Myeloid-derived interleukin-1β drives oncogenic KRAS-NF-κB addiction in malignant pleural effusion

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Malignant pleural effusion (MPE) is a frequent metastatic manifestation of human cancers. While we previously identified KRAS mutations as molecular culprits of MPE formation, the underlying mechanism remained unknown. Here, we determine that non-canonical IKKα-RelB pathway activation of KRAS-mutant tumor cells mediates MPE development and this is fueled by host-provided interleukin IL-1β. Indeed, IKKα is required for the MPE-competence of KRAS-mutant tumor cells by activating non-canonical NF-κB signaling. IL-1β fuels addiction of mutant KRAS to IKKα resulting in increased CXCL1 secretion that fosters MPE-associated inflammation. Importantly, IL-1β-mediated NF-κB induction in KRAS-mutant tumor cells, as well as their resulting MPE-competence, can only be blocked by co-inhibition of both KRAS and IKKα, a strategy that overcomes drug resistance to individual treatments. Hence we show that mutant KRAS facilitates IKKα-mediated responsiveness of tumor cells to host IL-1β, thereby establishing a host-to-tumor signaling circuit that culminates in inflammatory MPE development and drug resistance.

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Malignant pleural effusion (MPE) is one of the most challenging cancer-related disorders. It ranks among the top prevalent metastatic manifestations of tumors of the lungs, breast, pleura, gastrointestinal tract, urogenital tract, and hematopoietic tissues, killing an estimated two million patients worldwide every year and causing 126,825 admissions in U.S. hospitals in 2012 alone.12–14 The presence of a MPE at diagnosis is an independent negative prognostic factor in patients with lung cancer and mesothelioma.3,4 In addition, current therapies are non-etiological and often ineffective, may cause further morbidity and mortality, and have not yielded significant improvements in survival.5,6

To meet the pressing need for mechanistic insights into the pathobiology of MPE, we developed immunocompetent mouse models of the condition that unveiled inflammatory tumor-host signaling networks causing active plasma extravasation into the pleural space.7 Nuclear factor (NF)-κB activity in tumor cells was pivotal for MPE formation in preclinical models, driving pro-inflammatory gene expression and promoting pleural tumor cell survival.5,6 However, the mechanism of oncogenic NF-κB activation of MPE-competent pleural tumor cells remained unknown. In parallel, we recently pinned mutant KRAS as a molecular determinant of the propensity of pleural-metastasized tumor cells for MPE formation: mutant KRAS delivered its pro-MPE effects by directly promoting C-C chemokine motif ligand 2 (CCL2) secretion by pleural tumor cells, resulting in pleural accumulation of MPE-fostering myeloid cells.11 However, a molecular determinant of the propensity of pleural-metastasized tumor cells to promote pleural effusion in vivo remains elusive and diverse, and different studies indicate that IKK activity is pivotal for MPE formation in preclinical models, driving pro-inflammatory gene expression and promoting pleural tumor cell survival.5,6

Kras mutations have been previously linked to elevated or aberrant NF-κB activity via cell-autonomous and paracrine mechanisms. KRAS-mutant tumors, including lung and pancreatic adenocarcinomas, require active NF-κB signaling.12–14 and NF-κB inhibition blocks KRAS-induced tumor growth.14–16 In turn, NF-κB activation of KRAS-mutant tumor cells has been associated with enhanced RAS signaling, drug resistance, and stemness.17,18 Despite significant research efforts, the NF-κB-activating kinases (IkB kinases, IKK) and pathways (canonical, involving IkBα, IKKβ, and RelA/P50, versus non-canonical, comprising IkBβ, IKKα, and RelB/P52) that mediate this oncogenic addiction between mutant KRAS and NF-κB signaling are still elusive and diverse, and different studies indicate that IKKα, IKKβ, IKKγ, IKKε, and/or TANK-binding kinase 1 (TBK1) are key for this17–24

Here we use immunocompetent mouse models of MPE to show that mutant KRAS determines the responsiveness of pleural tumor cells to host-delivered interleukin (IL)-1β signals by directly regulating IL-1 receptor 1 (IL1R1) expression. IKKα is further shown to critically mediate IL-1β signaling in KRAS-mutant tumor cells, culminating in marked MPE-promoting effects delivered by C-X-C chemokine motif ligand 1 (CCL1), and in oncogenic addiction with mutant KRAS evident as drug resistance. Importantly, simultaneous inhibition of IKKα and KRAS is effective in annihilating mutant KRAS-IKKα addiction in MPE.

Results
Non-canonical NF-κB signaling of KRAS-mutant cancer cells.

We first evaluated resting-state NF-κB activity of five mouse cancer cell lines with defined KRAS mutations and MPE capabilities in syngeneic C57BL/6J mice.11 Lewis lung carcinoma (LLC; MPE-competent; KrasG12C), MC38 colon adenocarcinoma (MPE-competent; KrasG12R), AE17 malignant pleural mesothelioma (MPE-competent; KrasG12C), B16F10 skin melanoma, and PANO2 pancreatic adenocarcinoma (both MPE-incompetent and KrasWT) cells. Parallel transient transfection of these cell lines with reporter plasmids encoding Photorus Pyralis LUC under control of either a constitutive (pCAG.LUC) or a NF-κB-dependent (pNF-κB.GFP.LUC, pNGL) promoter8,9 revealed that only PANO2 cells, a cell line with relatively low NF-κB activity, were transiently transfected with pKrasG12C, their NF-κB expression levels were elevated.11 Moreover, KRAS mutant (MUT) cells displayed elevated DNA-binding activity of non-canonical NF-κB subunits P52 and RelB by functional NF-κB enzyme-linked immunosorbent assay (ELISA) and enhanced nuclear immunofluorescent localization of RelB compared with KRASWT cells.8,9,11,25 Immunoblotting of cytoplasmic and nuclear extracts revealed that KRASMUT cells had increased levels of cytoplasmic RelA and IkBα and of nuclear RelB, IkBβ, and IKKα compared with KrasWT cells.8,9 This results suggest that KRASMUT cancer cells exhibit non-canonical endogenous NF-κB activity.

Resistance of KRAS-mutant cancer cells to IKKβ inhibition.

We next examined the effects of small molecule inhibitors of the proteasome (bortezomib),9 of IKKβ (IMD-035427), or of heat shock protein 90 (HSP90) (17-dimethylaminoethylamino-17-demethoxygeladanycin (17-DMAG))19 that display significant inhibitory activity against IKKβ and/or IKKα (of note, a specific IKKα inhibitor does not exist) on NF-κB reporter activity and cellular proliferation of our murine cancer cell lines (Fig. 1g, h; Supplementary Table 1). Bortezomib, an indirect inhibitor of IKKβ via cytoplasmic accumulation of non-degraded IkBα,9,20,21 attenuated endogenous NF-κB activity of KrasWT cells but paradoxically activated NF-κB in KRASMUT cells, at the same time more effectively killing KrasWT than KrasMUT cells in vitro. Similarly, IKKβ-selective IMD-035427 blocked NF-κB activity and cellular proliferation of unstimulated KrasWT cells but not of KRASMUT cells. Interestingly, the HSP90 and dual IKKα/IKKβ inhibitor 17-DMAG was equally effective in limiting NF-κB activity and cellular proliferation of all cell lines irrespective of KRAS mutation status. These results suggest the existence of endogenous resistance of KRAS-mutant cells to IKKβ inhibition, which can be overcome by combined HSP90/IKKα/IKKβ inhibition.

II-1-inducible NF-κB activation of KRAS-mutant cancer cells.

