclear IKKα, they do not develop SCCs. In addition to the outstanding difference in their susceptibility to the development of skin tumors, N-and C-IKKα mice display other opposite histological and molecular cutaneous characteristics, i.e., N-IKKα mice exhibit an atrophic and hyperkeratotic epidermis, induction of c-Myc and negative regulation of NF-κB and STAT3 signaling pathways, and of genes involved in inflammatory responses, while, on the contrary, C-IKKα mice present hyperproliferative skin with defects in epidermal differentiation, inhibition of c-Myc signaling and chronic activation of STAT-3 and NF-κB, and increased inflammatory responses.

Our findings extend our previous research [20], in which we conducted chemical carcinogenesis experiments on the skin of C-IKKα/TgAC and N-IKKα/TgAC mice carrying the activated *Ha-Ras* oncogene in keratinocytes. In that work we demonstrated that high levels of IKKα, both nuclear and cytoplasmic, acted as promoters of skin tumors that had developed in keratinocytes transformed by *Ha-Ras*, although the mechanisms through which IKKα favored the progression of skin tumors in C-IKKα/TgAC and N-IKKα/TgAC mice were different. Taken together, our data show that although high levels of IKKα both in the nucleus and cytoplasm of keratinocytes favor the progression of cutaneous SCCs developed as a consequence of an oncogenic event (the activation of *Ha-Ras*), only the expression of high levels of nuclear IKKα in keratinocytes is capable of inducing the de novo development of this type of tumors, in the absence of previous oncogenic events.

Therefore, our present results, together with previous data finding a direct correlation between elevated levels of nuclear IKKα and increased tumor progression of skin SCCs in mice and humans [20, 22], suggest the potential usefulness of targeting nuclear IKKα as a therapeutic approach against this type of tumor.

RESULTS

We have previously described the generation of C-IKKα and N-IKKα transgenic mice which express the human IKKα protein under the control of the Keratin 5 (K5) regulatory elements [20]. As we explained, the modifications that these models carry are either the elimination of the nuclear localization signal (NLS) in the cDNA of the human IKKα by directed point-mutation in C-IKKα mice or the insertion of an additional NLS sequence in the amino terminal in N-IKKα mice, outside of the kinase domain of the human *IKKα/CHUK* gene. As reported, C-IKKα and N-IKKα mice express the transgenic IKKα in the cytoplasm or in the nucleus of epithelial cells respectively, following the expression pattern of K5 [20]. Thus, they express the transgene in keratinocytes of the basal layer of the epidermis, the outer root sheath (ORS) of hair follicles (HF) and in the immature cells surrounding the sebaceous glands (SG) (Supplementary Fig. 1). Both lines of transgenic mice developed normally after birth. However, while N-IKKα mice remain indistinguishable from their non-transgenic (Control) littermates, transgenic C-IKKα mice showed a characteristic altered fur from very early age (14-day-old) due to a delay in hair growth, and from the age of 3 months they presented diffuse alopecia (Fig. 1A, B).

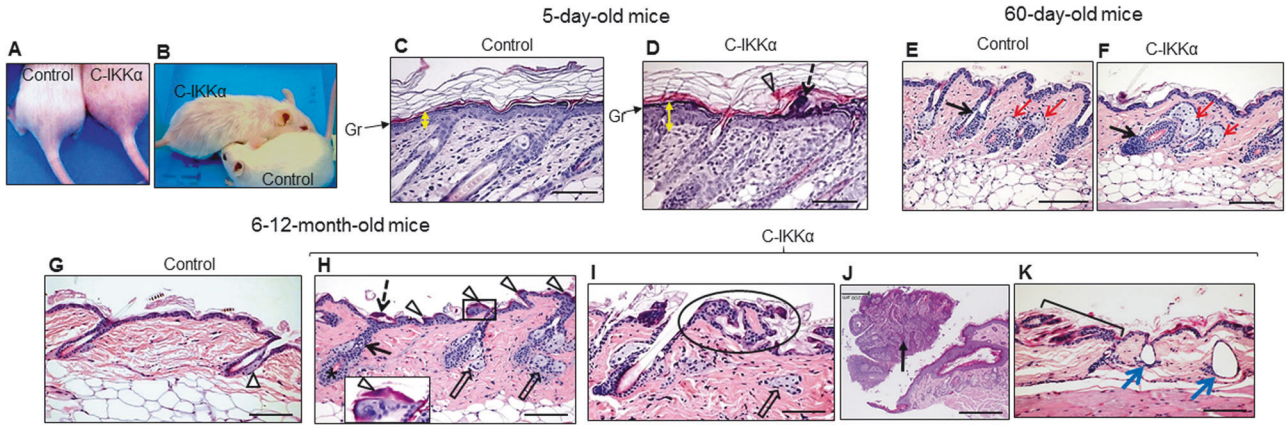


Fig. 1 External phenotype and histological alterations in the back skin of C-IKKα transgenic mice. A, **B** Diffuse alopecia in the back of 14-day-old (left) and 3-month-old (right) C-IKKα mice. H&E staining of back skin sections of 5-day-old Control (**C**) and C-IKKα (**D**) mice showing parakeratosis (arrowhead), dyskeratosis (dotted arrow), and epidermal hyperplasia (yellow double-headed arrow) (Gr; dark purple granulated keratinocytes) in C-IKKα mice. **E**, **F** Back skin sections from 60-day-old mice. Hyperplasia of the ORS (black arrows) and mature sebocytes of SG (red arrows) in telogenic HF of transgenic mice (**F**) versus normal ORS of HF and SGs in Control mice (**E**). **G**, **K** Observe the worsening of the skin phenotype of C-IKKα mice from 6 months of age; **G** Control mouse, note the presence of small SG (arrowhead); **H–K** C-IKKα mice. **H** Abundant foci of dyskeratosis and parakeratosis (dotted arrow and arrowheads, respectively); hyperplasia of the ORS of HF (black arrow); numerous orphan and hyperplastic SG (empty arrows). Inset shows parakeratosis at higher magnification. See notable papillomatous hyperplasia in the epidermis of transgenic mice (circle in **I**) and the formation of sporadic papillomas (black arrow in **J**). **K** Formation of follicular cysts (blue arrows) from degenerated HF and severe papillomatous hyperplasia of IFE with dyskeratosis and parakeratosis (bracket). 6–10 animals of each age and genotype were analyzed. Each photo represents a different section of skin from the same mouse or different mice. Scale bars: **C**, **D**: 50 µm; **E**, **F**, **G–I**, **K**: 100 µm; **J**: 200 µm.

C-IKKα mice exhibit hyperplasia of the epidermis and adnexal organs

Back skin sections of transgenic mice of different ages, from newborns to adults, were analyzed and revealed epidermal alterations that began soon after birth (Fig. 1C–K). Five-day-old C-IKKα mice showed hyperplasia of the interfollicular epidermis (IFE), mainly due to the increased number of cell layers in the *stratum granulosum* (Fig. 1C, D), as well as foci of dyskeratosis, i.e., abnormal keratinization occurring prematurely below the *stratum granulosum*. Additionally, the skin of these transgenic mice showed parakeratosis (anomalous retention of nuclei within the *stratum corneum*) suggesting alterations in epidermal terminal differentiation (Fig. 1D). These changes worsened with age and other alterations also appeared in older mice; thus, 31-and 60-day-old C-IKKα animals showed reduced numbers of HF (Fig. 1F; Supplementary Fig. 2A–C), hyperplasia of the ORS of the HF, as well as hyperplasia of the HF-associated SGs (Fig. 1F; Supplementary Fig. 2D–E). This phenotype was even more notable in C-IKKα mice from 6 months of age, which showed persistent dyskeratosis and parakeratosis of IFE, and papillomatous epidermal hyperplasia (Fig. 1H, I, K) that occasionally led to papilloma development (benign epidermal growths; observed in 3/23 C-IKKα versus 0/28 Control mice) (Fig. 1J). Also, follicular cysts (Fig. 1K), and "orphan" SGs (non-associated to HF) (Fig. 1H, I) were detected in the dermis of transgenic animals.

Similar alterations were also found in the skin of other locations in C-IKKα mice, such as lip, plantar sole, eyelid and tail skin (Supplementary Fig. 3).

The skin of N-IKKα mice exhibits epidermal atrophy, hyperkeratosis, and signs of premature aging

N-IKKα mice showed skin alterations from early ages that became more prominent with aging. Thus, five-day-old N-IKKα mice showed a stretched and thin epidermis with 3–4 layers of keratinocytes, which contrasted with the 6 layers of keratinocytes arranged along soft epidermal folds exhibited by Control animals (Fig. 2A, B). At 31 days of age, N-IKKα mice developed dysplastic HFs and presented foci of epidermal atrophy containing a single layer of keratinocytes (Supplementary Fig. 2F–H). Foci of atrophic epidermis alternated with normal epidermal thickness resembling the histological appearance of wrinkles in aged skin (Supplementary Fig. 2G). When these transgenic mice reached 60 days of age atrophic epidermal foci were more frequent along the IFE, whereas Control mice did not exhibit these changes (Fig. 2C, D). In addition, other alterations associated with premature aging were also detected in the skin of young N-IKKα animals, including a thinner layer of adipose tissue in the hypodermis and increased packing of collagen fibers (sclerosis) in the dermis (Fig. 2D, E; Supplementary Fig. 2G). In addition, 6-month-old and older N-IKKα mice showed altered epidermal differentiation, i.e., the epidermis of N-IKKα mice showed disorders in the maturation of suprabasal keratinocytes, which underwent an abrupt transition from the basal layer of keratinocytes to the *stratum corneum*, which appeared thickened, parakeratotic and with scales that retained the nucleus (Fig. 2G); this caused the presence in the skin of N-IKKα mice of extensive areas of the IFE that lacked the *spinosum* and *granulosum strata* (Fig. 2G, H). Hyperkeratosis and increased desquamation was detected in these transgenic animals (Fig. 2G–I). This altered and accelerated desquamation observed in N-IKKα mice contrasted with the normal shedding of anucleated keratin scales that occurred in Control mice (Fig. 2F). Similar abnormalities were found in the skin of N-IKKα mice in other localizations such as tail and eyelid skin (Supplementary Fig. 4).

Therefore, our data suggest that IKKα plays different functions in nucleus and cytoplasm of keratinocytes that are necessary for proper skin development, and that alterations in the subcellular localization of IKKα disrupt normal skin homeostasis, leading to the development of different and almost opposite skin phenotypes.

