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. IκB 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 (IkkαΔAD) 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 down-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 IkkαΔAD 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.

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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 antitumorigenic 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 KEAP1 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). Ikkαβ 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 IkkαΔLm mice (15) by intratracheal adenovirus.Cre (Ad.Cre) administration (IkkαΔLm). Conditional deletion of Ikkα resulted in spontaneous lung ADCs in 8 out of 48 IkkαΔLm mice at 13–20 mo of age (Fig. 1A, Top). No lung ADCs were detected in 30 WT mice. Activating Kras 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 KrasLG12DΔlu (KrasG12D) mice were used as positive controls (Fig. 1A, Bottom). With increasing age, ADC derived from IkkαΔLm mice metastasized to the spleen and other organs, as indicated by positivity for SP-C, a marker of II type lung epithelial cells (Fig. S1A).

To investigate the effect of Ikkα on KrasG12D-induced lung ADC, we crossed C57BL/6 IkkαΔLm mice or IkkαKA/KA mice with C57BL/6 KrasLG12D mice and used Ad.Cre to induce KrasG12D expression and simultaneously delete Ikkα. KrasG12D,IkkαΔLm and KrasG12D,IkkαKA/KA mice showed a significantly greater lung tumor burden compared with KrasG12D mice (Fig. 1B and C and Fig. S1B). ADCs derived from KrasG12D,IkkαΔLm and KrasG12D,IkkαKA/KA mice were positive for SP-C and CC10 (a marker of lung epithelial Clara cells), but negative for Ki67, an SCC marker (Fig. 1 D and E). We confirmed Ikkα deletion and KrasG12D activation in KrasG12D,IkkαΔLm lung ADCs and KrasG12D activation in KrasG12D,IkkαKA/KA ADCs (Fig. S1C).

Following Ad.Cre treatment, KrasG12D,Ikkαβm, KrasG12D,IkkαΔLm, and KrasG12D,IkkαKA/KA mice showed a significantly reduced life span compared with KrasG12D mice (Fig. 1F and Fig. S1D). Loss of the WT Ikkα allele (i.e., loss of heterozygosity [LOH], a tumor-suppressor hallmark) was detected in KrasG12D,IkkαΔLm lung ADCs (Fig. S1E). Ikkα LOH was previously reported in carcinogen-induced skin tumors in IkkαΔLm mice (26). Collectively, these results indicate that lung epithelial cell Ikkα ablation promotes KrasG12D-initiated lung ADC development. Although FVB IkkαΔ/KA 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 IkkαΔLm mice, KrasG12D,IkkαΔLm mice, or KrasG12D,IkkαKA/KA 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 CHUKG411 and CHUKG53 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 KrasG12C 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,IkkαΔLm and KrasG12D,IkkαKA/KA mice developed significantly enlarged lungs with markedly increased Ki67-positive bronchial epithelial cells, which can give rise to lung ADCs (Fig. 2 A and B and Fig. S2A), suggesting that Ikkα reductive or deletion promotes lung epithelial cell proliferation. The Ikkα mutation severely destabilizes Ikkα and also abolishes its catalytic activity (5). Indeed, Ikkα levels were decreased in KrasG12D,IkkαΔLm and KrasG12D,IkkαKA/KA lung ADCs compared with WT lungs and KrasG12D ADCs (Fig. S2B). Moreover, following intratracheal treatment with Ad.Cre, a small group of KrasG12D,IkkαΔLm and KrasG12D,IkkαKA/KA mice developed severe skin lesions, precluding their maintenance. Thus, we used IkkαΔLm 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,IkkαΔLm 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 p21Cip1 (p21) expression can overcome cell
Fig. 1. 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;IkkαΔLu mice served as a positive control. (Scale bar: 30 μm.) All images in this study were captured by a Nikon (Ver. 3.06) microscope. (B) Lung ADC burden in 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, KrasG12D;IkkαΔLu, and KrasG12D;IkkαKA/KA mice and WT lungs (n = 3 mice/group). DAPI, nuclear staining. (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) CHUK mutations/truncated proteins in human lung ADCs. CHUK mutations included truncating mutations with K5 or SP-C antibody (n = 3). (Scale bar: 30 μm.) (H) Survival curves for patients with CHUK alterations, including mutations (M) and hemizygous deletions (Hem). CHUK mutations, and KRAS mutations/CHUK hemizygous deletions. **P < 0.01, χ² test (comparisons between two groups).
cycle arrest and senescence and thereby promote tumor progression. Immunoblot (IB) analysis showed lower expression of p53 and p21 in Kras\(^{G12D};Ikk\^{ΔLu}\) tumors than in Kras\(^{G12D}\) tumors (Fig. 2D), which may account for the hyperproliferative phenotype in the lungs of Kras\(^{G12D};Ikk\^{ΔLu}\) mice compared with Kras\(^{G12D}\) mice. Of note, decreased IKK\(\alpha\) expression was seen in some Kras\(^{G12D}\)-lung ADCs and this was accompanied by reduced p53 and p21 expression (Fig. 2D). These results suggest that reduced IKK\(\alpha\) expression in lung ADCs is associated with increased cell proliferation and decreased cell senescence.