We next studied NF-κB activation patterns of our murine cancer cells in response to exogenous stimuli. For this, cells were stably transfected with pNGL, were pretreated with saline or bortezomib (1 μM), and incubated with 50% NF-κB inhibitory concentration obtained from KrasWT cells (Supplementary Table 1), were exposed to 60 different candidate NF-κB-pathway ligands at 1 nM concentration,9 and were longitudinally monitored for NF-κB-dependent LUC activity by bioluminescence imaging of live cells in vitro (Fig. 2a, b; Supplementary Table 2). Incubation with lipopolysaccharide (LPS) and tumor necrosis factor (TNF) resulted in markedly increased NF-κB activity in all cells irrespective of KRAS status, while lymphotixin-β activated NF-κB in all but PANO2 cells, effects that peaked by 4–8 h of incubation and subsided by 16–24 h. Uniquely, IL-1α and IL-β induced NF-κB exclusively in KRASMUT cells. In addition, bortezomib exaggerated endogenous and inducible NF-κB activation of KRASMUT cells, in contrast to KrasWT cells that displayed efficient NF-κB blockade by bortezomib. In line with the above, IIl1r1 (encoding IIl1R1, cognate to IL-1α/β) expression, but not Tnfrsf1a/Tnfrsf1b (encoding TNF receptors) or IIl1a/Ii1b expression (that was undetectable in all cell lines), was exclusively restricted to...
KRAS\textsuperscript{MUT} MPE-proficient tumor cells (Fig. 2c, d). We subsequently tested whether inducible NF-κB activation occurs in tumor cells entering the pleural space in vivo, simulating incipient pleural carcinomatosis\textsuperscript{4,7}. For this, naive C57BL/6 mice were pulsed with a million intrapleural pNGL-expressing tumor cells and were serially imaged for NF-κB-dependent bioluminescence. Amazingly, KRAS\textsuperscript{MUT} MPE-competent cells responded to the pleural environment with markedly escalated NF-κB activity within 4 h after injection, while KRAS\textsuperscript{WT} MPE-incompetent cells showed diminishing NF-κB signals (Fig. 3a). Interestingly, this
in vivo NF-κB response of KRAS\textsuperscript{MUT} cells was abolished in IL-1β-deficient (\textit{Il1β}−/−), but not in TNF-deficient (\textit{Tnf}−/−), mice (Fig. 3b), indicating that KRAS\textsuperscript{MUT} tumor cells selectively respond to IL-1β of the pleural environment by activating NF-κB.

**Mutant KRAS promotes non-canonical NF-κB signaling.** To define the role of mutant KRAS in the aberrant NF-κB activation patterns of KRAS\textsuperscript{MUT} tumor cells, including non-canonical endogenous NF-κB activity, resistance to IKKβ inhibition, and IL-1β-inducibility, we undertook short hairpin RNA (shRNA)-mediated KRAS silencing (shKras) and plasmid-mediated overexpression of a mutant dominant-negative form of IkBα (pIkBα-BudDN; inhibits canonical NF-κB signaling) in KRAS\textsuperscript{MUT} cell lines, as well as plasmid-mediated overexpression of mutant KRAS (pKras\textsuperscript{G12C}) in KRAS\textsuperscript{WT} cell lines.\textsuperscript{41,43} Stable pIkBα-BudDN expression in MC38 cells (Kras\textsuperscript{G13R}) resulted in decreased RelA and sustained RelB nuclear-binding activity, while shKras did not affect RelA but abolished RelB nuclear-binding activity (Fig. 4a). shKras also eliminated nuclear RelB localization in these cells without affecting RelA (Fig. 4b) and abolished nuclear IKKα immunoreactivity of LLC (Kras\textsuperscript{G12C}) and MC38 cells (Fig. 4c). shKras expression reversed the endogenous resistance of MC38 cells to bortezomib and IMD-0354, rendering them as sensitive as KRAS\textsuperscript{WT} cells (Fig. 4d, e). In addition, shKras annihilated IL-1β-induced NF-κB transcriptional activity of pNGL-expressing LLC, MC38, and AE17 (Kras\textsuperscript{G12C}) cells (Fig. 4f), and pKras\textsuperscript{G12C} transmitted this phenotype to KRAS\textsuperscript{WT} PANO2 cells (Fig. 4g). Importantly, shKras abrogated the in vivo NF-κB response of pleural-inoculated MC38 cells, which was reinstated in PANO2 cells by stable pKras\textsuperscript{G12C} expression (Fig. 4h, i). In parallel, KRAS silencing in KRAS\textsuperscript{MUT} cells significantly decreased, whereas pKras\textsuperscript{G12C} overexpression in KRAS\textsuperscript{WT} cells significantly increased Il1r1 expression, as well as resting-state and IL-1β-inducible nuclear immunoreactivity for RelB, IkBβ, and IKKα (Fig. 4j–l). Collectively, these data indicate that mutant KRAS induces non-canonical NF-κB signaling of cancer cells in unstressed and IL-1β-stimulated conditions.

IKKα in mutant KRAS-dependent MPE. To define the NF-κB-activating kinase responsible for aberrant NF-κB signaling of KRAS\textsuperscript{MUT} cancer cells, we stably expressed shRNAs specifically targeting IKKα, IKKβ, IKKe, and TBK1 transcripts (Chuk, Ikbbk, Ikbe, and Tbk1, respectively) in our pNGL-expressing cell lines and validated them (Fig. 5a). In addition, we cloned these murine transcripts into an eukaryotic expression vector and generated stable transfectants of our cell lines. Interestingly, resting-state NF-κB transcriptional activity across KRAS\textsuperscript{MUT} cells was markedly suppressed by shChuk but not by shIkbbk or shTbk1, while shIkbbk yielded minor NF-κB inhibition in MC38 and AE17 cells. On the contrary, endogenous NF-κB-mediated transcription of B16F10 cells was exclusively silenced by shIkbbk and, to a lesser extent, shIkbe, and of PANO2 cells by no shRNA (Fig. 5b). In a reverse approach, overexpression of any kinase resulted in enhanced NF-κB activity in all KRAS\textsuperscript{MUT} cell lines, of IKKβ only in B16F10 cells, and of no kinase in PANO2 cells (Fig. 5c). In addition to intrinsic, IKKα also mediated IL-1β-inducible NF-κB activity of KRAS\textsuperscript{MUT} tumor cells, since shChuk but not shIkbbk abolished IL-1β-induced NF-κB activity across KRAS\textsuperscript{MUT} cell lines (Fig. 5d). In line with the above, shChuk abolished the immunoreactivity of MC38 cell nuclear extracts for RelB, IkBβ, and IKKα, both at resting and IL-1β-stimulated states (Fig. 5e). Taken together, these data suggest that KRAS-mutant cancer cells respond to pleural IL-1β via IKKα-mediated non-canonical NF-κB activation. Based on these results and our previous identification of the importance of KRAS mutations and NF-κB signaling in MPE development,\textsuperscript{4,8–11} we hypothesized that IKKα is required for sustained NF-κB activation and MPE induction by pleural-homing KRAS\textsuperscript{MUT} cancer cells. To test this, we injected IKKα-silenced pNGL-expressing LLC cells (Kras\textsuperscript{G12C}; MPE-competent) into the pleural space of C57BL/6 mice. Indeed, recipients of IKKα-silenced LLC cells displayed significant reductions in MPE incidence and volume, pleural inflammatory cell influx, and pleural tumor NF-κB activity and prolonged survival. IKKe silencing delivered more modest and equivocal beneficial effects, while IKKβ and TBK1 silencing had no impact (Fig. 6a–c; Supplementary Table 3). These experiments were repeated with IKKα- and IKKβ-silenced MC38 cells (Kras\textsuperscript{G12R}; MPE-competent) stably expressing pNGL, confirming that IKKα is cardinal for oncogenic NF-κB activation and MPE precipitation by pleural-metastatic KRAS\textsuperscript{MUT} tumor cells (Fig. 6d–f; Supplementary Table 3). However, standalone overexpression of IKKα or IKKβ did not confer MPE competence to KRAS\textsuperscript{WT} PANO2 cells, as opposed to pKras\textsuperscript{G12C} (Fig. 6g–i; Supplementary Table 3), in accord with our previous observations.\textsuperscript{11} Collectively, these results suggest that mutant KRAS-potentiated IL-1β signaling results in KRAS\textsuperscript{MUT} addiction to IKKα activity, which is required but not sufficient for oncogenic NF-κB activation and MPE formation.