Highly proliferative epidermis with defective terminal differentiation in C-IKKα mice versus increased epidermal terminal differentiation in N-IKKα mice

Given the cutaneous phenotype of C-IKKα and N-IKKα mice that suggested alterations in the balance of proliferation and differentiation of keratinocytes, and considering the key role of IKKα in the regulation of these processes [4], we examined both the proliferation and differentiation properties of the skin of C-and

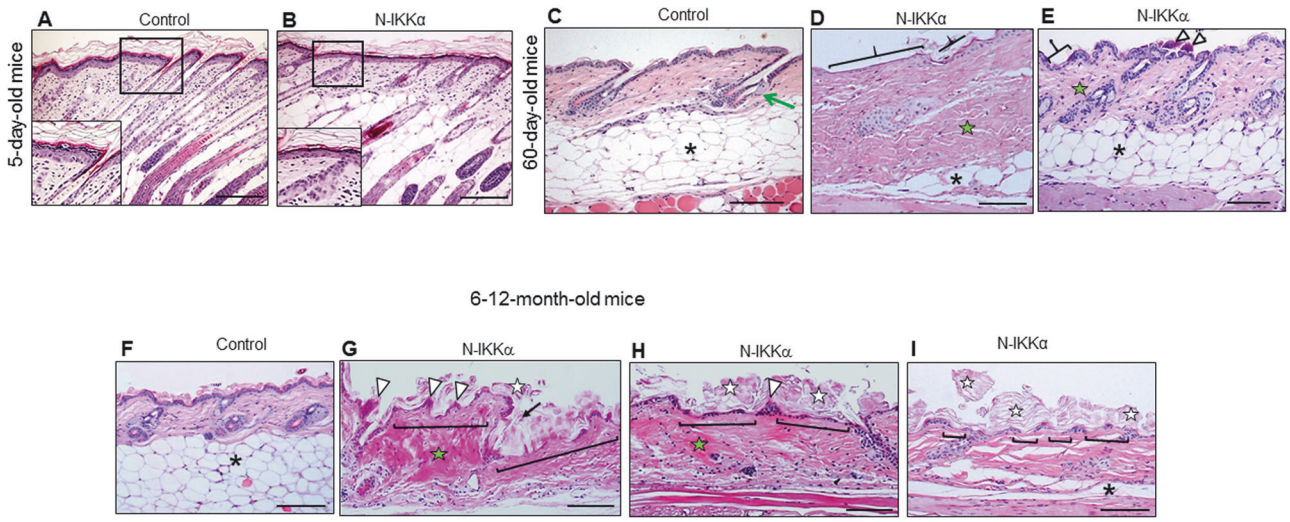


Fig. 2 Histological alterations in the back skin of N-IKKα mice. A, **B** Back skin sections of 5-day-old mice. **A** Representative image of the skin of Control mice showing the interfollicular epidermis (IFE) containing 6 layers of keratinocytes and forming soft folds versus the IFE of N-IKKα mice (**B**) that appears stretched and thinner, with only 3–4 layers of keratinocytes. Back skin of 60-day-old Control (**C**) and transgenic (**D**, **E**) mice. **C** See in skin sections of Control mice the presence of 2–4 layers of keratinocytes in the IFE, short and well differentiated telogenic HF and abundant adipose panicle (asterisk). **D**, **E** The skin of transgenic mice shows wide areas of epidermal atrophy (brackets), sclerosis of the dermis (green star), with foci of parakeratosis (arrowheads, **E**), and scarce adipose tissue (asterisk). **F–I** Representative sections of the back skin of 6-12-month-old Control (**F**) and transgenic (**G–I**) mice. **F** See in the skin of Control mice the presence of 2 cell layers of keratinocytes *and the stratum corneum* composed of anucleated, laminated keratin scales and adipose panicle is present (asterisk). **G**, **H** Severe sclerosis of the dermis (green stars), and striking hyperkeratosis (white stars). Arrowheads indicate parakeratotic foci. Note in (**G**) the extreme epidermal atrophy of the IFE (brackets), the absence of adipose panicle, and the presence of keratinocytes that experiment an abrupt transition from a flat basal layer to nucleated scale (black arrow). **I** Severe hyperkeratosis (stars), lack of adipose panicle (asterisk) and frequent regions of epidermal atrophy (brackets). 6–10 animals (males and females) of each age and genotype were analyzed. Each photo represents a different section of skin from the same mouse or different mice. Scale bars: **A**, **B**: 80 μm; **C–E**: 100 μm; **F–I**: 120 μm.

N-IKKα animals. The rate of proliferation was studied by analyzing BrdU incorporation and Ki67 immunostaining in the skin of 18-month-old mice, the age at which these mice exhibit the most intense phenotype. The results showed that, as expected, the epidermis of Control animals was sparsely proliferative, exhibiting a low number of Ki67-positive basal keratinocytes (Fig. 3A, and data not shown). By contrast, a significant number of basal and suprabasal keratinocytes proliferated in the epidermis of C-IKKα mice (Fig. 3B). Although no quantitative differences in the rate of epidermal proliferation were found in N-IKKα mice, suprabasal keratinocyte proliferation was nevertheless observed in isolated foci (Fig. 3C). The analysis of BrdU incorporation confirmed the increased number of proliferative cells only in the epidermis of C-IKKα mice (Fig. 3D). Consistent with these observations, K5 staining showed an increased number of basal and suprabasal keratinocyte layers that expressed this keratin in C-IKKα mice, as opposed to the single basal keratinocyte layer that express K5 in Control mice (Fig. 3E–G).

Examination of early epidermal differentiation by immunostaining with an anti-K10 antibody showed in the epidermis of C-IKKα mice areas of suprabasal layers of keratinocytes that expressed K5 instead of K10, which was absent (Fig. 3F, G). No changes in the expression of K5 and K10 were observed in the epidermis of N-IKKα mice (data not shown). Analysis of the expression of Filaggrin, a protein marker of epidermal terminal differentiation, showed a lower level of expression in the skin of C-IKKα mice, compared to that detected in Control mice, while it was increased in the skin of N-IKKα mice (Fig. 3H, I). To further confirm that changes in IKKα expression levels in the nucleus or cytoplasm of keratinocytes produced alterations in their capacity to differentiate, we performed in vitro differentiation assays of HaCaT human keratinocytes expressing exogenous IKKα in the nucleus or cytoplasm [19, 20]. After one week growing under serum-deprived conditions, HaCaT-Control-cells showed the first signs of differentiation, i.e., formed small domes of differentiated keratinocytes, while HaCaT-N-IKKα cells exhibited a great number of large differentiation domes and, HaCaT-C-IKKα cells did not yet show any sign of differentiation (Fig. 3J). Therefore, these results in HaCaT keratinocytes confirmed those obtained in vivo in the epidermis of N- and C-IKKα mice, indicating that nuclear expression of IKKα favored keratinocyte differentiation, while in the cytoplasm it caused defects in epidermal differentiation. Interestingly, the hyperplasia of the epidermis, the altered balance between keratinocyte proliferation and differentiation, and the aberrant expression of K5 and K10 in the epidermis of C-IKKα mice were partly reminiscent of the changes described in the skin of IKKα-deficient mice [4–6].

The analysis of the apoptotic rate in the epidermis of mice of the three genotypes by immunohistochemical staining and Western blot (WB) with an anti-Activated-Caspase 3 antibody showed no differences (data not shown).

Mechanisms involved in the development of the skin phenotype of the two types of IKKα-transgenic mice

To characterize the mechanisms leading to the development of the cutaneous phenotype in C- and N-IKKα mice, we analyzed the skin transcriptome of 6-month-old mice across the three genotypes using RNAseq. We chose this specific age because it allowed the analysis of changes in gene expression in the skin of mice that did not yet present significant alterations, since one of the objectives of the study was to determine, at the genetic level, if signs of premature aging were detected in N-IKKα animals. Gene set enrichment analysis (GSEA) revealed transcriptome changes characteristics of molecular signatures of aging in N-IKKα animals. Among them, we found reduced expression of genes that are downregulated at advanced ages, such as those involved in fatty acid metabolism, adipogenesis, oxidative phosphorylation and glycolysis (Fig. 4A; Supplementary Fig. 5A-D) [28–30]. In addition, we detected the underexpression of genes involved in myogenesis (Fig. 4A; Supplementary Fig. 5E), this inhibition being

