IKKα inactivation promotes Kras-initiated lung adenocarcinoma development through disrupting major redox regulatory pathways

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Lung adenocarcinoma (ADC) and squamous cell carcinoma (SCC) are two distinct and predominant types of human lung cancer. Kras kinase α (IKKα) has been shown to suppress lung SCC development, but its role in ADC is unknown. We found inactivating mutations and homologous or hemizygous deletions in the CHUK locus, which encodes IKKα, in human lung ADCs. The CHUK deletions significantly reduced the survival time of patients with lung ADCs harboring KRAS mutations. In mice, lung-specific Ikkα ablation (IkkαΔα) induces spontaneous ADCs and promotes KrasG12D-initiated ADC development, accompanied by increased cell proliferation, decreased cell senescence, and reactive oxygen species (ROS) accumulation. IKKα deletion up-regulates NOX2 and regulates NRF2, leading to ROS accumulation and blockade of cell senescence induction, which together accelerate ADC development. Pharmacologic inhibition of NADPH oxidase or ROS impairs KrasG12D-mediated ADC development in IkkαΔα mice. Therefore, IKKα modulates lung ADC development by controlling redox regulatory pathways. This study demonstrates that IKKα functions as a suppressor of lung ADC in human and mice through a unique mechanism that regulates tumor cell-associated ROS metabolism.

Significance

Reactive oxygen species (ROS) can promote tumorigenesis or kill cancer cells. How different cancer-associated genetic alterations regulate ROS balance and outcome is of great importance for the design of rational cancer treatments, many of which affect ROS metabolism and sensing. Kras activation induces a ROS defense system and cell senescence, which counteract its oncogenic activity. KRAS-activating mutations are accompanied by IKKα loss mutations that result in elevated NOX2 but decreased expression of the NRF2 ROS defense system. Thus, IKKα ablation turns the immunoregulatory effect of Kras-induced ROS to a protumorigenic effect that enhances Kras-induced progression of lung adenocarcinoma (ADC). Restoration of IKKα activity or inhibition of the pathways activated on its loss may offer new opportunities for ADC treatment.

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ROS are essential for maintaining cellular metabolism, survival, proliferation, and differentiation in normal cells. Cancer cells adapt to exist with elevated ROS levels compared with normal cells (19, 20). Numerous studies have documented that excessive ROS either promote tumor development or kill cancer cells via an apoptotic mechanism (21, 22). In response to ROS, NRF2 up-regulates the expression of antioxidants and detoxifying enzymes, thereby maintaining ROS homeostasis. NRF2 has been shown to inhibit KrasG12D-initiated early lung ADC but to accelerate advanced ADC (23); however, most human lung ADCs do not harbor KAP1 mutations that result in NRF2 accumulation (1, 24). Thus, there remains a need to identify additional NRF2 regulators and mechanisms underlying NRF2 accumulation or down-regulation in lung ADC.

Chemical carcinogens induce activating Hras mutations and ROS accumulation in mouse skin (25, 26). Deletion of NRF2 or NAD(P)H quinone dehydrogenase 1 (NQO1, an NRF2 target) enhances carcinogen-induced skin carcinogenesis in mice (27, 28). Ikka+/− mice develop many more skin papillomas and malignant carcinomas than wild-type (WT) mice in response to carcinogen administration (26). Given the known activities of NRF2 and NQO1 in scavenging ROS, these phenotypic similarities among NRF2, NQO1, and IKKα suggest that all may impact ROS accumulation and Hras activation during skin tumorigenesis. To date, the regulatory relationship between NRF2 and IKKα remains unclear. Moreover, activated Kras promotes ROS accumulation, which induces cell senescence (29–31), antagonizing Kras-initiated lung ADC progression. How NRF2 regulates the antitumorigenic effects of Kras-induced ROS merits further investigation.

The Cancer Genome Atlas (TCGA) database analysis has revealed the mutations and deletions in the CHUK locus, which encodes IKKα, in a subfraction of human lung ADC. Here we show that lung-specific IKKα ablation induces spontaneous lung ADC and promotes Kras-initiated lung ADC development in mice, and further demonstrate that IKKα controls ADC development through its unique effects on ROS metabolism, mediated through NRF2 and NOX2.

Results

Lung Epithelial Cell IKKα Suppresses ADC Development. To investigate the effect of IKKα on lung ADC development, we ablated IKKα in lungs of C57BL/6 IkkaΔf/f mice (15) by intratracheal Adenovirus.Cre (Ad.Cre) administration (IkkaΔf/f). Conditional deletion of IKKα resulted in spontaneous lung ADCs in 8 out of 48 Ikka−/− mice at 13–20 mo of age (Fig. L4, Top). No lung ADCs were detected in 30 WT mice. Activating KrasG12D mutations at amino acid 12 are commonly identified in human lung ADC (1), and KrasG12D activation induces spontaneous lung ADC in mice (32). Thus, ADC developed from C57BL/6 KrasG12D−/− (KrasG12D) mice were used as positive controls (Fig. 1A, Bottom). With increasing age, ADC derived from Ikka−/− mice metastasized to the spleen and other organs, as indicated by positivity for SP-C, a marker of type II lung epithelial cells (Fig. S1A).

To investigate the effect of IKKα on KrasG12D-induced lung ADC, we crossed C57BL/6 Ikka−/− mice or Ikka−/− mice with C57BL/6 KrasG12D−/− mice and used Ad.Cre to induce KRASG12D expression and simultaneously delete IKKα. KrasG12D−/− Ikka−/− and KrasG12D−/− IkkaΔf/f mice showed a significantly greater lung tumor burden compared with KrasG12D−/− mice (Fig. 1B and C and Fig. S1B), ADCs developed from KrasG12D−/− IkkaΔf/f, KrasG12D−/− IkkaΔf/f, and KrasG12D−/− Ikka+/− mice were positive for SP-C and CC10 (a marker of lung epithelial Clara cells), but negative for K5, an SCC marker (Fig. 1D and E). We confirmed Ikka deletion and KrasG12D activation in KrasG12D−/− Ikka−/− lung ADCs and KrasG12D activation in KrasG12D−/− IkkaΔf/f ADCs (Fig. S1C).