**Myeloid IL-1β fosters mutant KRAS-IKKα addiction in MPE.** To study the importance of host-delivered IL-1β in the proposed KRAS\textsuperscript{MUT}–IKKα addiction culminating in MPE, we delivered pNGL-expressing KRAS\textsuperscript{MUT} LLC and MC38 cells into the pleural space of Il1β−/−, Tnf−/−, and WT C57BL/6 mice. Interestingly, Il1β−/− but not Tnf−/− mice displayed decreased MPE incidence, volume, inflammatory cell influx, and oncogenic NK-B
activation (Fig. 7a–d; Supplementary Table 3). Host-provided IL-1β was of myeloid origin, since bone marrow (BM) transplantants from C57BL/6 and Tnf−/−, but not Il1b−/− donors, to lethally irradiated Il1b−/− recipients unable to foster MPE proficient LLC cells MPE proficient (Fig. 7e, f; Supplementary Table 3). To define which myeloid cells provide the bulk of IL-1β to fuel tumor cell NF-κB activity, we isolated BM cells from C57BL/6 mice and drove them toward monocyte and neutrophil differentiation by macrophage colony-stimulating factor (M-CSF) and granulocyte-colony-stimulating factor (G-CSF) culture, respectively. Both BM-derived monocytes and neutrophils secreted IL-1β upon 24-hour treatment with cell-free LLC supernatants as measured by ELISA, but monocytes secreted ~200 times higher cytokine levels than undifferentiated BM cells

**Fig. 2** Kras-mutant tumor cells possess IL-1β-inducible NF-κB activity. Five different C57BL/6 mouse tumor cell lines with (LLC, MC38, AE17) or without (B16F10, PANO2) Kras mutations were assessed for inducible NF-κB activation in response to exogenous stimuli and for the expression of relevant receptors in vitro. a, b, Representative bioluminescent images (a, shown are n = 3 replicates/data-point) and data summary (b, mean ± s.d. of n = 3 independent experiments) of cells stably expressing pGL3 and pretreated with saline or 1 μM bortezomib at different time points after addition of 1 nM of the indicated NF-κB ligands (arrows in a and legend in b). Note NF-κB inducibility by IL-1β and bortezomib exclusively in KrasW124C cells. ns and *** P < 0.001 and P < 0.001, respectively, for comparison between ligands indicated by colored arrows and PBS at 4 and 8 h on treatment by two-way ANOVA with Bonferroni post-tests. c, d, Tnfrsf1a, Tnfrsf1b, Il1r1, Il1a, and Il1b mRNA expression relative to Gusb by microarray (c) and qPCR (d). Shown are mean (c) and mean ± s.d. (d) of n = 5 independent technical replicates of one biologic sample. P, probability of no difference between cell lines by two-way (c) or one-way (d) ANOVA, ns, single, double, and triple asterisks (*, **, and ***): P > 0.05, P < 0.05, P < 0.01, and P < 0.001, respectively, for comparison with B16F10 cells (c) by Bonferroni post-tests.
and neutrophils (Fig. 7g). These data clearly show that the main source of IL-1β in the pleural space during MPE development likely are recruited myeloid monocyte cells.

Mutant KRAS-IKKα addiction promotes MPE via CXCL1 secretion. To identify the MPE effectors and transcriptional signatures of IL-1β/KRAS/IKKα-addicted tumor cells, we subjected KRAS-silenced, IKKα-silenced, and IL-1β-challenged LLC and MC38 cells to microarray analyses, seeking for transcripts altered heterodirectionally by stimulation/challenge. Thirty transcripts fulfilled these criteria in LLC (including Ppdp, encoding pro-platelet basic protein, PGBP, and Cxcl1, encoding CXCL1) and 20 in MC38 (including Cxcl1) cells, with Cxcl1 being the only common gene of these two signatures (Fig. 8a, b; Supplementary Tables 4, 5). Cxcl1 microarray results were validated by quantitative PCR (qPCR) and ELISA (Fig. 8c–e). Furthermore, chromatin immunoprecipitation (ChIP) was performed in LLC cells treated with phosphate-buffered saline (PBS) or IL-1β in order to specify whether and which NF-κB component directly binds to the promoter region of Cxcl1. The data indicate that only RelB and IKKα bind to the NF-κB element in the Cxcl1 promoter and that IL-1β significantly strengthens this binding (Fig. 8f). These findings are consistent with the enhanced transcriptional induction of Cxcl1. Moreover, Cxcl1 and Ppdp expression was pivotal for MPE induction by IL-1β/KRAS/IKKα-addicted LLC cells, since these were MPE incompetent in both C-C-X-C chemokine motif receptor 1 (CXCRI) and CXCRII gene-deficient mice36,37 that lack the genes encoding CXCL1/PDPB-cognate CXCR1 and CXCR2 receptors38 (Fig. 8g; Supplementary Table 3).

Notably, in MPEs from CXCRI and CXCR2 gene-deficient mice the predominant cell population was monocytes, whereas in MPEs from CCR2 gene-deficient mice11 the prevalent cell type was neutrophils. This result was not unexpected since the majority of myeloid cells recruited in the pleural space during MPE development in C57BL/6 mice consist of both neutrophils and monocytes (Fig. 8h). Of note, the monocyte population is the most prevalent during MPE development.

Combined targeting of KRAS/IKKα is effective against MPE. To explore the therapeutic implications of the proposed mechanism, we examined potential synergy of the KRAS inhibitor deltarasin39 with the IKKβ-specific inhibitor IMD-0354 or the HSP90/IKKα/IKKβ inhibitor 17-DMAG using TNF- or IL-1β-stimulated LLC murine and A549 human lung adenocarcinoma cells expressing pNGL (Fig. 9a, b). Interestingly, all inhibitors alone or in combination failed to block TNF-inducible NF-κB activation in both cell lines. In addition, all standalone drugs failed to inhibit IL-1β-inducible NF-κB activation in both cell lines, except from partial effects observed in A549 cells by 17-DMAG. However, deltarasin/17-DMAG but not deltarasin/IMD-0354 combination treatment completely abolished IL-1β-induced NF-κB activation in both cell types to unstimulated levels (Fig. 9a, b), indicating that drugging the KRAS/IKKα axis can halt IL-1β responsiveness. To determine the potential efficacy of this approach against MPE, standalone or combined deltarasin, and 17-DMAG treatments (both 15 mg/Kg) were delivered to mice with established pleural tumors. For this, C57BL/6 mice received pleural LLC cells and treatments commenced after 5 days to allow initial pleural tumor implantation41. At day 13 post-tumor cells, standalone deltarasin and 17-DMAG-treated mice had significantly decreased MPE volume compared with saline-treated controls (40% reductions for both groups; P < 0.05; one-way analysis of variance (ANOVA) with Bonferroni post-tests). However, combination-treated mice were markedly protected from MPE development (57% incidence) and progression (65% volume reduction; P < 0.001; one-way ANOVA with Bonferroni post-tests) (Fig. 9c; Supplementary Table 3). Hence combined targeting of mutant KRAS and IKKα is effective in halting oncogenic NF-κB activation and MPE in mice.

IL-1β-inducible NF-κB activity in human KRAS-mutant cells. To assess whether our findings are relevant to human cancer, we screened nine human cancer cell lines of known KRAS mutation status40 for Rel-β-binding activity of nuclear extracts. In accord with murine data, KRASMUT cells displayed enhanced nuclear RelB activity in human KRAS-mutant cells.

