

# The Pivotal Role of IKK $\alpha$ in the Development of Spontaneous Lung Squamous Cell Carcinomas

Zuoxiang Xiao,<sup>1,6</sup> Qun Jiang,<sup>1,6</sup> Jami Willette-Brown,<sup>1</sup> Sichuan Xi,<sup>2</sup> Feng Zhu,<sup>1</sup> Sandra Burkett,<sup>3</sup> Timothy Back,<sup>1</sup> Na-Young Song,<sup>1</sup> Mahesh Datla,<sup>1</sup> Zhonghe Sun,<sup>4</sup> Romina Goldszmid,<sup>1</sup> Fanching Lin,<sup>1</sup> Travis Cohoon,<sup>5</sup> Kristen Pike,<sup>4</sup> Xiaolin Wu,<sup>4</sup> David S. Schrupp,<sup>2</sup> Kwok-Kin Wong,<sup>5</sup> Howard A. Young,<sup>1</sup> Giorgio Trinchieri,<sup>1</sup> Robert H. Wilttrout,<sup>1,\*</sup> and Yinling Hu<sup>1,\*</sup>

<sup>1</sup>Cancer and Inflammation Program

<sup>2</sup>Thoracic Oncology Section, Surgery Branch

<sup>3</sup>Mouse Cancer Genetics Program

Center for Cancer Research, National Cancer Institute, Frederick, MD 21701, USA

<sup>4</sup>Laboratory of Molecular Technology, Frederick National Laboratory for Cancer Research, SAIC-Frederick, Inc., Frederick, MD 21702, USA

<sup>5</sup>Department of Medical Oncology, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115, USA

<sup>6</sup>These authors contributed equally to this work

\*Correspondence: [wilttrout@mail.nih.gov](mailto:wilttrout@mail.nih.gov) (R.H.W.), [huy2@mail.nih.gov](mailto:huy2@mail.nih.gov) (Y.H.)

<http://dx.doi.org/10.1016/j.ccr.2013.03.009>

## SUMMARY

Here, we report that kinase-dead IKK $\alpha$  knockin mice develop spontaneous lung squamous cell carcinomas (SCCs) associated with IKK $\alpha$  downregulation and marked pulmonary inflammation. IKK $\alpha$  reduction upregulated the expression of p63, Trim29, and keratin 5 (K5), which serve as diagnostic markers for human lung SCCs. IKK $\alpha^{\text{low}}\text{K5}^{\text{+}}\text{p63}^{\text{hi}}$  cell expansion and SCC formation were accompanied by inflammation-associated deregulation of oncogenes, tumor suppressors, and stem cell regulators. Reintroducing transgenic K5.IKK $\alpha$ , depleting macrophages, and reconstituting irradiated mutant animals with wild-type bone marrow (BM) prevented SCC development, suggesting that BM-derived IKK $\alpha$  mutant macrophages promote the transition of IKK $\alpha^{\text{low}}\text{K5}^{\text{+}}\text{p63}^{\text{hi}}$  cells to tumor cells. This mouse model resembles human lung SCCs, sheds light on the mechanisms underlying lung malignancy development, and identifies targets for therapy of lung SCCs.

## INTRODUCTION

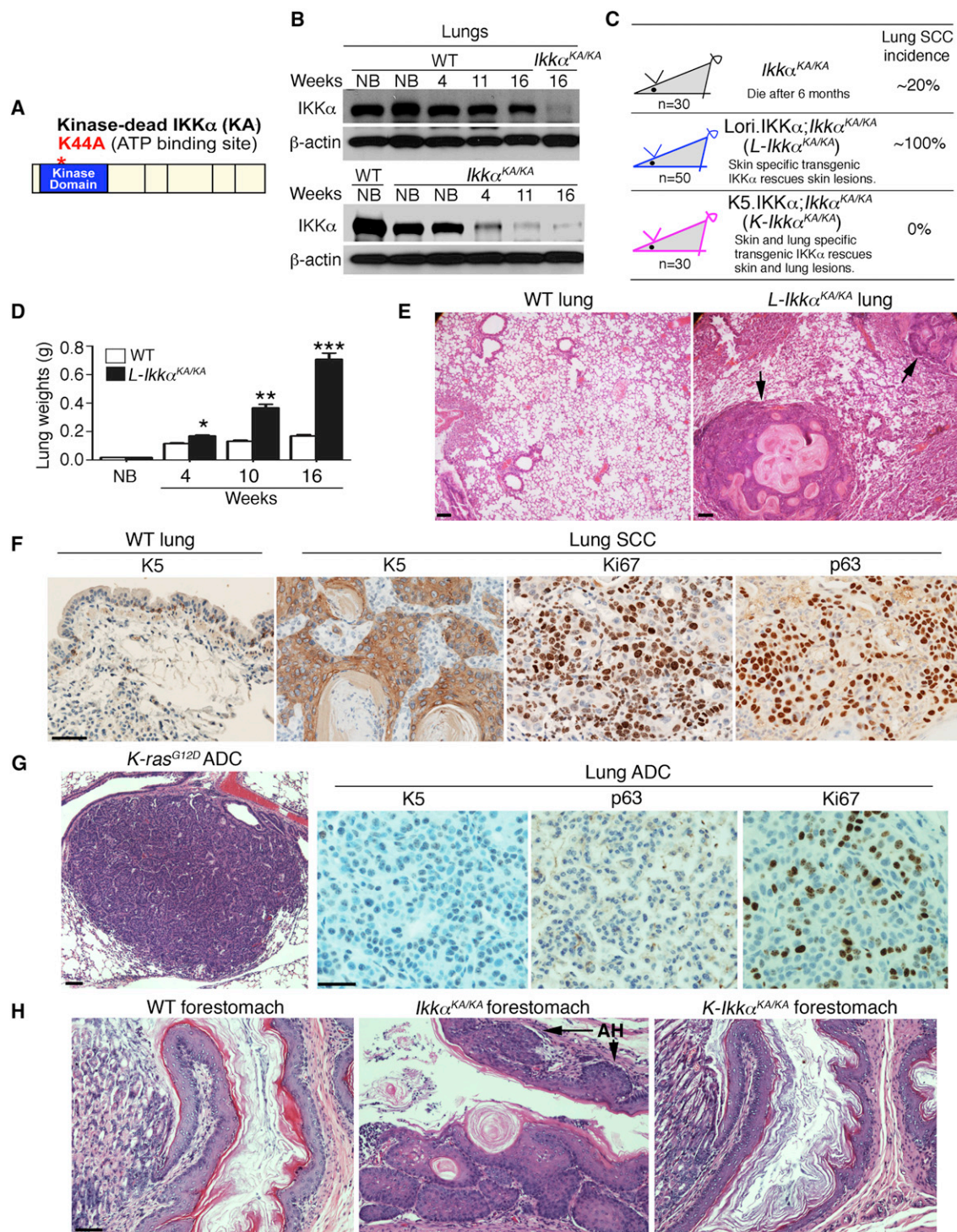
Lung cancer is the leading cause of cancer mortality worldwide, and the 5 year survival rate of patients with non-small cell lung carcinomas (NSCLCs) remains as low as 15% (Larsen and Minna, 2011). Therefore, new approaches to detect, cure, and prevent this devastating disease represent an urgent medical need. NSCLCs include adenocarcinomas (ADCs), squamous cell carcinomas (SCCs), and large cell carcinomas. Activating *K-ras* mutations have been identified in 10%–30% of human lung ADCs but in less than 5% of human lung SCCs (DuPage et al., 2009; Larsen and Minna, 2011). An oncogenic mutation in *K-ras* that changes a glycine at codon 12 to aspartic acid (*K-ras*<sup>G12D</sup>) induces spontaneous lung ADCs in mice (Johnson

et al., 2001). This mouse model has greatly enhanced our understanding of the pathogenesis, treatment, and prevention of lung cancer. Lung SCCs are strongly associated with smoking, suggesting that smoking-induced gene damage and inflammation are crucial for the development of this malignancy (Hecht, 2003; Larsen and Minna, 2011). Although many molecular alterations, such as deregulated epidermal growth factor receptor (*EGFR*), *PIK3CA*, *p53*, and *c-Myc*, have been identified in human lung SCCs (Hammerman et al., 2012; Hecht, 2003), lung SCCs have not been well recapitulated in animals.

Although *K-ras*<sup>G12D</sup> fails to induce spontaneous lung SCCs in mice, Ji et al. (2007) have shown that 56% of *K-ras*<sup>G12D</sup> mice lacking serine/threonine kinase 11 (also called LKB1) in the lungs develop mixed SCCs and ADCs. The *Lkb1* deletion alone does

### Significance

Lung cancer is the leading cause of cancer mortality worldwide. Lung squamous cell carcinoma (SCC), a major type of human lung cancer, is strongly associated with smoking. Although many oncogenes, tumor suppressors, and stem cell regulators have been found in human lung SCCs, mice overexpressing or lacking these genes do not well recapitulate the development of lung SCCs. A robust mouse lung SCC model is an urgent need for human health. Here, we established a robust lung SCC model in kinase-dead IKK $\alpha$  knockin mice and identified shared molecular alterations in human and mouse lung SCCs. Thus, this mouse model provides a suitable tool to study early diagnosis, treatment, and prevention of human lung SCCs.



**Figure 1. Identification of Lung SCCs in  $IkK\alpha^{KA/KA}$  Mice**

(A) The K at 44 is replaced by A within the IKK $\alpha$  kinase domain in  $IkK\alpha^{KA/KA}$  mice.

(B) Western blot shows IKK $\alpha$  levels in WT and  $IkK\alpha^{KA/KA}$  lungs. NB, newborn;  $\beta$ -actin, protein-loading control.

(C) Lung SCC incidence in  $IkK\alpha^{KA/KA}$ ,  $L-IkK\alpha^{KA/KA}$ , and  $K-IkK\alpha^{KA/KA}$  mice with FVB background. n, mouse numbers.

(D) WT and  $L-IkK\alpha^{KA/KA}$  lung weights (mean  $\pm$  SD of three mice per group). NB, newborn; g, gram. Statistical analysis: \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001 (Student's test).

(E) The histology of hematoxylin and eosin (H&E)-stained WT lungs and SCCs derived from  $L-IkK\alpha^{KA/KA}$  lungs. Arrows indicate SCC foci. Scale bar, 50  $\mu$ m.

(F) IHC-stained K5, p63, and Ki67 in paraffin sections of WT lungs and  $L-IkK\alpha^{KA/KA}$  lung SCCs. Brown, positive staining; blue, nuclear counterstaining. Scale bar, 50  $\mu$ m.

(legend continued on next page)

not induce lung tumors, suggesting that K-ras activation and LKB1 loss provide complementary pathways, which lead to lung SCC development. In human, germ-line mutations in *Lkb1* are associated with Peutz-Jeghers syndrome, and the *Lkb1* mutations have been also identified in a variety of human epithelial cancers, including lung SCCs (Hearle et al., 2006; Ji et al., 2007). Interestingly, *Lkb1*<sup>-/-</sup> mouse embryonic fibroblasts (MEFs) are resistant to oncogenic Ras-mediated cell proliferation and transformation (Bardeesy et al., 2002), indicating that LKB1 and Ras cooperate in promoting tumor development in a cell type-specific manner.

Lung SCCs are derived from keratin 5-positive (K5<sup>+</sup>) basal cells of the pseudostratified bronchial epithelium, while ADCs are derived from the epithelial cells of alveoli (Hackett et al., 2011; Woodworth et al., 1983). Antibodies against transcription factor p63, tripartite motif-containing 29 (Trim29) proteins, and K5 have been used to diagnose human lung SCCs and distinguish poorly differentiated lung SCCs from ADCs in the clinic (Ring et al., 2009). p63, a member of the tumor suppressor p53 family, is required for the formation of the epidermis, other stratified epithelia, and epithelial appendages (Vanbokhoven et al., 2011). The N-terminal-truncated form of p63 ( $\Delta$ Np63) is predominantly expressed in the epidermis and is overexpressed in various epithelial cancers, where it exerts oncogenic activities (Koster et al., 2007; Melino, 2011). In addition, induced bright-p63/K5<sup>+</sup> lung epithelial cells can give rise to alveoli, suggesting that the bright-p63/K5<sup>+</sup> cells are adult stem cells in the lungs (Kumar et al., 2011). Overexpressed Trim29 has been reported in human lung, bladder, colon, ovarian, endometrial, and gastric cancers. In these cell types, Trim29 promotes cell proliferation and inhibits p53 activity (Hatakeyama, 2011). These findings highlight that increased epithelial cell-specific p63 and Trim29 may also contribute to lung SCC development.