Following Ad.Cre treatment, KrasG12D−/− IkkaΔf/f, KrasG12D−/− IkkaΔf/f, and KrasG12D−/− IkkaΔf/f mice showed a significantly reduced life span compared with KrasG12D−/− mice (Fig. 1F and Fig. S1D). Loss of the WT Ikka allele [i.e., loss of heterozygosity (LOH), a tumor-suppressor hallmark] was detected in KrasG12D−/− Ikka−/− lung ADCs (Fig. S1E). Ikka LOH was previously reported in carcinogen-induced skin tumors in Ikka+/− mice (26). Collectively, these results indicate that lung epithelial cell IKKα ablation promotes KrasG12D-initiated lung ADC development. Although FVB −/− Ikka−/−/− mice, in which lysine is replaced by alanine at amino acid 44 of IKKα, develop spontaneous lung SCC (5), we did not detect lung SCC in FVB or C57BL/6 Ikka−/−/− mice, KrasG12D−/− Ikka−/−/− mice, or KrasG12D−/− Ikka−/−/− mice in this study.

We then examined the TCGA database (cBioPortal) of Human Cancer Genomics (1) and found a 2.2% mutation rate in the CHUK locus in lung ADC, including CHUK+1 and CHUK+3 point mutations, which generate the C-terminal truncated IKKα variants lacking its leucine zipper (LZ) and helix-loop-helix (HLH) domains, as well as CHUK homozygous deletions (Fig. 1G, Top). We also found CHUK hemizygous deletions in ∼22% of human lung ADCs (Fig. 1G, Bottom). The LZ and HLH motifs are required for IKKα activity (13, 15, 33, 34). Human lung ADCs carrying CHUK mutations had an activating KRAS mutation that causes an amino acid change at position 12, as well as TP53 mutations (Fig. 1G, Top and Fig. S1F). Eight out of 51 human lung ADCs bearing a CHUK hemizygous deletion also had an activating KRAS G12C or G12V mutation (Fig. 1G, Bottom), suggesting that some CHUK alterations have a positive correlation with activating KRAS mutations.

We also examined the effect of CHUK mutations on the survival of patients with lung ADC. The median survival of the patients in this cohort is 44.6 mo (1), compared with 19.5 mo for patients with CHUK mutations and 35.5 mo for patients with KRAS mutations. Although the number of patients with a CHUK mutation is limited, the data suggest that patients with lung ADC with CHUK mutations may have a tendency toward shorter survival. We further compared the survival curves among patients with CHUK alterations, including mutations and hemizygous deletions, KRAS mutations, and KRAS mutations/CHUK hemizygous deletions, and found that CHUK mutations or hemizygous deletions significantly reduced the survival time of patients with lung ADC carrying a KRAS mutation (Fig. 1H). Based on the foregoing animal results, IKKα inactivation may promote human lung ADC development.

Reduced IKKα Promotes Bronchial Epithelial Cell Proliferation and Attenuates Cell Senescence. Compared with KrasG12D−/− mice, KrasG12D−/− Ikka−/− and KrasG12D−/− IkkaΔf/f mice developed significantly enlarged lungs with markedly increased Ki67-positive bronchial epithelial cells, which can give rise to lung ADCs (Fig. 2A and B and Fig. S2A), suggesting that IKKα reduction or deletion promotes lung epithelial cell proliferation. The Ikka−/− mutation severely destabilizes IKKα and also abolishes its catalytic activity (5). Indeed, IKKα levels were decreased in KrasG12D−/− Ikka−/− lung ADCs compared with WT lungs and KrasG12D−/− ADCs (Fig. S2D). Moreover, following intratracheal treatment with Ad.Cre, a small group of KrasG12D−/− Ikka−/− mice developed severe skin lesions, precluding their maintenance. Thus, we used IkkaΔf/f mice for all subsequent studies.