Fig. 3 Pleural IL-1β activates NF-κB in Kas-mutant tumor cells in vivo. Five different C57BL/6 mouse tumor cell lines with (LLC, MC38, AE17) or without (B16F10, PANO2) Kas mutations were assessed for inducible NF-κB activation in response to the pleural environment in vivo. a Representative bioluminescent images and data summary (n = 6 mice/cell line) of C57BL/6 mice at 0 and 4 h after intrapleural injection of a million mouse tumor cells stably expressing pNGL. Note the marked induction of the bioluminescent signal emitted specifically by KasMUT cells after 4 h. Note also the diminishing signal emitted by KasWT cells. b Representative bioluminescent images and data summary (LLC: n = 6 mice/genotype; MC38: n = 7 mice/genotype) of C57BL/6, TNf−/− and Ilb−/− mice at 0 and 4 h after intrapleural injection of LLC or MC38 cells stably expressing pNGL. Note the marked induction of the bioluminescent signal in C57BL/6 mice, the borderline reduction of its inducibility in TNf−/− mice, and the disappearance of signal inducibility in Ilb−/− mice. Data are presented as mean ± s.d. P, probability of no difference between cell lines or genotypes by one-way ANOVA. ns, single, double, and triple asterisks (*, **, and ***): P > 0.05, P < 0.05, P < 0.01, and P < 0.001, respectively, for the indicated comparisons by Bonferroni post-tests.
compared with RelA binding (Fig. 10a). In addition, A549 (KRAS<sup>G12S</sup>) and NCI-H23 (KRAS<sup>G12C</sup>) cells displayed IL-1β-induced NF-κB activation, as opposed to HT-29 and SKMEL2 cells (both KRAS<sup>WT</sup>). Importantly, stable pKras<sup>G12C</sup> expression in SKMEL2 cells rendered them responsive to IL-1β (Fig. 10b, c).

In summary, KRAS mutations alter NF-κB signaling in tumor cells. KRAS<sup>WT</sup> cells preferentially utilize intrinsically or exogenously (i.e., by LPS, TNF) stimulated IKKα-mediated NF-κB signaling, display sensitivity to IKKβ inhibition, poor CXCR1/2 ligand secretion, and MPE incompetence. KRAS<sup>MUT</sup> cells...
predominantly use IKKα-mediated non-canonical NF-κB signaling at resting state and in response to myeloid IL-1β, display enhanced CXCR1/2 ligand secretion and MPE proficiency, and are addicted to sustained IKKα activity evident as resistance to IKKβ inhibitors.

**Discussion**

We provide a novel paradigm of how an oncogene can co-opt the host environment to foster addiction with a perturbed signaling pathway. KRAS-mutant cancer cells are shown to respond to host provided IL-1β in the pleural space by increasing non-canonical IKKα-RelB pathway activity. The co-existence of mutant KRAS and elevated IKKα-mediated non-canonical NF-κB signaling in the cancer cell, relentlessly driven by host IL-1β, leads to two important consequences. First, to enhanced transcription of CRBP secretion, and MPE. Finally, we show that this partnership activation, resistance to proteasome and IKK activity evident as resistance to IKKβ inhibitors. Indeed, KRAS-mutant cancer cells displayed non-canonical endogenous NF-κB activity evident by enhanced nuclear localization and/or DNA-binding activity of RelB, IkBα, and IKKα, which was further inducible by exogenous IL-1β. Importantly, non-canonical NF-κB utilization by KRAS-mutant cancer cells was IKKα-driven, involved RelB activation, and was required for MPE. Nuclear IKKα functions have been identified previously, including histone 3 modifications augmenting TNF receptor activator of NF-κB ligand–induced gene expression and repression of maspin, a metastasis gate-keeper. Our work links IKKα function with IL-1β-induced RelB activation and CXCL1/PPBP transcription. Moreover, we provide novel evidence that mutant KRAS is indirectly responsible for non-canonical NF-κB activation, which is IKKa and RelB based, via sensitization of cancer cells to host IL-1β. Finally, IKKα is found to be responsible for MPE, an important metastatic manifestation of various cancers. The findings concur with previous reports of a combined requirement for IKKα and IKKβ for oncogenic NF-κB activation, as well as with human observations of predominant non-canonical NF-κB activity of tumors with high incidence of KRAS mutations, such as lung adenocarcinoma. However, we demonstrate an isolated requirement for IKKα in KRAS-driven MPE, an important cancer phenotype.

In recent years, inflammation was established as a conditional tumor promoter. IL-1α/β are important components of the tumor microenvironment that stimulate tumor invasiveness and angiogenesis. Myeloid-derived IL-1β is implicated in the resistance to NF-κB inhibitors and IL-1β antagonism yielded beneficial effects in a mouse model of KRAS-mutant pancreatic cancer. We found previously that IL-1α/β are present in human and experimental MPE and that MPE-competent adenocarcinomas trigger myeloid cells to secrete IL-1β. Here the mechanism of pleural IL-1β function in MPE promotion is elucidated: CCL2-attracted monocyte–released IL-1β fosters NF-κB signaling via IKKα. Undoubtedly, IL-1β is not the sole NF-κB ligand expressed in the malignancy-affected pleural space: TNF, a known stimulator of canonical NF-κB signaling, is present in MPE and promotes disease progression. However, TNF likely originates from tumor cells in MPE and non-specifically triggers NF-κB activation in any tumor type irrespective of its KRAS status and MPE competence, suggesting...
Fig. 5 IL-1β-induced NF-κB signaling of KRAS-mutant cells is IKKα dependent. Five different C57BL/6 mouse tumor cell lines with (LLC, MC38, AE17) or without (B16F10, PANO2) Kras mutations were stably transfected with pGL-NFκB reporter and any of the following: control shRNA (shC) or shRNA targeting IKKα (shChuk), IKKβ (shIkbkb), IKKe (shIkbke), or TBK1 (shTbk1) transcripts; control plasmid (pC); or overexpression vectors encoding IKKα (pChuk), IKKβ (pIkbkb), IKKe (pIkbke), or TBK1 (pTbk1) transcripts. a Immunoblot of cytoplasmic protein extracts from LLC cells stably expressing shC, shChuk, shIkbkb, and shTbk1, (n = 3 independent experiments). b Bioluminescent quantification of NF-κB reporter activity of pGL cell lines stably expressing shC, shChuk, shIkbkb, shIkbke, and shTbk1, (n = 3 independent experiments). c Bioluminescent quantification of NF-κB reporter activity of pGL cell lines stably expressing pC, pChuk, pIkbkb, pIkbke, and pTbk1, (n = 3 independent experiments). d Bioluminescent detection of NF-κB reporter activity in LLC, MC38, and AE17 cells (Kras^mut^) stably expressing pGL and shC, shChuk, or shIkbkb (n = 3 independent experiments) during 4-h incubation with 1 nM IL-1β. Note IL-1β-induced NF-κB activity of shChuk and shIkbkb cells that is silenced in shIkbke cells. e Unbiased analysis identified cancer-elaborated CXCL1/PPBP, potent myeloid cell chemoattractants that drive inflammation and metastasis via CXCR1/CXCR2 on host cells, as the transcriptional targets of IL-1β-stimulated KRAS-IKKα addiction. Indeed, Cxcl1 expression was downregulated by Kras or Chuk silencing and IL-1β induced Cxcl1 expression by two different cancer cell lines and Ppbp by LLC cells (MC38 cells do not express Ppbp). Our experiments using CXCR1- and CXCR2-deficient mice support that pleural tumor cell-secreted CXCL1/PPBP is cardinal for MPE and are in line with a previous study demonstrating increased production of CXCL1 by tumor cells during human MPE that mobilizes regulatory T cells. In addition to the mechanistic insights into host environment-fostered co-option of IKKα activity by mutant KRAS, our data bear therapeutic implications for KRAS inhibitors. KRAS is notoriously undruggable, and proteasome and IKKβ inhibitors have yielded suboptimal results in mice and men with cancer. Focusing on lung cancer, a tumor with high KRAS mutation frequency, bortezomib has shown poor efficacy in clinical trials. In animal models of lung cancer, bortezomib and IKKβ inhibitors caused resistance or paradoxical tumor promotion via development of secondary mutations, NF-κB inhibition in myeloid cells, or enhanced IL-1β secretion by tumor-associated
neutrophils through an unknown mechanism\textsuperscript{15,16,33}. We show how KRAS-mutant cancer cells utilize myeloid-IL-1β in order to activate IKKα and alternative NF-κB signaling and to by-pass IKKβ canonical NF-κB dependence. We provide proof-of-concept data that KRAS-mutant cancer cells can be targeted by combined inhibition of KRAS and HSP90/IKKβ signaling, a strategy that blocks IL-1β-inducible oncogenic NF-κB activation and in vivo MPE development, a cancer phenotype that requires mutant KRAS-potentiated, IL-1β-induced IKKα activity. These results challenge the prevailing focus on IKKβ for the development of anti-tumor drugs and establish IL-1β and IKKα as important targets in KRAS-mutant tumors.

