Published OnlineFirst March 27, 2018; DOI: 10.1158/0008-5472.CAN-17-1944

Tumor Biology and Immunology

Cancer Research

# IκB Kinase α Is Required for Development and Progression of *KRAS*-Mutant Lung Adenocarcinoma

Malamati Vreka[1,2], Ioannis Lilis[1], Maria Papageorgopoulou[1,2], Georgia A. Giotopoulou[1,2], Marina Lianou[1], Ioanna Giopanou[1], Nikolaos I. Kanellakis[1], Magda Spella[1], Theodora Agalioti[1], Vasileios Armenis[1], Torsten Goldmann[3], Sebastian Marwitz[3], Fiona E. Yull[4], Timothy S. Blackwell[4], Manolis Pasparakis[5], Antonia Marazioti[1], and Georgios T. Stathopoulos[1,2]

## Abstract

Although oncogenic activation of NFκB has been identified in various tumors, the NFκB–activating kinases (inhibitor of NFκB kinases, IKK) responsible for this are elusive. In this study, we determined the role of IKKα and IKKβ in *KRAS*-mutant lung adenocarcinomas induced by the carcinogen urethane and by respiratory epithelial expression of oncogenic $KRAS^{G12D}$. Using NFκB reporter mice and conditional deletions of IKKα and IKKβ, we identified two distinct early and late activation phases of NFκB during chemical and genetic lung adenocarcinoma development, which were characterized by nuclear translocation of *Rel*B, IκBβ, and IKKα in tumor-initiated cells. IKKα was a cardinal tumor promoter in chemical and genetic *KRAS*-mutant lung adenocarcinoma, and respiratory epithelial IKKα-deficient mice were markedly protected from the disease. IKKα specifically cooperated with mutant *KRAS* for tumor induction in a cell-autonomous fashion, providing mutant cells with a survival advantage *in vitro* and *in vivo*. IKKα was highly expressed in human lung adenocarcinoma, and a heat shock protein 90 inhibitor that blocks IKK function delivered superior effects against *KRAS*-mutant lung adenocarcinoma compared with a specific IKKβ inhibitor. These results demonstrate an actionable requirement for IKKα in *KRAS*-mutant lung adenocarcinoma, marking the kinase as a therapeutic target against this disease.

**Significance:** These findings report a novel requirement for IKKα in mutant *KRAS* lung tumor formation, with potential therapeutic applications. *Cancer Res; 78(11); 2939–51. ©2018 AACR.*

## Introduction

Tumors harboring mutations in the V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) are notoriously resistant to current treatments (1). Lung adenocarcinoma (LADC), the number one cancer killer worldwide (2), harbors *KRAS* mutations in up to 30% to 40% of the cases diagnosed in Europe and North America (3). A cardinal mechanism of *KRAS* mutation–associated drug resistance appears to be the oncogene's addiction to transcriptional programs that facilitate sustained tumor-initiated cell survival, such as NFκB (4). To this end, mutant *KRAS* was recently shown to interact with NFκB–activating kinases [inhibitor of NFκB (IκB) kinases, IKK] to promote cancer cell survival, stemness, and drug resistance (5, 6).

NFκB is activated via the canonical (involving IκBα, IKKβ, and *Rel*A/P50) and noncanonical (comprising IκBβ, IKKα, and *Rel*B/P52) pathways (7). We and others previously documented NFκB activation in murine and human LADC (8–10). However, the IKKs responsible for this remain elusive, and most studies focused on IKKβ, IKKε, and TANK-binding kinase 1 (TBK1; refs. 11–14). IKKα participates in both canonical and noncanonical NFκB pathways and cooperates with IKKβ for tumor cell growth *in vitro* (11, 15), but its role in LADC development *in vivo* is uncharted.

We deployed NFκB reporter and conditional IKKα- and IKKβ-deleted mice to decipher the timing of NFκB activation and the mutual impact of IKKα and IKKβ on LADC development. In mouse models of tobacco carcinogen- and oncogenic $KRAS^{G12D}$-triggered LADC, IKKα was cardinal for disease initiation and progression. Moreover, IKKα selectively fostered cellular proliferation in the context of mutant *KRAS* and was also highly expressed in human LADC. Importantly, dual IKKα /IKKβ

[1]Laboratory for Molecular Respiratory Carcinogenesis, Department of Physiology, Faculty of Medicine, University of Patras, Rio, Greece. [2]Comprehensive Pneumology Center (CPC) and Institute for Lung Biology and Disease (iLBD), University Hospital, Ludwig-Maximilians University and Helmholtz Zentrum München, Member of the German Center for Lung Research (DZL), Munich, Germany. [3]Clinical and Experimental Pathology, Research Center Borstel, Airway Research Center North (ARCN), Member of the German Center for Lung Research (DZL), Borstel, Germany. [4]Department of Medicine, Division of Allergy, Pulmonary and Critical Care Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee. [5]Mouse Genetics and Inflammation Laboratory, Institute for Genetics, University of Cologne, Cologne, Germany.

**Note:** Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

M. Vreka, I. Lilis, and M. Papageorgopoulou share first authorship of this article.

A. Marazioti and G.T. Stathopoulos share senior authorship of this article.

**Corresponding Authors:** Georgios T. Stathopoulos, University of Patras, Basic Biomedical Sciences Research Building, 2nd Floor, 26504 Rio, Greece. Phone: 302610969155; Fax: 302610997215; E-mail: gstathop@upatras.gr; and A. Marazioti, amarazioti@upatras.gr

**doi:** 10.1158/0008-5472.CAN-17-1944

Published OnlineFirst March 27, 2018; DOI: 10.1158/0008-5472.CAN-17-1944



inhibition yielded promising results against *KRAS*-driven LADC, lending hope for translational applications of our findings.

## Materials and Methods

Additional Methods are described in the Online Supplement.

### Mice

*C57BL/6J* (#000664), *FVB/NJ* (#001800), B6.129(Cg)-*Gt(ROSA)26Sor*$^{tm4(ACTB-tdTomato,-EGFP)Luo}$/J (*mT/mG*; #007676; ref. 16), B6.129S4-*Kras*$^{tm4Tyj}$/J (LSL.KRAS$^{G12D}$; #008179; ref. 17), NOD.CB17-*Prkdc*$^{<scid>}$/J (*NOD/SCID*; #001303), and FVB.129S6(B6)-*Gt(ROSA)26Sor*$^{tm1(Luc)Kael}$/J (*LSL.R26.Luc*; #005125; ref. 18) mice were from The Jackson Laboratory. NFκB reporter mice (*NGL*; NF-κB.GFP.Luciferase), B6.B4B6-*Chuk*$^{<tm1Mpa>}$/Cgn (*Chukf/f*), and B6.B4B6-*Ikbkb*$^{<tm2.1Mpa>}$/Cgn (*Ikbkbf/f*), B6;CBA-Tg(Scgb1a1-cre)1Vart/Flmg (*Scgb1a1.Cre*), and Tg(Sftpc-cre)1Blh (*Sftpc.Cre*) mice have been described (8, 19–21). Mice were bred >F9 to the *C57BL/6* and/or *FVB* backgrounds at the University of Patras Center for Animal Models of Disease. The number of mice used for these studies ($n = 542$) is detailed in Supplementary Table S1.

### Reagents

Urethane (CAS#51-79-6) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay were from Sigma, adenoviruses from the Vector Development Lab of the Baylor College of Medicine (Houston, TX), D-luciferin from Gold Biotechnology, HEK293T cells from ATCC, and Lewis lung carcinoma (LLC) and A549 lung adenocarcinoma cells from the NCI Tumor Repository (Frederick, MD). Primers and antibodies are listed in Supplementary Tables S2 and S3. Lentiviral shRNA pools (Santa Cruz Biotechnology) are described in Supplementary Table S4.

### Mouse models of LADC

Chemical-induced LADC was induced in *FVB* and *C57BL/6* mice, respectively, by a single or by 10 consecutive weekly intraperitoneal exposures to 1 g/kg urethane (8, 22–24). *KRAS*$^{G12D}$-driven LADC was induced via intratracheal injections of $5 \times 10^8$ plaque-forming units (PFU) adenovirus type 5 encoding CRE recombinase (Ad-*Cre*) to *LSL.KRAS*$^{G12D}$ mice on the *C57BL/6* background (9, 17). *NOD/SCID* and *C57BL/6* mice were anesthetized by isoflurane and received $2 \times 10^6$ HEK293T and $0.5 \times 10^6$ tumor cells into the rear flank, and vertical tumor diameters (δ) were measured and mice were imaged for bioluminescent detection of cell mass weekly thereafter. Cell spot volume (V) was calculated as $V = \pi \times (\delta 1 \times \delta 2 \times \delta 3)/6$, and mice were killed after 6 weeks. Flank tumors were harvested and fixed with 4% paraformaldehyde or processed for immunoblotting.

### Drug treatments

*LSL.KrasG12D;LSL.R26.Luc* mice received $5 \times 10^8$ PFU intratracheal Ad-*Cre*, followed by daily intraperitoneal injections of 100 μL saline or 0.5 mg/Kg TPCA-1 or 17-DMAG in 100 μL saline at days 14 to 28 or 84 to 112 after Ad-*Cre*. Mice were imaged for bioluminescent detection of LADC burden at 0, 14, 28, 84, and 112 days after Ad-*Cre*. Mice were sacrificed and lungs were harvested at 112 days after Ad-*Cre*.