IKK $\alpha$ , one of subunits in the IKK complex (Ghosh and Karin, 2002), is required for the formation of the epidermis during mouse embryonic development and serves as an innate surveillance that prevents skin tumor development through suppressing the EGFR- and c-Myc-related pathways in adult mice (Descargues et al., 2008; Hu et al., 1999; Liu et al., 2008). It is known that K5<sup>+</sup> keratinocytes markedly expand in the skin of *Ikka*<sup>-/-</sup> mice compared to wild-type (WT) mice, *Ikka*<sup>+/-</sup> mice have enhanced susceptibility to chemical carcinogen-induced K5<sup>+</sup> SCCs associated with dedifferentiation in the skin, and inducible K5.CreER-mediated *Ikka* deletion in keratinocytes causes spontaneous skin papillomas and SCCs in *Ikka*-floxed mice (Hu et al., 1999, 2001; Liu et al., 2008; Park et al., 2007). These data suggest that K5<sup>+</sup> epithelial cells lacking IKK $\alpha$  may be the targets for SCC development. IKK $\alpha$  downregulation has been reported in human lung and skin SCCs (Kwak et al., 2011; Marinari et al., 2008); however, the role that IKK $\alpha$  plays in lung cancer has not been investigated. In eukaryotic cells, chromatin consists of packaged chromosomal DNA wound around nucleosome cores formed from histones (H). Modifications at these

histone proteins can alter chromatin structure, which facilitates or blocks transcription factor access to DNA, thereby regulating gene transcription without changing gene codes. Epigenetics plays a major role in embryonic development. Notably, the bivalent modifications of H3 lysine 4 trimethylation (H3K4me3), a positive transcription mark, and H3K27me3, a negative transcription mark, on the loci of genes regulate stem cell proliferation and differentiation (Bernstein et al., 2006). Nuclear IKK $\alpha$  has been shown to regulate the cell cycle checkpoint in keratinocytes in an epigenetic manner (Liu et al., 2008; Zhu et al., 2007). Whether altering the normal epigenetic control of the nuclear IKK $\alpha$  affects the establishment of tumor cells during carcinogenesis remains unknown.

Inflammatory cells are mobile, plastic, and able to produce many factors (Hanahan and Coussens, 2012). Thus, they orchestrate complex communications among different types of cells at pathological sites, regulating the development of various diseases. Chronic inflammation plays a crucial role in tumor development.

To understand the pathogenesis of lung SCCs, in this study, we attempt to establish a mouse lung SCC model, which may recapitulate human lung SCC development, and to identify crucial events, which may be used to prevent and treat this lung disease.

## RESULTS

### IKK $\alpha$ Reduction Is Associated with the Development of Spontaneous Lung SCCs in Kinase-Dead IKK $\alpha$ Knockin Mice

We generated kinase-dead IKK $\alpha$  knockin (*Ikka*<sup>K44A/K44A</sup>, *Ikka*<sup>KA/KA</sup>) mice, in which the lysine (K) at amino acid 44, an ATP-binding site, was replaced with alanine (A) (Figure 1A; Zhu et al., 2007). *Ikka*<sup>KA/KA</sup> newborn mice did not display any obvious abnormalities, indicating that IKK $\alpha$  kinase inactivation does not affect mouse embryonic development. However, after 3 months of age, mutant mice with an FVB background displayed severe skin lesions and developed systemic inflammation (these phenotypes are not discussed in this study) and the mice began to die after 6–10 months. Unexpectedly, spontaneous lung tumors appeared in FVB *Ikka*<sup>KA/KA</sup> mice from 4 to 10 months of age. Despite the fact that *Ikka*<sup>KA/KA</sup> mice with severe skin phenotypes have to be euthanized at early age, tumors were detected in 20% of the mutant mice. To determine the relationship between IKK $\alpha$  and lung tumor development, we examined IKK $\alpha$  levels in mouse lungs. Western blotting showed a strong expression of IKK $\alpha$  in WT mouse lungs and that IKK $\alpha$  expression was slightly reduced in elder mice (Figure 1B, top panel). IKK $\alpha$  levels were lower in the lungs of *Ikka*<sup>KA/KA</sup> newborns and markedly decreased in the lungs of 4-month-old *Ikka*<sup>KA/KA</sup> mice (Figure 1B, bottom panel), indicating that IKK $\alpha$  reduction is associated with lung tumor development. To determine whether the K44A mutation may contribute to the reduction of the IKK $\alpha$  protein, we used

(G) H&E-stained and K5-, p63-, and Ki67 IHC-stained mouse lung ADC induced by *K-ras*<sup>G12D</sup>. Brown, positive staining; blue, nuclear counterstaining. Scale bar, 50  $\mu$ m.

(H) H&E-stained forestomach paraffin sections of WT, *K-Ikka*<sup>KA/KA</sup>, and *Ikka*<sup>KA/KA</sup> (with SCC in situ; see also Figure S1F) mice at 1.2 years of age. AH, atypical hyperplasia. Scale bar, 50  $\mu$ m.

See also Figure S1.



pulse-chase analysis to show that this K44A mutation promoted IKK $\alpha$  protein degradation compared to WT IKK $\alpha$  (Figure S1A available online). We previously reported that IKK $\alpha$  RNA levels were decreased in *Ikk $\alpha$ <sup>KA/KA</sup>* mice (Balkhi et al., 2012). Thus, IKK $\alpha$  is downregulated both at the level of messenger RNA (mRNA) expression and posttranslationally in *Ikk $\alpha$ <sup>KA/KA</sup>* mice. In order to keep the *Ikk $\alpha$ <sup>KA/KA</sup>* mice alive long enough to study lung cancer development, the skin phenotype of *Ikk $\alpha$ <sup>KA/KA</sup>* mice was corrected by expression of WT IKK $\alpha$  complementary DNA (cDNA) in the epidermis under the control of a truncated loricrin promoter (Lori.IKK $\alpha$ ) (Liu et al., 2006; Figure 1C). Although Lori.IKK $\alpha$ ;*Ikk $\alpha$ <sup>KA/KA</sup>* (*L-Ikk $\alpha$ <sup>KA/KA</sup>*) mice displayed almost no skin phenotype, all of 50 *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice developed spontaneous lung tumors. Next, we introduced the WT IKK $\alpha$  transgene under the control of the K5 promoter (Liu et al., 2008), which is expressed in the basal epidermal keratinocytes and the basal lung epithelial cells of bronchia, into *Ikk $\alpha$ <sup>KA/KA</sup>* mice (Figure 1C). Although K5.IKK $\alpha$ ;*Ikk $\alpha$ <sup>KA/KA</sup>* (*K-Ikk $\alpha$ <sup>KA/KA</sup>*) mice survived longer than *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice, we did not observe lung tumors in all of 30 *K-Ikk $\alpha$ <sup>KA/KA</sup>* mice at more than 1 year of age. Analysis by RT-PCR confirmed that Lori.IKK $\alpha$  was expressed in the skin and that K5.IKK $\alpha$  was expressed in the skin and lungs (Figure S1B). These results demonstrate that epithelial cell-derived IKK $\alpha$  prevents lung tumor development.

The weight of *L-Ikk $\alpha$ <sup>KA/KA</sup>* lungs compared to WT lungs gradually increased with age (Figure 1D), indicating that lung tumor development is associated with increased lung size. Most FVB *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice were not able to live longer than 7 or 8 months, because their lung weights continued to increase with age, and most lung SCCs were observed in *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice at 4–6 months of age. Lung tumor foci with the typical SCC features of keratin pearls and squamous cellular morphology were observed on the mutant lung surface and interior (Figures 1E and S1C). Similar to human lung SCCs (Hackett et al., 2011), lung tumors in *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice expressed K5, p63, and Ki67 (Figures 1F and S1D), indicating that the spontaneous tumors in *L-Ikk $\alpha$ <sup>KA/KA</sup>* (*Ikk $\alpha$ <sup>KA/KA</sup>*) mice are SCCs. *K-ras*<sup>G12D</sup>-induced lung ADCs were negative for K5 and p63 immunostaining but showed increased Ki67-positive cells (Figures 1G, S1D, and S1E). We did not observe lung SCC metastases in *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice. It was also reported that metastases from lung ADCs but not from SCCs were seen in *K-ras*<sup>G12D</sup>;*Lkb1*<sup>-/-</sup> mice (Ji et al., 2007). In addition, *Ikk $\alpha$ <sup>KA/KA</sup>* mice lack lymph nodes (Balkhi et al., 2012). Whether these conditions affect lung SCC metastases remains to be investigated in the future.

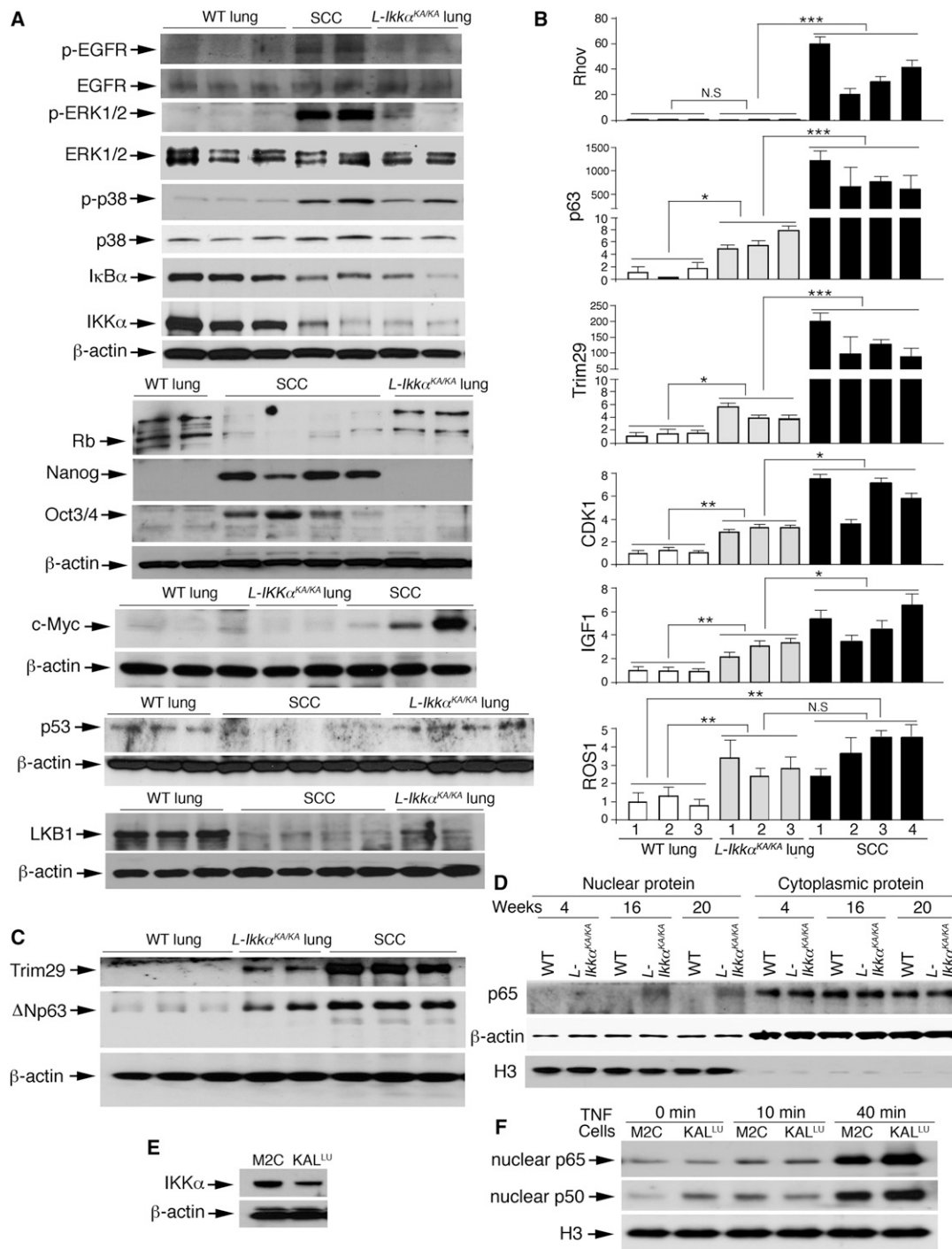
To determine whether IKK $\alpha$  reduction is associated with squamous cell hyperproliferation and malignant development in the stratified epithelium of *Ikk $\alpha$ <sup>KA/KA</sup>* mice, we histologically examined the forestomach, esophagus, and skin in BL6 *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice. All of the 16 *Ikk $\alpha$ <sup>KA/KA</sup>* mice at 5 months–1.2 years of age developed atypical squamous hyperplasia in the forestomach, and two of three *Ikk $\alpha$ <sup>KA/KA</sup>* mice at 1.2 years of age developed forestomach SCCs in situ (Figures 1H and S1F). Reintroduced K5.IKK $\alpha$  rescued the forestomach phenotype in *K-Ikk $\alpha$ <sup>KA/KA</sup>* mice. We observed esophageal hyperplasia in all three BL6 *Ikk $\alpha$ <sup>KA/KA</sup>* mice at 1.2 years of age, but we observed no esophageal phenotypes in all four *Ikk $\alpha$ <sup>KA/KA</sup>* mice at 5–7 months of age (Figure S1G). Furthermore, approximately 24% of 25 BL6 *Ikk $\alpha$ <sup>KA/KA</sup>* mice at 5–10 months of age developed skin SCCs

