Inhibition of KRAS-Driven Tumorigenicity by Interruption of an Autocrine Cytokine Circuit

INTRODUCTION

Oncogenic mutations in KRAS and receptor tyrosine kinases (RTK) drive tumor growth by engaging multiple downstream mitogenic pathways, including RAF–MAPK and PI3K–AKT (1). KRAS also activates RAL–GEF (2, 3) and downstream mitogenic pathways, including RAF–MAPK and receptor tyrosine kinases (RTK) drive tumor growth by engaging multiple cytoplasmatic cancer cell proliferation and survival (17). The TBK1 homolog inhibitor of IkB kinase (IKK)–ε (IKK ε; encoded by IkBKE) also promotes NF-kB activation downstream of KRAS (23), substitutes for AKT to drive cell transformation (24), and is induced by RAS-associated cytokines such as IL-1 and IL-6 (25). Thus, TBK1/IKKε signaling is coopted by oncogenic KRAS and facilitates tumorogenesis.

Following viral infection, TBK1 and IKKε amplify IFN-β production via an autocrine loop (26). Here, we identify a similar circuit involving CCL5 and IL-6 required for KRAS-driven lung tumorogenesis and potently suppressed by CYT387, a novel TBK1/IKKε and Janus-activated kinase (JAK) inhibitor.

RESULTS

TBK1-Regulated Cell Survival Involves Autocrine CCL5 and IL-6 and STAT3 Signaling

Expression of Tbk1 is required for transformation by oncogenic KRAS (18, 20, 21). Although Tbk1−/− mouse embryonic fibroblasts (MEF) proliferate in standard culture, we noted marked impairment of Tbk1−/− MEF proliferation in a clonogenic assay compared with wild-type (WT) littermate control.

ABSTRACT

Although the roles of mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) signaling in KRAS-driven tumorigenesis are well established, KRAS activates additional downstream pathways for tumor maintenance, the inhibition of which are likely to be necessary for effective KRAS-directed therapy. Here, we show that the IkB kinase (IKK)-related kinases Tank-binding kinase-1 (TBK1) and IKKε promote KRAS-driven tumorigenesis by regulating autocrine CCL5 and interleukin (IL)-6 and identity CYT387 as a potent JAK/TBK1/IKKε inhibitor. CYT387 treatment ablates RAS-associated cytokine signaling and impairs Kras-driven murine lung cancer growth. Combined CYT387 treatment and MAPK pathway inhibition induces regression of aggressive murine lung adenocarcinomas driven by Kras mutation and p53 loss. These observations reveal that TBK1/IKKε promote tumor survival by activating CCL5 and IL-6 and identify concurrent inhibition of TBK1/IKKε, Janus-activated kinase (JAK), and MEK signaling as an effective approach to inhibit the actions of oncogenic KRAS.

SIGNIFICANCE: In addition to activating MAPK and PI3K, oncogenic KRAS engages cytokine signaling to promote tumorigenesis. CYT387, originally described as a selective JAK inhibitor, is also a potent TBK1/IKKε inhibitor that uniquely disrupts a cytokine circuit involving CCL5, IL-6, and STAT3. The efficacy of CYT387-based treatment in murine Kras-driven lung cancer models uncovers a novel therapeutic approach for these refractory tumors with immediate translational implications. Cancer Discov; 4(4): 452–65. © 2014 AACR.
MEFs (Fig. 1A). To assess the role of cell contact versus a secreted factor, we plated Tbk1−/− MEFs in the clonogenic assay with conditioned medium from WT or Tbk1−/− MEFs propagated at high density (Fig. 1B). Conditioned medium from WT but not Tbk1−/− MEFs rescued colony formation, revealing that Tbk1 regulates secreted factors that promote cell proliferation and may contribute to KRAS-driven tumorigenesis.