Oncogenic KrasG12D induces premalignant lesions by increasing cell senescence, as indicated by senescence-associated β-galactosidase (SA-β-gal) staining (30). KrasG12D−/− Ikka−/− lung ADCs displayed substantially less SA-β-gal staining and more Ki67 than KrasG12D−/− ADCs (Fig. 2C and Fig. S2C). The tumor suppressor p53 is essential for induction of cell senescence (30). Decreased p53 and p21Δup (p21) expression can overcome cell
IKKα deletion induces spontaneous lung ADCs and promotes Kras-initiated lung ADCs, and somatic CHUK aberrations are detected in human lung ADCs. (A, Top) Lung-specific IKKα ablation by intratracheal Ad.Cre injection induced spontaneous lung ADCs in 8 of 48 IkkαΔLu mice and in 0 of 30 WT mice. ADCs stained with hematoxylin and eosin (H&E) in IkkαΔLu mice at age 13 mo. (A, Bottom) H&E-stained ADCs from KrasG12D and KrasG12D;IkkαΔLu mice at 4 mo after Ad.Cre treatment (n = 6 mice/group) and a representative H&E-stained ADC. ***P < 0.001, Student’s t test. (Scale bar: 25 μm.) (C) Lung ADC burden in KrasG12D and KrasG12D;IkkαKA/KA mice at 4.5 mo after Ad.Cre treatment (n = 4 mice/group) and a representative H&E-stained ADC. **P < 0.01, Student’s t test. (Scale bar: 25 μm.) (D) Immunofluorescence (IF) staining with anti–SP-C or anti–CC10 antibody showing the tissue origins of ADCs in KrasG12D, KrasG12D;IkkαΔLu, and KrasG12D;IkkαKA/KA mice and WT lungs (n = 3 mice/group). DAPI, nuclear staining. (Scale bar: 30 μm.) (E) ADCs from KrasG12D;IkkαΔLu mice were stained by immunohistochemistry (IHC) with K5 or SP-C antibody (n = 3). (Scale bar: 30 μm.) (F) Survival of KrasG12D mice compared with several IKKα mutants crossed with KrasG12D mice. **P < 0.01; *P < 0.05, Mantel–Cox log-rank test. Mouse numbers and P values are shown. The red asterisk indicates IKKα reduction; the red #, LOH. (G) Top) CHUK mutations and deletion were found in 230 human lung ADCs (cBioPortal for Cancer Genomics) (1) that generate truncated IKKα proteins labeled with red numbers. aa, amino acid; HLH, helix-loop-helix; KD, kinase domain; LZ, leucine zipper. (G, Bottom) Analysis of CHUK (IKKα) mRNA expression (RNA sequence V2 RSEM) in 230 human lung ADCs (1). Putative copy number calls on 230 cases were determined using GISTIC 2.0. Values: −2, homozygous deletion; −1, hemizygous (shallow) deletion; 0, neutral/no change; 1, gain; 2, high-level amplification. (H) Survival curves for patients with CHUK alterations, including mutations (M) and hemizygous deletions (Hem), Kras mutations, and Kras mutations/CHUK hemizygous deletions. **P < 0.01, χ² test (comparisons between two groups).
Fig. 2. IKKa deletion promotes cell growth but reduces cell senescence. (A) Lung appearance and weights of Kras\textsuperscript{G12D} mice (n = 8) and Kras\textsuperscript{G12D},Ikk\textalpha\textalpha\textalpha\textalpha\textalpha\textalpha\texttext{ΔLu} mice (n = 7) and Ki67-stained bronchial epithelial cells in the lungs of these mice (n = 3 mice/10 slides/group; Right) at 4 mo after Ad.Cre treatment. **P < 0.01; ***P < 0.001, Student’s t test. (B) Lung appearance and weight in four Kras\textsuperscript{G12D} and three Kras\textsuperscript{G12D},Ikk\textalpha\textalpha\textalpha\textalpha\textalpha\textalpha\texttext{ΔLu} mice (Left and Center) and Ki67-stained bronchial epithelial cells in the lungs of these mice (n = 3 mice/group; Right) at 4.5 mo after Ad.Cre treatment. **P < 0.01, Student’s t test. (C) Comparison of SA-β-gal staining intensities between Kras\textsuperscript{G12D},Ikk\textalpha\textalpha\texttext{ΔLu} and Kras\textsuperscript{G12D} tumors (n = 3 mice/group). SA, senescence-associated. **P < 0.01, Fisher’s exact test. (D) IB analysis of IKKα, p53, and p21 expression in WT lungs and Kras\textsuperscript{G12D} and Kras\textsuperscript{G12D},Ikk\textalpha\textalpha\textalpha\textalpha\textalpha\textalpha\texttext{ΔLu} ADCs. β-actin served as a protein-loading control. (E) A scheme for generating Kras-CL and Kras\textsuperscript{G12D} cells involving intratracheal injections of these cells into WT mice with a C57BL/6 background. ADCs generated by these cells are stained with H&E. (Scale bar: 25 μm.) (F) IB analysis of IKKα in Kras-CL and Kras\textsuperscript{G12D} cells. β-actin served as a protein-loading control (Top Left). Shown are lung appearance (Bottom Left) and tumor burden (Right) in WT mice receiving intratracheal injections of Kras-CL (n = 4) or Kras\textsuperscript{G12D},Ikk\textalpha\textalpha\textalpha\textalpha\textalpha\textalpha\texttext{ΔLu} (n = 5) cells (5 × 10\textsuperscript{6} cells/mouse), as analyzed statistically using Student’s t test. ***P < 0.001. (G) The growth of tumors in nude mice receiving s.c. injection of Ikk\textalpha\textalpha\textalpha\textalpha\textalpha\texttext{ΔLu} or control vector-transfected Kras\textsuperscript{G12D} cells (n = 5 mice/group) for 2 wk. Data represent mean ± SD. **P < 0.01, Student’s t test.

cycle arrest and senescence and thereby promote tumor progression. Immunoblot (IB) analysis showed lower expression of p53 and p21 in Kras\textsuperscript{G12D},Ikk\textalpha\textalpha\textalpha\textalpha\textalpha\textalpha\texttext{ΔLu} tumors than in Kras\textsuperscript{G12D} tumors (Fig. 2D), which may account for the hyperproliferative phenotype in the lungs of Kras\textsuperscript{G12D},Ikk\textalpha\textalpha\textalpha\textalpha\textalpha\textalpha\texttext{ΔLu} mice compared with Kras\textsuperscript{G12D} mice. Of note, decreased IKKα expression was seen in some Kras\textsuperscript{G12D}-lung ADCs and this was accompanied by reduced p53 and p21 expression (Fig. 2D). These results suggest that reduced IKKα expression in lung ADCs is associated with increased cell proliferation and decreased cell senescence.