In conclusion, we show that KRAS-mutant cancer cells use host IL-1β to sustain IKKα-mediated non-canonical NF-κB activity responsible for MPE development and primary drug resistance. We identify CXCL1/PPBP as effectors of MPE downstream of KRAS/IKKα addiction. Finally, we provide proof-of-concept data suggesting that KRAS/IKKα addiction may occur in human cancers and may be targeted by combined KRAS/IKKα inhibition.

**Methods**

**Study approval.** All mouse experiments were prospectively approved by the Veterinary Administration of Western Greece (approval # 276134/14873/2) and were conducted according to Directive 2010/63/EU (http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex%3A32010L0063).

**Reagents.** D-Luciferin was from Gold Biotechnology (St. Louis, MO); lentiviral shRNA and puromycin from Santa Cruz (Dallas, TX); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and Hoechst 33528 from Sigma-Aldrich (St. Louis, MO); mouse gene ST2.0 microarrays and relevant reagents from Affymetrix (Santa Clara, CA); recombinant cytokines and growth factors from Immunotools (Friesoythe, Germany); NF-κB-binding ELISA from Active Motif (La Hulpe, Belgium); hortizomib, IMD-0354, 17-DMAG, and deltarasin from Selleckchem (Houston, TX); G418 from Applichem (Darmstadt, Germany); IL-1β and CXCL1 ELISA from Peprotech (London, UK); and primers from VBC Biotech (Vienna, Austria). Primers, antibodies, and lentiviral shRNA pools are listed in Supplementary Tables 6–8.

**Cells.** LLC, B16F10, PANO2, and A549 cells were from the National Cancer Institute Tumor Repository (Frederick, MD); MC38 cells were a gift from Dr. Barbara Fingleton (Vanderbilt University, Nashville, TN)\textsuperscript{34,35}, and AE17 cells from Dr. YC Gary Lee (University of Western Australia, Perth, Australia)\textsuperscript{11,25}. All cell lines were cultured at 37°C in 5% CO\textsubscript{2}–95% air using Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, 2 mM L-glutamine, 1 mM pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cell lines were tested according to Directive 2010/63/EU (http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex%3A32010L0063).

**Fig. 6.** IKKα is required for mutant KRAS-induced malignant pleural effusion. a–c Malignant pleural disease induced by LLC cells (Kras\textsuperscript{G12C}) stably expressing pNGL NF-κB reporter and control shRNA (shC) or shRNA targeting IKKα (shChuk), IKKβ (shIkbb), IKKε (shIkbe), or TBK1 (shTbk1) transcripts (n is given in Table 3). Shown are Kaplan–Meier survival plot (a), data summaries of effusion volume and pleural fluid cells (b), and representative images of effusions (dashed lines) and pleural tumors (t) as well as bioluminescent images at day 13 after pleural injections of the indicated tumor cells (c). d–f Malignant pleural disease induced by MC38 cells (Kras\textsuperscript{G12C}) stably expressing pNGL NF-κB reporter and shC, shChuk, or shIkbb (n is given in Supplementary Table 3). Shown are immunoblots of cytoplasmic extracts (d), data summaries of effusion volume and pleural fluid cells (e), and representative images of effusions (dashed lines) and pleural tumors (t) as well as representative bioluminescent images at day 13 after pleural injections of the indicated tumor cells (f). g–i Malignant pleural disease induced by PANO2 cells (Kras\textsuperscript{WT}) stably expressing pNGL NF-κB reporter and control plasmid (shC) or plasmid encoding IKKα (pChuk), IKKβ (pIkbb), or mutant (pKras\textsuperscript{G12C}) transcripts (n is given in Supplementary Table 3). Shown are Kaplan–Meier survival plot (g), data summary of effusion volume (h), and representative images of effusions (dashed lines) and pleural tumors (t), hearts (h), and lungs (l), as well as representative bioluminescent images at day 14 after pleural injections of the indicated tumor cells (i). Data are presented as mean ± s.d., P, probability of no difference between cell lines by overall log-rank test (a, g) or one-way ANOVA (b, e, h), ns, single, double, and triple asterisks (*, **, and ***): P > 0.05, P < 0.05, P < 0.01, and P < 0.001, respectively, for the indicated comparisons with control cells by Bonferroni post-tests. Scale bars, 0.5 cm.
Mouse models and drug treatments. C57BL/6 (#000664), B6.129P2-Ccex1^{G12C} (Ccex1^{G12C}/Ccex1^{G12C}), B6.129 S1(C)-Ccex1^{G12C}/Ccex1^{G12C}(Tnf^{−/−}−/−/−), B6.129S1-Tnf^{−/−}Il1b^{+/−} (Tnf^{−/−}Il1b^{+/−}/Il1b^{+/−}/Il1b^{+/−}), B6.129S2(C)-Ccex1^{G12C}/Ccex1^{G12C}(Ikbke^{−/−}−/−/−), and MGL\#6129S2(C)-Ccex1^{G12C}/Ccex1^{G12C}(CAG.LUC\#6129S2(C)-Ccex1^{G12C}/Ccex1^{G12C}) have been described elsewhere.\(^2\)\(^3\)\(^4\)\(^5\)\(^6\)\(^7\)\(^8\)\(^9\)\(^10\)\(^11\)\(^12\)\(^13\)\(^14\)\(^15\)\(^16\)\(^17\)\(^18\)\(^19\)\(^20\)\(^21\)\(^22\)\(^23\)\(^24\)\(^25\)\(^26\)\(^27\)\(^28\)\(^29\)\(^30\)\(^31\)\(^32\)\(^33\)\(^34\)\(^35\)\(^36\)\(^37\)\(^38\)\(^39\)\(^40\)\(^41\)\(^42\)\(^43\)\(^44\)\(^45\)\(^46\)\(^47\)\(^48\)\(^49\)\(^50\)\(^51\)\(^52\)\(^53\)\(^54\)\(^55\)\(^56\)\(^57\)\(^58\)\(^59\). Lentiviral shRNA pools (Santa Cruz) are described in Supplementary Table 8. A pMIGRI-based (#74409) have been described elsewhere.\(^42\)\(^55\)\(^57\)\(^58\) Drug treatments were initiated 5 days post-tumor cells and consisted of daily intraperitoneal injections of 100 μL PBS containing no drug, deltarasin\(^35\), 17-DMAG\(^28\), or both at 15 mg/kg.