To determine the epithelial cell-autonomous role of IKK\(\alpha\) in lung ADC development, we generated a Kras\(^{G12D}\) 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\(^{IKK\alpha}\)−, that expressed less IKK\(\alpha\) than the parental Kras-CL cells (Fig. 2E and Fig. 2F, Top Left). Kras\(^{IKK\alpha}\)− cells generated more lung ADCs than the parental Kras-CL cells after transplantation into C57BL/6 WT mice, although both cell lines contained an activated Kras\(^{G12D}\) allele (Fig. 2F, Bottom Left and Right and Fig. S2D). To verify the inhibitory effect of IKK\(\alpha\) on tumorigenesis, we reexpressed IKK\(\alpha\) into Kras\(^{IKK\alpha}\)− cells and found that reintroduction of IKK\(\alpha\) 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 IKK\(\alpha\) expression in lung ADC cells promotes tumorigenesis.

**IKK\(\alpha\) Ablation Enhances ROS in Lung ADCs, and Treatment with Apocynin Attenuates ROS and Lung Tumorigenesis.** ROS induce tumors (Ref. 7) and Ki67-stained bronchial epithelial cells in the lungs of these mice (n = 3 mice for 10 slides/group; Right) at 4 mo after Ad.Cre treatment. **P < 0.01; ***P < 0.001, Student’s t test. (A) Lung appearance and weight in four Kras\(^{G12D}\) and three Kras\(^{G12D};Ikk\^{ΔLu}\) 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\(^{G12D};Ikk\^{ΔLu}\) and Kras\(^{G12D}\) tumors (n = 3 mice/group). SA, senescence-associated. **P < 0.01, Fisher’s exact test. (D) IB analysis of IKK\(\alpha\), p53, and p21 expression in WT lungs and Kras\(^{G12D}\) and Kras\(^{G12D};Ikk\^{ΔLu}\) ADCs. β-actin served as a protein-loading control. (E) A scheme for generating Kras-CL and Kras\(^{IKK\alpha}\)− cell lines 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 IKK\(\alpha\) in Kras-CL and Kras\(^{IKK\alpha}\)− 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\(^{IKK\alpha}\)− (n = 5) cells (5 × 10\(^6\) cells/mouse), as analyzed statistically using Student’s t test. **P < 0.01. (G) The growth of tumors in nude mice receiving s.c. injection of IKK\(\alpha\)− or control vector-transfected Kras\(^{IKK\alpha}\)− cells (n = 5 mice/group) for 2 wk. Data represent mean ± SD. **P < 0.01, Student’s t test.
Kras<sup>G12D</sup>, Ikkα<sup>ΔLZ</sup> mice (Fig. 3H, Left); however, treatment with apocynin did not decrease the lung ADC burden in Kras<sup>G12D</sup> 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 Kras<sup>IKKαΔ</sup> cells than in Kras-CL cells and verified that treatment with apocynin reduced ROS levels in Kras<sup>IKKαΔ</sup> cells (Fig. 4 A and B). Because Kras-CL cells required more than 3 mo to generate lung ADCs in WT mice, we used Kras<sup>IKKαΔ</sup> cells to determine the effect of NOX2 and ROS on ADC formation. Consistently, a 6-wk course of treatment with apocynin reduced Kras<sup>IKKαΔ</sup> cell–generated lung ADC numbers and lung weights in C56BL/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 Kras<sup>IKKαΔ</sup> cells.