To determine the epithelial cell-autonomous role of IKKα in lung ADC development, we generated a Kras\textsuperscript{G12D} ADC (Kras-CL) cell line (Fig. 2E) and transplanted these cells into the lungs of C57BL/6 WT mice. From the resulting lung ADCs, we isolated another cell line, Kras\textsuperscript{IKKα ΔLu}, that expressed less IKKα than the parental Kras-CL cells (Fig. 2E and Fig. 2F, Top Left). Kras\textsuperscript{IKKα ΔLu} cells generated many more ADCs than the parental Kras-CL cells after transplantation into C57BL/6 WT mice, although both cell lines contained an activated Kras\textsuperscript{G12D} allele (Fig. 2F, Bottom Left and Right and Fig. S2D). To verify the inhibitory effect of IKKα on tumorigenesis, we reexpressed IKKα into Kras\textsuperscript{IKKα ΔLu} cells and found that reintroduction of IKKα reduced tumor sizes compared with controls when these cells were injected s.c. into nude mice (Fig. 2G and Fig. S2E). These results indicate that reduced IKKα expression in lung ADC cells promotes tumorigenesis.

IKKα Ablation Enhances ROS in Lung ADCs, and Treatment with Apocynin Attenuates ROS and Lung Tumorigenesis. ROS induce p53 expression (35). Unexpectedly, however, we detected more ROS in Kras\textsuperscript{G12D},Ikk\textalpha\textalpha\textalpha\textalpha\textalpha\textalpha\texttext{ΔLu} ADCs than in Kras\textsuperscript{G12D} ADCs (Fig. 3A). Using gene expression array analysis (GSE84159), we found increased expression of genes encoding the NADPH oxidase (NOX) complex subunits that are involved in ROS generation (36), such as Cyba, Ncf2, Ncf1, and Cybb, which encodes NOX2 (37), in Kras\textsuperscript{G12D},Ikk\textalpha\textalpha\textalpha\textalpha\texttext{ΔLu} lungs compared with Kras\textsuperscript{G12D} lungs (Fig. 3B). We did not observe increased expression of other NOX types. RT-PCR confirmed the significantly higher levels of NOX2 in Kras\textsuperscript{G12D},Ikk\textalpha\textalpha\textalpha\textalpha\texttext{ΔLu} lungs compared with Kras\textsuperscript{G12D} lungs (Fig. 3C). Moreover, IB analysis showed significantly higher NOX2 levels in Kras\textsuperscript{G12D},Ikk\textalpha\textalpha\textalpha\textalpha\texttext{ΔLu} ADCs compared with Kras\textsuperscript{G12D} ADCs (Fig. 3D and Fig. S34). Indeed, some human
Fig. 3. IKKα ablation increases NOX2 expression, and NOX inhibition reduces lung tumor burden. (A) Increased levels of ROS stained with DCF-DA (in green) in tumors derived from KasR\textsuperscript{G12D};Ikkα\textsuperscript{ΔLz} mice compared with KasR\textsuperscript{G12D} tumors and WT lungs (Right). n = 6/group. ***p < 0.001, Student’s t test. (B) Comparison of expression levels of genes encoding Cyba, Ncf2, Ncf1, and Cyb in KasR\textsuperscript{G12D};Ikkα\textsuperscript{ΔLz} lungs vs. WT (red) and in KasR\textsuperscript{G12D} lungs vs. WT (green), as determined by gene array analysis. Red asterisk: Cyb is also the name of the Nox2 gene. (C) RT-PCR analysis of Nox2 mRNA expression in KasR\textsuperscript{G12D} and KasR\textsuperscript{G12D};Ikkα\textsuperscript{ΔLz} lungs (n = 6). Data represent mean ± SEM (three repeats). *p < 0.05, Student’s t test. (D) IB analysis of NOX2 expression in four KasR\textsuperscript{G12D} and five KasR\textsuperscript{G12D};Ikkα\textsuperscript{ΔLz} lung ADCs (Fig. S3A). Data represent mean ± SD (three repeats). *p < 0.05, Student’s t test. (E) IB analysis of IKKα, NOX2, and Nrf2 expression in human lung ADCs. Red asterisks indicate a relationship between IKKα and NOX2. β-actin served as a protein-loading control. (F and G) Apocynin (100 μM) or NAC (10 mM) reduces ROS (stained with DCF-DA) induced by IKKα down-regulation in human A549 cells (F) and mouse KasR\textsuperscript{CL} cells (G). (H) The effect of apocynin on lung tumor burden in KasR\textsuperscript{G12D} (Right) and KasR\textsuperscript{G12D};Ikkα\textsuperscript{ΔLz} (Left) mice. n = 6 for untreated mice; n = 9 for treated mice. *p < 0.05, Student’s t test. n.s., not significant.

lungs. A549 cells were treated with apocynin, a NOX inhibitor, for 4 mo significantly reduced the lung ADC burden in KasR\textsuperscript{G12D},Ikkα\textsuperscript{ΔLz} mice compared with vehicle-treated KasR\textsuperscript{G12D} mice (Fig. 3H, Left); however, treatment with apocynin did not decrease the lung ADC burden in KasR\textsuperscript{G12D} mice compared with controls (Fig. 3H, Right). These results suggest that IKKα reduction results in increased amounts of NOX2 and intratumoral ROS. IKKα is part of the IKK complex, but knockdown of IKKα did not alter NF-κB activity in A549 cells (Fig. S3E), suggesting that IKKα may regulate NOX2 expression and ROS levels via an NF-κB-independent mechanism.

Knockdown of NOX2 in Lung ADC Cells Inhibits Lung ADC Growth, and IKKα Regulates NOX2 Expression via the Nox2 Promoter. To verify the relationships among epithelial cell IKKα, NOX2, and ROS in lung tumorigenesis, we confirmed higher ROS levels in KasR\textsuperscript{IKKα} cells than in KasR\textsuperscript{CL} cells and verified that treatment with apocynin reduced ROS levels in KasR\textsuperscript{IKKα} cells (Fig. 4A and B). Because KasR\textsuperscript{CL} cells required more than 3 mo to generate lung ADCs in WT mice, we used KasR\textsuperscript{IKKα} cells to determine the effect of NOX2 and ROS on ADC formation. Consistently, a 6-wk course of treatment with apocynin reduced KasR\textsuperscript{IKKα} cell-generated lung ADC numbers and lung weights in C57BL/6 WT mice compared with controls (Fig. 4C). These results demonstrate that IKKα levels in lung ADC cells are inversely correlated with ROS levels and lung tumor development and that increased ROS enhance the tumorigenic potential of KasR\textsuperscript{IKKα} cells.