and all *Ikk $\alpha$ <sup>KA/KA</sup>* mice at more than 3 months of age developed epidermal keratinocyte hyperproliferation (Figure S1H; data not shown). Collectively, these results showed a good correlation between the development of SCC and squamous hyperplasia and increased *Ikk $\alpha$ <sup>KA/KA</sup>* mouse age. Thus, the squamous cell hyperproliferation and malignancies are associated with IKK $\alpha$  reduction.

### Specific Molecular Alterations in the Lungs and Lung SCCs of *L-Ikk $\alpha$ <sup>KA/KA</sup>* Mice and Similar Molecular Changes in Human and Mouse Lung SCCs

Because *K-ras*<sup>G12D</sup> induces lung ADCs in mice (DuPage et al., 2009), we sequenced K-, H-, and N-ras cDNA isolated from mouse lung SCCs and found only a low number of random mutations and insertions and no activating mutations in K-, H-, and N-ras (Figure S2A). Using western blot and quantitative real-time PCR (qPCR), we found increases in the stem cell regulators Nanog, Oct3/4, and c-Myc, and small G protein Rhov/Chp (Cdc42 homologous protein) (Aspenström et al., 2007) and decreases in the tumor suppressors p53 and Rb in lung SCCs but not in adjacent lung tissues (Figures 2A and 2B). Similarly to human (Ji et al., 2007; Ring et al., 2009), the levels of insulin growth factor 1, cyclin-dependent kinase 1 (CDK1), Trim29, and p63, and the activities of EGFR, extracellular signal-regulated kinase (ERK), and p38 were elevated in *L-Ikk $\alpha$ <sup>KA/KA</sup>* lungs and were dramatically increased in lung SCCs compared to WT lungs, while LKB1 levels were significantly decreased in *L-Ikk $\alpha$ <sup>KA/KA</sup>* lung SCCs compared to WT lungs. In addition, I $\kappa$ B $\alpha$ , an inhibitor of nuclear factor  $\kappa$ B (NF- $\kappa$ B) (Ghosh and Karin, 2002), and IKK $\alpha$  levels were reduced, and c-ros-1 receptor tyrosine kinase (ROS1), a pro-oncogene receptor tyrosine kinase (Rikova et al., 2007; Takeuchi et al., 2012), was increased to a similar extent in both *L-Ikk $\alpha$ <sup>KA/KA</sup>* lungs and SCCs compared to WT lungs, suggesting that the alterations in IKK $\alpha$ , I $\kappa$ B $\alpha$ , and ROS1 levels are ubiquitous rather than epithelial cell-specific in *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice (Figures 2A and 2B). Because both Trim29 and p63 are specifically expressed in human lung SCCs, we examined their protein levels and found that Trim29 and  $\Delta$ Np63 levels were increased in *L-Ikk $\alpha$ <sup>KA/KA</sup>* lungs and further increased in lung SCCs compared to WT lungs (Figure 2C). To determine NF- $\kappa$ B activity, we examined the nuclear p65 levels in the lungs of WT and *L-Ikk $\alpha$ <sup>KA/KA</sup>* at 4, 16, and 20 weeks of age after depleting CD45<sup>+</sup> cells and found elevated nuclear p65 levels in *L-Ikk $\alpha$ <sup>KA/KA</sup>* lungs with increasing age compared to WT lungs (Figure 2D). We also observed increased p65 levels in the lungs of 16- and 20-week-old *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice compared to WT using immunohistochemistry (data not shown). Increased nuclear p65 and p50 were detected in a *L-Ikk $\alpha$ <sup>KA/KA</sup>* lung SCC cell line (KAL<sup>LU</sup>) compared to a WT lung epithelial cell line M2C (Padilla-Nash et al., 2012) following tumor necrosis factor (TNF) stimulation, and the IKK $\alpha$  level was lower in KAL<sup>LU</sup> than in M2C cells (Figures 2E and 2F), suggesting that the canonical NF- $\kappa$ B activity was increased in IKK $\alpha$ -deficient lung SCC cells. Collectively, we identified multiple molecular alterations that can be classified into three groups: (1) specifically deregulated in SCCs; (2) deregulated prior to tumor formation; and (3) ubiquitously deregulated in *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice.

To compare the similarity of molecular alterations between mouse and human lung SCCs, we examined alterations in



**Figure 2. Molecular Alterations in *L-Ikkα<sup>KA/KA</sup>* Lungs and Lung SCCs**

(A) Western blot shows indicated protein levels in WT lungs, *L-Ikkα<sup>KA/KA</sup>* lung SCCs, and SCC-adjacent lung tissues (*L-Ikkα<sup>KA/KA</sup>* lungs). β-actin, protein-loading control.

(B) qPCR shows the expression levels (fold) of indicated genes (mean ± SD of three or four mice per group). Each column represents an individual sample that was tested three times. WT and *L-Ikkα<sup>KA/KA</sup>* lungs were obtained from 9- to 10-week-old mice. \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001 (Student's test). N.S., not statistically significant.

(C) Western blot shows Trim29 and ΔNp63 levels in WT and *L-Ikkα<sup>KA/KA</sup>* lungs and lung SCCs. β-actin, protein-loading control.

(D) Western blot shows nuclear and cytoplasmic p65 levels in lung cells (CD45<sup>-</sup>) isolated from WT and *L-Ikkα<sup>KA/KA</sup>* mice at 4, 16, and 20 weeks of age.

(E) Western blot shows IKKα levels in a WT mouse lung epithelial cell line M2C and an *L-Ikkα<sup>KA/KA</sup>* lung SCC cell line KAL<sup>LU</sup>. β-actin, protein-loading control.

(F) Western blot shows nuclear p65 and p50 levels in M2C and KAL<sup>LU</sup> cells following TNF stimulation (10 ng/ml). H3, histone H3 as nuclear protein loading control; nuclear, nuclear proteins.

See also Figure S2 and Tables S1 and S2.

IKK $\alpha$ , *c-Myc*, and Trim29 in human lung SCCs. Several studies have shown that IKK $\alpha$  is downregulated in a large proportion of human lung SCCs (Kwak et al., 2011; Marinari et al., 2008). We confirmed these findings by showing that IKK $\alpha$  levels as analyzed by western blot were significantly higher in normal human lungs than in all of the eight human lung SCCs and their adjacent lung tissues (Figure S2B). Comparative genomic hybridization revealed *c-Myc* amplification in three (37%) of the eight human lung SCCs (Figure S2C). Moreover, we examined the expression of Trim29 in human lung SCCs compared to cancer-adjacent lung tissues in human tissue array using immunohistochemical (IHC) staining (Figure S2D). Twenty-four (49%) of forty-nine human SCCs strongly expressed Trim29 versus 0 (0%) of 50 in cancer-adjacent lung tissues. Four (8%) of forty-nine human SCCs weakly expressed Trim29 versus 25 (50%) of 50 cancer-adjacent lung tissues. A moderate level of Trim29 immunostaining was detected in 21 (43%) of 49 human SCCs and 25 (50%) of 50 cancer-adjacent lung tissues. In addition to high levels of Trim29 in human and mouse lung SCCs, the localization of Trim29 expression in the cells of human and mouse SCCs was similar (Figures S2E and S2F). These results demonstrate that human and mouse lung SCCs share similar molecular changes.

In addition, we sequenced the genes of *Sox2*, *PIK3CA* (exons 9 and 20), and *DDR2* (exons 4, 7, 12, 13, 14, and 16) in ten lung SCCs derived from *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice, because the mutations in these genes have been reported in human lung SCCs (Hammerman et al., 2012). We detected several silent mutations in *DDR2* and *Sox2* and some small insertions and deletions in the introns and UTRs of *DDR2* and *Sox2* but no mutations in *PIK3CA* (Tables S1 and S2). Whether these genetic alterations affect the function of these genes remains to be elucidated in the future.

### IKK $\alpha$ Suppresses the Expression of $\Delta$ Np63 and Trim29 in an Epigenetic Manner

Although  $\Delta$ Np63 levels were increased in *L-Ikk $\alpha$ <sup>KA/KA</sup>* lungs compared to WT, the levels were still lower compared to SCCs (Figure 2C). The tumor cells represent the majority of cells in SCCs, whereas the cells in WT and *L-Ikk $\alpha$ <sup>KA/KA</sup>* lungs are a mixture of epithelial and other cells. Thus, it is possible that the p63 level was already elevated in the K5<sup>+</sup> epithelial cells in *L-Ikk $\alpha$ <sup>KA/KA</sup>* lungs. To test this hypothesis, we compared the intensity of  $\Delta$ Np63 and K5 in WT and *L-Ikk $\alpha$ <sup>KA/KA</sup>* lungs and lung SCCs using immunofluorescent (IF) staining with anti- $\Delta$ Np63 and anti-K5 antibodies.  $\Delta$ Np63 levels were specifically elevated in the K5<sup>+</sup> cells of *L-Ikk $\alpha$ <sup>KA/KA</sup>* lungs compared to WT, and the K5<sup>+</sup>p63<sup>hi</sup> cells expanded with the formation of lung SCCs (Figure 3A). The IF-stained  $\Delta$ Np63 levels were very weak in WT lungs, which is consistent with the results shown in Figures 2B and 2C. Also, Kumar et al. (2011) reported that p63 was undetectable in the lungs of WT mice using IF staining. The intensity of K5 was also elevated in the epithelial cells of *L-Ikk $\alpha$ <sup>KA/KA</sup>* lungs compared to WT (Figure 3A).