### Cellular assays

Mouse primary lung adenocarcinoma cells and airway epithelial cells were derived from the lungs of urethane (single dose 1 g/kg) or saline-treated *FVB* or *C57BL/6* mice by simple tumor or large airway dissection or epithelial stripping, respectively, and 5-month or 5-day culture, respectively, as described elsewhere (25). These cell lines were named XYLA#, with X signifying the mouse strain (F, *FVB*; C, *C57BL/6*), Y the carcinogen used (U, urethane), LA lung adenocarcinoma, and # their serial number by derivation date. Cells were cultured at 37°C in 5% $CO_2$–95% air using DMEM supplemented with 10% FBS, 2 mmol/L L-glutamine, 1 mmol/L pyruvate, 100 U/mL penicillin, and 100 mg/mL streptomycin. Cells were tested biannually for identity (by the short tandem repeat method) and for *Mycoplasma spp.* (by PCR). For experiments, frozen cells were reconstituted and were passaged 2 to 5 times for less than 2 weeks. *In vitro* cancer cell proliferation was determined using the MTT assay. For this, $2 \times 10^4$ cells/well were plated onto 96-well plates. Daily thereafter, 15 μL of 5 mmol/L MTT working solution in PBS was added to wells to be measured that day. The plate was incubated for 4 hours at 37°C in a 5% $CO_2$ humidified incubator, followed by the addition of 100 μL acidified isopropanol per well for sediment solubilization and absorbance measurement at 492 nm on an MR-96A photometer (Mindray). For soft-agar colony formation assay, $7.5 \times 10^3$ cells were plated in 60-mm culture vessels in semisolid 0.7% agarose in full culture medium and were incubated for 30 days at 37°C in a 5% $CO_2$ humidified incubator. Fresh culture medium (2 mL) was added to each vessel biweekly. After incubation, 500 μL MTT working solution was added to each vessel and plates were dried, inverted, photographed, and colonies were counted, as described elsewhere (25).

### Human samples

Matched tumor and normal lung tissue RNA and sections of 23 and 35, respectively, previously reported patients with LADC from Institution 3 were used for microarray and immunohistochemistry for IKKα and IKKβ (26). Human studies were approved *a priori* by the ethics committee of the University of Lübeck, Germany (approval #AZ 12-220) and were conducted according to the Declaration of Helsinki. Written informed consent was obtained from all patients. IKK score was 0, 1, 2, or 3 for no, cytoplasmic only, cytoplasmic and nuclear, and nuclear only immunoreactivity, respectively (modified from ref. 11).

### Statistical analysis

Sample size ($n$; always biological) was determined using G*power (http://www.gpower.hhu.de/), assuming $\alpha = 0.05$, $\beta = 0.05$, and $d = 1.5$. Data were acquired by two blinded readers, reevaluated if >20% deviant (no data were excluded), examined for normality by the Kolmogorov–Smirnov test and presented as median (interquartile range) or mean ± SEM. Differences in frequencies were examined by Fisher exact/$\chi^2$ tests, in means of normally distributed variables by $t$ test or one-way ANOVA/Bonferroni posttests, and in medians of nonnormally distributed variables by Mann–Whitney test or Kruskal–Wallis/Dunn posttests. Survival and flank tumor volumes were examined by Kaplan–Meier estimates/log-rank tests and two-way ANOVA/Bonferroni posttests. Probability ($P$) is two-tailed; $P < 0.05$ was considered statistically significant. Statistics and plots were done on Prism v5.0 (GraphPad).

### Study approval

All animal experiments were approved *a priori* by the Veterinary Administration of Western Greece according to a full and detailed

Published OnlineFirst March 27, 2018; DOI: 10.1158/0008-5472.CAN-17-1944



protocol (approval #276134/14873/2). Male and female mice were sex-, weight (20–25 g)-, and age (6–12 weeks)-matched. Human studies were approved *a priori* by the ethics committee of the University of Lübeck, Germany (approval #AZ 12-220).

## Results

### NFκB is activated in *KRAS*-mutant LADC

To map pulmonary NFκB activity during *KRAS*-driven neoplasia, NFκB reporter mice (*NGL*) on the carcinogen-susceptible *FVB* background expressing NFκB–driven *Photinus Pyralis* luciferase (LUC) in-frame with EGFP (8, 23) received a single intraperitoneal injection of saline or the tobacco carcinogen urethane (1 g/kg) and were serially imaged for bioluminescence. Urethane causes respiratory epithelial $Kras^{G12V/Q61R}$ mutations and progressive inflammation, hyperplasias, adenomas, and adenocarcinomas in *FVB* mice (22–25) that in this experiment also expressed the *NGL* reporter (Fig. 1A and B). In addition to the baseline signals of these mice, markedly increased light emission from the chest was exclusively detected in urethane-treated mice at early and late time points corresponding to carcinogen-induced inflammation and LADC, respectively (8). Enhanced NFκB activation indicated by the EGFP reporter emanated exclusively from LADC (Fig. 1C–F). In a second approach, *NGL* mice were intercrossed with mice carrying a conditional loxP-STOP-loxP.$KRAS^{G12D}$ allele (*LSL.*$KRAS^{G12D}$; ref. 17), and *NGL* and *NGL;LSL.*$KRAS^{G12D}$ offspring (all *C57BL/6* background) received intratracheal Ad-*Cre* and were longitudinally imaged. In *LSL.*$KRAS^{G12D}$ mice, progressive inflammation, hyperplasia, adenomas, and adenocarcinomas carrying the *KRAS* mutation are inflicted by Ad-*Cre* (9, 17). To titrate Ad-*Cre*, *mT/mG* CRE reporters that switch from membranous Tomato (mT) to EGFP (mG) fluorescence upon CRE recombination (16) received 0, $5 \times 10^7$, $5 \times 10^8$, or $5 \times 10^9$ PFU intratracheal Ad-*Luc* or Ad-*Cre* and were killed upon subsidence of transient Ad-mediated transgene expression at 2 weeks after injection (27). The low, intermediate, and high Ad-*Cre* titers, respectively, caused infrequent, stochastic, and ubiquitous respiratory epithelial recombination (Supplementary Figs. S1A and S1B). We selected $5 \times 10^8$ PFU Ad-*Cre* to stochastically induce recombination into the respiratory epithelium of *NGL* and *NGL;LSL.*$KRAS^{G12D}$ mice (Supplementary Fig. S1C). Similar to the urethane model, two phases of enhanced chest light emission by *NGL;LSL.*$KRAS^{G12D}$ but not *NGL* mice were observed, coinciding with early inflammation and late LADC development (8, 22–24, 28). Again, NFκB–dependent EGFP expression was confined to LADC (Supplementary Fig. S1D–S1G). These data demonstrate biphasic pulmonary NFκB activation during *KRAS*-driven LADC development.

### *KRAS*-mutant LADC displays both canonical and noncanonical NFκB activity

To investigate the NFκB pathway at play during *KRAS*-mutant inflammation, hyperproliferation, and LADC formation, the immunoreactivity of nuclear and cytoplasmic protein extracts of whole lungs of urethane-treated *FVB* mice and of Ad-*Cre*–treated *LSL.*$KRAS^{G12D}$ mice for NFκB subunits, kinases, and inhibitors were probed longitudinally (Fig. 2A–D). In the urethane model, marked Rel*B* and Rel*A* immunoreactivity was detected in nuclear extracts and enhanced IκBα, IκBβ, IKKα, IKKβ, and TBK1 immunoreactivity in cytoplasmic extracts of the neoplastic stage. Some immunoreactivity was also present in early stages but their expression peaked in tumor-bearing lungs, while no IKKε signal was evident at any time point. In the *LSL.*$KRAS^{G12D}$ model, enhanced nuclear Rel*B* and P52 and modest Rel*A* immunoreactivity was detected in nuclear extracts of tumor-bearing lungs, together with some cytoplasmic immunoreactivity for IκBβ, IKKα, and TBK1 (120 days). In addition, some Rel*A*, Rel*B*, P52, IκBα, IκBβ, and TBK1 immunoreactivity was evident in same-day–treated lungs (0 days), some Rel*A*, Rel*B*, P52, IKKα, and TBK1 immunoreactivity in inflammatory and proliferative lungs (30 and 60 days), and no IKKβ and IKKε signal at any stage. IKK expression patterns were corroborated using immunofluorescent detection of IKKα/IKKβ on lung sections of urethane-treated *FVB* and Ad-*Cre*–treated *LSL.*$KRAS^{G12D}$ mice at 240 and 120 days after treatment, respectively. In both models, IKKα was expressed by a significant proportion of LADC cells, while minimal IKKβ expression was detectable (Fig. 2E and F). To further characterize NFκB activity of LADC, LADC cells were derived from the lungs of saline and urethane-treated *FVB* mice, according to established methods (Supplementary Fig. S2A and S2B; refs. 25, 29). LADC cells exhibited enhanced nuclear *Rel*B (but not *Rel*A) localization and activity compared with saline- and urethane-treated lungs (Supplementary Fig. S2C and S2D). Taken together, these results indicate coactivation of the canonical and noncanonical NFκB pathways in LADC.