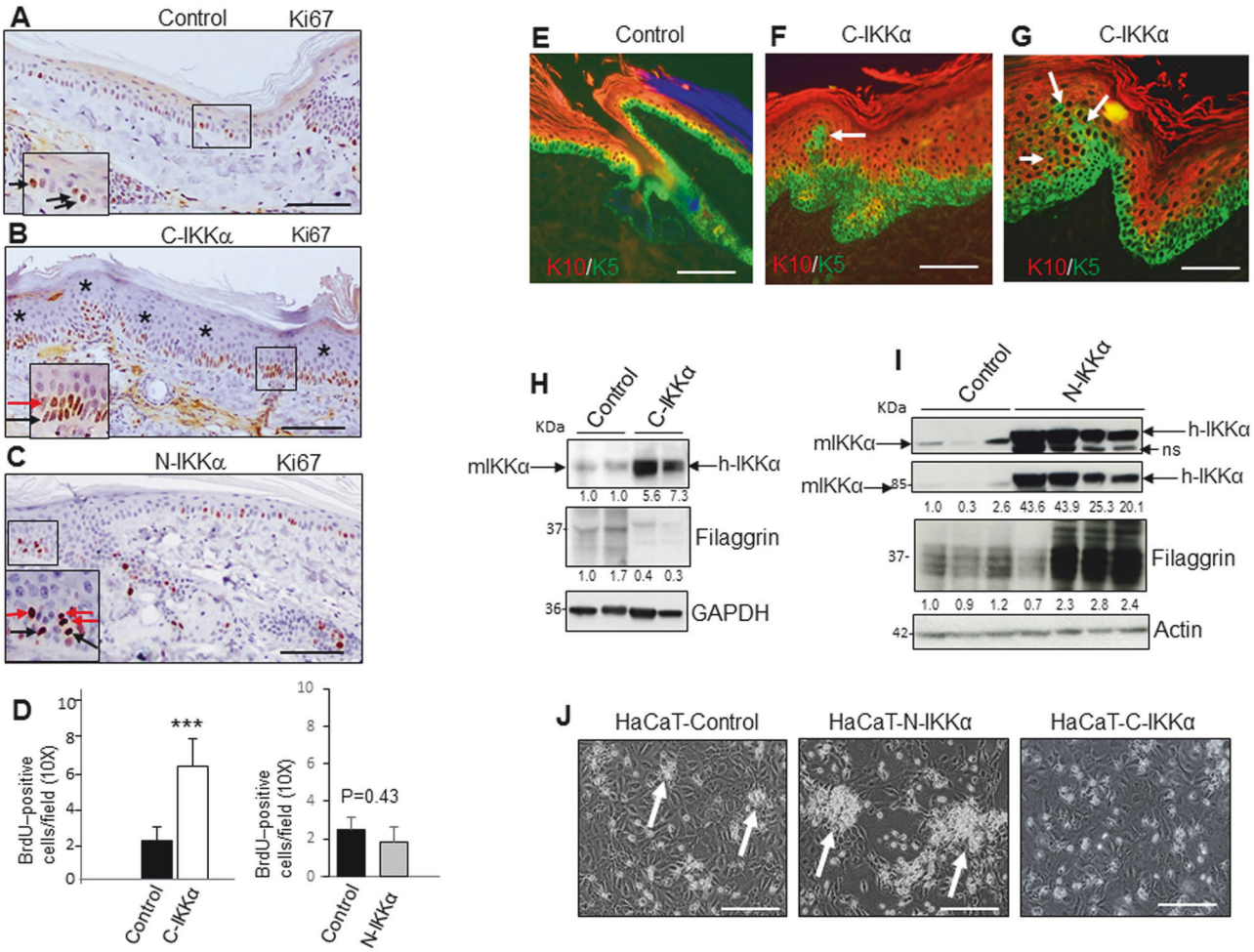


Fig. 3 Analysis of epidermal proliferation and differentiation in IKKα-transgenic mice. A–C Anti-Ki67 immunohistochemistry of tail skin sections of 18- month-old mice showing proliferating keratinocytes; $n = 6$ animals of each genotype were analyzed. **A** Scarce proliferative basal keratinocytes in the epidermis of Control mice (black arrows). **B** Increased number of proliferative basal keratinocytes (black arrow) and abundant proliferative suprabasal keratinocytes (red arrow) in C-IKKα mice. **C** Low numbers of positive basal keratinocytes (black arrows) and foci of proliferative suprabasal keratinocytes (red arrows) in N-IKKα mice. Insets: higher magnification of the section framed by the box. **D** Graph showing the significant increase in the number of proliferative keratinocytes, positive for BrdU incorporation, in the IFE of the skin of C-IKKα mice (***$p < 0.001$). Three independent researchers analyzed and counted 4–5 animals of each genotype. Double immunofluorescence of tail skin sections from Control (**E**) and C-IKKα (**F**, **G**) mice with anti-K10 (red) and anti-K5 (green) antibodies showing K5 expression in the basal layer of keratinocytes in Control mice and K10 expression in keratinocytes of the suprabasal layers (**E**). **F**, **G** Note the aberrant expression of K5 in suprabasal layers of keratinocytes from C-IKKα mice, including the most upper epidermal differentiation layers (white arrows in **F**, **G**). Note the absence of K10 in several layers of suprabasal keratinocytes in C-IKKα mice. Western blots showing decreased levels of Filaggrin expression in the skin of C-IKKα mice (**H**) and increased levels in the skin of N-IKKα mice (**I**). See in (**I**) two different times of exposition of the same hybridization with the anti-IKKα antibody: the longer exposition allows seeing the endogenous IKKα; the lower exposition was used to quantify levels of IKKα expression. $n = 8$ mice of each genotype. Anti-IKKα from Novus Biologicals (Supplementary information) that in western blot recognizes mouse and human IKKα was used. Arrows = mIKKα: mouse IKKα; hIKKα: transgenic human IKKα (C-IKKα (**H**) or N-IKKα (**I**)). **J** Day 7 of in vitro differentiation of human HaCaT keratinocytes of the three genotypes. Note the appearance of the domes of stratified HaCaT-Control cells (white arrows). Larger and more abundant domes of differentiation were formed in HaCaT-N-IKKα cells (white arrows) whereas they were not detected in HaCaT-C-IKKα cells. Scale bars: **A–C**: 150 µm, **E–G**: 70 µm; **J**: 50 µm.

characteristic in elderly mammals, in which a reduction in muscle mass and its regenerative potential occurs [31]. Taken together, these GSEA results were consistent with histological observations in the skin of N-IKKα mice, which showed a severe decrease in hypodermic adipose tissue along with other signs of accelerated skin aging. In addition, we observed an enrichment of Myc target genes in N-IKKα mice (Fig. 4A; Supplementary Fig. 5F). To further verify these data, we analyzed c-Myc expression by WB and found increased levels in the skin of N-IKKα mice (Fig. 4B), which is in agreement with previous results showing that IKKα increased c-Myc protein stability and therefore c-Myc levels [32, 33], although our data now suggest that this action is only exerted by nuclear IKKα. Both overexpression of c-Myc and the underexpression of lipid-related genes in the skin of N-IKKα mice could explain the hyperkeratosis observed in these animals, since c-Myc is induced as a compensatory mechanism to repair altered terminal epidermal differentiation [34]. In addition, defects in lipid metabolism enzymes are linked to aged skin and could predispose to the development of an aberrant *stratum corneum* [35].

GSEA also revealed that C-IKKα mouse skin showed increased enrichment of target genes of the canonical NF-κB, and STAT3 pathways (Fig. 5A; Supplementary Fig. 6A, B). We analyzed the activation of these pathways by WB and found that NF-κB signaling was overactivated in the skin of C-IKKα mice, as demonstrated by increased levels of NF-κB activation (measured as P-p65 levels) at basal conditions, in untreated skin (Fig. 5B), and

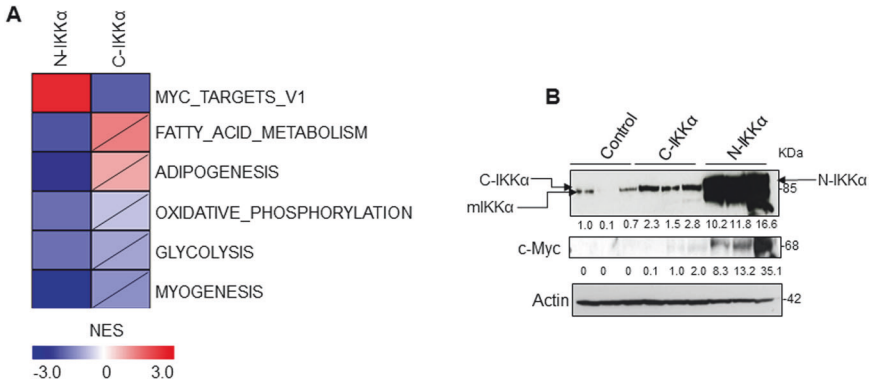


Fig. 4 Opposite regulation of fatty acid metabolism and c-Myc pathways in N-IKKα and C-IKKα mice. A GSEA analysis was performed comparing "N-IKKα versus Control skin" or "C-IKKα versus Control skin" and using the Hallmarks gene sets from the MSigDB database. 6 mice of each genotype were analyzed in the RNAseq study. Heatmap represents normalized enrichment scores (NES) of gene sets with significant deregulation (p val < 0.05 and FDR < 0.05). Hatched boxes indicate that the genes are not significantly regulated in the skin of C-IKKα versus the skin of Control mice. Positive values (red color): overexpression in transgenic mice skin (N-IKKα or C-IKKα) versus Control skin; negative values (blue color): underexpression in transgenic mice skin (N-IKKα or C-IKKα) versus Control skin. Results show down- and up-regulation of genes involved in fatty acid metabolism in the skin of N-IKKα mice and C-IKKα mice, respectively. GSEA analysis also shows downregulation of genes of adipogenesis, oxidative phosphorylation, glycolysis and myogenesis pathways in N-IKKα mice. c-Myc target genes are up- and down-regulated in the skin of N-IKKα mice and C-IKKα mice, respectively. **B** WB showing the increased expression of c-Myc in the skin of N-IKKα mice. Anti-IKKα from Novus Biologicals (Supplementary information) that in western blot recognizes mouse and human IKKα was used. Arrows = mIKKα: mouse IKKα; C-IKKα, N-IKKα: transgenic human IKKα. Actin was used as a loading control.

after stimulation with tumor necrosis factor-α (TNF-α), where activation was observed to last for longer times, i.e., up to 40 min post-inoculation of TNF-α (Fig. 5C); IKKβ and IKKγ levels showed no significant changes after TNF-α stimulation (Supplementary Fig. 7). Total levels of p65 were comparable in mice of all three genotypes (Fig. 5B) and did not change with TNF-α stimulation (Fig. 5C); an increase in the expression of IKKβ and IKKγ was observed in C-IKKα mice (Fig. 5B). No changes in the kinetics of p65 phosphorylation were detected in the skin of N-IKKα mice (Supplementary Fig. 8). To determine whether the overactivation of NF-κB in C-IKKα mice was maintained at older ages, we analyzed the kinetics of p65 phosphorylation in the skin of 20-month-old C-IKKα mice and also found higher levels of P-p65 in the skin of these old transgenic mice (Fig. 5D), also verifying that NF-κB hyperactivation increased with age (compare the densitometric data in Fig. 5C, D). Therefore, our data show that there was chronic overactivation of the canonical NF-κB pathway in the skin of C-IKKα mice and that the C-IKKα construct is functional throughout the life of C-IKKα mice.

Next, we analyzed the activation of STAT3 signaling and, consistent with the RNAseq data, found that it was increased in the skin of C-IKKα mice (no differences in total levels of STAT3 were found between mice of the three genotypes) (Fig. 5E). In agreement with the chronic activation of these two main inflammatory pathways, NF-κB and STAT3, in the skin of C-IKKα mice, GSEA analysis also showed enrichment in genes involved in the inflammatory response as well as in interferon γ (IFNγ) and IFNα responses, and host versus graft disease (Fig. 5A; Supplementary Fig. 6C, D). Accordingly, we detected infiltration of $CD45^+$ and $CD3^+$ cells in the skin of C-IKKα animals (Fig. 6A–L) and elevated levels of proinflammatory cytokine genes encoding proteins TNF-α, IL6, IL23, IL1β and IL17 (Fig. 6M). These findings were corroborated by a detailed histological analysis that showed infiltration foci of polymorphonuclear neutrophils, mainly intraepidermal or located in the ORS of HFs, in the skin (mainly in the tail and eyelids) of C-IKKα mice (Fig. 6N, O). By contrast, gene sets related to inflammatory and IFNγ and IFNα responses were significantly underexpressed in the skin of N-IKKα mice (Fig. 5A; Supplementary Fig. 6C, D). Overactivation of NF-κB and STAT3 was linked to epidermal hyperplasia and defects in terminal epidermal differentiation [36, 37], so the activation of these pathways could explain the development of the skin abnormalities seen in C-IKKα mice. Interestingly, activation of these signaling pathways as well as increased inflammation were also features of the skin of $IKKα^{-/-}$ mice [24, 38].

Remarkably, as can be seen, the GSEA showed the opposite enrichment of well-defined biological states (Hallmarks) in each type of transgenic mouse. Thus, some pathways, such as NF-κB and STAT3, and responses to inflammation, IFN-γ and IFN-α, were significantly upregulated in the skin of C-IKKα mice and downregulated in that of N-IKKα mice; and Myc target genes that were increased in the skin of N-IKKα mice appeared significantly downregulated in C-IKKα mice. Other pathways related to adipose metabolism that were significantly downregulated in the skin of N-IKKα mice had a marked tendency to be upregulated in that of C-IKKα animals.

N-IKKα mice develop squamous skin carcinomas

The histological and molecular changes in the skin of N-IKKα mice suggested that these animals could present a greater predisposition to the development of SCCs, i.e., they showed premature signs of skin aging, with aging being the main cause of NMSC development, and, furthermore, they exhibited a marked overexpression of c-Myc in the skin, which plays a causal role in the genesis of several types of murine and human tumors [39], including the development of spontaneous cutaneous SCCs in K5-cMyc transgenic mice [40]. Therefore, we analyzed mice over 18 months old for possible tumor development. Our studies showed that, in addition to the changes explained in the previous paragraphs, preneoplastic changes occurred in the back and tail skin of N-IKKα mice. These alterations included severe atypia of keratinocytes that began within the basal layer of the epidermis (Fig.7B) and extended above, in the suprabasal layers (Fig. 7C, F); these abnormal keratinocytes lost their polarity, and showed small, rounded and pyknotic nuclei arranged parallel to the basement membrane, in sharp contrast to those of Control animals that were elongated and arranged in a palisade, perpendicular to the basement membrane (compare Fig. 7A, B); these regions of severe atypia showed poor definition of the basement membrane profile (compare Fig. 7A with B, C; and 7D with E). In addition, wide areas of severe epidermal hyperplasia with elongated rete ridges were observed in the skin of N-IKKα mice (Fig. 7C). Increased number of dysplastic, severely dedifferentiated, and abortive HFs were also detected (Fig. 7F, H, I).