Because TBK1/IKKε regulates cytokine production, we assessed conditioned medium from WT or Tbk1−/− MEFs using a cytokine antibody array. Tbk1−/− MEF conditioned medium lacked CCL5 and exhibited decreased CXCL10 levels compared with WT MEF conditioned medium (Fig. 1C and Supplementary Fig. 1A). CCL5 and CXCL10 were also absent in Tbk1−/− MEF clonogenic culture media (Supplementary Fig. 1B). Because Tbk1 also regulates IL-6 (27), we measured CCL5, CXCL10, and IL6 mRNA levels and observed reduced expression of each cytokine/chemokine in Tbk1−/− MEFs (Fig. 1D), whereas others such as Cxcl1 were increased (Supplementary Fig. S1C and S1D). Reintroduction of WT but not kinase-dead (KD) TBK1 restored CCL5 production by Tbk1−/− MEFs, revealing kinase-dependent regulation of this chemokine (Fig. 1E).

To examine the contribution of CCL5, CXCL10, and/or IL-6 to Tbk1−/−-regulated survival, we supplemented media with each factor and measured Tbk1−/− MEF colony formation. CCL5 (10 ng/mL) rescued Tbk1−/− MEFs colonies comparably with WT MEF conditioned medium, whereas IL-6 had a modest effect and CXCL10 failed to rescue colony formation (Fig. 1F and Supplementary Fig. S1E). Adding IL-6 or CXCL10 to CCL5 did not increase Tbk1−/− MEF colonies. Thus, autocrine CCL5 and IL-6 signaling promote TBK1-regulated proliferation/survival.

Because CCL5 and IL-6 induce prosurvival JAK–STAT signaling, we next measured Y705 pSTAT3 phosphorylation in
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Cytokine Suppression

**Figure 2.** CYT387 inhibits JAK and TBK1/IKKe signaling. A, in vitro kinase assay with His-TBK1 (4 nmol/L) or GST–IKKe (2 nmol/L). 100 μmol/L IKKe–Tide, 100 μmol/L ATP, and CYT387. Assays performed in duplicate, ADP generated normalized to dimethyl sulfoxide (DMSO) control, mean ± SD shown. B, immunoblot of S418 pCYLD, CYLD, TBK1, IKKe, and β-actin levels in 293T cells 24 hours following transient cotransfection of Myc-tagged CYLD with TBK1-WT, TBK1-KD, IKKe-WT, or IKKe-KD and 4-hour treatment with DMSO or 5 μmol/L MRT67307, CYT387, or ruxolitinib. C, immunoblot of S396 pIRF3, IRF3, S172 pTBK1, TBK1, IKKe, and β-actin levels in macrophages stimulated with lipopolysaccharide (LPS) (100 ng/mL) for 2 hours ± DMSO or inhibitor pretreatment at the indicated concentrations for 1 hour. D, mRNA levels of IFNB1, CCL5, and IL-6 in macrophages stimulated with LPS (100 ng/mL) for 2 hours ± pretreatment with inhibitors for 1 hour. Mean and SEM of triplicate samples shown.

WT or Tbk1+/− MEFs. Tbk1+/− MEFs exhibited low pSTAT3 levels following release from serum starvation (Fig. 1G). Oncogenic KRASG12V expression or stimulation of RAS activity with EGF failed to rescue STAT3 signaling in Tbk1+/− MEFS (Fig. 1H and I). Supplementation of Tbk1+/− MEF media with CCL5 completely restored pSTAT3 activation under basal conditions and following EGF stimulation (Fig. II). Thus, Tbk1-regulated CCL5 promotes both clonogenic proliferation and autocrine STAT3 activation.