We further investigated whether IKK $\alpha$  regulates the expression of Trim29 and  $\Delta$ Np63 at the transcriptional level, because mRNA levels of Trim29 and p63 were increased in *L-Ikk $\alpha$ <sup>KA/KA</sup>* lungs and SCCs compared to WT lungs (Figure 2B). Using the chromatin immunoprecipitation (ChIP) assay with an anti-IKK $\alpha$  antibody, we detected IKK $\alpha$  binding to the promoter regions of *Trim29*

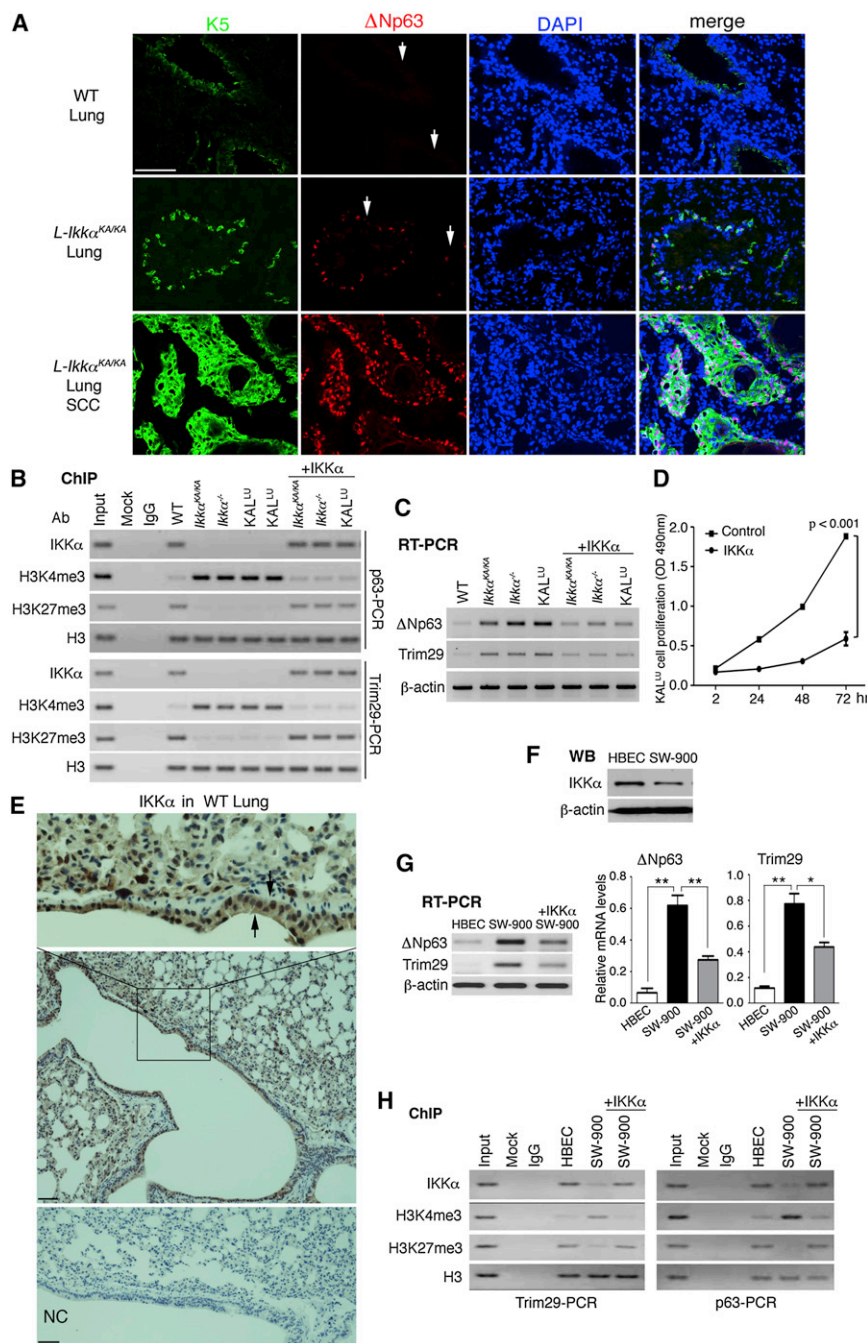
and *p63* genes, which was associated with high levels of H3K27me3, a negative transcription modifier, and low levels of H3K4me3, a positive transcription modifier, in WT MEFs; conversely, in *Ikk $\alpha$ <sup>KA/KA</sup>* and *Ikk $\alpha$ <sup>-/-</sup>* MEFs and KAL<sup>LU</sup> lung SCC cells, increased H3K4me3 levels and reduced H3K27me3 levels were associated with the *Trim29* and *p63* loci (Figure 3B). The mRNA levels of Trim29 and  $\Delta$ Np63 were higher in *Ikk $\alpha$ <sup>KA/KA</sup>*, *Ikk $\alpha$ <sup>-/-</sup>*, and KAL<sup>LU</sup> cells than in WT cells (Figure 3C). Reintroducing IKK $\alpha$  decreased H3K4me3 levels and increased H3K27me3 levels on *Trim29* and *p63* loci and repressed the expression of Trim29 and p63 in *Ikk $\alpha$ <sup>KA/KA</sup>*, *Ikk $\alpha$ <sup>-/-</sup>*, and KAL<sup>LU</sup> cells (Figures 3B and 3C). Although *Ikk $\alpha$ <sup>KA/KA</sup>* MEFs maintained a low level of IKK $\alpha$ , we found that the nuclear kinase-dead IKK $\alpha$  (KA) level was reduced compared to WT IKK $\alpha$  when WT IKK $\alpha$  and IKK $\alpha$ -KA were transfected into cells (data not shown). Thus, the expression levels of Trim29 and  $\Delta$ Np63 were comparable in *Ikk $\alpha$ <sup>KA/KA</sup>* and *Ikk $\alpha$ <sup>-/-</sup>* MEFs. These results suggest that IKK $\alpha$  regulates the expression of both Trim29 and  $\Delta$ Np63 at the transcription level by modifying the chromatin structure of *Trim29* and *p63* in an epigenetic manner. Furthermore, re-expressed IKK $\alpha$  was found to repress KAL<sup>LU</sup> cell proliferation compared to the control vector (Figure 3D). Thus, IKK $\alpha$  also inhibited lung epithelial cell proliferation. IHC staining further showed strongly stained nuclear IKK $\alpha$  in the bronchial epithelial cells of WT mice (Figure 3E), which supports the result that IKK $\alpha$  suppressed the expression of  $\Delta$ Np63 and Trim29 in the nucleus.

Moreover, we examined the relationship between IKK $\alpha$  and the expression of p63 and Trim29 in a WT human bronchial epithelial cell line HBEC (Xi et al., 2010) and a human lung SCC cell line SW-900. Western blot showed a higher IKK $\alpha$  level in HBEC cells than in SW-900 cells (Figure 3F). The expression levels of  $\Delta$ Np63 and Trim29 were higher in SW-900 cells than in HBEC cells, and reintroducing IKK $\alpha$  repressed the expression of  $\Delta$ Np63 and Trim29 in SW-900 cells (Figure 3G). ChIP analysis showed higher H3K4me3 levels and lower H3K27me3 levels on *p63* and *Trim29* loci in SW-900 SCC cells compared to HBEC cells (Figure 3H). Reintroducing IKK $\alpha$  reversed H3K4me3 and H3K27me3 levels on *p63* and *Trim29* genes and decreased the expression levels of p63 and Trim29 in SW-900 SCC cells (Figures 3G and 3H). Together, these results indicated a similar regulatory mechanism by which IKK $\alpha$  regulates p63 and Trim29 expression in human and mouse lung epithelial cells.

### Excessive Inflammatory Cells, Cytokines, and Chemokines Are Present in the Lungs of *L-Ikk $\alpha$ <sup>KA/KA</sup>* Mice

To evaluate the effect of the inflammatory microenvironment on lung tumorigenesis, we examined leukocyte infiltration and expression of cytokines and chemokines. We found significantly increased absolute numbers of CD4<sup>+</sup> cells and macrophages (F4/80<sup>+</sup>) and moderately increased CD8<sup>+</sup> cells and neutrophils (Ly6G<sup>+</sup>/CD11b<sup>+</sup>) in the lungs of *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice compared to WT mice at 4 and 16 weeks of age (Figure 4A). B cells did not significantly increase (data not shown), which is consistent with previous results (Balkhi et al., 2012). Significantly increased expression (qPCR analysis) of tumor necrosis factor- $\alpha$ , interleukin (IL)-1 $\beta$ , IL-6, IL-4, IL-13, IL-10, chemokine (C-C motif) ligand 2 (CCL2), chemokine (C-X-C motif) ligand 5, CCL11, and CCL8 was observed in *L-Ikk $\alpha$ <sup>KA/KA</sup>* lungs compared to WT





**Figure 3. IKK $\alpha$  Regulates Trim29 and p63 Expression in an Epigenetic Manner**

(A) Immunofluorescent staining shows K5 (green) and  $\Delta$ Np63 (red) in WT lungs, *L-Ikk $\alpha$ <sup>KA/KA</sup>* lungs, and *L-Ikk $\alpha$ <sup>KA/KA</sup>* lung SCCs. Blue color, DAPI for nuclear staining; arrows indicate  $\Delta$ Np63 staining. Scale bar, 50  $\mu$ m.

(B) ChIP assay was performed with indicated antibodies (Abs) and PCR with Trim29 and p63 primers in WT, *Ikk $\alpha$ <sup>KA/KA</sup>*, *Ikk $\alpha$ <sup>-/-</sup>* MEFs, and a cell line KAL<sup>LU</sup> from *L-Ikk $\alpha$ <sup>KA/KA</sup>* lung SCCs. Mock, immunoglobulin G (IgG) as negative controls; +IKK $\alpha$ , reintroducing IKK $\alpha$  into cells; H3, control for ChIP assay.

(C) RT-PCR with  $\Delta$ Np63 and Trim29 primers from indicated cells. +IKK $\alpha$ , reintroducing IKK $\alpha$  into cells.

(D) The proliferation of KAL<sup>LU</sup> cells was examined at 2, 24, 48, and 72 hr following transfection with IKK $\alpha$  vector or control vector, using the kit of CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) and mean  $\pm$  SD of four samples per group.

(E) IHC staining with an anti-IKK $\alpha$  antibody shows strong nuclear IKK $\alpha$  in the cells of bronchial epithelium of adult WT mice. The area in the box of the middle panel was amplified at the top panel. Brown, positive staining; arrows, bronchial epithelium; blue, nuclear counterstaining; NC, negative control. Scale bar, 50  $\mu$ m.

(F) Western blotting shows IKK $\alpha$  levels in a WT human lung cell line HBEC and a human lung SCC cell line SW-900.  $\beta$ -actin, protein-loading control.

(G) Left panel: RT-PCR with  $\Delta$ Np63 and Trim29 primers from indicated cells. +IKK $\alpha$ , reintroducing IKK $\alpha$  into cells. Right panel: the comparison of mRNA levels of  $\Delta$ Np63 and Trim29 in HBEC and SW-900 cells (mean  $\pm$  SD of three samples per group). \* $p$  < 0.05; \*\* $p$  < 0.01; \*\*\* $p$  < 0.01 (Student's test).

(H) ChIP assay was performed with indicated Abs and PCR with Trim29 and p63 primers in a normal human lung cell line and two human lung SCC cell lines. Mock, IgG as negative controls; +IKK $\alpha$ , reintroducing IKK $\alpha$  into cells; H3, control for ChIP assay.