Furthermore, KasR\textsuperscript{IKKα} cells expressed higher levels of Nox2 mRNA compared with KasR\textsuperscript{CL} cells (Fig. 4D), and silencing IKKα resulted in elevated NOX2 expression in A549 cells (Fig. S4A and B). In contrast, knockdown of NOX2 significantly attenuated ROS levels in KasR\textsuperscript{IKKα} cells and impaired KasR\textsuperscript{IKKα} cell-generated lung tumors in C57BL/6 WT mice at 6 wk after the transplantation of Nox2-SiRNA– or control-SiRNA–treated KasR\textsuperscript{IKKα} cells (Fig. 4E and F), although NOX2 knockdown had less effect on lung weight than apocynin, suggesting that increased NOX2 expression enhances the tumorigenic potential of KasR\textsuperscript{IKKα} cells by elevating ROS levels.

We then investigated the mechanism underlying the regulation of NOX2 expression by IKKα. The aryl hydrocarbon receptor (AhR) is known to repress Nox2 transcription (39). We postulated that IKKα may regulate Nox2 transcription via its effects on AhR activity. Indeed, an interaction between IKKα and AhR was detected by pull-down assays with an anti-AhR or an anti-IKKα antibody in A549 cells (Fig. S4C). In addition, kinase-inactive IKKα (IKKα-ΔLZ), but not a mutant IKKα with its LZ deletion from amino acids 441–531 (IKKα-ΔLZ), interacted with AhR (Fig. S4D), suggesting that IKKα may regulate Nox2 expression independent of its kinase activity. Chromatin immunoprecipitation (ChiP) assays demonstrated that both IKKα and AhR were associated with the xenobiotic response element-containing region of the Nox2 promoter in KasR\textsuperscript{CL} cells and in human A549 cells (Fig. 4G and Fig. S4 E and F). In contrast, IKKα depletion decreased the recruitment of AhR to the Nox2 promoter, and reintroduction of WT IKKα or IKKα-ΔLZ, but not of IKKα-ΔLZ, recruited AhR to the Nox2 promoter in IKKα-deficient KasR\textsuperscript{CL} and A549 cells (Fig. 4G and Fig. S4E, Left, and Fig. S4F). Furthermore, silencing IKKα elevated Nox2 expression in KasR\textsuperscript{CL} cells, while reintroducing IKKα or IKKα-ΔLZ, but not IKKα-ΔLZ, elevated NOX2 expression in IKKα-deficient KasR\textsuperscript{CL} cells (Fig. 4H and Fig. S4E, Right), although a slight reduction in IKKα-ΔLZ binding to the Nox2 promoter was seen, suggesting that IKKα integrity, but not its kinase activity, is required for the regulation of NOX2 expression. These results indicate that IKKα suppresses NOX2 expression by recruiting AhR to the Nox2 promoter, whereas IKKα deletion diminishes AhR binding to the Nox2 promoter, leading to increased Nox2 expression and ROS production (Fig. 4I).
ADCs Express Reduced NRF2, and NAC Treatment Inhibits Lung ADC Burden in Kras\textsuperscript{G12D},Ikk\textalpha\textsubscript{ΔLz} Mice. A feedback loop between ROS production and elimination balances physiological ROS amounts. We expected to find that increased ROS resulted in NRF2 activation. Surprisingly, however, the expression of NRF2 target genes encoding antioxidants and detoxifying enzymes was lower in Kras\textsuperscript{G12D},Ikk\textalpha\textsubscript{ΔLz} lungs than in Kras\textsuperscript{G12D} lungs (Fig. S5A). IB analysis showed that Kras\textsuperscript{G12D} ADCs expressed more NRF2 than WT lungs, whereas Kras\textsuperscript{G12D},Ikk\textalpha\textsubscript{ΔLz} ADCs expressed less NRF2 than WT lungs (Fig. 5A). Among Kras\textsuperscript{G12D} ADCs, those expressing less Ikk\textalpha consistently showed lower NRF2 and p21 expression (Fig. 5B). Importantly, IB analysis showed reduced Ikk\textalpha and NRF2 expression in a subfraction of human lung ADCs, and indeed, some human lung ADCs showed reduced Ikk\textalpha and NRF2 expression and increased NOX2 expression (Fig. 3E) and found that a subgroup of these ADCs expressed significantly less Ikk\textalpha and NRF2 compared with another ADC group (Fig. 5C), suggesting clinical relevance of the reduced Ikk\textalpha and NRF2 expression in human lung ADC.

If reduced NRF2 expression promotes ROS accumulation, which further contributes to increased tumorogenesis, then treatment with NAC should inhibit lung ADC burden in Kras\textsuperscript{G12D},Ikk\textalpha\textsubscript{ΔLz} mice. Indeed, NAC treatment significantly decreased lung weights and ADC burden in Kras\textsuperscript{G12D},Ikk\textalpha\textsubscript{ΔLz} mice compared with controls, but this treatment did not significantly affect the ADC burden in Kras\textsuperscript{G12D} mice (Fig. 5D and E). The oxidative DNA damage (8-OHdG) marker was higher in Kras\textsuperscript{G12D},Ikk\textalpha\textsubscript{ΔLz} ADCs than in Kras\textsuperscript{G12D} ADCs, and NAC treatment decreased DNA damage (Fig. 5F), suggesting that accumulated ROS cause DNA damage, which is associated with enhanced lung tumorogenesis. As expected, the expression levels of NRF2 targets Nqo1 and Gpx2 were significantly lower in Kras\textsuperscript{G12D},Ikk\textalpha\textsubscript{ΔLz} ADCs than in Kras\textsuperscript{G12D} ADCs (Fig. 5G), suggesting that ROS scavengers regulate lung ADC development in the absence of Ikk\textalpha.