Constructs. pNGL, pB8DsN, and pCAG.LUC (#74409) have been described elsewhere.\(^42\)\(^55\)\(^57\)\(^58\) Lentiviral shRNA pools (Santa Cruz) are described in Supplementary Table 8. A pMIGRI-based (#74409) bicistronic retroviel expression vector was generated by replacing eGFP sequences with puromycin resistance gene (#58250). Ras\(^{H12C}\), Chuk, Ilkbb, Hifense, and Tbk1 cDNAs were cloned via reverse transcriptase-PCR (RT-PCR) from LLC or MC38 RNA using specific primers.

Fig. 7 Myeloid cell-derived IL-1β drives mutant KRAS-1Kαx addiction in malignant pleural effusion. a Malignant pleural disease induced by LLC cells (Kras\(^{G12C}\)) stably expressing pNGL NF-κB reporter plasmid in wild-type C57BL/6 mice (black) and TNF (blue) and IL-1β (red)-deficient mice (Tnf^{−/−}−/− and Il1b^{−/−}−/−, respectively; both C57BL/6 background; n is given in Supplementary Table 3). Shown are data summaries of effusion volume, pleural fluid cells, and NF-κB-dependent thoracic bioluminescent signal. b–d Malignant pleural disease induced by MC38 cells (Kras\(^{G12C}\)) stably expressing pNGL NF-κB reporter plasmid in wild-type C57BL/6 (black), Tnf^{−/−}−/− (blue), and Il1b^{−/−}−/− (red) mice (all C57BL/6 background; n is given in Supplementary Table 3). Shown are data summaries of effusion volume, pleural fluid cells, and NF-κB-dependent thoracic bioluminescent signal (b), representative images of effusions (dashed lines), pleural tumors (t), and lungs (l) (c), as well as representative bioluminescent images at day 13 after pleural injections of the indicated tumor cells (d). e, f Malignant pleural disease induced by LLC cells in Il1b^{−/−}−/− mice (C57BL/6 background; n is given in Supplementary Table 3) that received total body irradiation (1100 Rad), same-day bone marrow transplants (10 million cells) from C57BL/6 (black), Tnf^{−/−}−/− (blue), or Il1b^{−/−}−/− (red) donors, and pleural tumors after 1 month. Shown are data summaries of effusion volume and pleural fluid cells (e) and representative images of effusions (dashed lines), pleural tumors (t), lungs (l), and hearts (h) (f). g IL-1β protein secretion by C57BL/6 mouse bone marrow-isolated myeloid cells 24 h after treatment with LLC supernatants; undifferentiated cells (day 0), neutrophils (day 2 after addition of 20 ng/ml G-CSF), and macrophages (day 6 after addition of 20 ng/ml M-CSF; n = 3 independent experiments). Data are presented as mean ± s.d. P, probability of no difference by one-way ANOVA. ns, single, double, and triple asterisks (*, **, and ***): P > 0.05, P < 0.05, P < 0.01, and P < 0.001, respectively, for the indicated comparisons by Bonferroni post-tests. Scale bars, 1 cm (c, f) and 100 μM (g).
Fig. 8 CXCL1/PPBP are the downstream effectors of KRAS/IL-1β/IKKα signaling in malignant pleural effusion. a-c LLC and MC38 cells were stably transfected with shC or shKras or shChuk or were stimulated with 1 nM IL-1β for 4 h, and total cellular RNA was examined by Affymetrix mouse gene ST2.0 microarrays. a Venn diagram of analytic strategy employed: transcripts altered >1.3-fold in one direction by shKras and shChuk and in the other by IL-1β were filtered for each cell line and are given in Supplementary Tables S1–S2. These gene sets, coined KRAS/IL-1β/IKKα signatures, were crossexamined and only Cxcl1 was common to both. b Unsupervised hierarchical clustering of LLC cell results by the 29-gene KRAS/IL-1β/IKKα signature accurately clustered three control samples together, shKras and shChuk samples together, and IL-1β-stimulated cells apart. c Cxcl1 mRNA normalization levels of microarray (n = 2 independent experiments). d Cxcl1 mRNA expression by qPCR relative to Gusb (n = 3 independent experiments). e CXCL1 protein secretion by LLC cells stably expressing shC, shChuk, or shKras after 24 h of stimulation with PBS or 1 nM TNF or IL-1β (n = 3 independent experiments). f Chromatin immunoprecipitation (ChIP) was performed in PBS- or IL-1β-treated LLC cells, followed by immunoprecipitation with the indicated antibodies. The immunoprecipitates were then detected by qPCR. Data are shown as fold enrichment of Cxcl1 or Gusb promoter in each antibody immunoprecipitate over control IgG immunoprecipitate. g Malignant pleural disease induced by LLC cells in C57BL/6, Cxcr1−/−, and Cxcr2−/− mice (n is given in Supplementary Table S3). Shown are data summaries of effusion volume and pleural fluid cells, as well as representative images of effusions (dashed lines), pleural tumors (t), lungs (l), and hearts (h). h Data summaries of C57BL/6, Cxcr1−/−, Cxcr2−/− and Cxcr2−/− pleural neutrophils and monocytes, accompanied by microphotographs. Data are presented as mean ± s.d. P, probability of no difference by two-way (c–e) or one-way (f–h) ANOVA. ns, single, double, and triple asterisks (*** and ****): P > 0.05, P < 0.05, P < 0.01, and P < 0.001, respectively, for comparison with PBS (e) or indicated (g, h) by Bonferroni post-tests. Scale bars 1 cm (g) and 200 μM (h).
Fig. 9 Combined targeting of mutant KRAS and I KKα abolishes IL-1β-induced NF-κB activation and malignant pleural effusion development. a, b Bioluminescent detection of NF-κB reporter activity in LLC (a; C57BL/6 Lewis lung carcinoma, KrasG12D) and A549 (b; human lung adenocarcinoma, KrasG12S) cells stably expressing pNGL under PBS or 1 nM TNF-α or IL-1β-stimulated conditions (4 h), with or without pretreatment with 1 μM deltarasin, IMD-0354, or 17-DMAG alone or in combination (n = 3 independent experiments). Note four-fold induction of NF-κB reporter activity by both TNF-α and IL-1β. Note also inability of any treatment to block TNF-induced NF-κB activation and of any standalone treatment except 17-DMAG to inhibit IL-1β-induced NF-κB activation. Finally, note complete abrogation of IL-1β-induced NF-κB activation in both cell lines by deltarasin/17-DMAG combination. Data are presented as mean ± s.d., probability of no difference by one-way ANOVA (PBS group excluded). Double and triple asterisks (** and ***); P < 0.01 and P < 0.001, respectively, for comparison with PBS by Student’s t-tests. Single and double section symbols (§ and §§); P < 0.05 and P < 0.01, respectively, for comparison with TNF-α or IL-1β by Bonferroni post-tests. c Malignant pleural disease induced by LLC cells in wild-type C57BL/6 mice treated with deltarasin and 17-DMAG. Mice received pleural LLC cells, were allowed 5 days for pleural tumor development, and were randomized to daily intraperitoneal treatments with saline (100 μL), deltarasin, 17-DMAG, or both (both at 15 mg/kg in 100 μL saline; n is given in Supplementary Table 3). Shown are data summaries of effusion volume and pleural fluid cells and Kaplan–Meier survival plots, as well as representative images of effusions (dashed lines), pleural tumors (t), lungs (l), and hearts (h). Data are presented as mean ± s.d., probability of no difference by one-way ANOVA or log-rank test. ns, single, double, and triple (**, ***, and ****); P > 0.05, P < 0.05, P < 0.01, and P < 0.001, respectively, for the indicated comparisons by Bonferroni post-tests. Scale bars, 1 cm.