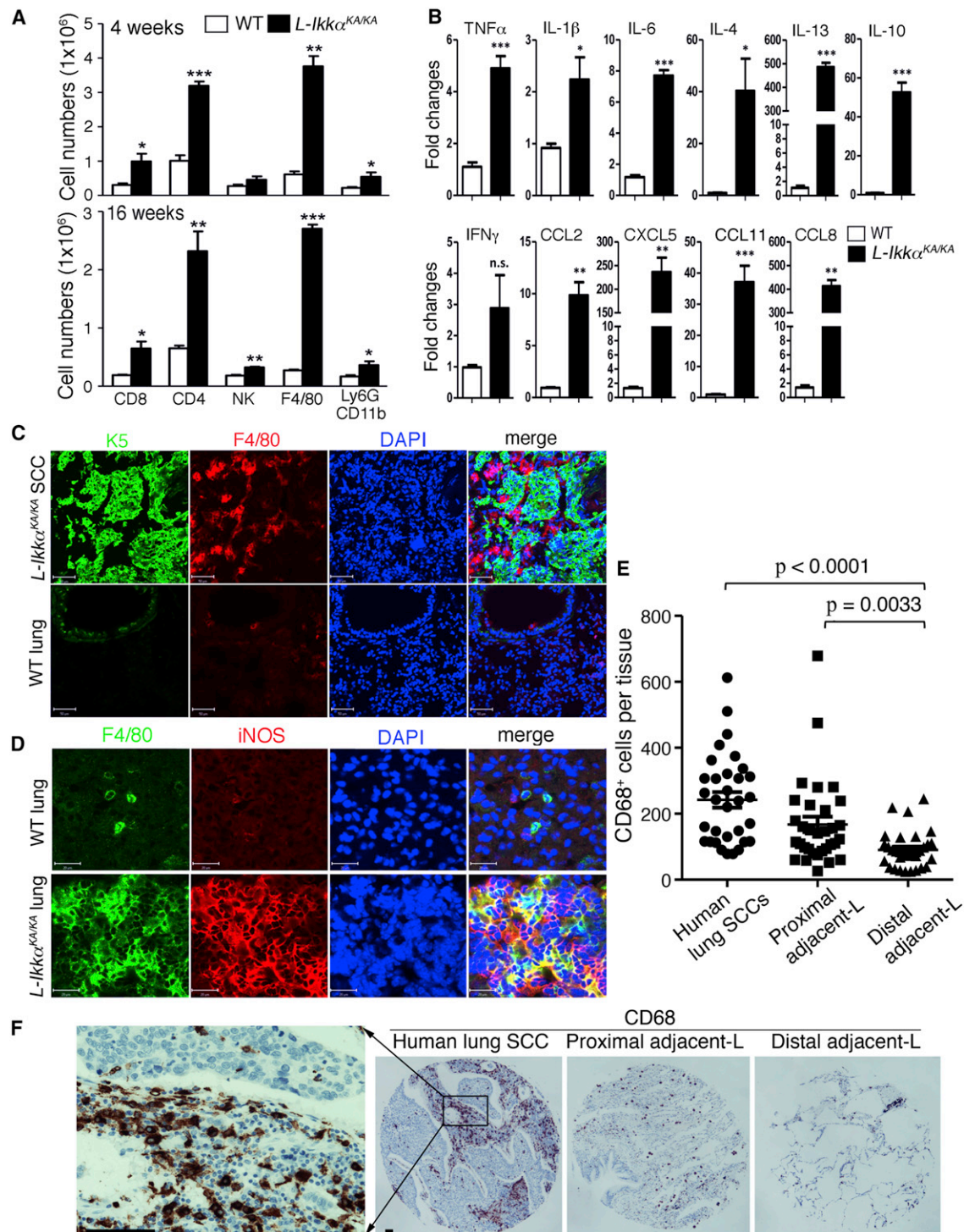
(Figure 4B; Table S3). IF staining showed extensive macrophage infiltration into SCCs and elevated expression of inducible nitric oxide synthase (iNOS) (Lechner et al., 2005) in *L-Ikk $\alpha$ <sup>KA/KA</sup>* macrophages (Figures 4C and 4D). These results showed that marked inflammation and oxidants were present in *L-Ikk $\alpha$ <sup>KA/KA</sup>* lungs.

To determine whether increased numbers of macrophages are present in human lung SCCs, we used IHC staining with an anti-CD68 antibody to examine macrophages in a human array containing 32 human lung SCCs with their proximal adjacent lung tissues and distal adjacent lung tissues and found increased

macrophages in lung SCCs and proximal adjacent lung tissues compared to distal adjacent lungs, indicating a good correlation between increased macrophages and lung SCC development (Figures 4E and 4F), which is consistent with the observation in the lungs and lung SCCs of *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice.

### Depleting Macrophages Reduces Inflammation and Epithelial Proliferation and Prevents Lung SCC Development

To determine whether inflammation regulates lung SCC development, we depleted macrophages in 7-week-old *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice by injection of clodronate-loaded liposomes, which induce macrophage apoptosis (Jenkins et al., 2011). A single treatment significantly reduced macrophages in treated *L-Ikk $\alpha$ <sup>KA/KA</sup>* lungs



**Figure 4. Marked Inflammation in the Lungs of  $L-Ikk\alpha^{KA/KA}$  Mice**

(A) The comparison of indicated leukocytes in the lungs of WT and  $L-Ikk\alpha^{KA/KA}$  mice at 4 and 16 weeks of age examined with flow cytometry analysis (mean  $\pm$  SD of four samples per group). \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

(B) The expression levels (fold changes) of various cytokines and chemokines in the lungs of  $L-Ikk\alpha^{KA/KA}$  mice at 4 weeks of age were compared to WT lungs using qPCR. The fold changes were calculated with delta-delta cycle threshold methods (mean  $\pm$  SD of four samples per group). n.s., not statistically significant.

(C) Macrophages, lung SCCs, and WT lungs were immunofluorescently stained with anti-F4/80 (red for macrophages) and anti-K5 (green for epithelial cells) antibodies. Blue, DAPI for nuclear staining. Scale bar, 50  $\mu$ m.

(D) The expression of iNOS (red) in macrophages (green) in the lungs of WT and  $L-Ikk\alpha^{KA/KA}$  mice at 16 weeks of age detected using immunofluorescent staining. Blue, DAPI for nuclear staining. Scale bar, 50  $\mu$ m.

(legend continued on next page)



compared to untreated *L-Ikk $\alpha$ <sup>KA/KA</sup>* lungs (Figure 5A). After 3 months of liposome treatment, the weights of *L-Ikk $\alpha$ <sup>KA/KA</sup>* lungs were significantly reduced in comparison to untreated *L-Ikk $\alpha$ <sup>KA/KA</sup>* lungs (Figure 5B, right and left panels). The macrophage depletion also decreased Trim29 and  $\Delta$ Np63 levels, p38 activity, and DNA damage that was indicated by 8-hydroxydeoxyguanosine (Yang et al., 2009) in treated *L-Ikk $\alpha$ <sup>KA/KA</sup>* lungs (Figures 5C and 5D). Using histological examination, we did not detect lung SCCs in any of the six liposome-treated *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice.

We analyzed the effect of macrophage depletion on global gene expression by comparing gene expression profiles of *L-Ikk $\alpha$ <sup>KA/KA</sup>* versus WT lungs and liposome-treated *L-Ikk $\alpha$ <sup>KA/KA</sup>* versus WT lungs (Figures 5E and S3A); the full gene names are reported in Table S4. K6 and K16, which are highly expressed in hyperproliferative squamous epithelial cells and SCCs (Hackett et al., 2011), as well as K14, K5, and other keratins, were highly expressed in *L-Ikk $\alpha$ <sup>KA/KA</sup>* lungs compared to WT. Macrophage depletion dramatically reduced the expression of these keratins (Figure 5E, left panel). Macrophage depletion also repressed the expression of cell cycle regulators, metalloproteinases, a disintegrin and metalloproteinases, growth factors, early growth response genes, cytokines, and chemokines, and altered the expression of oxidases in *L-Ikk $\alpha$ <sup>KA/KA</sup>* lungs (Figures 5E, middle and right panels, and S3A). Although the clodronate-loaded liposome treatment reduced macrophages and prevented lung SCCs, we observed infiltrating lymphocytes in treated *L-Ikk $\alpha$ <sup>KA/KA</sup>* lungs (Figure S3B). These results indicate that the excessive macrophages increased inflammation, epithelial cell proliferation, DNA damage, and activities of many pathways that may contribute to lung carcinogenesis in *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice; in contrast, macrophage reduction reverses these abnormal responses.

Because *K-ras<sup>G12D</sup>;Lkb1<sup>-/-</sup>* mice developed mixed lung SCCs and ADCs (Ji et al., 2007) and LKB1 downregulation was found in *L-Ikk $\alpha$ <sup>KA/KA</sup>* lung SCCs, we examined whether increased numbers of macrophages were present in the lungs of *K-ras<sup>G12D</sup>;Lkb1<sup>-/-</sup>* mice. The IHC staining showed significantly more macrophages in *K-ras<sup>G12D</sup>;Lkb1<sup>-/-</sup>* lungs than in WT lungs but more macrophages in *L-Ikk $\alpha$ <sup>KA/KA</sup>* lungs than in *K-ras<sup>G12D</sup>;Lkb1<sup>-/-</sup>* lungs (Figures 5F and S3C). These results demonstrated the presence of increased macrophages in the lung SCCs derived from *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice and *K-ras<sup>G12D</sup>;Lkb1<sup>-/-</sup>* mice, as well as in human lung SCCs.

#### ***L-Ikk $\alpha$ <sup>KA/KA</sup>* Bone Marrow Reconstitutes Lung SCCs in Irradiated *L-Ikk $\alpha$ <sup>KA/KA</sup>* Mice**

To investigate the effects of bone marrow (BM)-derived macrophages from WT and *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice on lung SCC development, we performed BM transplant experiments by intravenously injecting either *L-Ikk $\alpha$ <sup>KA/KA</sup>* BM into irradiated *L-Ikk $\alpha$ <sup>KA/KA</sup>* and WT mice or WT BM into irradiated *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice (Figure S4A). The irradiated WT mice receiving WT BM

were similar to WT mice (data not shown). At 3 months after the BM injection, all of the ten irradiated *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice receiving *L-Ikk $\alpha$ <sup>KA/KA</sup>* BM had enlarged lungs infiltrated with many macrophages and lymphocytes and developed lung SCCs; all of the seven irradiated WT mice receiving *L-Ikk $\alpha$ <sup>KA/KA</sup>* BM were SCC-free, and their lungs were infiltrated with a few macrophages and lymphocytes (Figures 6A–6C and S4B). Six out of seven irradiated *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice receiving WT BM were SCC-free; their lungs, which were infiltrated with many lymphocytes but reduced macrophages, were smaller than the lungs of *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice receiving *L-Ikk $\alpha$ <sup>KA/KA</sup>* BM but larger than the lungs of WT mice receiving *L-Ikk $\alpha$ <sup>KA/KA</sup>* BM (Figures 6A–6C and S4B). However, one out of seven irradiated *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice receiving WT BM developed a very large lung SCC (Figure S4B). Because we performed BM transplant experiments in mice at 4 or 5 weeks of age and increased pulmonary inflammation was already observed in the lungs of 4-week-old *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice (Figure 4A), it is possible that the development of this tumor began prior to BM transfer. These results further showed that *L-Ikk $\alpha$ <sup>KA/KA</sup>*-derived macrophages are associated with lung SCC development.