### Respiratory epithelial IKKα promotes *KRAS*-driven LADC

We next functionally assessed the role of IKKα and IKKβ in LADC development, utilizing conditional IKKα and IKKβ gene–deleted mice (*Chukf/f* and *Ikbkbf/f*) that feature loxP-flanked alleles excised upon CRE expression (19). In a first line of experiments, *mT/mG* CRE reporter (control), *Chukf/f*, and *Ikbkbf/f* mice on the urethane-resistant *C57BL/6* background (8) received $5 \times 10^9$ PFU intratracheal Ad-*Cre* (a titer causing recombination in ∼75% of the respiratory epithelium within 2 weeks; Supplementary Fig. S1A and S1B), and were started 2 weeks thereafter on 10 weekly doses of 1 g/kg intraperitoneal urethane, a regimen that reproducibly induces LADC in *C57BL/6* mice (23, 29). In this multihit model, stochastic *KRAS* mutations, inflammation, apoptosis, and regeneration were repeatedly inflicted across IKK-deleted and nondeleted respiratory epithelium (Fig. 3A). Interestingly, *Ikbkbf/f* mice displayed decreased survival during repeated urethane exposures, suggesting a role for IKKβ in epithelial repair (Fig. 3B). However, at 6 months after urethane start, IKKα-deleted mice had markedly decreased LADC incidence, multiplicity, and burden per lung compared with controls, while IKKβ-deleted mice displayed only minor reductions in tumor multiplicity but not burden (Fig. 3C–G). These experiments were replicated on *Chukf/f* and *Ikbkbf/f* mice backcrossed >F9 to the single-hit *FVB* model that recapitulates the mutation spectrum of human LADC and allows separate insights into the effects of IKK deletion on tumor initiation and progression via observations on LADC number and size after 6 months (8, 22–24). For this, *WT* control, *Chukf/f*, and *Ikbkbf/f* mice (all *FVB*) received $5 \times 10^9$ PFU intratracheal Ad-*Cre*, followed by a single intraperitoneal exposure to 1 g/kg urethane (Supplementary Fig. S3A). All genotypes comparably survived single-hit urethane (Supplementary Fig. S3B). Again, *Chukf/f* mice developed fewer and smaller LADC compared with controls, indicating marked tumor-initiating and promoting effects of IKKα, but *Ikbkbf/f* mice displayed tumor incidence, number, size, and load closely resembling *WT* littermates, suggesting that the minor

Published OnlineFirst March 27, 2018; DOI: 10.1158/0008-5472.CAN-17-1944



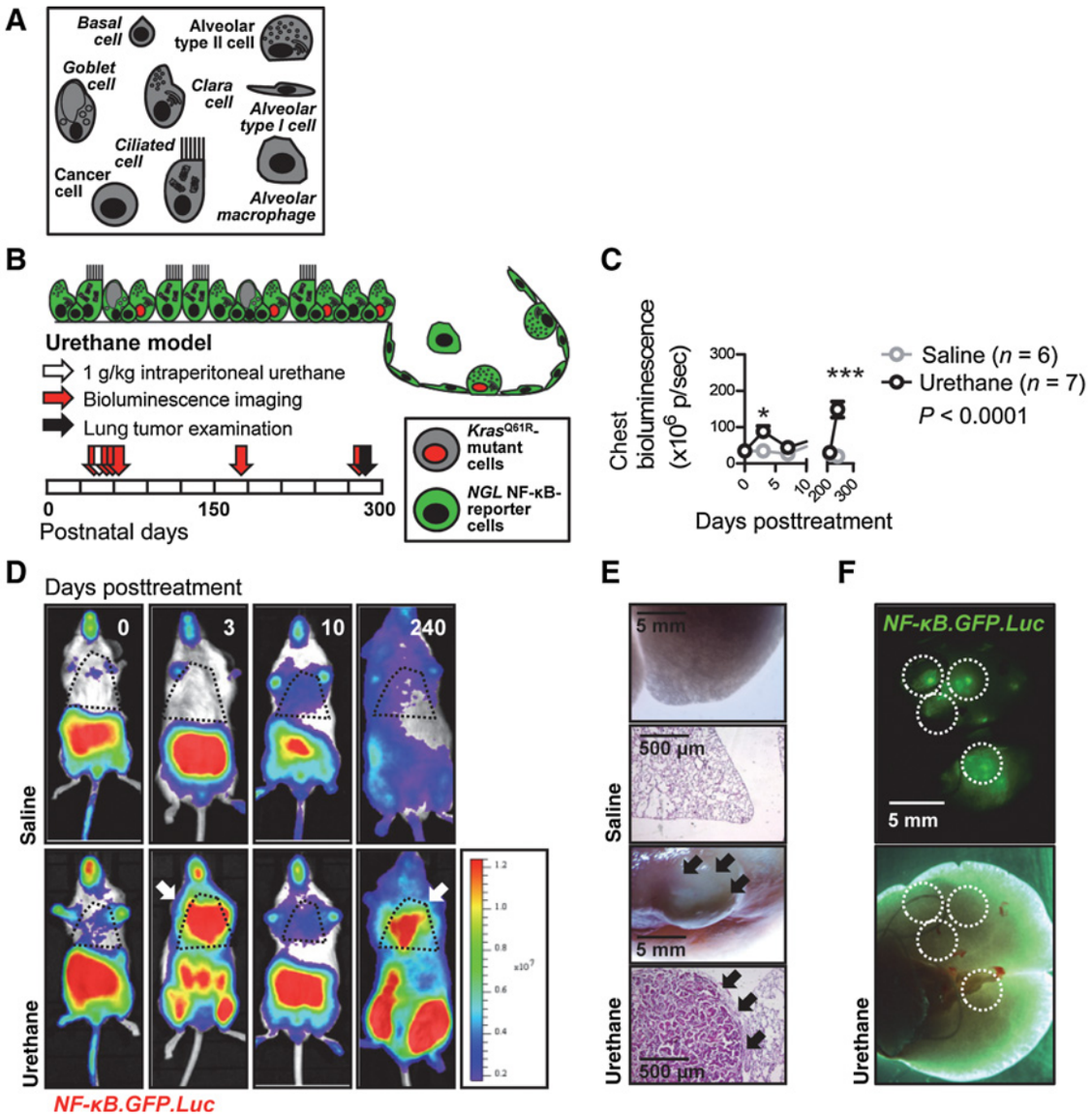


**Figure 1.**

NFκB activation in urethane-induced lung adenocarcinoma. **A–F,** *NGL* mice were backcrossed > F9 to the carcinogen-susceptible *FVB* strain and received single intraperitoneal injections of saline ($n = 6$) or the tobacco-contained carcinogen urethane (1 g/kg; $n = 7$) and were imaged longitudinally for bioluminescence. **A,** Legend to respiratory epithelial cells used in schematics throughout. **B,** Schematic of experimental time course (boxes, months) and topology of NFκB–reporter (green, cytosol) versus *Kras*$^{Q61R}$-mutant (red, nucleus) cells in this model. **C,** Data summary of chest bioluminescence shown as mean (points), SEM (bars), and two-way ANOVA *P* value. *, $P < 0.05$; ***, $P < 0.001$, respectively, for urethane-treated compared with saline-treated mice by Bonferroni posttests. **D,** Representative merged bioluminescent/photographic images with pseudocolor scale showing increased chest light emission of urethane-treated mice at early and late time points after carcinogen injection (arrows). **E,** Representative images of gross lungs and hematoxylin and eosin–stained lung sections of saline-treated and urethane-treated mice at 8 months after injection showing lung adenocarcinomas in the latter (arrows). **F,** Light-optic and green fluorescent lung images of representative urethane-treated mouse at 8 months after injection showing NFκB–driven GFP expression in lung adenocarcinomas (dashed lines).

tumor-promoting effects of IKKβ require repetitive carcinogen challenge to become evident (Supplementary Fig. S3C–S3G). *Chukf/f* and *Ikbkbf/f* mice were also intercrossed with *Scgb1a1.Cre* (20) and *Sftpc.Cre* (21) CRE drivers (all *C57BL/6*) and their offspring received 10 consecutive weekly intraperitoneal injections of 1 g/kg urethane starting at 6 weeks of age (Supplementary Fig. S4A). Interestingly, both *Scgb1a1.Cre*- and *Sftpc.Cre*-driven IKKα-deletion was equally protective against LADC, while IKKβ-deletion had no effect (hence pooled *Scgb1a1.Cre* and *Sftpc.Cre* data are presented; Supplementary Fig. S4B–S4E). To solidify the link between IKKα and mutant *KRAS* and to discriminate between cell-autonomous and paracrine IKKα effects, *Chukf/f* and *Ikbkbf/f* mice were intercrossed with *LSL.KRAS*$^{G12D}$ mice (all *C57BL/6*) and their offspring received $5 \times 10^8$ PFU intratracheal Ad-*Cre*, a