Ikk\textalpha Loss Down-Regulates NRF2 Expression in an Epigenetic Manner. Treatment with NAC decreased the number of ROS in Kras\textsuperscript{Ikk\textalpha} cells and also inhibited Kras\textsuperscript{Ikk\textalpha} cell-generated lung tumor growth in WT mice compared with controls (Fig. 6A and B), suggesting a reciprocal correlation between Ikk\textalpha-regulated NRF2 expression and ROS levels during lung tumorigenesis. KEAP1 is a major negative regulator of NRF2 stability (40), Kras-CL and Kras\textsuperscript{Ikk\textalpha}\textsubscript{ΔLz} cells expressed similar amounts of
KEAP1, however (Fig. S6A). Expression of Nrf2 mRNA was lower in Kras\(^{G12D,\Delta\text{IKK}_{LZ}}\) lungs than in Kras\(^{G12D}\) lungs (Fig. 6C). Knockdown of IKK\(\alpha\) attenuated Nrf2 expression, and reintroduction of IKK\(\alpha\) rescued Nrf2 expression (Fig. S6B), suggesting that IKK\(\alpha\) regulates Nrf2 gene transcription.

Trimethylation at lysine 9 of histone H3 (H3-K9) represses gene expression by recruiting DNA methyltransferases, and trimethyltransferase Suv39h1 is required for H3-K9 trimethylation (13, 41, 42). We previously reported that IKK\(\alpha\) interacts directly with H3 protein, which in turn shields chromatin-associated H3 from H3-K9 trimethylation by preventing SuV39h1 from accessing the Stratifin chromatin (13). Several CpG islands are present in the Nrf2 promoter region. Nrf2 mRNA levels were significantly lower in Kras\(^{\Delta\text{IKK}_{LZ}}\) cells than in Kras-CL cells (Fig. 6D). Treatment with 5-azacytidine, a DNA methyltransferase inhibitor, increased Nrf2 mRNA levels in Kras\(^{\Delta\text{IKK}_{LZ}}\) cells (Fig. 6E), suggesting that IKK\(\alpha\) modulates the Nrf2 promoter activity in an epigenetic manner.

Using ChIP assays, we found IKK\(\alpha\) to be associated with the Nrf2 promoter in Kras-CL cells (Fig. 6F and Fig. S6C). With the loss of IKK\(\alpha\) from the Nrf2 promoter in Kras\(^{\Delta\text{IKK}_{LZ}}\) cells, increased levels of trimethylated-H3-K9, SuV39h1, and Dnmt3a were found at the Nrf2 promoter compared with Kras-CL cells (Fig. 6F and Fig. S6C). Reintroducing WT IKK\(\alpha\) or IKK\(\alpha\)-K\(\alpha\), but not IKK\(\alpha\)-\(\Delta\text{LZ}\), formed the complex with Nrf2 promoter in Kras\(^{\Delta\text{IKK}_{LZ}}\) cells (Fig. S6D). Consistently, silencing IKK\(\alpha\) decreased Nrf2 expression in Kras-CL cells, and reintroducing WT IKK\(\alpha\) or IKK\(\alpha\)-K\(\alpha\), but not IKK\(\alpha\)-\(\Delta\text{LZ}\), increased Nrf2 expression in IKK\(\alpha\)-deficient Kras-CL cells (Fig. 6G). These results suggest that IKK\(\alpha\) suppresses H3-K9 trimethylation on the Nrf2 promoter, thereby increasing its transcription. In contrast, IKK\(\alpha\) loss elevates H3-K9 trimethylation on the Nrf2 promoter, inhibiting Nrf2 expression (Fig. 6H and Fig. S6E).

To elucidate how IKK\(\alpha\) expression is down-regulated in Kras\(^{\Delta\text{IKK}_{LZ}}\) cells, we sequenced full-length IKK\(\alpha\) cDNA and identified a missense mutation at nucleotide 2054 (amino acid 685) in Kras\(^{\Delta\text{IKK}_{LZ}}\) cells, Kras-CL cells, and Kras\(^{G12D}\)-induced ADCs (Fig.
Interestingly, Kras<sup>IKKα−/−</sup> cells exhibited many additional Ikkα mutations surrounding the nucleotide 2054 genetic lesion, suggesting that these mutations confer a growth advantage, possibly by destabilizing the IKKα protein. Accordingly, IKKα immunoprecipitation from Kras-CL and Kras<sup>IKKα−/−</sup> cells, followed by IB analysis with an anti-ubiquitin antibody, showed more ubiquitinated IKKα in Kras-CL cells compared with Kras-CL cells (Fig. S6G, Top). Treatment with MG132, a proteasome inhibitor, elevated Ikkα and NRF2 levels in Kras<sup>IKKα−/−</sup> cells (Fig. S6G, Bottom), suggesting that tumor-associated mutations promote proteasomal degradation of IKKα. We previously detected the same Ikkα mutations and deletions in the C-terminal region of IKKα in skin SCCs derived from carcinogen-treated Ikkα<sup>−/−</sup> and WT mice (13, 15, 26). These mutations impaired the IKKα activity that controls the G2/M cell cycle checkpoint in response to DNA damage and keratinocyte growth. Thus, the DNA encoding the IKKα C-terminal region behaves like a mutational “hot spot” in different types of cancers.