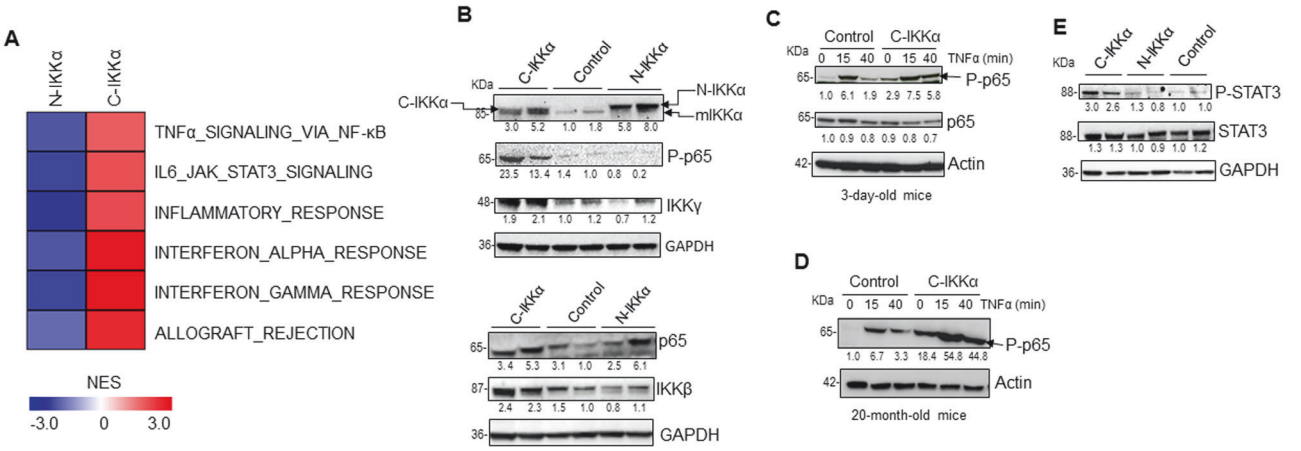


Fig. 5 Up- and down-regulation of NF-κB and STAT3 pathways and inflammation-related responses in the skin of C-IKKα and N-IKKα mice, respectively. A GSEA analysis was performed comparing "N-IKKα versus Control skin" or "C-IKKα versus Control skin" and using the Hallmarks gene sets from the MSigDB database. 6 mice of each genotype were analyzed in the RNAseq study. Heatmap represents normalized enrichment scores (NES) of gene sets with significant deregulation (p val < 0.05 and FDR < 0.05). Positive values (red color): overexpression in the skin of C-IKKα mice versus Control skin; negative values (blue color): underexpression in the skin of N-IKKα animals versus Control mice. GSEA shows the up- and down-regulation of genes of the canonical NF-κB pathway, and STAT3 pathway in the skin of C- and N-IKKα mice, respectively. GSEA analysis also shows the up- and down-regulation of genes related to inflammatory responses in the skin of C-IKKα and N-IKKα mice, respectively. **B**–**E** WBs of total protein extracts from the back skin of mice of the three genotypes. **B** Representative WB showing increased activation of NF-κB, measured as P-p65, in the skin of C-IKKα mice, in the basal state, in absence of activation, although no important changes in total p65 levels are detected. Also note the increased levels of IKKβ and IKKγ in the skin of C-IKKα animals. Anti-IKKα from Novus Biologicals (Supplementary information) that in western blot recognizes mouse and human IKKα was used. Arrows = mIKKα: mouse IKKα; C-IKKα, N-IKKα: transgenic human IKKα. **C** Augmented levels of P-p65 in the skin of 3-day-old C-IKKα mice at the indicated times after TNF-α stimulation. No relevant differences in total levels of p65 were observed. **D** Increased P-p65 levels in the skin of 20-month-old C-IKKα mice at the indicated times after TNF-α stimulation. **E** WB showing increased levels of P-STAT3 in the skin of 1-month-old C-IKKα mice. No differences in total levels of STAT3 are detected. GAPDH and Actin: loading controls.

All these changes, together with the areas of epidermal atrophy and parakeratosis, the disruption of the normal sequence of keratinocyte maturation, and the hyperkeratosis described above (Fig. 2), were reminiscent of the characteristics of the skin of patients with actinic keratosis (AK), a disease recognized as a precursor to the development of in situ SCCs (Bowen's disease) and invasive SCCs [41]. Consistent with these premalignant lesions, N-IKKα mice developed carcinomas in situ (observed in 3/24 N-IKKα versus 1/28 Control mice; $p = 0.3$) (Fig. 7J–K'), which are characterized by the cellular atypia described before associated with a high mitotic index (Fig. 7K, K') without breakage of the basal membrane; as well as skin SCCs (identified in 5/24 N-IKKα versus 0/28 Control mice; $p = 0.01$) with varying degrees of malignancy, ranging from well-differentiated to undifferentiated SCCs (Fig. 7L–N; Supplementary Fig. 9Q, R). Well differentiated SCCs developed in N-IKKα mice (see examples in Fig. 7L, M), were infiltrating, malignant tumors, composed of irregular nests or cords of keratinocytes invading the adjacent dermis (arrows in Fig. 7L) that progressively formed characteristic foci of keratin pearls, consisting of concentric laminated masses of keratin (Fig. 7M). Both carcinomas in situ and infiltrating and well differentiated SCCs retained the expression of keratins, characteristic of keratinocytes (Supplementary Fig. 9A, C, E), although a decrease in the expression of another epithelial marker, E-Cadherin, was observed in invasive SCCs (arrow in Supplementary Fig. 9F). In contrast, undifferentiated SCCs (an example is showed in Fig. 7N) that presented solid masses of anaplastic cells with spindle cell-shaped keratinocytes invading the adjacent dermis, showed minimal or absent squamous differentiation and expressed low levels of keratins or had lost the expression of epithelial markers (Supplementary Fig. 9G, H, P). We verified that the transgene was expressed in the nucleus of epithelial cells of pretumoral and tumoral lesions (Supplementary Fig. 9, I-P).

However, although chronic inflammation and increased activation of STAT3 and NF-κB signaling pathways have been linked to cancer development [42–44] C-IKKα mice did not develop cutaneous SCCs, even in very old (2.5 years) mice analyzed over several generations. Skin SCCs were also not observed in old Control mice.

Thus, our results identify in vivo the pro-oncogenic role of nuclear IKKα in keratinocytes for the development of skin SCCs. However, other studies, using different study models in which the function of nuclear IKKα in keratinocytes was not specifically analyzed, suggested that by inhibiting EGFR/ERK signaling in keratinocytes and inducing the expression of c-Myc inhibitors, such as Ovol1, through the Smad2/3 pathway, nuclear IKKα acted as a tumor suppressor of skin SCCs [14, 27]. Therefore, we analyzed these proteins in the skin of both types of IKKα-transgenic mice. Our data showed similar levels of Ovol1 in the skin of mice of all three genotypes (Supplementary Fig. 10A). Higher levels of P-Smad2/3, which have been linked to skin tumor progression [45], were detected in the skin of N-IKKα mice (Supplementary Fig. 10B, C). No significant changes in the activation of the EGFR/ERK pathway were detected in the skin of N-IKKα mice nor in that of C-IKKα animals (Supplementary Fig. 10D). The different western blots analyzed showed variability in the levels of exogenous IKKα expression between different transgenic animals (see examples in Fig. 5B and Supplementary Fig. 10A), however, despite this fact, all animals exhibited the described skin alterations, supporting that high levels of the exogenous IKKα are not needed to observe the skin phenotype.

Therefore, our results suggest that accelerated cutaneous aging, c-Myc overexpression, and increased P-Smad2/3 levels in the skin of N-IKKα mice could be responsible for the de novo development and progression of skin SCCs in these transgenic mice. However, we found that neither the EGFR/ERK pathway nor changes in Ovol1 expression were involved in the protumoral phenotype of the skin of N-IKKα mice.

Decreased endogenous IKKα expression and scarce nuclear localization of IKKα in the skin of C-IKKα mice

Skin alterations found in C-IKKα have revealed some similarities with those described in the skin of IKKα$^{-/-}$ mice, including

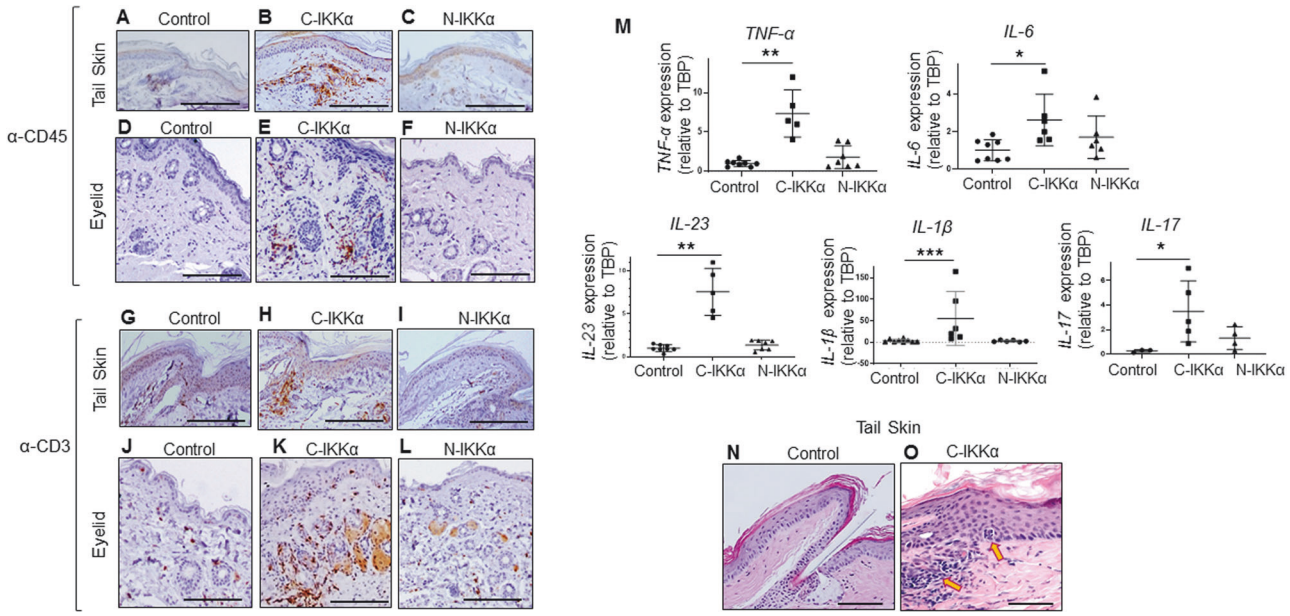


Fig. 6 Inflammation features of the skin of C-IKKα mice. A–F CD45 immunostaining showing inflammatory cell infiltration in the skin of C-IKKα mice (**B**, **E**). **G–L** CD3 immunostaining showing CD3 + T cell infiltration in the skin of C-IKKα mice (**H**, **K**). $n = 5–6$ mice of each genotype were examined (12-month-old). **M** Graphs showing increased levels of proinflammatory cytokine genes in the eyelid skin of 9 to 12-month-old C-IKKα mice. mRNA levels of expression were analyzed by qPCR analysis. Each circle, square and triangle correspond to a different animal examined. Representative sections of the tail skin of 12-month-old Control (**N**) and transgenic (**O**) mice. Arrows in O: infiltration foci of polymorphonuclear neutrophils. Scale bars: **A**, **B**, **N**: 200 μm; **C**, **D**, **F**: 50 μm; **E**, **O**: 40 μm; **G**, **H**: 100 μm; **I–L**: 200 μm.