## **DISCUSSION**

### **IKK $\alpha$ Downregulation Is Associated with Lung Enlargement and Lung SCC Development**

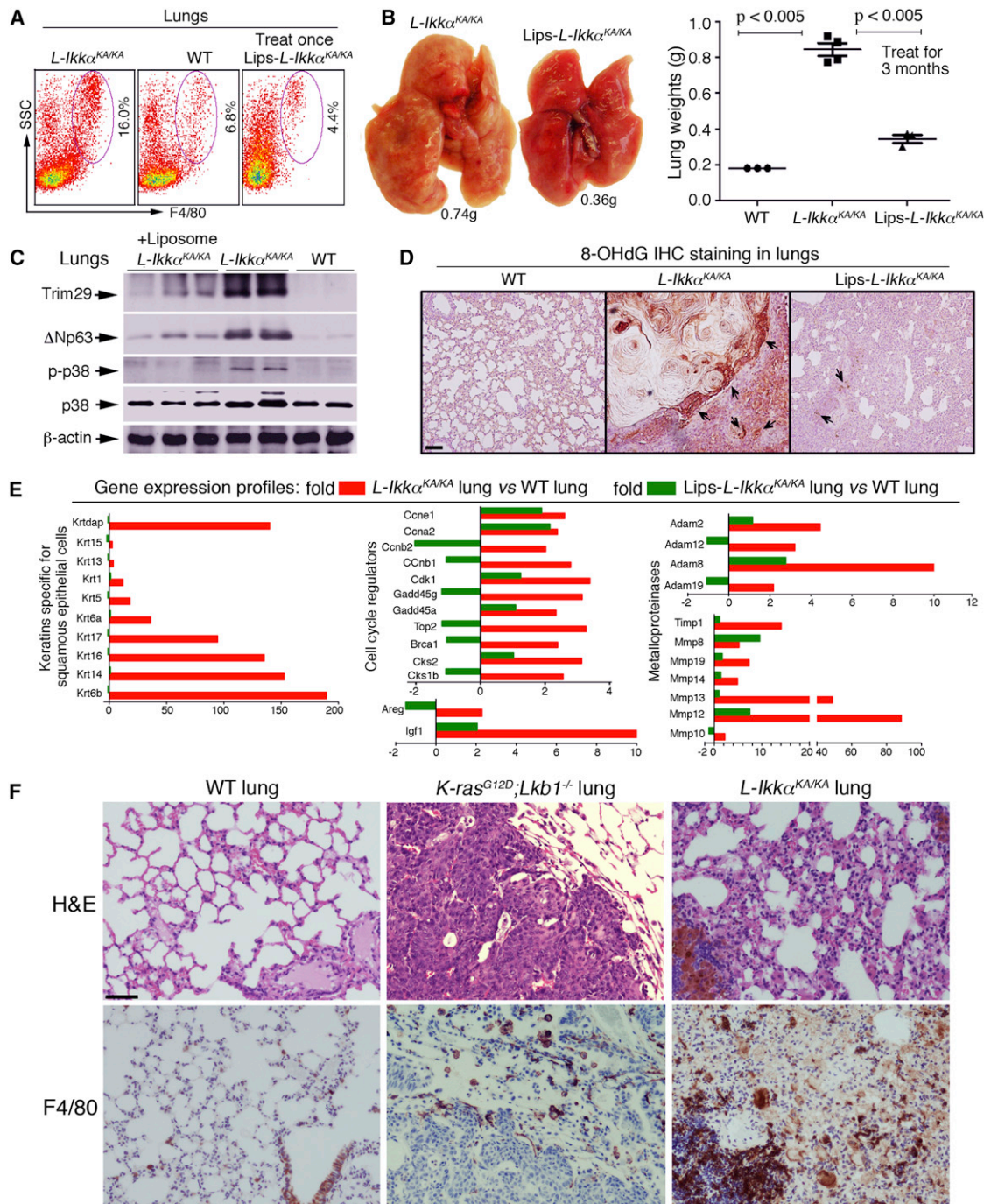
In this study, we showed that the lungs of *L-Ikk $\alpha$ <sup>KA/KA</sup>* newborn mice were relatively normal. The K44A mutation in IKK $\alpha$  destabilized the protein. With increasing age and decreasing IKK $\alpha$  levels, the lungs became enlarged and lung SCCs occurred in *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice. Reintroducing transgenic K5.IKK $\alpha$  prevented lung enlargement and tumorigenesis, and re-expressing WT IKK $\alpha$  repressed the proliferation of KAL<sup>LU</sup> lung SCC cells, suggesting that IKK $\alpha$  is important for the tumor initiation and maintenance. The elevated activities of EGFR/ERK, cell cycle regulators, and growth factors may largely contribute to cell proliferation in the lungs of *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice. We previously reported that IKK $\alpha$  loss promotes keratinocyte proliferation and induces spontaneous skin SCCs (Liu et al., 2008). In the current study of *Ikk $\alpha$ <sup>KA/KA</sup>* mice, we found squamous cell hyperproliferation and SCCs in the forestomach, esophagus, and skin, which were associated with IKK $\alpha$  reduction. Again, reintroducing K5.IKK $\alpha$  rescued these phenotypes. These findings demonstrate that IKK $\alpha$  is required for maintaining the normal function of squamous cells in multiple epithelial organs.

Furthermore, we found significant downregulation of LKB1, a tumor suppressor, in *L-Ikk $\alpha$ <sup>KA/KA</sup>* lung SCCs and adjacent lung tissues compared to WT lungs. It is known that *Lkb1* deletion promotes SCC development in both the lungs and skin (Gurumurthy et al., 2008; Ji et al., 2007). *Lkb1<sup>-/-</sup>* knockout mice died at midgestation with vascular and neural tube defects (Ylikorkala et al., 2001). Thus, the role of LKB1 in the formation of stratified epithelial organs is unclear. K14.Cre-mediated *Lkb1*

(E) Comparison of IHC-stained CD68<sup>+</sup> cell counts in each tissue of the human array slide (LC991) among three groups (mean  $\pm$  SD of 32 tissues per group). The p value was examined by Student's test.

(F) One representative case [see (E)] was shown, and the region in the box was amplified in the left panel, indicated by lines. Brown, CD68 positive staining; blue, nuclear counterstaining; -L, lung tissue. Scale bar, 50  $\mu$ m.

See also Table S3.



**Figure 5. Depletion of Macrophages Prevents Lung SCC Development**

(A) Flow cytometry analysis shows the number of macrophages (F4/80) in the lungs of WT, *L-Ikk $\alpha$ <sup>KA/KA</sup>*, and liposome-treated (*Lips-*, one treatment) *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice.

(B) Left panel: the lungs (g, gram) of *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice after 3 months of treatment and untreated *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice. Right panel: the comparison of the lung weights of treated *L-Ikk $\alpha$ <sup>KA/KA</sup>*, untreated *L-Ikk $\alpha$ <sup>KA/KA</sup>*, and WT mice (mean  $\pm$  SD of three or four mice per group).

(C) Trim29,  $\Delta$ Np63, p38, and p-p38 levels in WT, *L-Ikk $\alpha$ <sup>KA/KA</sup>*, and *Lips-L-Ikk $\alpha$ <sup>KA/KA</sup>* lungs detected using western blotting.  $\beta$ -actin, protein-loading control.

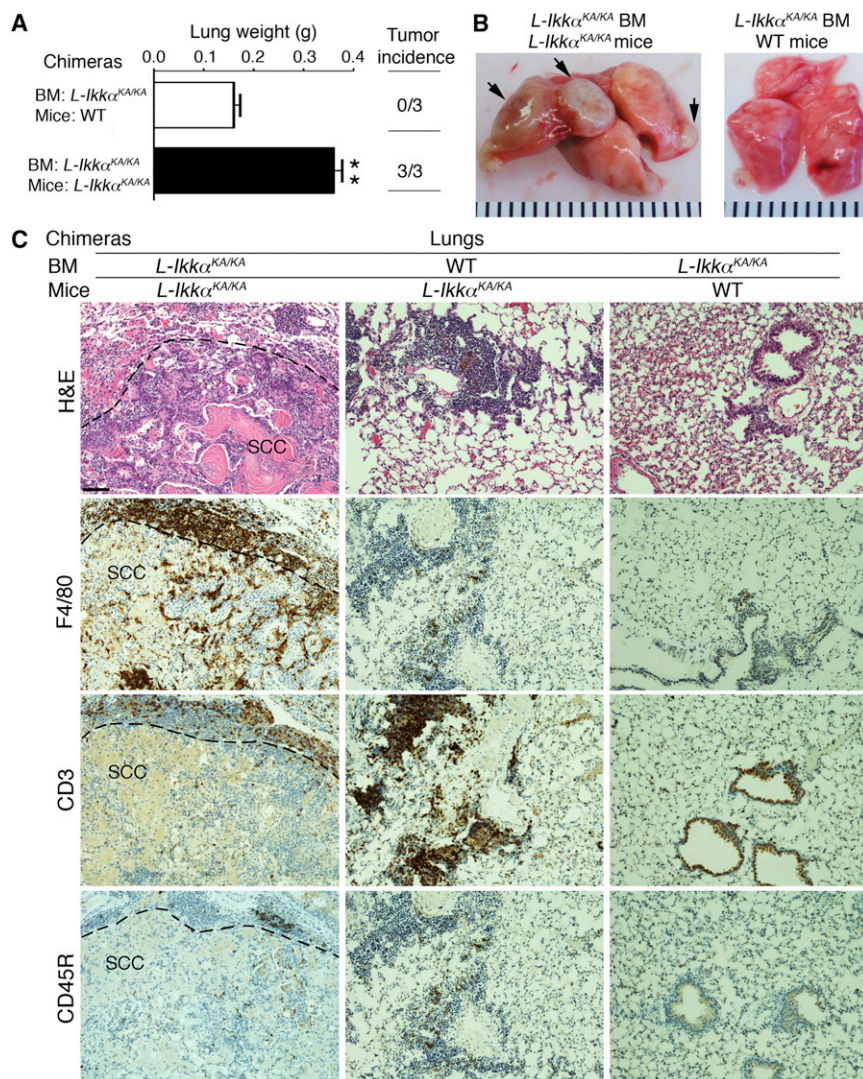
(D) The IHC-stained 8-hydroxydeoxyguanosine in WT, *L-Ikk $\alpha$ <sup>KA/KA</sup>*, and *Lips-L-Ikk $\alpha$ <sup>KA/KA</sup>* lungs. Brown, positive staining. Scale bar, 50  $\mu$ m.

(E) The comparison of the gene expression profiles (fold) of *L-Ikk $\alpha$ <sup>KA/KA</sup>* versus WT (red bars) and *Lips-L-Ikk $\alpha$ <sup>KA/KA</sup>* versus WT lungs (green bars) using microarray.

(F) H&E- or F4/80-IHC-stained lungs of WT, *K-ras<sup>G12D</sup>;Lkb1<sup>-/-</sup>*, and *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice. Blue, nuclear-counterstaining; brown, positive staining. Scale bar, 50  $\mu$ m.

See also Figure S3 and Table S4.





**Figure 6. The Effect of BM Cells on Inflammation and SCC Development in the Lungs of Mice**

(A) Left panel: the lung weights (g, gram) of irradiated WT and *L-Ikk $\alpha$ <sup>KA/KA</sup>* chimeras at 16 weeks of age (mean  $\pm$  SD of three mice per group). Right panel: tumor incidence in indicated irradiated chimeras. \*\*p < 0.01.

(B) The appearance of indicated mice at 4 months of age.

(C) H&E staining and IHC-CD3, -F4/80, and -CD45R staining in the lungs of indicated irradiated chimera. Brown, positive staining; blue, nuclear counterstaining. Scale bar, 50  $\mu$ m.

See also Figure S4.

Trim29 and  $\Delta$ Np63 by modifying H3K4me3 and H3K27me3 levels on the *Trim29* and *p63* loci, indicating that Trim29 and  $\Delta$ Np63 are IKK $\alpha$  targets in the lung epithelial cells. Human lung SCC cells are K5<sup>+</sup> and overexpress p63 and Trim29 (Ring et al., 2009). In mice, *Ikk $\alpha$*  loss promotes K5<sup>+</sup> keratinocyte proliferation (Hu et al., 1999, 2001; Liu et al., 2008). Here, we observed K5<sup>+</sup>p63<sup>hi</sup> cells in the lungs of *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice as well as in lung SCCs but not in WT mice, and re-expression of IKK $\alpha$  under the K5 promoter prevented lung SCC development in *Ikk $\alpha$ <sup>KA/KA</sup>* mice. We will further investigate whether IKK $\alpha$ <sup>low</sup>K5<sup>+</sup>p63<sup>hi</sup> cells have stem cell features and whether IKK $\alpha$ <sup>low</sup>K5<sup>+</sup>p63<sup>hi</sup> cells serve as tumor-initiating cells.