**A ROS-Mediated NRF2-NQO1 Pathway Leads to the Induction of p53** p21 and Cell Senescence, and IKKα Inactivation Reverses This Pathway. Reduction of NOQ1, an NRF2 target, results in p53 degradation independent of MDM2 (43, 44), suggesting that along with antioxidative activity, the ROS-mediated NRF2-NQO1 pathway may promote tumor progression by up-regulating p53, p21, and cell senescence (30). We hypothesized that reduced Ikkα down-regulates NRF2 and NOQ1 expression, which attenuates p53 and p21 expression and cell senescence. Indeed, Kras<sup>IKKα−/−</sup> cells expressed reduced Ikkα, NRF2, NOQ1, p53, and p21 and showed attenuated cell senescence compared with Kras-CL cells (Fig. 7A, Left and B). Silencing of Ikkα repressed NRF2, NOQ1, p53, and p21 expression and attenuated cell senescence in Kras-CL and A549 cells (Fig. 7A, Center and C and Fig. S7A and B). In addition, silencing of NRF2 or NOQ1 repressed NOQ1, p53, and p21 expression and attenuated cell senescence in Kras-CL cells (Fig. 7A, Right, D and E and Fig. S7C). These results suggest that IKKα reduction blocks cell cycle arrest by decreasing NRF2, NOQ1, and p21 expression. Importantly, silencing of Ikkα, NRF2, or NOQ1 in Kras-CL cells promoted tumor growth compared with the control when these cells were injected s.c. into nude mice (Fig. 7F–H).

To demonstrate links among Ikkα action, ROS, and ROS-meditated cell senescence, we examined the effect of NAC and apocynin on cell senescence (p53/p21) in Kras<sup>IKKα−/−</sup> and Kras-CL cells (Fig. 7I). Indeed, treatment with NAC or apocynin induced p53/p21 expression in Kras<sup>IKKα−/−</sup> cells. This induction was stronger in Kras<sup>IKKα−/−</sup> cells than in Kras-CL cells (Fig. 7I). Taken together, these findings show that IKKα ablation not only elevates NOX2 expression, but also blocks the induction of NRF2 and NOQ1, resulting in accumulated ROS and attenuated cell senescence, both of which promote lung tumor development (Fig. 7I).

Furthermore, we examined NF-xB activity in Kras-CL and Kras<sup>IKKα−/−</sup> cells following TNFα treatment, and found that NF-xB activity was not decreased in Kras<sup>IKKα−/−</sup> cells compared with Kras-CL cells (Fig. S7D). However, relative to Kras-CL, Kras<sup>IKKα−/−</sup> cells showed increased expression of the regulators for stem cell properties, mitogenic activity, and inflammation and reduced expression of the regulators for apoptosis and antioxidant/detoxification functions, as analyzed by a microarray assay (GSE84163; Fig. S7E). Among these alterations, Ikkα down-regulates Fgf13, Adam12, and Egfr (14, 15) and ROS elevate Jak2, Egrf, and Notch1 expression (45–47). These changes may also contribute to the enhanced tumorigenic potential of Kras<sup>IKKα−/−</sup> cells compared with Kras-CL cells.

**Discussion**

Here we demonstrate that lung-specific Ikkα deletion promotes Kras<sup>G12D</sup>-mediated lung ADC development in association with elevated NOX2, down-regulated NRF2, accumulated ROS, and attenuated cell senescence. Pharmacologic inhibition of NOX or ROS attenuates lung ADC development in Kras<sup>G12D−/−</sup> mice. These results define a previously undescribed role of Ikkα, in which dual Ikkα-NOX2 and Ikkα-NRF2 pathways control ROS homeostasis and proliferation/survival that regulate Kras<sup>G12D</sup>-mediated lung ADC growth. Importantly, a fraction of human lung ADCs harbor CHUK locus mutations and deletions or express reduced Ikkα, some of which coexpress activated KRAS. During malignancy development, the activation of oncogenes is a ubiquitous phenomenon. Human lung ADCs express different oncogenes that induce mitogenic stress and ROS (29). Therefore, the mechanism identified in this study may apply in those CHUK-deficient human ADCs that do not carry KRAS alterations. Furthermore, KRAS mutations frequently occur in human pancreatic and colon cancers (cBioPortal). CHUK mutations and hemizygous deletions are also found in these patients, suggesting that IKKα inactivation or reduction may promote KRAS mutation-involved pancreatic and colon cancer development through a mechanism provided in this study.

FVB 1-Ikkα<sup>−/−</sup> mice develop spontaneous lung SCCs, in which no activating Kras mutations are detected, but not ADCs (5). l-Ikkα<sup>−/−</sup> mice develop systemic inflammation, marked pulmonary macrophage infiltration, and reduced epithelial cell IKKα levels before lung SCC formation. Restoration of IKKα in K5-expressing lung epithelial cells or depleting macrophages prevents lung SCC development. In this study, we detected lung ADCs, but not SCCs, in Kras<sup>G12D</sup>, Ikkα<sup>−/−</sup>, and Kras<sup>G12D</sup>;Ikkα<sup>−/−</sup> mice. These mice have a WT background before Ad.Cre treatment. Furthermore, Kras<sup>G12D</sup>, Ikkα<sup>−/−</sup> mice only developed lung ADCs. Notably, activating KRAS mutations are detected in ~35% and 5% of human lung ADCs and SCCs, respectively (1, 2), suggesting that activated Kras may predominantly induce ADCs in the lung, and that inflammatory conditions may also determine the formation of lung cancer, either ADC or SCC (4). The detailed mechanism remains to be revealed. Moreover, lung-specific Ikkα ablation induced spontaneous lung ADCs. Reintroduction of Ikkα inhibited Kras<sup>IKKα−/−</sup> cell-generated tumor growth, and silencing of Ikkα promoted Kras-CL cell-generated tumor growth. Hence, the epithelial-intrinsic Ikkα is critical for suppressing lung ADC development.