histological resemblances and increased activation of the NF-κB and STAT3 pathways [24, 38]. These findings, together with our previous observations suggesting that keratinocytes overexpressing cytoplasmic IKKα have impeded certain IKKα nuclear functions, such as VEGF repression [20], prompted us to analyze whether there was any alteration in the expression of endogenous IKKα in keratinocytes of C-IKKα animals that could explain the similarities between the skin of our transgenic mice and that of IKKα$^{-/-}$ animals. Analysis by qPCR of the expression of endogenous *Chuk* in the skin of the three types of mice showed a marked decrease in the expression of murine *Chuk*-mRNA in the skin of C-IKKα mice compared to Control and N-IKKα mice (Fig. 8A, B). Accordingly, WB analysis showed reduced levels of endogenous IKKα protein in the skin of C-IKKα mice that further decreased with aging (Fig. 8C, D) (no reduction was seen in IKKβ or p65 levels, Supplementary Fig. 11). Immunohistochemical staining showed endogenous IKKα expression both in the nucleus and cytoplasm of keratinocytes in Control mice, with nuclear IKKα staining being mainly restricted to suprabasal keratinocytes (Fig. 8E) which was consistent with observations by Sil et al, who described the need for nuclear expression of IKKα in suprabasal keratinocytes for proper terminal epidermal differentiation to occur [25]. Nuclear and cytoplasmic IKKα expression was also detected in the epidermis of N-IKKα mice, with cytoplasmic staining being due to endogenous IKKα expression (Fig. 8F). However, in the epidermis of C-IKKα mice almost all IKKα was localized in the cytoplasm of keratinocytes, and very few suprabasal keratinocytes expressed endogenous nuclear IKKα (Fig. 8G). Therefore, these results indicate that C-IKKα mice hardly express endogenous IKKα and have almost no IKKα in the nucleus of keratinocytes. Thus, our data suggest that keratinocytes from C-IKKα and IKKα$^{-/-}$ mice lack nuclear IKKα and that this is a likely reason why they develop similar skin phenotypes. Moreover, these results further reinforce our above data showing that nuclear IKKα is not a tumor suppressor of skin SCC development, as C-IKKα mice almost lack nuclear IKKα in keratinocytes and do not develop SCCs.

DISCUSSION

We have dissected the roles that IKKα plays in vivo in non-melanoma skin cancer development through the analysis of transgenic mice expressing exogenous IKKα in the nucleus or in the cytoplasm of basal keratinocytes. Our results reveal that alterations in the level of IKKα in each subcellular localization of keratinocytes result in different cutaneous changes that, in the case of nuclear IKKα oversignaling, lead to premature skin aging, the appearance of premalignant lesions reminiscent of those of actinic keratosis patients and skin SCC development. Notably, we found that IKKα regulates in an opposite way the expression of genes essential for inflammatory and cancerous processes when localized in the nucleus or cytoplasm of keratinocytes that could ultimately determine the development or not of cutaneous SCCs.

Altered levels of nuclear and cytoplasmic IKKα in keratinocytes lead to opposite skin changes and inflammatory responses

A remarkable finding of our study is the verification that altered levels of IKKα in the nucleus or cytoplasm of keratinocytes lead to opposite cutaneous changes, i.e., atrophic epidermis with exacerbated terminal differentiation of the epidermis in N-IKKα mice versus epidermal hyperplasia with defective epidermal differentiation in C-IKKα animals. These opposite phenotypes could be the consequence of the opposite regulation of c-Myc, and NF-κB and STAT3 pathways in the skin of each type of transgenic mice. Unlike its role in other cell types in which c-Myc promotes cell proliferation, in keratinocytes c-Myc causes a progressive reduction in the growth rate (without inducing apoptosis), and a marked stimulation of terminal epidermal differentiation [46]. Thus, the skin atrophy and hyperkeratosis exhibited by N-IKKα mice could be due to c-Myc overexpression. Also, overexpression of c-Myc in actinic keratosis [47] has been described, being possible that c-Myc overexpression in the skin of N-IKKα mice lead to the development of the actinic keratosis-like premalignant lesions observed in these animals. Moreover, it might be possible that nuclear IKKα translocation/accumulation in

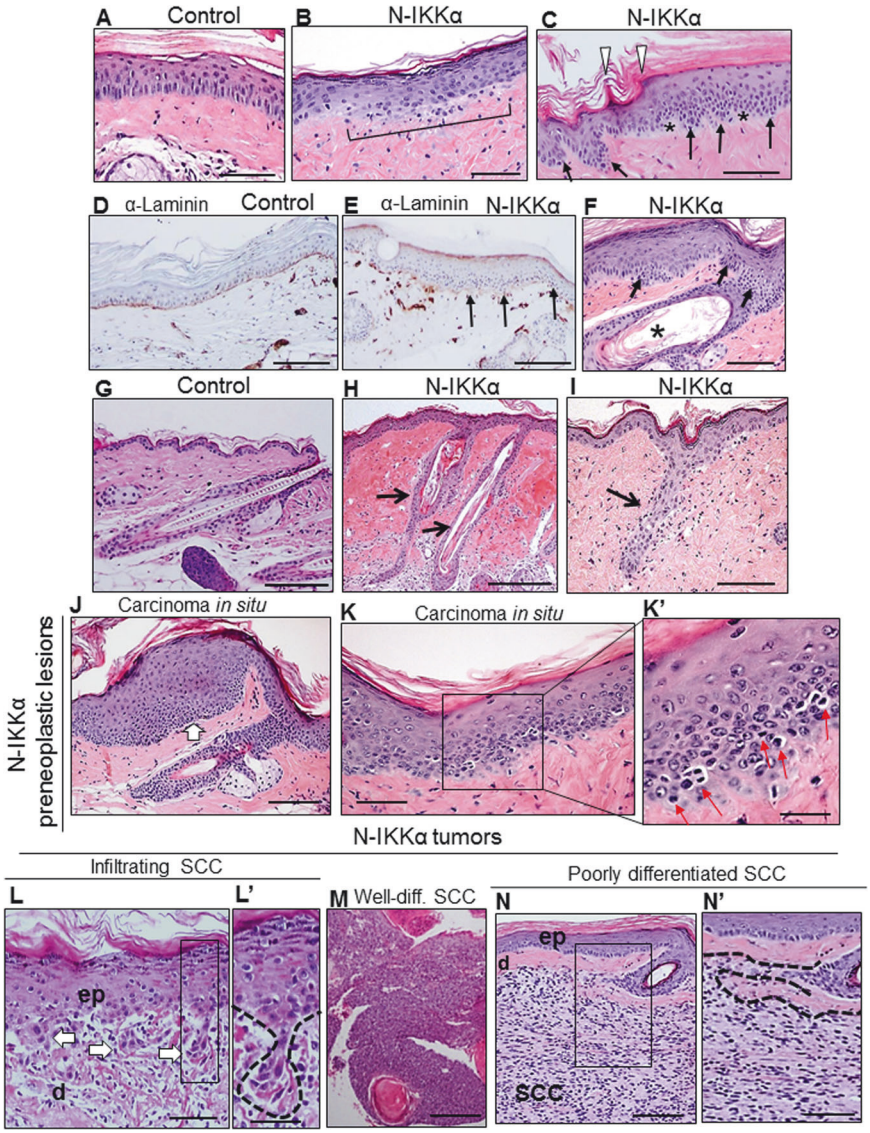


Fig. 7 Development of premalignant lesions resembling those of patients with actinic keratosis, and cutaneous squamous cell carcinomas in the skin of N-IKKα mice. Tail skin sections of Control (**A**, **D**) and transgenic (**B**, **C**, **E**, **F**) mice. $n = 8$ female and male mice of each genotype (18–24 months old). **A** Note the arrangement of the nucleus of the basal layer of keratinocytes perpendicular to the basal membrane in Control mice. **B** Representative example of loss of nuclear polarity of keratinocytes with pyknotic nuclei in the basal layer of the epidermis of transgenic mice, and basal cell atypia (bracket). **C** Indented basal layer of keratinocytes, with pseudocarcinomatous basal hyperplasia forming rete ridges (arrows), showing a clear separation with the dermis (asterisks). Observe the atypia of keratinocytes of basal and suprabasal epidermal layers. **D**, **E** Immunostaining with an anti-Laminin antibody. Observe the discontinuous pattern of expression in the basal membrane of N-IKKα mice (arrows). **F** Dysplastic and hyperkeratotic HF (asterisk) and atypia of keratinocytes which appear pyknotic (arrows). Back skin section of Control (**G**) and N-IKKα (**H**, **I**) mice. **H** Severely dysplastic HFs (arrows); (**I**) Abortive, undifferentiated and dysplastic HF (arrow). **J–K** Carcinomas in situ in the tail skin of 18-and 20-month-old transgenic mice. **K'** Amplification of the rectangle in (**K**) showing the presence of frequent mitosis in basal and suprabasal keratinocytes (red arrows). Note that the basal membrane is not yet interrupted. **L–N** Malignant tumors developed in N-IKKα mice. **L** Invasive (infiltrating) squamous cell carcinoma (SCC). Observe the erased limit of the basal membrane and how small undifferentiated tumor cells form cords that infiltrate the dermis (d) (arrows) (ep: epidermis); (**L'**): amplification of the rectangle in (**L**) showing how keratinocytes invade the dermis (area delimited by the dashed line). Observe the presence of keratinocytes with loose unions between them at the advancing front. **M** Well differentiated SCC in the back skin of a 19-month-old N-IKKα mice; note the concentric laminated masses of keratin (keratin pearl). **N** Undifferentiated SCC developed in the tail skin of a 22-month-old transgenic mouse. d: dermis, ep: epidermis. **N'** Amplification of the rectangle in (**N**) showing the contrast between the differentiated morphology of the epidermis and the dedifferentiated morphology of keratinocytes invading the dermis (area between dashed lines). Scale bars: **A**, **B**: 40 μm; **C**, **F**, **I**, **J**: 50 μm; **D**, **E**, **G**, **H**, **M**: 100 μm; **K**: 40; **K'**: 20 μm; **L**: 30 μm; **N**: 150 μm; **L'**, **N'**: 25 μm.

keratinocytes could serve as an early warning sign for the risk of developing Bowen's disease and, subsequently, SCC in certain cases. In addition, RNAseq analysis revealed the downregulation of genes involved in fatty acid metabolism and adipogenesis in the skin of N-IKKα mice, which could cause the profound changes in keratinization and the premature skin aging of these animals.