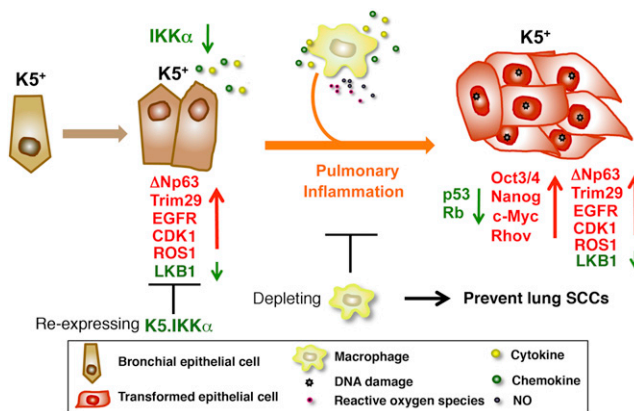
In addition to the further changes in EGFR/ERK activity and Trim29,  $\Delta$ Np63, IGF, CDK1, and LKB1 levels in SCCs, only lung SCCs, not precancerous lungs,

deletion does not cause epidermal hyperplasia and does not promote the development of chemical carcinogen-induced benign papillomas, but it does enhance malignant skin SCC development (Gurumurthy et al., 2008), suggesting that LKB1 loss does not provide a selective growth advantage. Interestingly, however, LKB1 loss-associated SCCs showed increased EGFR and ERK activities; thus, an indirect mechanism may activate EGFR/ERK pathways, which cooperate with LKB1 deficiency to promote carcinogenesis. Although the mechanism of how IKK $\alpha$  regulates LKB1 expression in different cell types remains to be elucidated, the phenotypic similarities and differences between IKK $\alpha$  and LKB1 suggest that LKB1 reduction provides an important pathway to promote IKK $\alpha$  deficiency-associated tumorigenesis.

In this study, IHC staining showed that the majority of IKK $\alpha$  was located in the nuclei of lung epithelial cells, underscoring the importance of nuclear IKK $\alpha$  in maintaining the normality of lung epithelial cells. We found that IKK $\alpha$  deficiency increased the transcription levels of Trim29 and  $\Delta$ Np63 and that reintroduced WT IKK $\alpha$  repressed the expression of

from *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice showed decreased p53 and Rb and increased Nanog, Oct3/4, c-Myc, and Rhov traits, which have also been reported in human lung SCCs (Larsen and Minna, 2011; Yuan et al., 2010). It is known that p53 loss can promote tumorigenesis by accelerating pluripotent cell generation induced by stem cell regulators (Utikal et al., 2009). Elevated small G proteins can regulate the cytoskeleton and cellular polarity and promote mitogen-activated protein kinase and EGFR activities, tumor cell proliferation, and abnormal polarity, resulting in tumor genomic heterogeneity (Iden and Collard, 2008). These combined abnormal properties may be critical for maintaining the specific oncogenic cell lineage during lung SCC development in *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice. Although p53 genetic alterations are most frequently detected in human lung SCCs (Hammerman et al., 2012), most human lung SCCs bear multiple genetic alterations. Mice lacking p53 and Rb or overexpressing the oncogenes and stem-like genes do not develop spontaneous lung SCCs. Therefore, these specific molecular alterations in *L-Ikk $\alpha$ <sup>KA/KA</sup>* lungs and lung SCCs may be important for lung SCC development.





**Figure 7. A Working Model of Lung SCC Development in *L-Ikk $\alpha$ <sup>KA/KA</sup>* Mice**

IKK $\alpha$  downregulation dysregulates the expression of multiple oncogenes and tumor suppressors in K5<sup>+</sup> lung epithelial cells. The mutant macrophages increase inflammatory responses and oxidative stress to promote DNA damage in IKK $\alpha$  mutant K5<sup>+</sup> lung epithelial cells, which further dysregulate the levels of multiple oncogenes, tumor suppressors, and stem cell genes, thereby promoting the IKK $\alpha$ <sup>low</sup>K5<sup>+</sup>p63<sup>hi</sup> cell transition to tumor cells in *L-Ikk $\alpha$ <sup>KA/KA</sup>* lungs. Arrow down (green), downregulation; arrow up (red), upregulation; crossing two lines, inhibition.

#### Inflammation Is Required for Lung SCC Development in *L-Ikk $\alpha$ <sup>KA/KA</sup>* Mice

Notably, adult *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice retain a low level of IKK $\alpha$ . Previously, we showed that *Ikk $\alpha$ <sup>+/-</sup>* mice were relatively normal but were haploid insufficient in chemical carcinogen-induced skin carcinogenesis (Park et al., 2007), suggesting that reduced IKK $\alpha$  fails to protect skin cells from environmental inducers. Thus, it is possible that microenvironmental signals may trigger and accelerate the development of lung malignancy in *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice. Most human lung SCC patients have a long history of smoking, which is known to induce DNA damage and inflammation and to affect immune functions (Hecht, 2003). Defining these inflammatory inducers may facilitate the development of immunotherapy for human lung SCCs.

In this study, we found markedly higher infiltration of macrophages and lymphocytes and expression of cytokines and chemokines in *L-Ikk $\alpha$ <sup>KA/KA</sup>* lungs compared to WT lungs. The pulmonary inflammation and lung enlargement occurred prior to SCC formation. Macrophage depletion using liposome treatment significantly reduced lung weights and prevented SCC formation in *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice, indicating that increased macrophages promote lung SCC development. Although macrophages were reduced, infiltrating lymphocytes were still present in the lungs of treated *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice, suggesting that the lymphocytes alone were not sufficient to promote lung tumorigenesis. The precise functions of lymphocytes in the lung SCC development in *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice remain to be further investigated.

Previously, we demonstrated that kinase-dead IKK $\alpha$  downregulates the expression of Pax5, a B cell regulator, thereby impairing early B cell development and promoting myeloid-cell differentiation in *Ikk $\alpha$ <sup>KA/KA</sup>* BM (Balkhi et al., 2012). It is possible that increased mutant macrophages may be one of the inducers for lung SCC development. The BM transfer experiments

showed that six of seven irradiated *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice receiving WT BM were SCC-free; all irradiated *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice receiving *L-Ikk $\alpha$ <sup>KA/KA</sup>* BM developed lung SCCs. Although we observed markedly increased lymphocytes in the lungs of both irradiated *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice, there were more macrophages in the lungs of irradiated *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice receiving *L-Ikk $\alpha$ <sup>KA/KA</sup>* BM than in the lungs of irradiated *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice receiving WT BM. Together, these results suggest that *L-Ikk $\alpha$ <sup>KA/KA</sup>* BM, not WT BM, strongly promotes lung SCC development. In addition, we observed a good correlation between increased macrophages and human lung SCCs or/and lungs bearing SCCs and ADCs in *K-ras*<sup>G12D</sup>;*Lkb1*<sup>-/-</sup> mice, although the effect of increased macrophages on lung tumorigenesis in different systems remains to be determined in the future.

It is well known that macrophages generate oxidative stresses that can induce DNA damage (Hanahan and Coussens, 2012). We observed increased iNOS, a source of oxidative stress, in *L-Ikk $\alpha$ <sup>KA/KA</sup>* macrophages and increased DNA damage in *L-Ikk $\alpha$ <sup>KA/KA</sup>* lungs compared to WT. These IKK $\alpha$  mutant macrophages were surrounding the SCCs as well as infiltrating into SCCs. Depleting macrophages not only repressed  $\Delta$ Np63 and Trim29 expression, K5<sup>+</sup> epithelial cell expansion, and oxidative stress-associated DNA damage, but also prevented SCCs in the *L-Ikk $\alpha$ <sup>KA/KA</sup>* lungs. Thus, the inflammation-mediated DNA damage may facilitate to select and promote the IKK $\alpha$ <sup>low</sup>K5<sup>+</sup>p63<sup>hi</sup> cell transition to SCC cells by deregulating tumor suppressors, oncogenic proteins, and stem cell regulators (Figure 7). In the future, it will be important to precisely define the critical molecular events in *L-Ikk $\alpha$ <sup>KA/KA</sup>* macrophages that promote lung SCC development and the specific signature of the inflammation-mediated DNA damage in lung SCCs, and whether IKK $\alpha$  kinase inactivation plays a role in promoting gene instability in the lung epithelial cells surrounded by an inflamed microenvironment. In addition, we found IKK $\alpha$  downregulation in SCC adjacent lung tissues. Thus, further investigation is needed to determine whether reduced IKK $\alpha$  contributes to the inflammatory microenvironment.

The results obtained in BM transfer experiments and in those in which IKK $\alpha$  was re-expressed in K5-expressing cells showed that the IKK $\alpha$  mutant lung epithelium contributed to the development of inflammation and SCCs in *L-Ikk $\alpha$ <sup>KA/KA</sup>* mice. Many overexpressed factors, such as IL-13 and IL-4, may educate macrophages in inflamed *L-Ikk $\alpha$ <sup>KA/KA</sup>* lungs (Biswas and Manto-vani, 2010). Thus, identifying crucial inflammatory mediators may help to develop immunotherapy for lung SCCs. IKK $\alpha$  regulates canonical and noncanonical NF- $\kappa$ B signaling pathways (Sun, 2011), and its activity is different for these NF- $\kappa$ B pathways in different types of cells, due to specific cell receptors (Balkhi et al., 2012). Previously, we showed that IKK $\alpha$  loss reduces I $\kappa$ B $\alpha$  levels and elevates TNF-mediated NF- $\kappa$ B activity in keratinocytes, because the stronger IKK $\beta$  kinase may replace IKK $\alpha$  in the IKK complex (Hu et al., 2001; Park et al., 2007). On the other hand, canonical and noncanonical NF- $\kappa$ B activities are decreased in the BM B cells of *Ikk $\alpha$ <sup>KA/KA</sup>* mice (Balkhi et al., 2012). In this study, we found that the levels of I $\kappa$ B $\alpha$  were reduced in *L-Ikk $\alpha$ <sup>KA/KA</sup>* lungs compared to WT. The canonical NF- $\kappa$ B activity was higher in *L-Ikk $\alpha$ <sup>KA/KA</sup>* lungs (CD45<sup>-</sup> cells), lung SCCs, and *L-Ikk $\alpha$ <sup>KA/KA</sup>* lung SCC cell line than in WT lungs and a WT lung epithelial cell line. Some cytokines and

chemokines overexpressed in *L-Ikk $\alpha^{KA/KA}$*  lungs are NF- $\kappa$ B targets and may help with recruiting leukocytes to the lungs. Also, increased NF- $\kappa$ B activity can reduce cell apoptosis (Ghosh and Karin, 2002). Therefore, we will further determine whether the increased canonical NF- $\kappa$ B pathway contributes to lung SCC development in *L-Ikk $\alpha^{KA/KA}$*  mice and whether the NF- $\kappa$ B pathway can be used as a therapeutic target to prevent and treat lung SCCs.

## EXPERIMENTAL PROCEDURES

### Animal Experiments, Human Samples, and Microarray Data

All mice used in this study were cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the National Institutes of Health, and all animal experiments were approved by IACUC (protocols 08-074, 08-075, 11-051, and 11-052). *Ikk $\alpha^{KA/KA}$*  (Zhu et al., 2007), *Lori.IKK $\alpha$*  (Liu et al., 2006), and *K5.IKK $\alpha$*  (Liu et al., 2011) mice with FVB or BL6 background were used in this study. Human lung tumors were obtained from Dr. David Schrupp at the Surgery Branch, National Cancer Institute. All human samples used in this study were approved by National Institutes of Health Internal Review Board (protocol 06-C-0014), and informed consent has been obtained from patients. Human normal lung tissue lysates were obtained from abcam (ab43320, ab42178, and ab42527, Cambridge, MA). The tissue array (LC991) containing 32 human SCCs with their proximal and distal adjacent lung tissues was purchased from US Biomax. The human lung squamous cell carcinoma cell line SW-900 (HTB-59) was purchased from American Type Culture Collection. Dr. Kwok-Kin Wong provided the paraffin lung sections of five *K-ras<sup>G12D</sup>;Lkb1<sup>-/-</sup>* mice, and this animal experiment was approved by IACUC of Harvard Medical School (protocol 04-094) (Ji et al., 2007).

### ACCESSION NUMBERS

Microarray results (accession number: GSE37655) have been deposited at the NCBI Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>).

### SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, four tables, and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.ccr.2013.03.009>.

### ACKNOWLEDGMENTS

We thank Teri Plona, Scott Coccodrilli, Arati Raziuddin, Hue Vuong, Vika Grinberg, and Robin Stewart from Laboratory of Molecular Technology, SAIC-Frederick, Inc., Frederick National Laboratory for Cancer Research for sequencing *DDR2*, *Sox2*, and *PIK3CA* genes in mouse lung SCCs and Dr. Hesus Padilla-Nash and Dr. Thomas Ried from National Institutes of Health for kindly providing a spontaneously transformed lung murine epithelial cell line M2C. This work was supported by the National Cancer Institute (ZIA BC 011212 and ZIA BC 011391 to Y.H.).