Published OnlineFirst March 27, 2018; DOI: 10.1158/0008-5472.CAN-17-1944



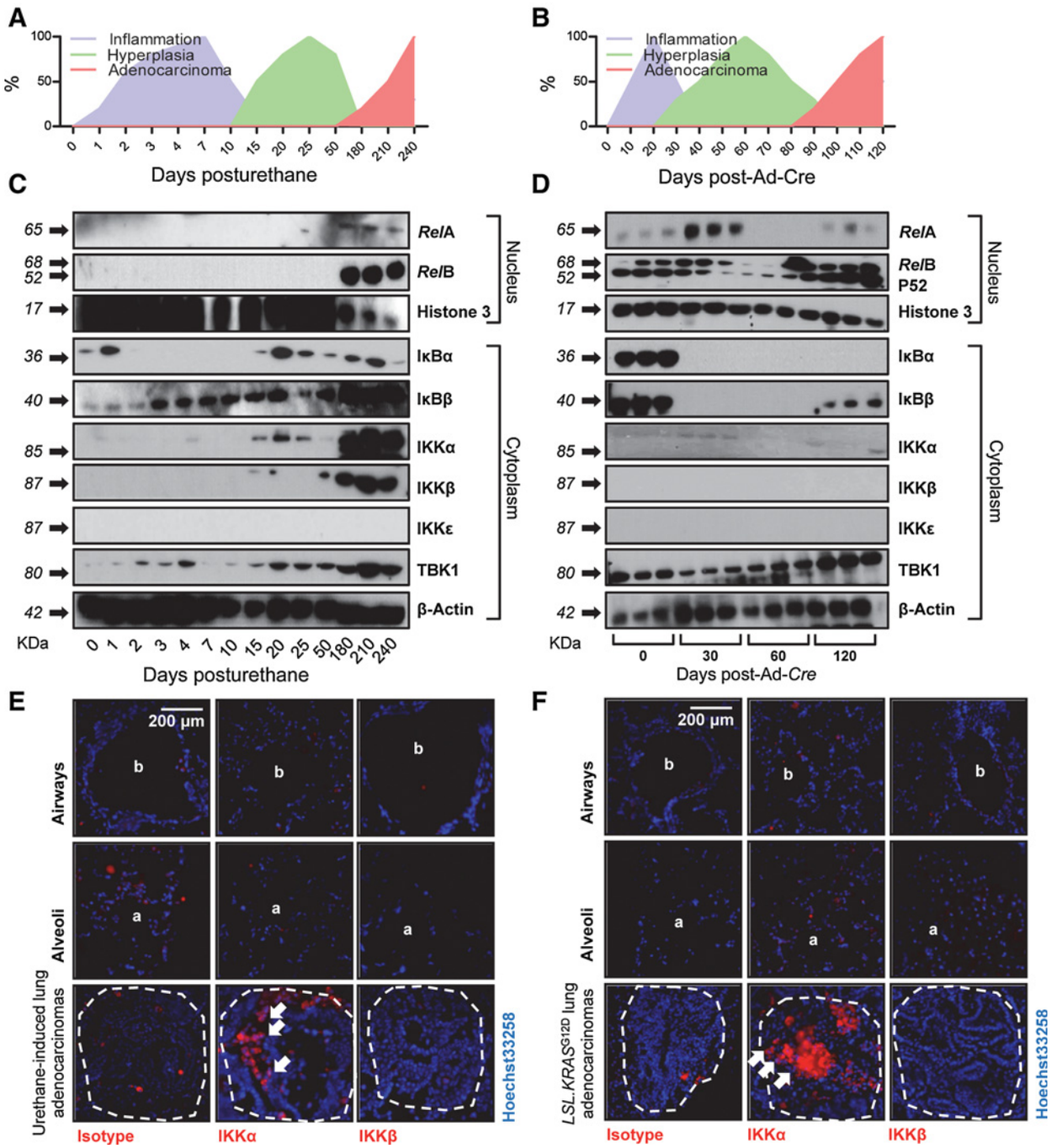


**Figure 2.**

Increased NFκB activity and enhanced IKKα expression of *KRAS*-driven lung adenocarcinoma. **A, C,** and **E,** *FVB* mice ($n = 14$) received 1 g/kg intraperitoneal urethane and were sacrificed after the indicated time intervals. **B, D,** and **F,** Mice carrying a conditional loxP-STOP-loxP.*KRAS*$^{G12D}$ allele (LSL.*KRAS*$^{G12D}$; *C57BL/6* strain; $n = 12$) received $5 \times 10^8$ intratracheal PFU Ad-*Cre* and were sacrificed after the indicated time intervals. **A** and **B,** Schematic representations of intensity and time course of inflammation, hyperplasia, and tumorigenesis in the two models (8, 20–22, 25). **C** and **D,** Immunoblots of whole lung nuclear and cytoplasmic extracts for NFκB pathway components. **C,** Note the increased expression of *Rel*A, *Rel*B, IκBβ, IKKα, IKKβ, and TBK1 at late time points post-urethane, when lung adenocarcinomas have developed. **D,** Note the increased expression of *Rel*B, P52, and TBK1 at 4 months after Ad-*Cre*, when lung adenocarcinomas have developed. **E,** *FVB* mice ($n = 5$) received 1 g/kg intraperitoneal urethane and were sacrificed after 8 months for fluorescent detection of IKKα and IKKβ immunoreactivity on cryosections of lungs with bronchi (b) and alveoli (a) and lung tumors (dashed lines). **F,** LSL.*KRAS*$^{G12D}$ mice (*C57BL/6* strain; $n = 5$) received $5 \times 10^8$ intratracheal PFU Ad-*Cre* and were sacrificed after 4 months for fluorescent detection of IKKα and IKKβ immunoreactivity on cryosections of lungs with bronchi (b) and alveoli (a) and lung tumors (dashed lines). Note the increased immunoreactivity of lung adenocarcinomas for IKKα (arrows). *Rel*, v-rel avian reticuloendotheliosis viral oncogene homolog.

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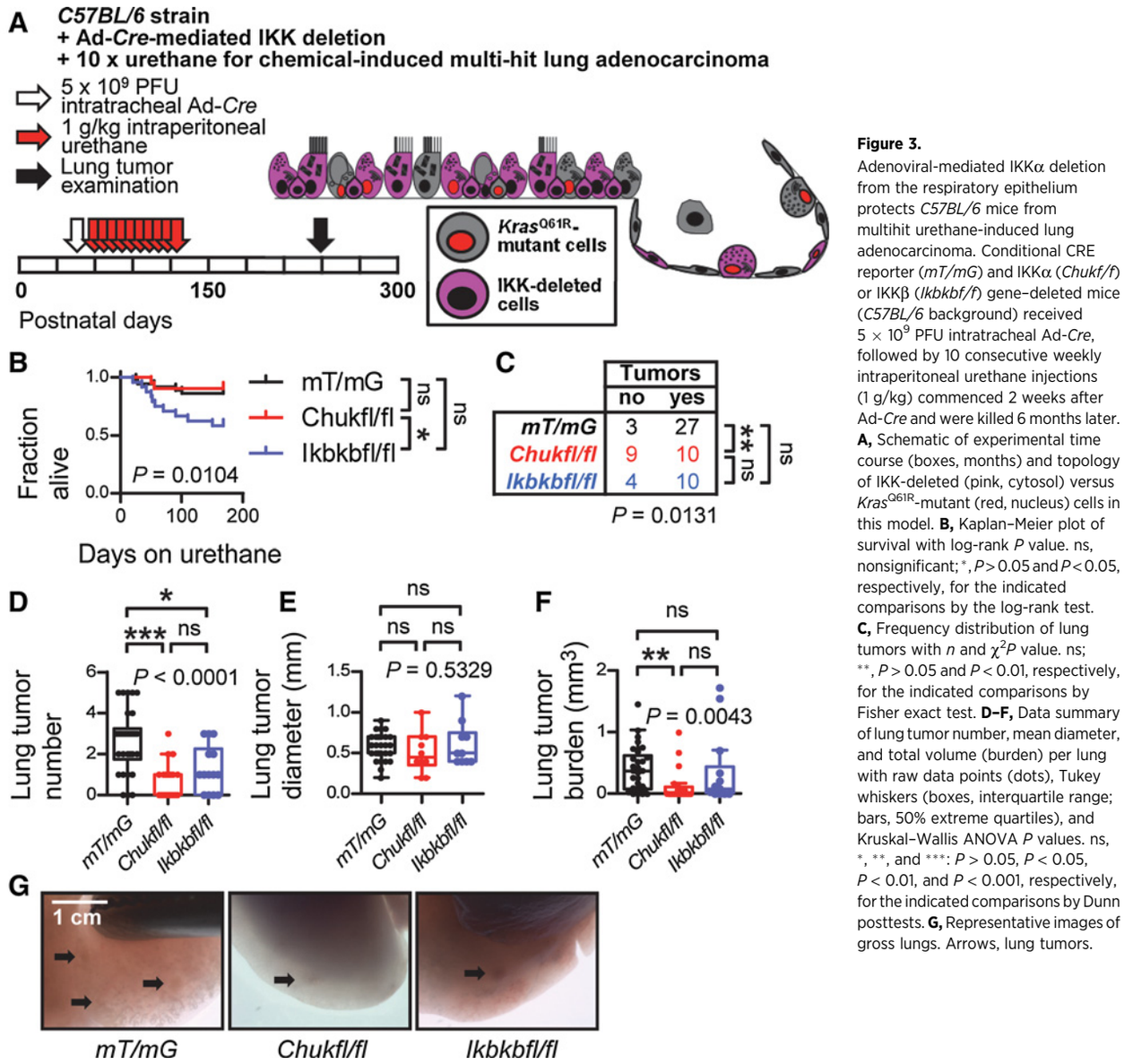


**Figure 3.**

Adenoviral-mediated IKKα deletion from the respiratory epithelium protects *C57BL/6* mice from multihit urethane-induced lung adenocarcinoma. Conditional CRE reporter (*mT/mG*) and IKKα (*Chukf/f*) or IKKβ (*Ikbkbf/f*) gene–deleted mice (*C57BL/6* background) received $5 \times 10^9$ PFU intratracheal Ad-*Cre*, followed by 10 consecutive weekly intraperitoneal urethane injections (1 g/kg) commenced 2 weeks after Ad-*Cre* and were killed 6 months later. **A,** Schematic of experimental time course (boxes, months) and topology of IKK-deleted (pink, cytosol) versus $Kras^{Q61R}$-mutant (red, nucleus) cells in this model. **B,** Kaplan–Meier plot of survival with log-rank *P* value. ns, nonsignificant; *, $P > 0.05$ and $P < 0.05$, respectively, for the indicated comparisons by the log-rank test. **C,** Frequency distribution of lung tumors with *n* and $\chi^2 P$ value. ns; **, $P > 0.05$ and $P < 0.01$, respectively, for the indicated comparisons by Fisher exact test. **D–F,** Data summary of lung tumor number, mean diameter, and total volume (burden) per lung with raw data points (dots), Tukey whiskers (boxes, interquartile range; bars, 50% extreme quartiles), and Kruskal–Wallis ANOVA *P* values. ns, *, **, and ***: $P > 0.05$, $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, for the indicated comparisons by Dunn posttests. **G,** Representative images of gross lungs. Arrows, lung tumors.

model where $KRAS^{G12D}$ expression and IKK deletion coincide (Fig. 4A). Lung morphometry (30) at 4 months after Ad-*Cre* showed that IKKα-deleted mice had markedly decreased LADC burden compared with controls, while IKKβ-deleted mice displayed an intermediate phenotype (Fig. 4B–E). Collectively, these findings show that IKKα promotes *KRAS*-mutant LADC in a cell-autonomous fashion, independent from and more pronounced than IKKβ.