Furthermore, Kras<sup>IKKαΔ</sup> cells expressed higher levels of Nox2 mRNA compared with Kras-CL cells (Fig. 4D), and silencing IKKα resulted in elevated NOX2 expression in A549 cells (Fig. S4 A and B). In contrast, knockdown of NOX2 significantly attenuated ROS levels in Kras<sup>IKKαΔ</sup> cells and impaired Kras<sup>IKKαΔ</sup> cell–generated lung tumors in C57BL/6 WT mice at 6 wk after the transplantation of Nox2 Si-RNA– or control Si-RNA–treated Kras<sup>IKKαΔ</sup> cells (Fig. 4 E and F), although NOX2 knockdown had less effect on lung weight than apocynin, suggesting that increased NOX2 expression enhances the tumorigenic potential of Kras<sup>IKKαΔ</sup> 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<sup>ΔKA</sup>) did not reduce AhR protein levels from amino acids 441–531 (IKKα<sup>ΔLZ</sup>) in A549 cells, which 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 Kras-CL cells and in human A549 cells (Fig. 4G and Fig. S4 E and F). In contrast, IKKα protein depletion decreased the recruitment of AhR to the Nox2 promoter, and reintroduction of WT IKKα or IKKα<sup>ΔKA</sup>, but not IKKα<sup>ΔLZ</sup>, recruited AhR to the Nox2 promoter in IKKα-deficient Kras-CL and A549 cells (Fig. 4G and Fig. S4E, Left, and Fig. S4F). Furthermore, silencing IKKα elevated NOX2 expression in Kras-CL cells, while reintroducing IKKα or IKKα<sup>ΔKA</sup>, but not IKKα<sup>ΔLZ</sup>, elevated NOX2 expression in IKKα-deficient Kras-CL cells (Fig. 4H and Fig. S4E, Right), although a slight reduction in IKKα-KA 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 depletion 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<sup>G12D</sup>;Ikkα<sup>ΔLZ</sup> 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<sup>G12D</sup>;Ikkα<sup>ΔLZ</sup> lungs than in Kras<sup>G12D</sup> lungs (Fig. S5A). IB analysis showed that Kras<sup>G12D</sup> ADCs expressed more NRF2 than WT lungs, whereas Kras<sup>G12D</sup>, Ikkα<sup>ΔLZ</sup> ADCs expressed less NRF2 than WT lungs (Fig. S5A). Among Kras<sup>G12D</sup> ADCs, those expressing less Ikkα consistently showed lower NRF2 and p21 expression (Fig. S5B). Importantly, IB analysis showed reduced Ikkα and NRF2 expression in a subfraction of human lung ADCs, and indeed, some human lung ADCs showed reduced Ikkα and NRF2 expression and increased NOX2 expression (Fig. 3E and Fig. S5B). Moreover, using RT-PCR, we examined additional 47 human lung ADCs (stage II–IV) and found that a subgroup of these ADCs expressed significantly less Ikkα and NRF2 compared with another ADC group (Fig. 5C), suggesting clinical relevance of the reduced Ikkα and NRF2 expression in human lung ADC.

If reduced NRF2 expression promotes ROS accumulation, which further contributes to increased tumorigenesis, then treatment with NAC should inhibit lung ADC burden in Kras<sup>G12D</sup>;Ikkα<sup>ΔLZ</sup> mice. Indeed, NAC treatment significantly decreased lung weights and ADC burden in Kras<sup>G12D</sup>;Ikkα<sup>ΔLZ</sup> mice compared with controls, but this treatment did not significantly affect the ADC burden in Kras<sup>G12D</sup> mice (Fig. 5D and E). The oxidative DNA damage (8-OHdG) marker was higher in Kras<sup>G12D</sup>;Ikkα<sup>ΔLZ</sup> ADCs than in Kras<sup>G12D</sup> ADCs, and NAC treatment decreased DNA damage (Fig. 5F), suggesting that accumulated ROS cause more DNA damage, which is associated with enhanced lung tumorigenesis. As expected, the expression levels of NRF2 targets Nqo1 and Gpx2 were significantly lower in Kras<sup>G12D</sup>;Ikkα<sup>ΔLZ</sup> lungs (Fig. 5E and F).

IKKα Loss Down-Regulates NRF2 Expression in an Epigenetic Manner. Treatment with NAC decreased the number of ROS in Kras<sup>Ikkα</sup> cells and also inhibited Kras<sup>Ikkα</sup> cell-generated lung tumor growth in WT mice compared with controls (Fig. 6A and B), suggesting a reciprocal correlation between IKKα-regulated NRF2 expression and ROS levels during lung tumorigenesis. KEAP1 is a major negative regulator of NRF2 stability (40). Kras-CL and Kras<sup>Ikkα</sup> cells expressed similar amounts of...
KEAP1, however (Fig. S6d). Expression of Nrf2 mRNA was lower in Kras\(^{G12D}\), Ikk\(\alpha\)Δ\(\alpha\)/Δ\(\alpha\) 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.