Down-regulation of NF-xB can cause apoptosis of Kras<sup>G12D</sup> ADC cells expressing reduced p53 (17, 18). Here, we showed that Kras<sup>G12D</sup>;Ikkα<sup>−/−</sup> and Kras<sup>G12D</sup> ADCs expressed comparable amounts of nuclear NF-xB proteins, although p53 expression was lower in Kras<sup>G12D</sup>;Ikkα<sup>−/−</sup> ADCs than in Kras<sup>G12D</sup> ADCs, suggesting that a basal NF-xB activity is sufficient for maintaining tumor cell survival. Furthermore, Kras<sup>G12D</sup>;Ikkα<sup>−/−</sup> ADCs showed increased proliferating cells and reduced p53/p21/senescence. NOQ1 has been shown to stabilize the p53 protein independent of MDM2, while reduced NOQ1 destabilizes p53 (43, 44). We found that Ikkα deletion decreased expression of NRF2 and NOQ1, which led to reduced p53/p21 and cell senescence in lung cancer cells, suggesting that Ikkα is required to maintain NRF2, NOQ1, and p53/p21 pathways for establishment of a barrier that antagonizes tumor progression.

On the other hand, silencing of Ikkα was found to down-regulate NRF2 and NOQ1 expression, resulting in reduced p53/p21 expression and cell senescence. Therefore, a reduction in Ikkα changes the antitumorigenic effect of Kras-induced ROS to a protumorigenic effect that enhances Kras-induced ADC progression. Although it has been reported that NRF2 deletion alone promotes the Kras<sup>G12D</sup>-mediated early ADCs and inhibits the advanced Kras<sup>G12D</sup>-mediated ADCs (23, 48), in this study, along with reduced NRF2, Ikkα deletion also promoted NOX2 expression, leading to further ROS accumulation and oxidative damage. Most likely, the ROS scavenging system induced by NRF2 becomes more...
An antagonizing relationship between accumulating ROS pathways and senescence. (A) IB analysis of IKKα, NRF2, NQO1, p53, p21, and MDM2 expression in Kras-CL and KrasIKKαL cells (Left), as well as Kras-CL cells treated with Si-Control, Si-IKKα (Center), or Si-NQO1 (Right). β-actin served as a protein-loading control. (B) SA-β-gal-positive cells in Kras-CL and KrasIKKαL cells (n = 3/group). Data represent mean ± SD (three repeats). ***P < 0.001, Student's t test. (C) The effect of IKKα knockdown on SA-β-gal levels in Kras-CL cells (Left) and A549 cells (Right) (n = 3/group). Data represent mean ± SD (three repeats). **P < 0.01, Student's t test. (D) The effect of NRF2 knockdown on SA-β-gal levels in Kras-CL cells (n = 3/group). Data represent mean ± SD (three repeats). ***P < 0.001, Student's t test. (E) The effect of NQO1 knockdown on SA-β-gal levels in Kras-CL cells (n = 3/group). Data represent mean ± SD (three repeats). ***P < 0.001, Student's t test. (F) Appearance (Left) and weight (Right) of tumors in nude mice receiving s.c. injections of Si-control or Si-IKKα-transfected Kras-CL cells for 3 wk (n = 10 tumors from 5 mice/group). Data represent mean ± SD. **P < 0.01, Student's t test. (G) Appearance (Left) and weight (Right) of tumors in nude mice receiving s.c. injections of Si-control or Si-NQO1-transfected Kras-CL cells for 3 wk (n = 10 tumors from 5 mice/group). Data represent mean ± SD. *P < 0.05, Student's t test. (H) IB analysis of p53 and p21 expression in Kras-CL and KrasIKKαL cells treated with NAC or apocynin (Apocy). Cont, untreated cells. β-actin served as a protein-loading control. (J) A working model for regulation of NOX2 or NRF2, their pathways, and biological consequences, regulated by IKKα in KrasIKKαL and KrasIKKαADC cells. Blue circle, trimethylation; white circle, no trimethylation; S39h1, Suv39h1; H3, histone H3; arrow, promotion or forward/maintaining; cross lines, inhibition; dashed line, no response.

Cell senescence, Cell cycle arrest Pre-malignant tumor

Cell proliferation, DNA damage Tumor progression

IKKα, KrasG12D

IKKα

KrasG12D

AHR

KrasG12D

p53

p21

p53

p21

ROS

NQO1

NOX2

S39h1

H3

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crucial for reducing oxidative damage in \textit{Kras}^{G12D}, \textit{Ikappa}^{ΔLU} mice than in \textit{Kras}^{G12D} mice. Overall, IKK\textalpha\ provides a protective role that suppresses excessive ROS and also ensures a pathway for ROS-induced antitumorigenic activity, thereby preventing ADC initiation and progression.

Materials and Methods
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. All animal experiments (protocols 14-051 and 14-052) were approved by the IACUC. \textit{Ikappa}^{ΔKAK}, and \textit{Ikappa}^{ΔKAK} mice (12, 15, and 14) were obtained from Dr. David Schrump, Thoracic and Gastrointestinal Oncology Branch, National Cancer Institute and from Sun Yat-Sen University Cancer Center, Guangzhou, China. All human samples used in this study were approved by the National Institutes of Health Internal Review Board (protocol 06-C-0014) and by the Ethics Committee and Institutional Review Board of Sun Yat-Sen University Cancer Center (YB2017-023), and informed consent was obtained from all patients.

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