### IKKα selectively fosters *KRAS*-mutant cell prevalence *in vitro* and *in vivo*

We next stably transfected HEK293T benign human embryonic kidney cells with vectors encoding control random sequence (p*C*), RFP (p*RFP*), EGFP (p*eGFP*), wild-type (p*eGFP.Kras*$^{WT}$), or mutant (p*eGFP.Kras*$^{G12C}$) murine *Kras* in-frame with EGFP, and murine IKKα (p*Chuk*) or IKKβ (p*Ikbkb*) in various combinations. After transgene expression was validated, RFP-expressing control cells and EGFP-expressing intervention cells cotransfected with various combinations of p*eGFP.Kras*$^{WT}$/p*eGFP.Kras*$^{G12C}$ and p*Chuk*/p*Ikbkb* were mixed at equal ratios and cocultured for 1 week, followed by quantification by fluorescent microscopy and flow cytometry (Supplementary Fig. S5A and S5B). Of note, as opposed to successful p*Ikbkb* coexpression with p*eGFP.Kras*$^{WT}$, p*Ikbkb* coexpression with p*eGFP.Kras*$^{G12C}$ was repeatedly impossible ($n = 5$), indicating mutual repulsion of mutant $Kras^{G12C}$ and IKKβ, similar to what was previously observed with other RAS/IκB-like GTPases called κB-RAS (Supplementary Fig. S5B; ref. 31). Despite this, IKKβ provided a proliferative advantage to HEK293T cells expressing $Kras^{WT}$, whereas $Kras^{G12C}$-expressing HEK293T cells proliferated more efficiently upon IKKα overexpression (Supplementary Fig. S5C and S5D). Subsequently, HEK293T cells were stably transfected with p*CAG.Luc*, followed by various

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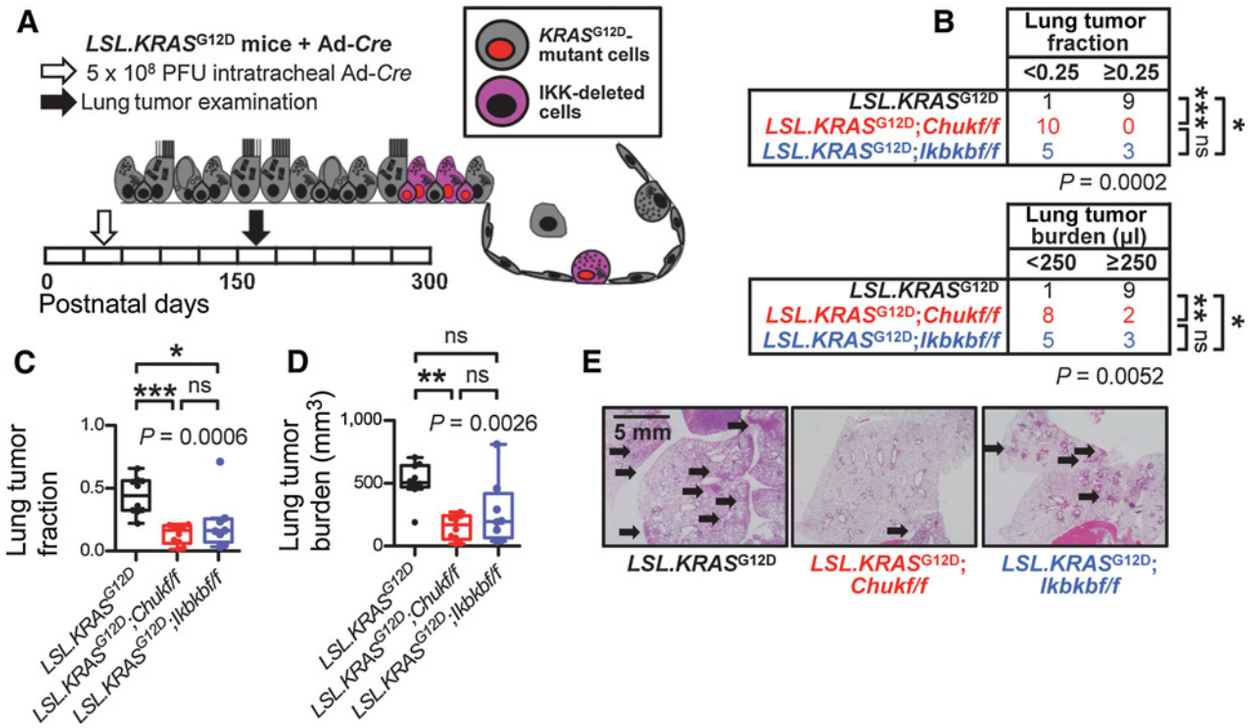


**Figure 4.**

IKKα deletion ameliorates respiratory epithelial oncogenicity of mutant *KRAS*$^{G12D}$. Conditional IKKα (*Chukf/f*) or IKKβ (*Ikbkbf/f*) gene–deleted mice were intercrossed with mice carrying a loxP-STOP-loxP.*KRAS*G12D conditional allele (LSL.*KRAS*G12D; all *C57BL/6*) and their offspring received 5 × 10$^8$ PFU intratracheal Ad-*Cre* and was killed 4 months later. **A,** Schematic of experimental time course (boxes, months) and topology of IKK-deleted (pink, cytosol) versus *KRAS*$^{G12D}$-mutant (red, nucleus) cells in this model, where IKK deletion and oncogenic *KRAS*$^{G12D}$ expression uniquely coincide in the same cells. **B,** Frequency distribution of lung tumorigenesis with *n* and $\chi^2$ *P* values. ns, nonsignificant; *, **, and ***: $P > 0.05$, $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, for the indicated comparisons by Fisher exact test. **C** and **D,** Data summary of relative lung tumor fraction and total lung tumor volume (burden) per lung with raw data points (dots), Tukey whiskers (boxes, interquartile range; bars, 50% extreme quartiles), and Kruskal–Wallis ANOVA *P* values. ns, *, **, and ***: $P > 0.05$, $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, for the indicated comparisons by Dunn posttests. **E,** Representative hematoxylin and eosin–stained lung tissue sections. Arrows, lung tumors.

combinations of p*C*, p*eGFP.Kras*$^{WT}$, p*eGFP.Kras*$^{G12C}$, p*Chuk*, and/or p*Ikbkb* were validated, and two million cells were injected at different dorsal skin spots of *NOD/SCID* mice, followed by serial spot volume assessment and bioluminescence imaging. Again, IKKβ boosted *in vivo* growth of HEK293T cells expressing *Kras*$^{WT}$, while *Kras*$^{G12C}$-expressing HEK293T cells were rendered more tumorigenic upon IKKα overexpression (Fig. 5A–C). Interestingly, none of 8 *NOD/SCID* mice bearing subcutaneous *Kras*$^{WT}$ cells developed pulmonary lesions, while 5 of 8 mice with subcutaneous *Kras*$^{G12C}$ cells developed lung metastases ($P = 0.0256$, $\chi^2$ test; Fig. 5D). We next examined HEK293T spots that had grown into tumors for NFκB pathway component immunoreactivity. By immunoblotting, we observed nuclear localization of IKKα but not IKKβ in control tumors expressing *Kras*$^{WT}$ that was further enhanced by coexpression of IKKβ. *Kras*$^{G12C}$ tumors showed both IKKα and IKKβ nuclear immunoreactivity, while *Kras*$^{G12C}$-IKKα-expressing tumors had enhanced IKKα and diminished IKKβ nuclear signals, and *Kras*$^{G12C}$-IKKβ-expressing tumors displayed loss of both nuclear signals (Fig. 5E). The nuclear localization of IKKα in *Kras*$^{G12C}$-IKKα coexpressing tumors was also evident on tissue sections by immunofluorescence (Fig. 5F). In addition to KRAS-IKK coexpression in benign cells, we stably expressed shRNAs specifically targeting IKKα and IKKβ transcripts (*Chuk* and *Ikbkb*, respectively) in different lung adenocarcinoma cell lines [LLC, Lewis lung adenocarcinoma cells; and primary lung adenocarcinoma cells derived from urethane-induced lung tumors of *FVB* (FULA) and *C57BL/6* (CULA) mice] bearing wild-type *Kras*$^{WT}$ (CULA cells), *Kras*$^{G12C}$ (LLC cells), *Kras*$^{Q61R}$ (FULA1 and FULA3 cells), or silenced *Kras*$^{Q61R}$ (FULA3 cells stably expressing sh*Kras*; refs. 25, 29). Interestingly, IKKα silencing resulted in decreased clonogenic capacity *in vitro* and decreased tumor growth *in vivo* specifically of *KRAS*-mutant tumor cells (Supplementary Fig. 6A–C). Moreover, this effect was not obvious *in vitro*, in line with recent observations on the *in vivo*–restricted effects of the oncogene (32). Collectively, these results indicated selective addiction of mutant *KRAS* to IKKα during carcinogenesis, possibly via nuclear IKKα functions reported elsewhere (33, 34).