(Supplementary Table 6) and were subcloned into pcGFP-C1 (Takara, Mountain View, CA). eGFP, eGFP.KrasG12D, eGFP.KrasG12C, eGFP.KrasG12C, eGFP.KrasG12C; eGFP.KrasG12C, eGFP.KrasG12C; eGFP.KrasG12C, eGFP.KrasG12C, and eGFP.TKγ dDNAs were subcloned into the new retrovector expression vector (#58249, #64372.87035, #58251, #87444, and #87443, respectively). Retrovector particles were obtained by co-transfecting HEK293T cells with retrovector vectors, pMD2.G (#12259), and pCMV-Gag-Pol (Cell Biolabs, San Diego, CA) at 1:5:1 stoichiometry using CaCl2/BES. After 2 days, culture media were collected and applied to cancer cells. After 48 h, media were replaced by selection medium containing 2–10 μg/mL puromycin. Stable clones were selected and subcultured.11 For stable plasmid/shRNA transfection, 105 tumor cells in six-well culture vessels were transfected with 5 μg DNA using Xfect (Takara), and clones were selected by G418 (400–800 μg/mL) or puromycin (2–10 μg/mL).

Cellular assays. In vitro cancer cell proliferation was determined using MTT assay. Nuclear extracts were assayed for RelA, RelB, c-Rel, P50, and P52 DNA-binding activity using a commercially available ELISA kit (Transam, Active Motif, Belgium). All cellular experiments were independently repeated at least thrice.

Bioluminescence imaging. Living cells and mice were imaged 0, 4, 8, 24, and 48 h after cellular treatments and 0 h, 4 h, and 12–14 days after pleural delivery of pNGL-expressing cells on a Xenogen Lumina II (Perkin-Elmer, Waltharm, MA) after addition of 300 μg/mL D-luciferin to culture media or isoflurane anesthesia and delivery of 1 mg intravenous D-luciferin to the retro-orbital veins.11,13,14,35 Data were analyzed using Living Image v.4.2 (Perkin-Elmer).

qPCR and microarray. RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) and RNAeasy (Qiagen, Hilden, Germany) was reverse transcribed using SuperScript III (Invitrogen), and RT-PCR or qPCR was performed using SYBR Green Master Mix in a StepOnePlus (Applied Biosystems, Carlsbad, CA) and specific primers (Supplementary Table 6). Ct values from triplicate qPCR reactions were analyzed by the 2ΔΔCt method36 relative to Gusb mRNA levels. For microarray, RNA was extracted from triplicate cultures of 105 cells. Five micrograms pooled total RNA were quality tested on an ABI 2000 (Agilent Technologies, Sta. Clara, CA), labeled, and hybridized to GeneChip Mouse Gene 2.0 ST arrays (Affymetrix, St. Clara, CA). For analysis of differential gene expression (DGE) and unsupervised hierarchical clustering, Affymetrix Expression and Transcriptome Analysis Consoules were used.

Chromatin immunoprecipitation. LLC cells were treated with PBS or 1 nM IL-1β, and 30 min later, cells were fixed sequentially with 2 mM dN(N-succinimidyl) glutarate (Sigma) and 1% formaldehyde (Sigma) and quenched with 0.125 M glycine, followed by lysis with 1% sodium dodecyl sulfate (SDS), 10 mM EDTA,
and 50 mM Tris pH 8. Sonication was performed in a Bioruptor (Diagenode) for 40 cycles (30 s on/off) power settings high), using 3 x 10^6 cells; 20 μg of chromatin was precipitated with 5 μg of RelA, RelB, IKKα, or IKKβ antibody or a mouse control immunoglobulin G (IgG). Immunoprecipitates were retrieved with 50 μl of magnetic Dynabeads conjugated to protein G (Invitrogen) and subjected to quantitative real-time PCR (Applied Biosystems StepOne), using the Kapa SYBR Fast qPCR Kit (Kapa Biosystems, KK4805) for amplification of the Cxcl1 promoter or Gusb as control. The sequences of the primers used for Cxcl1 promoter are: 5′-CAGCAGGGTAGGGATGC, 3′-CATTTTTAAAGTGCACCC, 5′-TTACTTTTAAGACGCTGATCACC, 3′-ACAATGGATGACACATGTC.

BM cell derivation and transfer. For adoptive BM replacement, Ilβ−/− mice (C57BL/6 background) received 10 million BM cells flushed from the femurs and tibias of C57BL/6, Tnf−/−, or Ilβ−/− donors (C57BL/6 background) intravenously after total-body irradiation (1100 Rad)12,25,43,45. One mouse in each experiment was not engrafted (sentinel) and was observed till moribund between days 5 and 15 post-irradiation. The mice were left to be engrafted for 1 month, when full BM reconstitution is complete, before experimental induction of pleural carcinomatosis via intrapleural injection of LLC cells. For BM cell retrieval, BM cells were flushed from C57BL/6 femurs and tibias using full DMEM and were simply cultured in full culture media (the same used for cancer cell line cultures), supplemented with 20 ng/ml M-CSF or G-CSF in order for cells to differentiate to monocytes or neutrophils, respectively. Supernatants and cyto centrifuged specimens were obtained at day 0 for undifferentiated cells, day 2 for neutrophils, and at day 6 for monocytes/macrophages.

Immunoblotting. Nuclear and cytoplasmic extracts were prepared using the NE-PER Extraction Kit (Thermo, Waltham, MA), separated by 12% SDS polyacrylamide gel electrophoresis, and electrophblotted to polyvinylidene difluoride membranes (Merck Millipore, Darmstadt, Germany). Membranes were probed with specific antibodies (Supplementary Table 7) and were visualized by film exposure after incubation with enhanced chemiluminesence substrate (Merck Millipore, Darmstadt, Germany).

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared using the NE-PER Extraction Kit. Proteins (10 μg) were incubated with NF-κB biotin-labeled probe using a commercially available non-radioactive EMSA Kit (Signosis Inc., Santa Clara, USA). DNA–protein complexes were electrophoresed in a preinsrned 6.5% polyacrylamide gel, transferred to a positively charged nylon membrane, and were visualized by film exposure after incubation with enhanced chemiluminesence substrate. For gel shift reactions, proteins were incubated with the specific antibody for 1 h at 4 °C before probe incubation. The antibodies used for observing the supershifted bands were RelA and RelB. IgG antibody served as negative control for super-shift assays.

Immunofluorescence. For immunofluorescence, cells were fixed in 4% paraformaldehyde overnight at 4 °C and were labeled with the indicated primary antibodies (Supplementary Table 7) followed by incubation with fluorescent secondary antibodies (Invitrogen, Waltham, MA; Supplementary Table 7). Cells were then counterstained with Hoechst 33258 (Sigma-Aldrich, St. Louis, MO) and mounted with Mowiol 4-88 (Calbiochem, Gibbstown, NJ). For isotype control, the primary antibody was omitted. Fluorescent microscopy was carried out on an AxioObserver.D1 inverted microscope (Zeiss, Jena, Germany) connected to an AxioCam MRc 5 s camera (Zeiss), and digital images were processed with the Fiji academic imaging freeware46.

Statistics. Sample size was calculated using G–power (http://www.gpower.hhu.de/)44 assuming α=0.05, β=0.05, and d=1.5, tailored to detect 30% differences between means with 20–30% SD spans, yielding n=13/group. Animals were allocated to groups by alternation (treatments or cells) or case-control-wise (transgenic animals). Data acquisition was blinded on samples coded by non-blinded investigators. No data were excluded. All data were examined for normality by Kolmogorov–Smirnov test and were normally distributed. Values are given as mean±SD. Sample size (n) refers to biological replicates. Differences in means were examined by t-test and one-way or two-way ANOVA with Bonferroni post-tests, in frequencies by Fischer’s exact or χ² tests, and in Kaplan–Meier survival estimates by log–rank test, as appropriate. P-values are two-tailed. P<0.05 was considered significant. Analyses and plots were done on Prism v5.0 (GraphPad, La Jolla, CA).