Trimethylatation at lysine 9 of histone H3 (H3-K9) represses gene expression by recruiting DNA methyltransferases, and trimethylatation Suvs9H1 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 methylating H3-K9 trimethylated-H3-K9, Suv39h1, and Dnmt3a (Fig. 6f). Using ChIP assays, we found IKK\(\alpha\) to be associated with the Nrf2 promoter in Kras\(-\beta\) cells (Fig. 6f and Fig. S6c). With the loss of IKK\(\alpha\) from the Nrf2 promoter in Kras\(-\beta\) 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\(\alpha\)-KA, but not IKK\(\alpha\)-Δ\(\alpha\), formed the complex with Nrf2 promoter in Kras\(-\beta\) cells (Fig. S6d). Consistently, silencing IKK\(\alpha\) decreased Nrf2 expression in Kras-CL cells, and reintroducing WT IKK\(\alpha\) or IKK\(\alpha\)-KA, but not IKK\(\alpha\)-Δ\(\alpha\), 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. 6f and Fig. S6e).

To elucidate how IKK\(\alpha\) expression is down-regulated in Kras\(-\beta\) cells, we sequenced full-length IKK\(\alpha\) cDNA and identified a missense mutation at nucleotide 2054 (amino acid 685) in Kras\(-\beta\) cells, Kras-CL cells, and Kras\(^{G12D}\)-induced ADCs (Fig. 6c).
cells exhibited many additional Ifkα mutations surrounding the nucleotide 2054 genetic lesion, suggesting that these mutations confer a growth advantage, possibly by destabilizing the IkKa protein. Accordingly, IkKa immunoprecipitation from Kras-CL and KrasΔκ/Δκ cells, followed by IB analysis with an anti-ubiquitin antibody, showed more ubiquitinated IkKa in KrasΔκ/Δκ cells compared with Kras-CL cells (Fig. S6G, Top). Treatment with MG132, a proteasome inhibitor, elevated IkKa and NRF2 levels in KrasΔκ/Δκ cells (Fig. S6G, Bottom), suggesting that tumor-associated mutations promote proteasomal degradation of IkKa. We previously detected the same Ikka mutations and deletions in the C-terminal region of IkKa in skin SCCs derived from carcinogen-treated IkaΔκ/Δκ and WT mice (13, 15, 26). These mutations impaired the IkKa activity that controls the G2/M cell cycle checkpoint in response to DNA damage and keratinocyte growth. Thus, the DNA encoding the IkKa 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 IkKa Inactivation Reverses This Pathway. Reduction of NQO1, 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 prevent tumor progression by up-regulating p53, p21, and cell senescence (30). We hypothesized that reduced IkKa down-regulates NRF2 and NQO1 expression, which attenuates p53 and p21 expression and cell senescence. Indeed, KrasΔκ/Δκ cells expressed reduced IkKa, NRF2, NQO1, p53, and p21 and showed attenuated cell senescence compared with Kras-CL cells (Fig. 7A, Left and B). Silencing of IkKa repressed NRF2, NQO1, 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 NQO1 repressed NQO1, 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 IkKa reduction blocks cell cycle arrest by decreasing NRF2, NQO1, and p21 expression. Importantly, silencing of IkKa, NRF2, or NQO1 in Kras-CL cells promoted tumor growth compared with the control when these cells were injected s.c. into nude mice (Fig. 7 F–H).

To demonstrate links among IkKa action, ROS, and ROS-mediated cell senescence, we examined the effect of NAC and apocynin on cell senescence (p53/p21) in KrasΔκ/Δκ and Kras-CL cells (Fig. 7I). Indeed, treatment with NAC or apocynin induced p53/p21 expression in KrasΔκ/Δκ cells. This induction was stronger in KrasΔκ/Δκ cells than in Kras-CL cells (Fig. 7J). Taken together, these findings show that IkKa ablation not only elevates NOX2 expression, but also blocks the induction of NRF2 and NQO1, resulting in accumulated ROS and attenuated cell senescence, both of which promote lung tumor development (Fig. 7J).