### Combined targeting of IKKα/IKKβ is effective against LADC

We subsequently evaluated the therapeutic potential of our findings using cellular and animal systems tailored to noninvasively monitor tumor growth and NFκB activity. For this, three *KRAS*-mutant LADC cell lines (mouse primary LADC, *Kras*$^{Q61R}$; murine LLC, *Kras*$^{G12C}$; A549 human LADC, *KRAS*$^{G12S}$) were stably transfected with constitutive (p*CAG.Luc*)

Published OnlineFirst March 27, 2018; DOI: 10.1158/0008-5472.CAN-17-1944



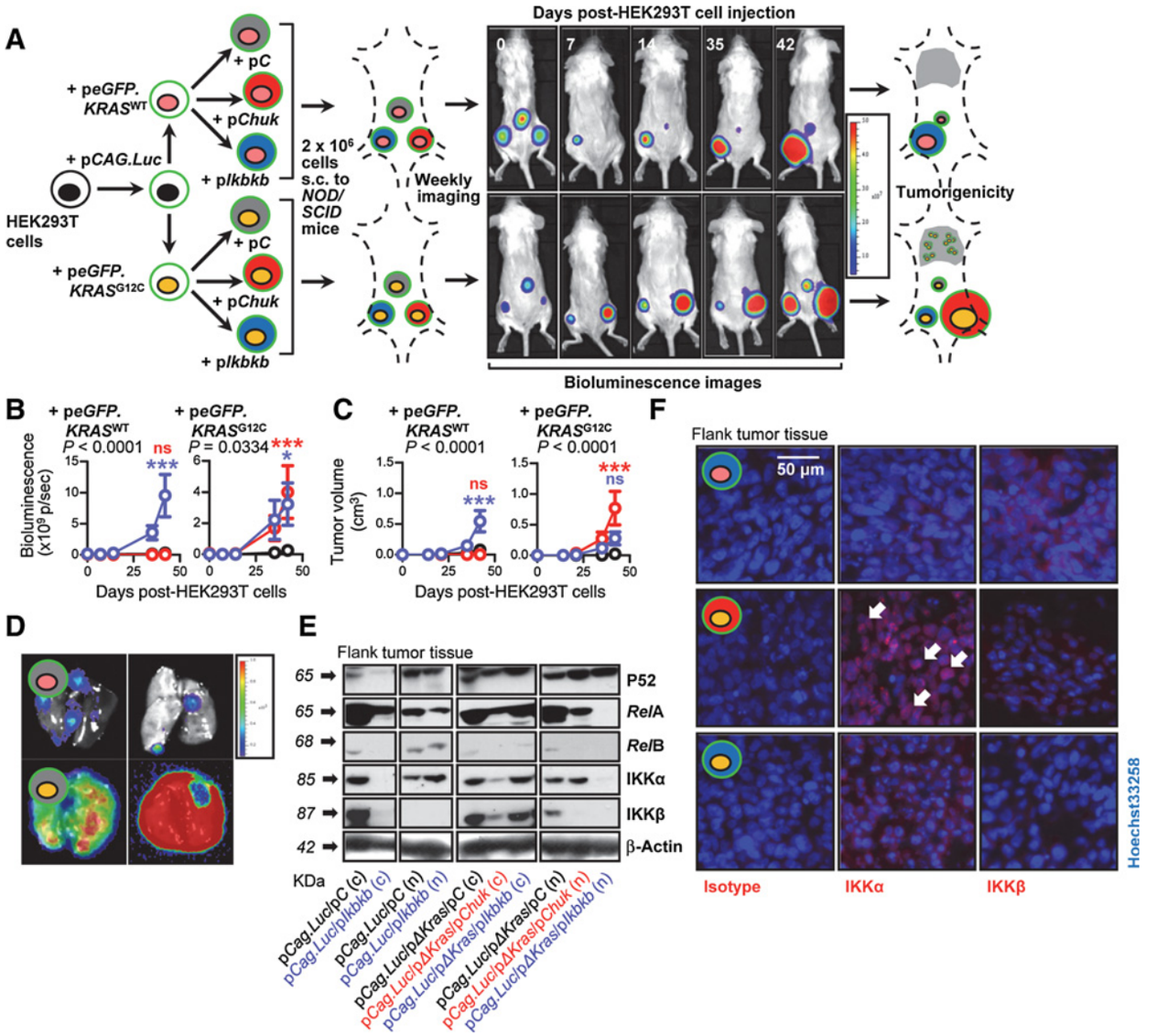


**Figure 5.**

IKKα selectively promotes the growth of *KRAS*-mutant cells *in vivo*. HEK293T cells were stably transfected with a constitutive luciferase reporter (p*CAG.Luc*), followed by plasmids encoding control random sequence (p*C*), wild-type (p*eGFP.Kras*$^{WT}$), or mutant (p*eGFP.Kras*$^{G12C}$) murine *Kras* in-frame with eGFP, and murine IKKα (p*Chuk*) or IKKβ (p*Ikbkb*) in various combinations. Two million cells were injected at different spots of the skin of *NOD/SCID* mice ($n = 16$), followed by serial spot volume assessment and bioluminescent imaging of spot cell mass. Mice were killed after 6 weeks for assessment of primary spots and lungs for tumorigenicity of the injected cells. **A,** Schematic of *in vivo* competition studies between bioluminescent cells expressing combinations of p*eGFP.Kras*WT, p*eGFP.Kras*G12C, p*Chuk*, and p*Ikbkb* and representative bioluminescent images. **B** and **C,** Data summary of spot bioluminescence (**B**) and volume (**C**) of p*C* (gray), p*Chuk* (red), and p*Ikbkb* (blue)-expressing cells shown as mean (points), SEM (bars), and two-way ANOVA *P* values. ns, nonsignificant; *, and ***: $P > 0.05$, $P < 0.05$, and $P < 0.001$, respectively, for comparisons of the indicated color-matched data points to p*C*-expressing cells at the same time point by Bonferroni posttests. **D,** Representative lung images of mice carrying p*eGFP.Kras*WT (top) and p*eGFP.Kras*G12C (bottom) tumors showing lung metastases of luminescent cells in the latter. **E,** Immunoblots of tumor nuclear and cytoplasmic extracts for NFκB pathway components. **F,** IKKα and IKKβ immunoreactivity of flank tumor cryosections showing increased immunoreactivity of p*eGFP.Kras*G12C/p*Chuk* tumors for IKKα (arrows). *Rel*, v-rel avian reticuloendotheliosis viral oncogene homolog.

and NFκB–dependent (p*NGL*) LUC reporters, inducibility of the NFκB reporter was validated, and cells were treated with increasing concentrations of the selective IKKβ inhibitor TPCA-1 {2-[(aminocarbonyl)amino]-5-[4-fluorophenyl]-3-thiophenecarboxamide; ref. 35) or the heat shock protein 90 inhibitor 17-DMAG (alvespimycin; 17-dimethylaminoethylamino-17-demethoxygeldanamycin; refs. 36, 37) that blocks, among other targets, IKKα and IKKβ function; bioluminescence imaging of live p*CAG.Luc* cells after 48-hour treatments was used to determine cell killing and bioluminescence imaging of live p*NGL* cells after 4-hour treatments was used to determine NFκB inhibition. Intriguingly, 17-DMAG displayed superior efficacy in

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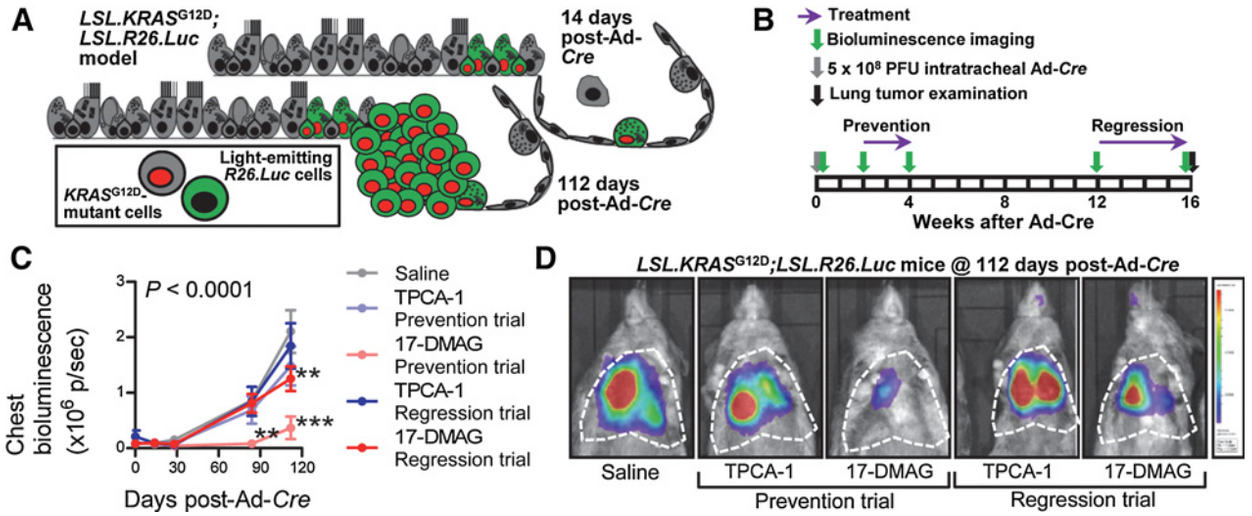