Data availability. All new plasmids have been deposited at the Addgene plasmid repository (https://www.addgene.org/search/advanced/?q=statophoulois) and their IDs (#) are given in the text. Microarray data are available at the GEO (http://www.ncbi.nlm.nih.gov/geo; Accession IDs: GSE10789 and GSE93370). The authors declare that all the other data supporting the findings of this study are available.
radioreistance in low-dose irradiated mouse skin epithelial cells. Cancer Res. 67, 3220–3228 (2007).
28. Rastelli, G., Tian, Z. Q., Wang, Z., Mylés, D. & Liu, Y. Structure-based design of 7-carbamate analogs of geldanamycin. Bioorg. Med. Chem. Lett. 15, 5016–5021 (2005).
29. Hertlein, E. et al. 17-DMAG targets the nuclear factor-kappaB family of proteins to induce apoptosis in chronic lymphocytic leukemia: clinical implications of HSP90 inhibition. Blood 116, 45–53 (2010).
30. Pahl, H. L. Activators and target genes of Rel/NF-kappaB transcription factors. Oncogene 18, 6853–6866 (1999).
31. Horai, R. et al. Production of mice deficient in genes for interleukin (IL)-1, IL-1beta, IL-1alpha and IL-1 receptor antagonist shows that IL-1beta is crucial in turpentine-induced fever development and glucocorticoid secretion. J. Exp. Med. 167, 1463–1475 (1998).
32. Keffer, J. et al. Transgenic mice expressing human tumour necrosis factor: a predictive genetic model of arthritis. EMBO J. 10, 4025–4031 (1991).
33. Stathopoulos, G. T. et al. Use of bioluminescent imaging to investigate the role of nuclear factor-kappaB in experimental non-small cell lung cancer metastasis. Clin. Exp. Metastasis 25, 43–51 (2008).
34. Marazioti, A. et al. Beneficial impact of CCL2 and CCL12 neutralization on experimental malignant pleural effusion. PLoS ONE 8, e71207 (2013).
35. Giannou, A. D. et al. Mast cells mediate malignant pleural effusion formation. J. Clin. Invest. 125, 2317–2334 (2015).
36. Sakai, N. et al. CXCR3 depletion does not alter liver regeneration after partial hepatectomy in mice. Transpl. Proc. 43, 1967–1970 (2011).
37. Cacalano, G. et al. Neutrophil and B cell expansion in mice that lack the murine IL-8 receptor homolog. Science 265, 682–684 (1994).
38. Zlotnik, A. & Yoshie, O. The chemokine superfamily revisited. Immunity 66, 471–486 (2012).
39. Zimmermann, G. et al. Small molecule inhibition of the KRAS-PDE5 interaction impairs oncocogenic KRAS signalling. Nature 497, 638–642 (2013).
40. Ikedoh, O. N. et al. Mutation analysis of 24 known cancer genes in the NCI-60 cell line set. Mol. Cancer Ther. 5, 2606–2612 (2006).
41. Schubbert, S., Shannon, K. & Bollag, G. Hyperactive Ras in developmental disorders and cancer. Nat. Rev. Cancer 7, 295–308 (2007).
42. Stephen, A. G., Espósito, D., Bagni, R. K. & McCormick, F. DraggIng rAs back in the ring. Cancer Cell 25, 272–281 (2014).
43. Starczynowski, D. T. et al. TRAF6 is an amplified oncocene bridging the RAS and NF-kB pathways in human lung cancer. J. Clin. Invest. 121, 4095–4105 (2011).
44. Kampf, A. & Bar-Sagi, D. Ras-induced interleukin-8 expression plays a critical role in tumor growth and angiogenesis. Cancer Cell 6, 447–458 (2004).
45. Ji, H. et al. K-ras activation generates an inflammatory response in tumors. Nature 455, 2105–2112 (2009).
46. Ortiz, E. et al. Differential usage of NF-kB activating signals by IL-1β and TNF-α in pancreatic beta cells. FEBS Lett. 586, 984–989 (2012).
47. Anest, V. et al. A nucleosomal function for IKKa/b kinase-alpha in NF-kappaB-dependent gene expression. Nature 423, 659–663 (2003).
48. Yamamoto, Y., Verma, U. N., Prajapati, S., Kwak, Y. T. & Gaynor, R. B. Histone H3 phosphorylation by IKK-α is critical for cytokine-induced gene expression. Nature 435, 655–659 (2003).
49. Luo, J. L. et al. Nuclear cytokine-activated IKKα/β controls prostate cancer metastasis by repressing Maspin. Nature 446, 690–694 (2007).
50. Giopanou, I. et al. Comprehensive evaluation of nuclear factor-kappaB-dependent gene expression in pancreatic cancer. Cancer Res. 66, 6720–6729 (2006).
51. Shalapour, S. & Karin, M. Immunity, inflammation, and cancer: an eternal struggle between good and evil. J. Clin. Invest. 125, 3347–3355 (2015).
52. Voronov, E. et al. IL-1 is required for tumor invasiveness and angiogenesis. Proc. Natl. Acad. Sci. USA 100, 2645–2650 (2003).
53. McLeod, A. G. et al. Neutrophil-derived IL-1β impairs the efficacy of NF-κB inhibitors against lung cancer. Cell. Rep. 16, 120–132 (2016).
54. Zhang, Z. et al. IL1 receptor antagonist inhibits pancreatic cancer growth by abrogating NF-κB activation. Clin. Cancer Res. 22, 1432–1444 (2016).
55. Solt, L. A., Madge, L. A., Orange, J. S. & May, M. J. Interleukin-1-induced NF-κB activation is NEMO-dependent but does not require IKKbeta. J. Biol. Chem. 282, 8724–8733 (2007).
56. Dong, G., Chen, Z., Kato, T. & Van Waes, C. The host environment promotes the constitutive activation of nuclear factor-kappaB and proinflammatory cytokine expression during metastatic tumor progression of murine squamous cell carcinoma. Cancer Res. 59, 3495–3504 (1999).
57. Ginsteler, C. et al. CXCR1 blockade selectively targets human breast cancer stem cells in vitro and xenografts. J. Clin. Invest. 120, 485–497 (2010).
58. Jamieson, T. et al. Inhibition of CXCR2 profoundly suppresses inflammation-driven and spontaneous tumorigenesis. J. Clin. Invest. 122, 3127–3144 (2012).
59. Iw, M. et al. miR141-CXCL1-CXCR2 signaling-induced Treg recruitment regulates metastasis and survival of non-small cell lung cancer. Mol. Cancer Ther. 13, 3152–3162 (2014).
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Author contributions
A.M. designed and performed NF-κB ELISA, immunoblotting, EMSA, drug testing, transfections, reporter assays, and most in vivo experiments, quantified and analyzed the data, provided critical intellectual input, and wrote the paper draft; I.L. isolated BMMCs; M.V. designed and performed reporter assays and in vivo experiments including bioluminescent imaging, quantified and analyzed the data, and provided critical intellectual input; H.A. and A.D.G. performed pNGL induction studies, mutant KRAS and IKK silencing/overexpression and relevant in vitro assays, and drug testing; A.K. performed CHIP experiments; I.G. did qPCR experiments; G.A.G. and A.C.K. performed in vivo deltarsalin/17-DMAG treatment experiments; M.I. did pleural fluid cell counts; N.I.K. analyzed microarray; T.A. cloned eukaryotic expression vectors; C.J.-P. performed NF-κB ELISA; Y.I. provided analytical tools and critical intellectual input; D.K. performed total body irradiation; T.S.B. provided pNGL and critical intellectual input; S.T. provided analytical tools and critical intellectual input; M.S. performed immunofluorescence; G.T. S. conceived the idea and supervised the study, designed experiments, analyzed the data, wrote the paper, and is the guarantor of the study’s integrity. All authors reviewed, edited, and concur with the submitted manuscript.

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