Received: October 27, 2012

Revised: January 21, 2013

Accepted: March 12, 2013

Published: April 15, 2013

## REFERENCES

Aspenström, P., Ruusala, A., and Pacholsky, D. (2007). Taking Rho GTPases to the next level: the cellular functions of atypical Rho GTPases. *Exp. Cell Res.* 313, 3673–3679.

Balkhi, M.Y., Willette-Brown, J., Zhu, F., Chen, Z., Liu, S., Guttridge, D.C., Karin, M., and Hu, Y. (2012). IKK $\alpha$ -mediated signaling circuitry regulates early B lymphopoiesis during hematopoiesis. *Blood* 119, 5467–5477.

Bardeesy, N., Sinha, M., Hezel, A.F., Signoretti, S., Hathaway, N.A., Sharpless, N.E., Loda, M., Carrasco, D.R., and DePinho, R.A. (2002). Loss of the *Lkb1* tumour suppressor provokes intestinal polyposis but resistance to transformation. *Nature* 419, 162–167.

Bernstein, B.E., Mikkelsen, T.S., Xie, X., Kamal, M., Huebert, D.J., Cuff, J., Fry, B., Meissner, A., Wernig, M., Plath, K., et al. (2006). A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell* 125, 315–326.

Biswas, S.K., and Mantovani, A. (2010). Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat. Immunol.* 11, 889–896.

Descargues, P., Sil, A.K., Sano, Y., Korchynskyi, O., Han, G., Owens, P., Wang, X.J., and Karin, M. (2008). IKK $\alpha$  is a critical coregulator of a Smad4-independent TGF $\beta$ -Smad2/3 signaling pathway that controls keratinocyte differentiation. *Proc. Natl. Acad. Sci. USA* 105, 2487–2492.

DuPage, M., Dooley, A.L., and Jacks, T. (2009). Conditional mouse lung cancer models using adenoviral or lentiviral delivery of Cre recombinase. *Nat. Protoc.* 4, 1064–1072.

Ghosh, S., and Karin, M. (2002). Missing pieces in the NF- $\kappa$ B puzzle. *Cell Suppl.* 109, S81–S96.

Gurumurthy, S., Hezel, A.F., Sahin, E., Berger, J.H., Bosenberg, M.W., and Bardeesy, N. (2008). LKB1 deficiency sensitizes mice to carcinogen-induced tumorigenesis. *Cancer Res.* 68, 55–63.

Hackett, N.R., Shaykhiev, R., Walters, M.S., Wang, R., Zwick, R.K., Ferris, B., Witover, B., Salit, J., and Crystal, R.G. (2011). The human airway epithelial basal cell transcriptome. *PLoS ONE* 6, e18378.

Hammerman, P.S., Hayes, D.N., Wilkerson, M.D., Schultz, N., Bose, R., Chu, A., Collisson, E.A., Cope, L., Creighton, C.J., Getz, G., et al.; Cancer Genome Atlas Research Network. (2012). Comprehensive genomic characterization of squamous cell lung cancers. *Nature* 489, 519–525.

Hanahan, D., and Coussens, L.M. (2012). Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell* 21, 309–322.

Hatakeyama, S. (2011). TRIM proteins and cancer. *Nat. Rev. Cancer* 11, 792–804.

Hearle, N., Schumacher, V., Menko, F.H., Olschwang, S., Boardman, L.A., Gille, J.J., Keller, J.J., Westerman, A.M., Scott, R.J., Lim, W., et al. (2006). Frequency and spectrum of cancers in the Peutz-Jeghers syndrome. *Clin. Cancer Res.* 12, 3209–3215.

Hecht, S.S. (2003). Tobacco carcinogens, their biomarkers and tobacco-induced cancer. *Nat. Rev. Cancer* 3, 733–744.

Hu, Y., Baud, V., Delhase, M., Zhang, P., Deerincq, T., Ellisman, M., Johnson, R., and Karin, M. (1999). Abnormal morphogenesis but intact IKK activation in mice lacking the IKK $\alpha$  subunit of IkappaB kinase. *Science* 284, 316–320.

Hu, Y., Baud, V., Oga, T., Kim, K.I., Yoshida, K., and Karin, M. (2001). IKK $\alpha$  controls formation of the epidermis independently of NF- $\kappa$ B. *Nature* 410, 710–714.

Iden, S., and Collard, J.G. (2008). Crosstalk between small GTPases and polarity proteins in cell polarization. *Nat. Rev. Mol. Cell Biol.* 9, 846–859.

Jenkins, S.J., Ruckerl, D., Cook, P.C., Jones, L.H., Finkelman, F.D., van Rooijen, N., MacDonald, A.S., and Allen, J.E. (2011). Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation. *Science* 332, 1284–1288.

Ji, H., Ramsey, M.R., Hayes, D.N., Fan, C., McNamara, K., Kozlowski, P., Torrice, C., Wu, M.C., Shimamura, T., Perera, S.A., et al. (2007). LKB1 modulates lung cancer differentiation and metastasis. *Nature* 448, 807–810.

Johnson, L., Mercer, K., Greenbaum, D., Bronson, R.T., Crowley, D., Tuveson, D.A., and Jacks, T. (2001). Somatic activation of the K-ras oncogene causes early onset lung cancer in mice. *Nature* 410, 1111–1116.

Koster, M.I., Dai, D., Marinari, B., Sano, Y., Costanzo, A., Karin, M., and Roop, D.R. (2007). p63 induces key target genes required for epidermal morphogenesis. *Proc. Natl. Acad. Sci. USA* 104, 3255–3260.

Kumar, P.A., Hu, Y., Yamamoto, Y., Hoe, N.B., Wei, T.S., Mu, D., Sun, Y., Joo, L.S., Dagher, R., Zielonka, E.M., et al. (2011). Distal airway stem cells yield alveoli in vitro and during lung regeneration following H1N1 influenza infection. *Cell* 147, 525–538.

- Kwak, Y.T., Radaideh, S.M., Ding, L., Li, R., Frenkel, E., Story, M.D., Girard, L., Minna, J., and Verma, U.N. (2011). Cells lacking IKK $\alpha$  show nuclear cyclin D1 overexpression and a neoplastic phenotype: role of IKK $\alpha$  as a tumor suppressor. *Mol. Cancer Res.* 9, 341–349.
- Larsen, J.E., and Minna, J.D. (2011). Molecular biology of lung cancer: clinical implications. *Clin. Chest Med.* 32, 703–740.
- Lechner, M., Lirk, P., and Rieder, J. (2005). Inducible nitric oxide synthase (iNOS) in tumor biology: the two sides of the same coin. *Semin. Cancer Biol.* 15, 277–289.
- Liu, B., Park, E., Zhu, F., Bustos, T., Liu, J., Shen, J., Fischer, S.M., and Hu, Y. (2006). A critical role for I kappaB kinase alpha in the development of human and mouse squamous cell carcinomas. *Proc. Natl. Acad. Sci. USA* 103, 17202–17207.
- Liu, B., Xia, X., Zhu, F., Park, E., Carbajal, S., Kiguchi, K., DiGiovanni, J., Fischer, S.M., and Hu, Y. (2008). IKKalpha is required to maintain skin homeostasis and prevent skin cancer. *Cancer Cell* 14, 212–225.
- Liu, B., Willette-Brown, J., Liu, S., Chen, X., Fischer, S.M., and Hu, Y. (2011). IKK $\alpha$  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.* 18, 1854–1864.
- Marinari, B., Moretti, F., Botti, E., Giustizieri, M.L., Descargues, P., Giunta, A., Stolfi, C., Ballaro, C., Papoutsaki, M., Alemà, S., et al. (2008). The tumor suppressor activity of IKKalpha in stratified epithelia is exerted in part via the TGF-beta antiproliferative pathway. *Proc. Natl. Acad. Sci. USA* 105, 17091–17096.
- Melino, G. (2011). p63 is a suppressor of tumorigenesis and metastasis interacting with mutant p53. *Cell Death Differ.* 18, 1487–1499.
- Padilla-Nash, H.M., Hathcock, K., McNeil, N.E., Mack, D., Hoepfner, D., Ravin, R., Knutsen, T., Yonescu, R., Wangsa, D., Dorritie, K., et al. (2012). Spontaneous transformation of murine epithelial cells requires the early acquisition of specific chromosomal aneuploidies and genomic imbalances. *Genes Chromosomes Cancer* 51, 353–374.
- Park, E., Zhu, F., Liu, B., Xia, X., Shen, J., Bustos, T., Fischer, S.M., and Hu, Y. (2007). Reduction in I kappaB kinase alpha expression promotes the development of skin papillomas and carcinomas. *Cancer Res.* 67, 9158–9168.
- Rikova, K., Guo, A., Zeng, Q., Possemato, A., Yu, J., Haack, H., Nardone, J., Lee, K., Reeves, C., Li, Y., et al. (2007). Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. *Cell* 131, 1190–1203.
- Ring, B.Z., Seitz, R.S., Beck, R.A., Shasteen, W.J., Soltermann, A., Arbogast, S., Robert, F., Schreeder, M.T., and Ross, D.T. (2009). A novel five-antibody immunohistochemical test for subclassification of lung carcinoma. *Mod. Pathol.* 22, 1032–1043.
- Sun, S.C. (2011). Non-canonical NF- $\kappa$ B signaling pathway. *Cell Res.* 21, 71–85.
- Takeuchi, K., Soda, M., Togashi, Y., Suzuki, R., Sakata, S., Hatano, S., Asaka, R., Hamanaka, W., Ninomiya, H., Uehara, H., et al. (2012). RET, ROS1 and ALK fusions in lung cancer. *Nat. Med.* 18, 378–381.
- Utikal, J., Polo, J.M., Stadtfeld, M., Maherali, N., Kulalert, W., Walsh, R.M., Khalil, A., Rheinwald, J.G., and Hochedlinger, K. (2009). Immortalization eliminates a roadblock during cellular reprogramming into iPS cells. *Nature* 460, 1145–1148.
- Vanbokhoven, H., Melino, G., Candi, E., and Declercq, W. (2011). p63, a story of mice and men. *J. Invest. Dermatol.* 131, 1196–1207.
- Woodworth, C.D., Mossman, B.T., and Craighead, J.E. (1983). Induction of squamous metaplasia in organ cultures of hamster trachea by naturally occurring and synthetic fibers. *Cancer Res.* 43, 4906–4912.
- Xi, S., Yang, M., Tao, Y., Xu, H., Shan, J., Inchauste, S., Zhang, M., Mercedes, L., Hong, J.A., Rao, M., and Schrupp, D.S. (2010). Cigarette smoke induces C/EBP- $\beta$ -mediated activation of miR-31 in normal human respiratory epithelia and lung cancer cells. *PLoS ONE* 5, e13764.
- Yang, G.Y., Taboada, S., and Liao, J. (2009). Induced nitric oxide synthase as a major player in the oncogenic transformation of inflamed tissue. *Methods Mol. Biol.* 512, 119–156.
- Ylikorkala, A., Rossi, D.J., Korsisaari, N., Luukko, K., Alitalo, K., Henkemeyer, M., and Mäkelä, T.P. (2001). Vascular abnormalities and deregulation of VEGF in Lkb1-deficient mice. *Science* 293, 1323–1326.
- Yuan, P., Kadara, H., Behrens, C., Tang, X., Woods, D., Solis, L.M., Huang, J., Spinola, M., Dong, W., Yin, G., et al. (2010). Sex determining region Y-Box 2 (SOX2) is a potential cell-lineage gene highly expressed in the pathogenesis of squamous cell carcinomas of the lung. *PLoS ONE* 5, e9112.
- Zhu, F., Xia, X., Liu, B., Shen, J., Hu, Y., Person, M., and Hu, Y. (2007). IKKalpha shields 14-3-3sigma, a G2/M cell cycle checkpoint gene, from hypermethylation, preventing its silencing. *Mol. Cell* 27, 214–227.