**Figure 6.**

Dual blockade of IKKα and IKKβ is effective against *KRAS*$^{G12D}$-driven lung adenocarcinoma *in vivo*. Mice harboring a conditional loxP-STOP-loxP.*R26.Luc* allele constitutively expressed in the ROSA locus (LSL.*R26.Luc*) were intercrossed with conditional mice carrying a loxP-STOP-loxP.*KRAS*G12D allele (LSL.*KRAS*G12D; all *C57BL/6*). Double transgenic LSL.*KRAS*G12D;LSL.*R26.Luc* mice ($n = 30$) received $5 \times 10^8$ intratracheal PFU Ad-*Cre* and were allocated to daily intraperitoneal treatment with the selective IKKβ inhibitor TPCA-1 or the dual IKKα/IKKβ inhibitor 17-DMAG (both at 0.5 mg/kg in 100 μL saline; approximately equivalent to 1 to 2 μmol/L by body volume extrapolation) before (prevention trial; days 14–28 after Ad-*Cre*) or after (regression trial; days 84–112 after Ad-*Cre*) lung adenocarcinoma establishment. Thereafter mice were imaged longitudinally for bioluminescence. **A,** Topology of luminescent *R26.Luc* (green, cytosol) versus *KRAS*G12D-expressing (red, nucleus) cells in this model. **B,** Schematic of experimental time course (boxes, weeks). **C,** Data summary of chest bioluminescence shown as mean (points), SEM (bars), and two-way ANOVA *P* value. **, $P < 0.01$; ***, $P < 0.001$, respectively, for comparisons of the indicated data points to saline-treated mice at the same time point by Bonferroni posttests. **D,** Representative merged bioluminescent/photographic images with pseudocolor scale showing decreased chest (dashed lines) light emission of 17-DMAG–treated mice at 112 days after Ad-*Cre*.

halting cell proliferation and NFκB activity in all three cell lines compared with TPCA-1, as evident by 4- to 5-fold lower 50% inhibitory concentrations of p*CAG.Luc* activity (mean ± SD: 28 ± 12 μmol/L for 17-DMAG and 114 ± 30 for TPCA-1) and 200- to 1,000-fold lower 50% inhibitory concentrations of p*NGL* activity (mean ± SD: 0.133 ± 0.068 μmol/L for 17-DMAG and 62 ± 30 for TPCA-1; Supplementary Fig. S7A–S7E). Based on these results and the data from *NGL* mice with *KRAS*$^{G12D}$ tumors (Supplementary Fig. S1), we designed an *in vivo* study where mice with *KRAS*$^{G12D}$-mutant LADCs received low doses of either drug tailored to inhibit NFκB activity rather than cell proliferation in both preventive and curative modes. To enable repetitive noninvasive bioluminescent quantification of tumor burden *in vivo*, mice harboring a conditional loxP-STOP-loxP.*R26.Luc* allele (*LSL.R26.Luc*; ref. 18) were intercrossed with *LSL.KRAS*$^{G12D}$ mice (all *C57BL/6*; ref. 17), yielding a model where CRE-recombination leads to simultaneous *KRAS*$^{G12D}$ and LUC expression (Fig. 6A; ref. 38). *LSL.KRAS*$^{G12D}$*;LSL.R26.Luc* mice ($n = 30$) received $5 \times 10^8$ intratracheal PFU Ad-*Cre* and were allocated to drug treatments during the two distinct phases of NFκB activation identified from *LSL.KRAS*$^{G12D}$*;NGL* mice (Supplementary Fig. S1): between days 14 and 28 after Ad-*Cre* (prevention trial) or between days 84 and 112 after Ad-*Cre* (regression trial; Fig. 6B). Treatments consisted of 100 μL daily intraperitoneal saline, TPCA-1, or 17-DMAG, both at 0.5 mg/Kg in 100 μL saline, equivalent by body volume extrapolation to maximal *in vivo* concentrations of 1.79 μmol/L for TPCA-1 and of 0.77 μmol/L for 17-DMAG, far inferior to cytotoxic concentrations (Supplementary Fig. S7). Bioluminescent detection of developing LADC revealed that TPCA-1 had no effect, while 17-DMAG prevention and regression regimens efficiently blocked tumor development compared with controls (Fig. 6C and D). Collectively, these results indicate that 17-DMAG exerts beneficial effects against *KRAS*-mutant LADCs *in vitro* and *in vivo*, even at low doses tailored to inhibit NFκB. On the contrary, a specific IKKβ inhibitor failed to show any effect, further supporting a druggable addiction of IKKα with mutant *KRAS* in LADC.

### IKKα in human LADC

The relevance of our findings with human LADC was subsequently addressed. For this, tumor and adjacent normal-appearing lung tissues of 23 patients with LADC were analyzed for *CHUK* and *IKBKB* expression by microarray and of another 35 patients from the same series for IKKα and IKKβ by immune labeling (26). *CHUK* mRNA was overrepresented in normal-appearing and LADC tissue compared with *IKBKB* mRNA, while the levels of both were not different between normal-appearing and tumor tissue (Fig. 7A). However, using a modified NFκB scoring system that examines staining intensity and localization (10), IKKα protein was significantly overexpressed in LADC compared with both normal-appearing tissues and with IKKβ (Fig. 7B and C), suggesting its possible involvement in the pathogenesis of human LADC.

## Discussion

We report an actionable requirement for IKKα in *KRAS*-mutant LADC. Using chemical and transgenic delivery of *KRAS* mutations to the respiratory tract in combination with NFκB reporter and conditional IKK-deleted mice, we map the patterns of NFκB activation in the lungs and identify the critical role of