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

Discussion

Here we demonstrate that lung-specific IkKa deletion promotes KrasΔκ/Δκ-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 KrasΔκ/Δκ;IkkaΔκ/Δκ mice. These results define a previously undescribed role of IkKa, in which dual IkKa-NOX2 and IkKa-NRF2 pathways control ROS homeostasis and proliferation/survival that regulate KrasΔκ/Δκ-mediated lung ADC growth. Importantly, a fraction of human lung ADCs harbor CHUK locus mutations and deletions or express reduced IkKa, 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 (eBioPortal). CHUK mutations and hemizygous deletions are also found in these patients, suggesting that IkKa inactivation or reduction may promote Kras mutation-involved pancreatic and colon cancer development through a mechanism provided in this study.

FVB l-Ikkalα/α/α mice develop spontaneous lung SCCs, in which no activating kras mutations are detected, but not ADCs (5). l-Ikkalα/α/α mice develop systemic inflammation, marked pulmonary macrophage infiltration, and reduced epithelial cell IkKa levels before lung SCC formation. Restoration of IkKa in K5-expressing lung epithelial cells or depleting macrophages prevents lung SCC development. In this study, we detected lung ADCs, but not SCCs, in KrasΔκ/Δκ;IkkaΔκ/Δκ and KrasΔκ/Δκ;IkkαΔκ/Δκ mice. These mice have a WT background before Ad.Cre treatment. Furthermore, KrasΔκ/Δκ;IkkaΔκ/Δκ 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 IkKa ablation induced spontaneous lung ADCs. Reintroduction of IkKa inhibited KrasΔκ/Δκ cell-generated tumor growth, and silencing of IkKa promoted Kras-CL cell-generated tumor growth. Hence, the epithelial-intrinsic IkKa is critical for suppressing lung ADC development.

Down-regulation of NF-xB can cause apoptosis of KrasΔκ/Δκ ADC cells expressing reduced p53 (17, 18). Here, we showed that KrasΔκ/Δκ;IkkaΔκ/Δκ and KrasΔκ/Δκ ADCs expressed comparable amounts of nuclear NF-xB proteins, although p53 expression was lower in KrasΔκ/Δκ;IkkaΔκ/Δκ ADCs than in KrasΔκ/Δκ ADCs, suggesting that a basal NF-xB activity is sufficient for maintaining tumor cell survival. Furthermore, KrasΔκ/Δκ;IkkaΔκ/Δκ ADCs showed increased proliferating cells and reduced p53/p21/senescence. NQO1 has been shown to stabilize the p53 protein independent of MDM2, while reduced NQO1 destabilizes p53 (43, 44). We found that IkKa deletion decreased expression of NRF2 and NQO1, which led to reduced p53/p21 and cell senescence in lung cancer cells, suggesting that IkKa is required to maintain NRF2, NQO1, and p53/p21 pathways for establishment of a barrier that antagonizes tumor progression.

On the other hand, silencing of IkKa was found to down-regulate NRF2 and NQO1 expression, resulting in reduced p53/p21 expression and cell senescence. Therefore, a reduction in IkKa 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 KrasΔκ/Δκ-mediated early ADCs and inhibits the advanced KrasΔκ/Δκ-mediated ADCs (23, 48), in this study, along with reduced NRF2, IkKa deletion also promoted NOX2 expression, leading to further ROS accumulation and oxidative damage. Most likely, the ROS scavenging system induced by NRF2 becomes more
**Fig. 7.** 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 Kras(ikka) 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 Kras(ikka) 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 A459 cells (Right) (n = 3/group). Data represent mean ± SD (three repeats). ***P < 0.001, 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-NRF2-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. (H) 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 = 9 tumors from 5 mice/group). Data represent mean ± SD. *P < 0.05, Student's t test. (I) IB analysis of p53 and p21 expression in Kras-CL and Kras(ikka) cells treated with NAC or apocynin (Apocy). 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 IKKα+KrasG12D and IKKα−KrasG12D ADC 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.
crucial for reducing oxidative damage in Kras^{G12D,Ikka^{ΔLU}} mice than in Kras^{G12D} mice. Overall, IKKα 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. Ikka^{Δ}, Ikka^{KAAA}, and Ikka^{ΔΔ} mice (12, 13, 15) and Kras^{G12D} mice (stock no. 008179; The Jackson Laboratory) were on a C57BL/6 background. Athymic nude mice were obtained from Charles River Laboratory (BALB/c; C57NUGN(Cr)-Foxn1nu^NεTm). Human lung adenocarcinomas 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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