The constitutive activation of the NF-κB and STAT3 pathways in the skin of C-IKKα mice could cause a large part of their skin alterations, since the overactivation of these two pathways has been linked to epidermal hyperplasia and defects in terminal epidermal differentiation [36, 37]. Accordingly, we have found that C-IKKα mice present NF-κB overactivation from 3-day-old, and

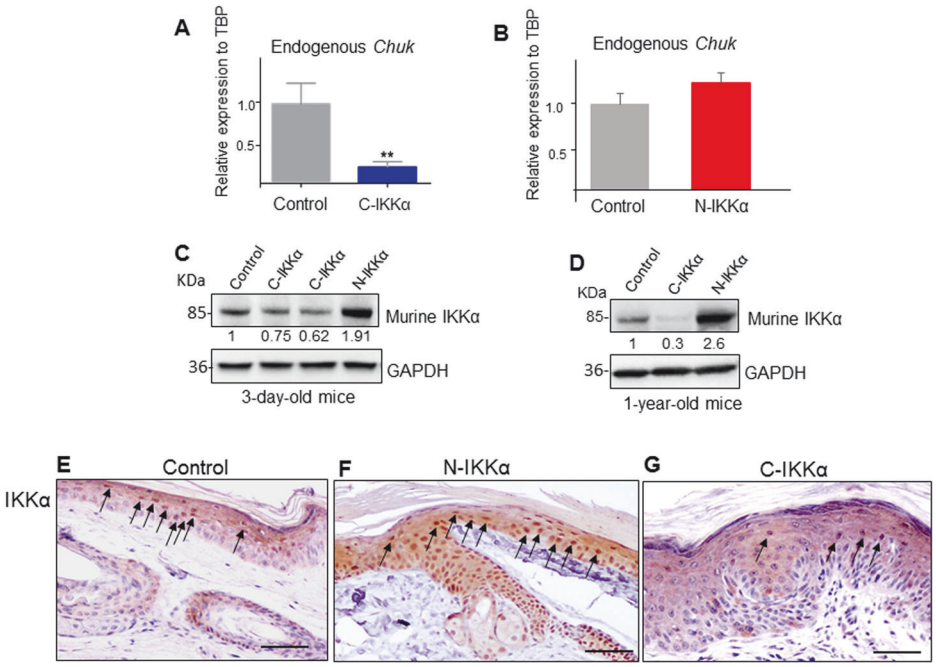


Fig. 8 Reduction of endogenous IKKα expression in the skin of C-IKKα mice. A, **B** The analysis by qPCR of the expression of murine *Chuk* gene in the skin of 1-year-old mice ($n = 4$) of the three genotypes shows a significant reduction of the endogenous *Chuk*-mRNA in C-IKKα mice. WB showing the diminished expression of murine IKKα in the skin of 3-day-old (**C**) and 1-year-old (**D**) C-IKKα mice that is even more notable with aging. $n = 3$ mice of each age and genotype were analyzed. **E–G** Immunostaining with the sc-7182 antibody that recognizes both mouse and human IKKα. Note the nuclear expression of IKKα in suprabasal keratinocytes of Control and N-IKKα and mice (**E**, **F**; black arrows), and the scarce nuclear expression of IKKα in (suprabasal) keratinocytes of C-IKKα mice (**G**). $n = 5$ mice (1-year-old) of each genotype were analyzed. Scale bars: **E–G**: 50 μm.

epidermal hyperplasia and defects in epidermal differentiation from 5-days of age. Furthermore, these transgenic mice develop papillomas, whose growth could be favored by the overexpression of VEGF in the skin of these animals that we described in a previous work [20], since VEGF promotes angiogenesis in addition to keratinocyte proliferation [48], being angiogenesis required in the earliest stages of papilloma formation [49]. Additionally, the activation of NF-κB and STAT3 pathways, and the induction of VEGF play a key role in the pathogenesis of pro-inflammatory responses, among others, NF-κB induces the expression of *IL6, IL1β* and *TNF-α* [43, 50], and STAT3 hyperactivation mediates the induction of *IL17* and *IL23* expression [51].

Other relevant data of our studies is that in addition to the enrichment in genes involved in signaling through the canonical NF-κB and STAT3 pathways in the skin of C-IKKα mice, the GSEA revealed significant increases in the expression of genes implicated in the inflammatory response and in the response to IFNγ and IFNα, while, on the contrary, these pathways were significantly repressed in the skin of N-IKKα animals. These are outstanding results as a previous work indicated that IKKα limited inflammation in macrophages through the inhibition of canonical NF-κB signaling [52]. However, our data show that, at least in keratinocytes, the role of IKKα in inflammation depends on its subcellular localization. In the cytoplasm, IKKα favors inflammation, inducing the expression of pro-inflammatory genes, while in the nucleus IKKα exerts the opposite function, limiting inflammation. Furthermore, it is worth mentioning that in contrast with the widely accepted dogma, which states that IKKα does not have a major role in NF-κB activation via the canonical pathway [53], our data show that, in keratinocytes, cytoplasmic IKKα stimulates NF-κB activation through the canonical pathway. This result agrees with recent research reporting the relevant role played by IKKα in the canonical NF-κB pathway in certain cell types, such as colorectal cells [54].

Expression of exogenous nuclear IKKα in keratinocytes induces the de novo development of skin SCCs, whereas cytoplasmic IKKα does not

Through the study of keratinocytes lacking IKKα, it was suggested that nuclear IKKα both repressed the transcription of genes encoding EGFR ligands and induced the expression of c-Myc antagonists in keratinocytes, and that the action of both mechanisms led to prevention of cell proliferation, promoting keratinocyte differentiation and preventing SCC development [14, 27]. However, whether nuclear IKKα activity in keratinocytes actually prevented skin tumor development remained to be proven. Now, our results indicate that nuclear IKKα activity in N-IKKα keratinocytes does not repress EGFR pathway activation or c-Myc expression, and, although nuclear IKKα promotes cell differentiation, it does not repress NMSC development. In contrast, our data show that nuclear IKKα induces c-Myc overexpression and leads to the development of skin SCCs. In addition, contrary to the assumption that the absence of nuclear IKKα in keratinocytes would cause SCCs development, we have discovered that loss of nuclear IKKα in keratinocytes from C-IKKα mice does not lead to SCC development, although the skin of these mice present epidermal hyperplasia and defective differentiation, which reinforces our data indicating that nuclear IKKα is not a suppressor of skin SCCs.

We also show that cutaneous SCCs developed in N-IKKα mice can be highly undifferentiated, which in this type of cancer correlates with high malignancy. This finding is consistent with studies showing that the most aggressive human skin SCCs were those expressing high levels of nuclear IKKα [22], although the mechanisms by which nuclear IKKα levels increase in these aggressive SCCs are still not known, neither is known how the subcellular localization of IKKα is normally regulated in each compartment in a normal physiological context. Our results also agree with our previous work showing that nuclear IKKα favors the

malignancy of skin tumors developed in chemical skin carcinogenesis in N-IKKα/TgAC mice [20].

Our biochemical and transcriptomic analysis suggest that c-Myc induction is the main mechanism through which nuclear IKKα signaling in keratinocytes leads to the de novo development of skin SCCs. A work demonstrated that nuclear IKKα stabilized c-Myc and enhanced its transcriptional activation [33], being c-Myc amplification considered a molecular hallmark of both the initiation and progression of cutaneous SCCs: K5-c-Myc transgenic mice, overexpressing c-Myc in basal keratinocytes, develop spontaneous skin SCCs [40] and, in humans, c-Myc amplification plays a causal role in the genesis and tumorigenic promotion of diverse tumors, including cutaneous SCCs [47, 55]. Therefore, the induction of c-Myc in the skin of N-IKKα keratinocytes could be a sufficient stimulus to trigger the development of SCCs in the skin of these mice. Activation of Smad2/3 in the skin of N-IKKα mice could contribute to the high malignancy of some of these SCCs, since Smad2/3 activation is considered a key event for tumor progression [45]. We have analyzed the expression of these proteins, IKKα, c-Myc and Smad2/3 in several human skin SCCs of different degrees of differentiation finding that the most undifferentiated and therefore more aggressive SCCs, show nuclear expression of IKKα, c-Myc and Smad2/3 while, well-to-moderately differentiated SCCs show cytoplasmic IKKα expression and do not show nuclear expression of c-Myc or Smad2/3 (Supplementary Fig. 12). These results agree with those described in other publications [22, 45, 47, 55]. We also studied the expression of activated (nuclear) STAT3 and p65, whose role in NMSC is not very clear [56–59], in these human SCCs and found nuclear expression of STAT3, and sporadically also of nuclear p65, in the poorly differentiated SCCs, while no nuclear staining of STAT3 or p65 was observed in any of the well-to-moderately differentiated SCCs analyzed (Supplementary Fig. 12).

Additionally, the development of signs of premature aging in the skin of N-IKKα mice, with its associated genetic changes, could also favor the development of skin SCCs, since NMSC mainly arises in the elderly population [9]. We will investigate whether the downregulation of genes related to the inflammatory response in the skin of N-IKKα mice also contributes to the formation of skin SCCs, since under normal conditions acute inflammation can promote cancer cell death by inducing an antitumor response [60]. Similarly, we will analyze whether increased inflammation in the skin of C-IKKα mice is involved in the prevention of SCC development in these transgenic mice, even though they have increased expression of VEGF in the skin and occasionally they develop papillomas. The absence of cutaneous SCC development in C-IKKα mice could also be influenced by elevated IKKβ levels and chronic activation of the canonical NF-κB pathway that they present in the skin, as our group found that K5-IKKβ mice, which overexpress IKKβ in basal keratinocytes, did not develop cutaneous SCCs and were also resistant to the development of SCCs in skin carcinogenesis assays [61, 62]. In addition, our data showing that C-IKKα mice hardly express IKKα in the nucleus of keratinocytes and present phenotypic and molecular changes similar to those observed in the skin of IKKα$^{-/-}$ mice [including their increased susceptibility to the development of papillomas [15], activation of the NF-κB [24] and STAT3 pathways and inflammation [38]] are particularly interesting since suggests that some of the skin abnormalities observed in IKKα-deficient mice could be due to the absence of nuclear IKKα in keratinocytes. It will be of interest to unravel the mechanisms through which the exogenous expression of cytoplasmic -but not nuclear IKKα- leads to the silencing of endogenous IKKα in keratinocytes. In this regard, it is worth mentioning that silencing of endogenous IKKα due to expression of exogenous IKKα has been previously reported: downregulation of murine IKKα was described in B cells from transgenic mice expressing the mutant IKKα^{KAKA}; and furthermore, the authors specifically demonstrated the marked decrease in murine nuclear IKKα in the B cells of these transgenic mice [63]. We also observed a decreased expression of endogenous *Chuk*-mRNA in the skin of K5-mIKKα mice expressing wild-type exogenous murine IKKα ([21] and unpublished results).

Altogether, our data reveal relevant information about the functions that IKKα plays in the nucleus or in the cytoplasm of keratinocytes, and, importantly, they show that in each subcellular localization IKKα plays almost opposite roles in essential processes such as cell proliferation and differentiation, inflammation, and skin SCC development. This implies the need to study the functions of IKKα, separately, in each subcellular compartment of each cell type. Otherwise, mistakes and misinterpretations will be made.

Our findings here show that increased nuclear IKKα signaling in keratinocytes leads to the initiation of skin SCCs, often of high malignancy. Previous data had shown that nuclear IKKα was necessary for the progression of cutaneous SCCs [20, 22]: our group and others showed the increased aggressiveness of skin SCCs expressing high levels of nuclear IKKα, i.e., tumors arisen in skin chemical carcinogenesis experiments in N-IKKα/TgAC mice (which carried activated *Ha-Ras* in keratinocytes) were more aggressive than those developed in Control/TgAC mice [20], and human cutaneous SCCs that exhibited higher levels of IKKα in the nucleus were at highest risk of malignancy [22]. This could suggest that nuclear IKKα would be a useful therapeutic target for the treatment of skin SCCs, but for this application it would be necessary to determine whether nuclear IKKα is also required for the maintenance of the advanced state of skin SCCs. This will be addressed in future studies. Our data showing that increased levels of nuclear IKKα in keratinocytes drive the development of skin SCCs highlight the relevance of testing the expression levels of IKKα using knock-in models to establish the physiological functions of cytoplasmic and nuclear IKKα in different types of cells.