Published OnlineFirst March 27, 2018; DOI: 10.1158/0008-5472.CAN-17-1944



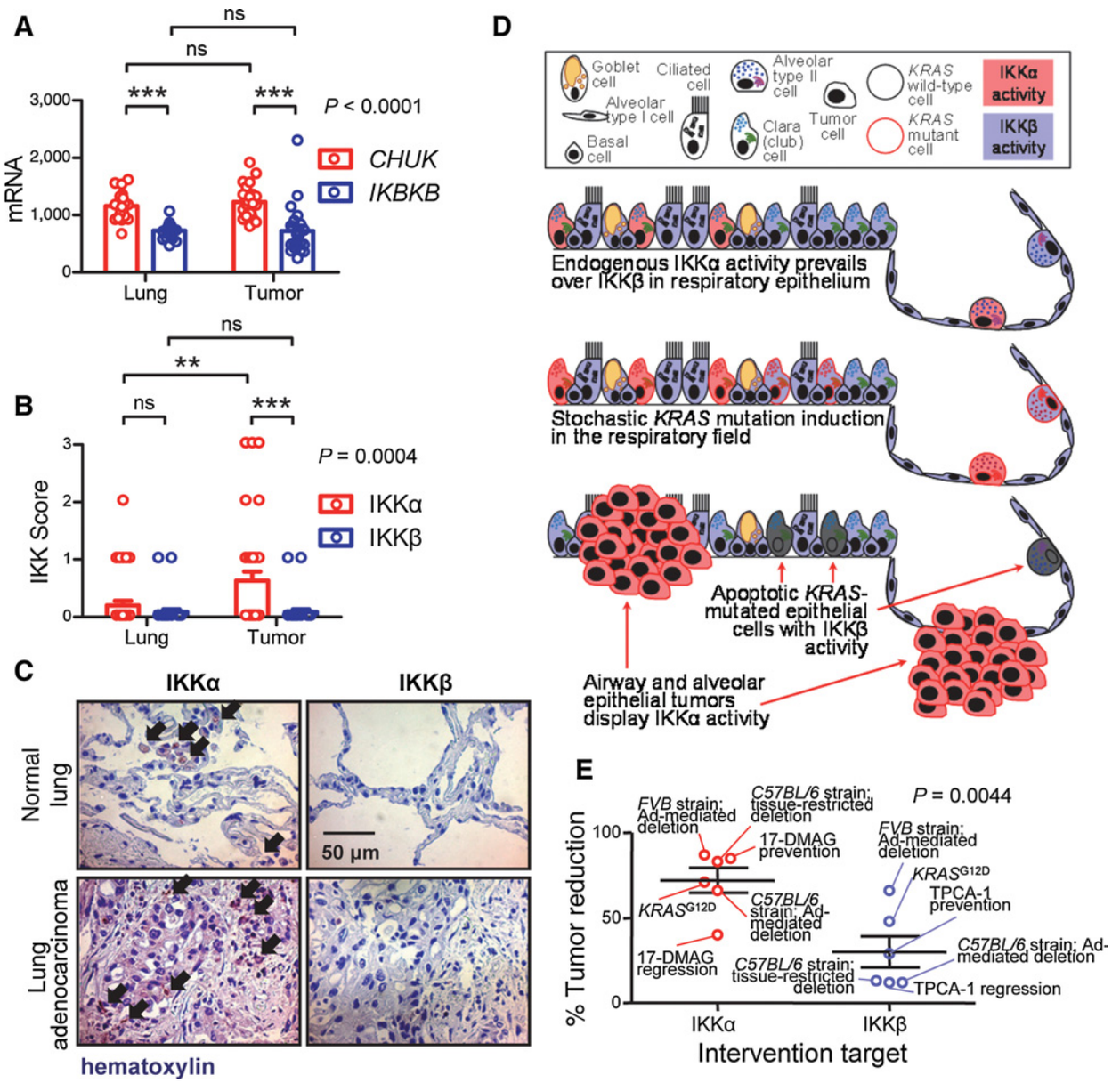


**Figure 7.**

IKKα in human lung adenocarcinoma. **A,** Data summary of normalized *CHUK* and *IKBKB* expression in tumor and adjacent normal-appearing lung tissues of 23 patients with lung cancer (43) by microarray. Data are shown as mean (columns), SEM (bars), raw data points (dots), and two-way repeated measures ANOVA *P* value. ns , nonsignificant; ***, $P > 0.05$ and $P < 0.001$ for the indicated comparisons by Bonferroni posttests. **B,** Data summary of IKKα and IKKβ immunoreactivity of tumor and adjacent normal-appearing lung tissues of 35 patients with lung cancer (43) by immunohistochemistry. Data are shown as mean (columns), SEM (bars), raw data points (dots), and two-way repeated measures ANOVA *P* value. ns, **, and ***, $P > 0.05$, $P < 0.01$, and $P < 0.001$ for the indicated comparisons by Bonferroni posttests. **C,** Representative IKKα- and IKKβ-immunostained lung and tumor tissue sections showing IKKα-immunoreactive cells (arrows). **D,** Schematic of proposed role of IKKα in *KRAS*-mutant lung adenocarcinoma. Endogenous IKKα activity sporadically prevails over IKKβ signaling across different cell types of the respiratory epithelium of smokers. Upon chemical induction of stochastic *KRAS* mutations across the respiratory field, preexisting IKKα activity fosters the survival of *KRAS*-mutant cells and is therefore addicted to the oncogene, while IKKβ signaling promotes the survival and maintenance of nonmutated cells and IKKβ-dependent cells that suffer *KRAS* mutations are destined to death. This opposing addiction of IKKα and IKKβ to mutant and wild-type *KRAS*, respectively, leads over time to the appearance of *KRAS*-mutant lung adenocarcinomas with enhanced IKKα activity. **E,** Summary of *in vivo* IKK deletion/targeting experiments shown as mean percentile reduction of lung tumor burden by IKKα- and IKKβ-targeted intervention (lines), SEM (bars), raw data (each dot represents one arm of an experiment), and paired Student *t* test *P* value.

Published OnlineFirst March 27, 2018; DOI: 10.1158/0008-5472.CAN-17-1944



IKKα. We show that IKKα drives LADC through cell-autonomous effects that are specifically exerted in the cellular context of mutant *KRAS*. These findings have implications for human disease, because IKKα is overexpressed in human LADC and oncogenic *KRAS*-IKKα addiction was annihilated by treatment with 17-DMAG.

The findings are novel and important on various counts. First, NFκB activity of *KRAS*-mutant LADC is charted in living mice and is shown to be activated early after *KRAS* mutation induction and late in established LADC. This pattern is in line with observations from smokers at risk for LADC that feature airway epithelial NFκB activation (39) and from patients with established LADC that display oncogenic NFκB activation (10). The results are consistent with the hypothesis that NFκB activation occurs together with field mutagenesis in the respiratory tract, persists in mutated cells, and reappears during clinical manifestation of late disease (40), bearing implications for NFκB–based therapy and prevention (Fig. 7D).

Second, noncanonical together with canonical NFκB pathway components are shown to be activated in *KRAS*-driven LADC. Canonical NFκB signaling is long known to be important in human and experimental LADC (8–10), but activity of the alternative pathway has not been described. This finding is in accord with our previous observations of enhanced *Rel*B activity of tumor cells in human LADC (10) and suggests important roles for alternative NFκB signaling in *KRAS*-driven LADC.

Importantly, IKKα is identified as the critical kinase for oncogenic NFκB activation of *KRAS*-mutant LADC. IKKα deletion provided beneficial effects in four different mouse models of combined *KRAS*-driven carcinogenesis and IKK depletion from the respiratory epithelium. In addition, 17-DMAG protected mice from $KRAS^{G12D}$-driven LADC when given early (preventive treatment) or late (regression trial), while the IKKβ blocker TPCA-1 did not. Although 17-DMAG likely suppresses a spectrum of targets broader than IKKα and IKKβ (41), inclusively targeting IKKα, utilizing even this nonspecific approach provided superior overall effects in reducing tumor burden compared with IKKβ-specific inhibition (Fig. 7E). We were the first to identify that indirect IKKβ blockade via overexpression of dominant-negative IκBα protects mice from urethane-induced LADC (8), a finding thereafter recapitulated in $KRAS^{G12D}$-mutant (9), and tobacco-smoke–induced (14) LADC. Urethane-triggered LADCs were recently genomically characterized and shown to harbor $Kras^{Q61R}$/$Kras^{G12V}$ mutations (22), similar to human LADC (42). Based on these findings and results from other tumor types, research and drug discovery focused on IKKβ yielding proteasome and IKKβ inhibitors (35, 43). However, these provide poor outcomes in human LADC (44) and cause resistance or paradoxical tumor promotion in animal LADC models via myeloid NFκB inhibition, secondary mutation development, and/or enhanced neutrophil-provided IL1β (23, 45, 46). In addition, recent evidence indicates that IKKβ might not be the only kinase responsible for oncogenic NFκB activation of *KRAS*-mutant LADC (47). To this end, TBK1 emerged as a KRAS addiction partner and was found to mediate EGFR-inhibitor resistance (5, 6), while IKKε promoted tumorigenesis together with TBK1 (13). Only one study addressed the role of IKKα depletion together with IKKβ in lung cancer cells *in vitro* and found both kinases to be important (11). Our results identify for the first time the pivotal role of IKKα in *de novo* development of *KRAS*-mutant LADC *in vivo* and position the kinase as a marked therapeutic target.

Using *in vitro* and *in vivo* competition studies, we determine that IKKα selectively fosters the survival of *KRAS*-mutant cells and is therefore addicted to the oncogene, while IKKβ promotes the survival and maintenance of nonmutated cells. We hypothesize that in a stochastically *KRAS*-mutated respiratory field, this opposing addiction of IKKα and IKKβ to mutant and wild-type *KRAS*, respectively, would lead over time via clonal selection to *KRAS*-mutant LADCs with enhanced IKKα activity (Fig. 7D). This cell-autonomous model is supported by the results from $KRAS^{G12D}$ mice (where IKKα was selectively deleted in *KRAS*-mutant cells) and from HEK293T cells (where IKK/KRAS combinations functioned similarly *in vitro* and *in vivo*), notwithstanding the possibility for autocrine IKKα-triggered cytokine networks identified elsewhere (48, 49). To this end, IKKα localized to the nucleus of our murine LADCs, a phenomenon that could enhance gene transcription or repress oncogenes (33, 34). Nuclear IKKα was also present in human LADC, which displayed enhanced nuclear IKKα immunoreactivity. The proposed IKKα function to site independently foster *KRAS*-mutant cells also emanates from tissue-restricted IKK-deletion studies where IKKα was critical in both airway and alveolar cells, a result of importance given the cellular and histologic diversity of human LADC (50).

Finally, a feasible approach for translation of the findings is explored. Treatment with 17-DMAG was tailored to target NFκB activation of *KRAS*-mutant LADC *in vitro* and was translated to a preclinical study, where it was well tolerated and effective against LADC *in vivo*, both preventively and therapeutically. The efficacy of 17-DMAG and the inefficacy of TPCA-1 strengthen the proposed link between mutant *KRAS* and IKKα and open up new avenues for therapy/prevention of *KRAS*-mutant LADC (1). In summary, we report a requirement for IKKα in *KRAS*-driven LADC, implicate IKKα as a *KRAS* nononcogene addiction partner, and show that targeting IKKα may confer beneficial effects against a currently untreatable disease that is the number one cancer killer in the world.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Authors' Contributions

**Conception and design:** M. Papageorgopoulou, T.S. Blackwell, A. Marazioti, G.T. Stathopoulos

**Development of methodology:** M. Vreka, I. Lilis, M. Papageorgopoulou, G.A. Giotopoulou, M. Lianou, I. Giopanou, M. Spella, T. Agalioti, G.T. Stathopoulos

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** M. Vreka, M. Papageorgopoulou, N.I. Kanellakis, M. Spella, T. Agalioti, V. Armenis, S. Marwitz, F.E. Yull, G.T. Stathopoulos

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** M. Vreka, I. Lilis, M. Papageorgopoulou, G.A. Giotopoulou, M. Lianou, I. Giopanou, N.I. Kanellakis, T. Agalioti, T. Goldmann, S. Marwitz, G.T. Stathopoulos

**Writing, review, and/or revision of the manuscript:** M. Vreka, M. Papageorgopoulou, T. Goldmann, S. Marwitz, F.E. Yull, A. Marazioti, G.T. Stathopoulos

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** M. Vreka, M. Papageorgopoulou, V. Armenis

**Study supervision:** M. Vreka, T. Goldmann, A. Marazioti, G.T. Stathopoulos

**Other (provided mouse models used in the study):** M. Pasparakis

## Acknowledgments

This work was supported by European Research Council 2010 Starting Independent Investigator and 2015 Proof of Concept Grants (260524 and 679345, respectively, awarded to G.T. Stathopoulos), as well as a Research Award by the Hellenic Thoracic Society (awarded to M. Vreka).

The authors thank the University of Patras Center for Animal Models of Disease and Advanced Light Microscopy Cores for experimental support.

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Received June 29, 2017; revised October 3, 2017; accepted March 22, 2018; published first March 27, 2018.

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Published OnlineFirst March 27, 2018; DOI: 10.1158/0008-5472.CAN-17-1944

Cancer Research
The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

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*Cancer Res* 2018;78:2939-2951. Published OnlineFirst March 27, 2018.