METHODS

Mice employed

The generation of C-IKKα and N-IKKα mice has been described [20]. Cloning of the K5-C-and N-IKKα constructs was performed as follows: For N-IKKα, an additional NLS sequence (DPKKKRKV), followed by a FLAG Tag (DYDDDDKA), were added after the initial methionine by PCR. For C-IKKα, the internal NLS sequence EKIKKKDPK found in exon 8 of the human *CHUK* gene [25] was modified by directed mutagenesis, changing AAGAAGAAG (KKK) to GCGGCAGCG (AAA), and a FLAG Tag was added in 5′ by PCR. All constructs were verified by sequencing. C-IKKα and N-IKKα mice used, female and male, are in FVB/N genetic background.

HaCaT cell culture and in vitro differentiation

Human HaCaT keratinocytes were kindly provided by Dr NE Fusenig (German Cancer Research Center (DKFZ), Germany), who generated the cell line. HaCaT cells were cultured in DMEM with 10%FCS and 0.4 mg/ml G418. HaCaT-N-IKKα, HaCaT-C-IKKα and HaCaT-Control cells were previously described [20], and checked for mycoplasma using Venor®GeM One Step (Minerva Biolabs, Berlin,Germany). To allow in vitro differentiation, cells were seeded and after reaching 60–80% confluence were grown in culture medium without FCS for 7 days, changing the medium every 2 days. Experiments were performed with two different clones of HaCaT cells overexpressing N-IKKα or C-IKKα constructs, or the empty plasmid in the case of HaCaT-Control cells. Differentiation assays in HaCaT cells were performed in triplicate on 3 different days.

BrdU labeling

Mice received an intraperitoneal injection of BrdU 120 mg/kg body weight 1 h before sample harvesting. BrdU incorporation was detected by immunohistochemistry of paraffin-embedded sections using an anti-BrdU monoclonal antibody (Abcam, Cambridge, UK; ab6326).

Histology and immunohistochemistry

Murine organs were fixed in 10% buffered formalin and embedded in paraffin. Sections were stained with H&E and histopathological evaluation was performed by experimented pathologists (AB, MJFA, and RAGF).

Information on the antibodies used is provided in Supplementary information. Immunohistochemical analysis were performed by three different investigators in a blinded manner. At least 5 mice (6-8 randomized images per mice) of each genotype were analyzed.

Western blot analysis

Protein extracts were obtained from pieces of back skin from mice of the three genotypes. Total protein extracts (40 µg) were subjected to SDS/PAGE and probed with primary antibodies. Proteins were visualized with the Supersignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Inc., Illinois, USA). Densitometric analysis was performed with Quantity One 1-D-Analysis software (Bio-Rad). Information on the antibodies used is provided in Supplementary information. Western blots were performed at least 3 times (with at least 2 replicates) with protein extracts from different mice and litters.

RNA isolation and quantitative RT-qPCR

Total RNA from back skin of male and female mice of the three genotypes was isolated using miRNeasy Mini Kit (Qiagen) and DNA was eliminated with an Rnase-Free DNase Set (Qiagen) according to the manufacturer's instructions. The reverse transcription reaction was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) for mRNA. Quantitative qRT-PCR was performed in a 7500 Fast Real Time PCR System using GoTaq qPCR Master Mix (Promega) for mRNA. The sequences of the oligonucleotides specific for mouse *IKKα* and cytokines used are listed in Supplementary Table 1. *TBP* was used for normalization of gene expression. Fold changes were calculated using the formula 2(log2B −log2A), being A the expression level of control skin and B the expression levels of skin from C-IKKα and N-IKKα mice.

RNA sequencing and analysis

RNA sequencing was performed after poly-A selection and using Illumina NovaSeq 2 × 150 bp, reads were aligned to GRCm39 mouse genome with "Rbowtie2", counts extracted with feature Counts function from "Rsubread" and gencode annotation release 27 (gencode.vM27.annotation.gft). Enrichment analysis was performed using Gene Set Enrichment Analysis (GSEA). Normalized counts were used to run comparisons between "N-IKKα versus Control skin" or "C-IKKα versus Control skin", and using well-defined biological states or processes genes sets (Hallmarks) of the MSigDB database from GSEA webpage. Significant deregulated expression of individual gene sets was considered when both p val < 0.05 and FDR < 0.05 threshold was found. $n = 6$ skin samples from the back of 6-month-old mice of each genotype, both males and females, were used in the study.

Quantification and statistical analysis

Sample size was estimated based on previous experience with the experimental approaches. Mice were randomly assigned to experimental groups without exclusions. Males and female mice of the different ages specified in each figure legend were used. Control and transgenic IKKα littermates of different litters were employed. Age and number of mice analyzed per experiment are indicated in each figure legend.

For proliferation assays, p values were determined by using the unpaired, two-tailed Student' t test. Statistical analysis of qPCR data was determined using unpaired two-tailed Student´t test for results that followed a normal distribution when they had equal variances; for different variances unpaired t test with Welch's correction; and, for the results that did not follow a normal distribution, the Mann–Whitney test. In all cases, p values < 0.05 were considered significant and data are expressed as means ± SEM ± SD.

Study approval

All animal experimental procedures were performed according to European and Spanish laws and regulations (2007/526/CE) and approved by the Ethics Committee for Animal Welfare of CIEMAT (OEBA) and by the legal authority "Consejería de Medio Ambiente y Ordenación del Territorio y Sostenibilidad" (protocol code PROEX182/15 and PROEX 297.2/20). Mice were housed at the CIEMAT laboratory Animal Facility (registration number ES280790000183). Human SCCs paraffin-samples (ref. 21011) were obtained from the Biobank of the Hospital Clínico San Carlos (Madrid) (registration number B.0000725). The Research Committee of the hospital approved the use of the samples in experiments (C.I. 19/146-E_BS) and prior informed consent was obtained from the patients.

DATA AVAILABILITY

All the RNAseq data have been uploaded to Gene Expression Omnibus (GEO) database; ID: GSE239533. Data generated or analyzed during this study are included in this published article and its supplementary information files, or available from the corresponding author on reasonable request.

REFERENCES

1. Hayden MS, Ghosh S. Signaling to NF-kappaB. Genes Dev. 2004;18:2195–224.
2. Li Q, Van Antwerp D, Mercurio F, Lee KF, Verma IM. Severe liver degeneration in mice lacking the IkappaB kinase 2 gene. Science. 1999;284:321–5.
3. Rudolph D, Yeh WC, Wakeham A, Rudolph B, Nallainathan D, Potter J, et al. Severe liver degeneration and lack of NF-κB activation in NEMO/IKKγ-deficient mice. Genes Dev. 2000;14:854–62.
4. Hu Y, Baud V, Delhase M, Zhang P, Deerinck T, Ellisman M, et al. Abnormal morphogenesis but intact IKK activation in mice lacking the IKKalpha subunit of IkappaB kinase. Science. 1999;284:316–20.
5. Li Q, Lu Q, Hwang JY, Buscher D, Lee KF, Izpisua-Belmonte JC, et al. IKK1-deficient mice exhibit abnormal development of skin and skeleton. Genes Dev. 1999;13:1322–8.
6. Takeda K, Takeuchi O, Tsujimura T, Itami S, Adachi O, Kawai T, et al. Limb and skin abnormalities in mice lacking IKKalpha. Science. 1999;284:313–6.
7. Fuchs EBC. The epidermis: rising to the surface. Curr Opin Genet Dev. 1994;4:725–36.
8. Ciążyńska M, Kamińska-Winciorek G, Lange D, Lewandowski B, Reich A, Sławińska M, et al. The incidence and clinical analysis of non-melanoma skin cancer. Sci Rep. 2021;11:4337.
9. Madan V, Lear JT, Szeimies RM. Non-melanoma skin cancer. Lancet. 2010;375:673–85.
10. Christenson LJ, Borrowman TA, Vachon CM, Tollefson MM, Otley CC, Weaver AL, et al. Incidence of basal cell and squamous cell carcinomas in a population younger than 40 years. Jama. 2005;294:681–90.
11. Moller R, Reymann F, Hou-Jensen K. Metastases in dermatological patients with squamous cell carcinoma. Arch Dermatol. 1979;115:703–5.
12. Sesto A, Navarro M, Burslem F, Jorcano JL. Analysis of the ultraviolet B response in primary human keratinocytes using oligonucleotide microarrays. Proc Natl Acad Sci USA. 2002;99:2965–70.
13. Liu B, Park E, Zhu F, Bustos T, Liu J, Shen J, et al. A critical role for I kappaB kinase alpha in the development of human and mouse squamous cell carcinomas. Proc Natl Acad Sci USA. 2006;103:17202–7.
14. Liu B, Xia X, Zhu F, Park E, Carbajal S, Kiguchi K, et al. IKKalpha is required to maintain skin homeostasis and prevent skin cancer. Cancer Cell. 2008;14:212–25.
15. Park E, Zhu F, Liu B, Xia X, Shen J, Bustos T, et al. Reduction in IkappaB kinase alpha expression promotes the development of skin papillomas and carcinomas. Cancer Res. 2007;67:9158–68.
16. Marinari B, Moretti F, Botti E, Giustizieri ML, Descargues P, Giunta A, et al. The tumor suppressor activity of IKKalpha in stratified epithelia is exerted in part via the TGF-beta antiproliferative pathway. Proc Natl Acad Sci USA. 2008;105:17091–6.
17. Xia X, Park E, Fischer SM, Hu Y. Mouse genetic models reveal surprising functions of IkB kinase alpha in skin development and skin carcinogenesis. Cancers. 2013;5:170–83.
18. García-García VA, Alameda JP, Page A, Mérida-García A, Navarro M, Tejero A, et al. IKKα induces epithelial-mesenchymal changes in mouse skin carcinoma cells that can be partially reversed by apigenin. Int J Mol Sci. 2022;23:1375–92.
19. Moreno-Maldonado R, Ramirez A, Navarro M, Fernandez-Acenero MJ, Villanueva C, Page A, et al. IKKalpha enhances human keratinocyte differentiation and determines the histological variant of epidermal squamous cell carcinomas. Cell Cycle. 2008;7:2021–9.
20. Alameda JP, Gaspar M, Ramirez A, Navarro M, Page A, Suarez-Cabrera C, et al. Deciphering the role of nuclear and cytoplasmic IKKalpha in skin cancer. Oncotarget. 2016;7:29531–47.
21. Alameda JP, Moreno-Maldonado R, Fernandez-Acenero MJ, Navarro M, Page A, Jorcano JL, et al. Increased IKKalpha expression in the basal layer of the epidermis of transgenic mice enhances the malignant potential of skin tumors. PLoS One. 2011;6:e21984.
22. Toll A, Margalef P, Masferrer E, Ferrándiz-Pulido C, Gimeno J, Pujol RM, et al. Active nuclear IKK correlates with metastatic risk in cutaneous squamous cell carcinoma. Arch Dermatol Res. 2015;307:721–9.
23. Huang WC, Hung MC. Beyond NF-κB activation: nuclear functions of IκB kinase α. J Biomed Sci. 2013;20:3.
24. Hu Y, Baud V, Oga T, Kim KI, Yoshida K, Karin M. IKKalpha controls formation of the epidermis independently of NF-kappaB. Nature. 2001;410:710–4.
25. Sil AK, Maeda S, Sano Y, Roop DR, Karin M. IkappaB kinase-alpha acts in the epidermis to control skeletal and craniofacial morphogenesis. Nature. 2004;428:660–4.
26. Descargues P, Sil AK, Karin M. IKKalpha, a critical regulator of epidermal differentiation and a suppressor of skin cancer. Embo J. 2008;27:2639–47.

27. Park E, Liu B, Xia X, Zhu F, Jami WB, Hu Y. Role of IKKα in skin squamous cell carcinomas. Fut Oncol. 2011;7:123–34.
28. Lee CK, Klopp RG, Weindruch R, Prolla TA. Gene expression profile of aging and its retardation by caloric restriction. Science. 1999;285:1390–3.
29. de Magalhães JP, Curado J, Church GM. Meta-analysis of age-related gene expression profiles identifies common signatures of aging. Bioinforma (Oxf, Engl). 2009;25:875–81.
30. Yang J, Huang T, Petralia F, Long Q, Zhang B, Argmann C, et al. Synchronized age-related gene expression changes across multiple tissues in human and the link to complex diseases. Sci Rep. 2015;5:1–16.
31. Kimmel JC, Yi N, Roy M, Hendrickson DG, Kelley DR. Differentiation reveals latent features of aging and an energy barrier in murine myogenesis. Cell Rep. 2021;35:109046.
32. Yeh P-Y, Lu Y-S, Ou D-L, Cheng A-L. IκB kinases increase Myc protein stability and enhance progression of breast cancer cells. Mol Cancer. 2011;10:1–12.
33. Moser B, Hochreiter B, Basílio J, Gleitsmann V, Panhuber A, Pardo-Garcia A, et al. The inflammatory kinase IKKα phosphorylates and stabilizes c-Myc and enhances its activity. Mol Cancer. 2021;20:16.
34. Schmuth M, Gruber R, Elias PM, Williams ML. Ichthyosis update: towards a function-driven model of pathogenesis of the disorders of cornification and the role of corneocyte proteins in these disorders. Adv Dermatol. 2007;23:231–56.
35. Knox S, O'Boyle NM. Skin lipids in health and disease: a review. Chem Phys Lipids. 2021;236:1–14.
36. Sławińska M, Lakomy J, Biernat W, Sokołowska-Wojdyło M, Karczewska J, Zabłotna M, et al. STAT3, STAT5A, STAT5B and STAT6 proteins are overexpressed in human basal cell carcinoma. Clin Exp Dermatol. 2020;45:165–71.
37. Weng H, Deng Y, Xie Y, Liu H, Gong F. Expression and significance of HMGB1, TLR4 and NF-κB p65 in human epidermal tumors. BMC Cancer. 2013;13:1–10.
38. Liu B, Willette-Brown J, Liu S, Chen X, Fischer SM, Hu Y. IKKalpha represses a network of inflammation and proliferation pathways and elevates c-Myc antagonists and differentiation in a dose-dependent manner in the skin. Cell Death Differ. 2011;18:1854–64.
39. Facchini LM, Penn LZ. The molecular role of Myc in growth and transformation: recent discoveries lead to new insights. FASEB J. 1998;12:633–51.
40. Rounbehler RJ, Schneider-Broussard R, Conti CJ, Johnson DG. Myc lacks E2F1's ability to suppress skin carcinogenesis. Oncogene. 2001;20:5341–9.
41. Smoller BR. Squamous cell carcinoma: from precursor lesions to high-risk variants. Mod Pathol. 2006;19:S88–92.
42. Grivennikov SI, Karin M. Dangerous liaisons: STAT3 and NF-kappaB collaboration and crosstalk in cancer. Cytokine Growth Factor Rev. 2010;21:11–9.
43. Fan Y, Mao R, Yang J. NF-κB and STAT3 signaling pathways collaboratively link inflammation to cancer. Protein cell. 2013;4:176–85.
44. Taniguchi K, Karin M. NF-κB, inflammation, immunity and cancer: coming of age. Nat Rev Immunol. 2018;18:309–24.
45. Kocic J, Bugarski D, Santibanez JF. SMAD3 is essential for transforming growth factor-β1-induced urokinase type plasminogen activator expression and migration in transformed keratinocytes. Eur J Cancer. 2012;48:1550–7.
46. Watt FM, Frye M, Benitah SA. MYC in mammalian epidermis: how can an oncogene stimulate differentiation? Nat Rev Cancer. 2008;8:234–42. https://doi.org/10.1038/nrc2328.
47. Toll A, Salgado R, Yébenes M, Martín-Ezquerra G, Gilaberte M, Baró T, et al. MYC gene numerical aberrations in actinic keratosis and cutaneous squamous cell carcinoma. Br J Dermatol. 2009;161:1112–8.
48. Johnson KE, Wilgus TA. Multiple roles for VEGF in non-melanoma skin cancer: angiogenesis and beyond. J Ski Cancer. 2012;2012:1–6.
49. Bolontrade MF, Stern MC, Binder RL, Zenklusen JC, Gimenez-Conti IB, Conti CJ. Angiogenesis is an early event in the development of chemically induced skin tumors. Carcinogenesis. 1998;19:2107–13.
50. Liu T, Zhang L, Joo D, Sun SC. NF-κB signaling in inflammation. Signal Transduct Target Ther. 2017;2:1–9.
51. Calautti E, Avalle L, Poli V. Psoriasis: A STAT3-centric view. Int J Mol Sci. 2018;19:171–84.
52. Lawrence T, Bebien M, Liu GY, Nizet V, Karin M. IKKalpha limits macrophage NF-kappaB activation and contributes to the resolution of inflammation. Nature. 2005;434:1138–43.
53. Israël A. The IKK complex, a central regulator of NF-kappaB activation. Cold Spring Harb Perspect Biol. 2010;2:a000158.
54. Prescott JA, Balmanno K, Mitchell JP, Okkenhaug H, Cook SJ. IKKα plays a major role in canonical NF-κB signalling in colorectal cells. Biochem J. 2022;479:305–25.
55. Little CD, Nau MM, Carney DN, Gazdar AF, Minna JD. Amplification and expression of the c-myc oncogene in human lung cancer cell lines. Nature. 1983;306:194–6.
56. Yin W, Cheepala S, Roberts JN, Syson-Chan K, DiGiovanni J, Clifford JL. Active Stat3 is required for survival of human squamous cell carcinoma cells in serum-free conditions. Mol Cancer. 2006;5:15.
57. Tolomeo M, Cascio A. The multifaced role of STAT3 in cancer and its implication for anticancer therapy. Int J Mol Sci. 2021;22:603–27.
58. Kim C, Pasparakis M. Epidermal p65/NF-κB signalling is essential for skin carcinogenesis. EMBO Mol Med. 2014;6:970–83.
59. van Hogerlinden M, Rozell BL, Ahrlund-Richter L, Toftgard R. Squamous cell carcinomas and increased apoptosis in skin with inhibited Rel/nuclear factor-kappaB signaling. Cancer Res. 1999;59:3299–303.
60. Ma Y, Adjemian S, Mattarollo SR, Yamazaki T, Aymeric L, Yang H, et al. Anticancer chemotherapy-induced intratumoral recruitment and differentiation of antigen-presenting cells. Immunity. 2013;38:729–41.
61. Page A, Bravo A, Suarez-Cabrera C, Alameda JP, Casanova ML, et al. IKKbeta-mediated resistance to skin cancer development Is Ink4a/Arf-dependent. Mol Cancer Res. 2017;15:1255–64.
62. Page A, Navarro M, Garin M, Perez P, Casanova ML, Moreno R, et al. IKKbeta leads to an inflammatory skin disease resembling interface dermatitis. J Invest Dermatol. 2010;130:1598–610.
63. Balkhi MY, Willette-Brown J, Zhu F, Chen Z, Liu S, Guttridge DC, et al. IKKα-mediated signaling circuitry regulates early B lymphopoiesis during hematopoiesis. Blood. 2012;119:5467–77.

ACKNOWLEDGEMENTS

We thank Federico Sánchez-Sierra, Pilar Hernández and Adrián Tejero (CIEMAT) for technical assistance; Jesús Martínez, Edilia Almeida and the personnel of the CIEMAT Animal Unit for care of the mice used. This work has been supported by the Instituto de Salud Carlos III/FEDER and by the Fundación de Investigación Biomédica Hospital 12 de Octubre (FIBH12O). The graphical abstract was elaborated with BioRender.

AUTHOR CONTRIBUTIONS

VAGG and JPA conducted most of the experiments with assistance from AP, RMG, MN, AR and MLC. VAGG, AP, JMP, RAGF, MJFA, MN, AB and MLC analyzed data. MN, VAGG and MLC designed research studies. RGE, MN, JMP and MLC performed genomic analysis. Writing the manuscript: MLC. AB, RAGF and MJFA wrote histological results. Revising the manuscript: MN, RGE, JMP, AR, RAGF, AB, MLC. The order of the co–first authors was assigned based on their efforts and contributions to the study.

FUNDING

This research was supported by grants from the Spanish Government and co-financed by Fondo Europeo de Desarrollo Regional (FEDER): PI19/01262 and PI23/00554 from the Instituto de Salud Carlos III (ISCIII) to MLC; PI20/01173 from the Instituto de Salud Carlos III to AR and MN; PI21/00208 to RGE; and FEDER cofounded MINECO grant PID2019-110758RB-I00 and Instituto de Salud Carlos III, CIBERONC n° CB16/12/00228 to JMP. It was also funded by the Fundación de Investigación Biomédica Hospital 12 de Octubre (FIBH12O) (i + 12), projects i12-AY201228-1/2021-0042 and i12-AY220-114-1/2022/0060 to MLC. This work was partially supported by the computing facilities of Extremadura Research Center for Advanced Technologies (CETA_CIEMAT), funded by the European Regional Development Fund (ERDF). CETA-CIEMAT belongs to CIEMAT and the Government of Spain. RMG is a recipient of an ISCII/HFIBH12O contract.

COMPETING INTERESTS

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41388-024-03203-0.

Correspondence and requests for materials should be addressed to M. Llanos Casanova.

[1]Molecular and Translational Oncology Unit, Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (CIEMAT), Avda. Complutense 40, 28040 Madrid, Spain. [2]Biomedical Research Institute, University Hospital "12 de Octubre", 28041 Madrid, Spain. [3]Centro de Investigación Biomédica en Red de Cáncer (CIBERONC), 28029 Madrid, Spain. [4]Pathological Anatomy Hospital Clínico San Carlos, 28040 Madrid, Spain. [5]Department of Animal Medicine and Surgery, Facultad de Veterinaria, UCM, 28040 Madrid, Spain. [6]Department of Anatomy, Animal Production and Veterinary Clinical Sciences, Faculty of Veterinary Medicine, University of Santiago de Compostela, Lugo, Spain. [7]These authors contributed equally: Verónica A. García-García, Josefa P. Alameda. ✉email: llanos.casanova@ciemat.es