**CYT387 Is a Potent TBK1/IKKe Inhibitor**

MRT67307 has been characterized as a potent TBK1 inhibitor (28). We noted that CYT387, a JAK1/2 kinase inhibitor in clinical trials for myelofibrosis, also inhibited TBK1 with low nanomolar potency (29). To explore the TBK1- and IKKe-specific activities of CYT387, we first measured TBK1/IKKe–mediated phosphorylation of a substrate motif peptide (IKKe-Tide) in vitro (30). CYT387 potently inhibited TBK1 (IC50 = 58 nmol/L) and IKKe (IC50 = 42 nmol/L) kinase activity in the presence of 100 μmol/L ATP (Fig. 2A). In contrast, another JAK1/2 inhibitor, ruxolitinib, failed to inhibit TBK1 or IKKe in this assay (IC50 > 1 μmol/L for both; Supplementary Fig. S2A). MRT67307 was comparable with CYT387 in the TBK1 assay (IC50 = 40 nmol/L), but inhibited IKKe less potently (IC50 = 242 nmol/L; Supplementary Fig. S2A). To confirm these observations in intact cells, we examined the effect of inhibitor treatment on TBK1/IKKe S418 CYLD phosphorylation, which mediates IKKe-induced transformation (30). Treatment with CYT387 abrogated TBK1/IKKe–induced CYLD phosphorylation in 293T cells, similar to MRT67307 and in contrast to ruxolitinib (Fig. 2B). These findings established CYT387 as a potent TBK1/IKKe inhibitor.

To determine activity of these inhibitors in a physiologic setting, we next measured IFN-γ-induced JAK activity or lipopolysaccharide (LPS)-induced TBK1/IKKe signaling in murine RAW macrophages. As expected, ruxolitinib treatment...
potently suppressed IFN-γ-induced Y701 pSTAT1, in contrast to MRT67307 (Supplementary Fig. S2B). CYT387 was less potent than ruxolitinib in these assays, but suppressed the STAT1 target gene Gbp1 (IC50 = 587 nmol/L) like ruxolitinib (IC50 = 20 nmol/L) and in contrast to MRT67307 (IC50 > 10 μmol/L; Supplementary Fig. S2C). Similar results were obtained for IFN-γ-induced Cxcl10 mRNA expression (Supplementary Fig. S2D). CYT387 treatment potently inhibited LPS-induced S396 IRF3 phosphorylation at concentrations < 1 μmol/L, similar to MRT67307 and in contrast to ruxolitinib (Fig. 2C). As previously reported, MRT67307 treatment paradoxically induced TBK1 S172 activation loop phosphorylation (28), which was less pronounced following CYT387 treatment in this assay. MRT67307 (IC50 = 228 nmol/L) or CYT387 (IC50 = 201 nmol/L) treatment also suppressed expression of the IRF3 target gene Ifnb1 (Fig. 2D).

MRT67307 or CYT387 further impaired LPS-induced expression of Ccl5 and Cxcl10, whereas ruxolitinib failed to suppress Ifnb1, Ccl5, or Cxcl10 expression (Fig. 2D and Supplementary Fig. S2E). MRT67307 (IC50 = 331 nmol/L) or ruxolitinib (IC50 = 589 nmol/L) each partially suppressed LPS-induced Il6, whereas CYT387 strongly inhibited Il6 mRNA levels (IC50 = 63 nmol/L; Fig. 2D). These findings confirmed CYT387 as a multitargeted JAK and TBK1/IKK inhibitor (Supplementary Fig. S2F) that potently suppresses multiple cytokines, including CCL5 and IL-6.

**CCL5 and IL-6 Promote Human KRAS-Dependent Lung Cancer Transformation**

To determine the role of these cytokines in human KRAS-driven lung cancer, we first measured TBK1 and STAT3 activation in a panel of human NSCLC lines. Because KRAS dependency does not strictly correlate with KRAS mutation status (19, 20, 31), we examined specific cell lines for their dependence on KRAS expression (Supplementary Fig. S3A). Although one group reported that A549 cells are KRAS independent (31), we observed sensitivity of this cell line to KRAS and TBK1 suppression, consistent with other reports (19, 22, 32, 33). The majority of other KRAS-dependent NSCLC cell lines also required TBK1 for survival (Supplementary Fig. S3A). We then compared activation of TBK1 and cytokine signaling in a panel of KRAS-mutant/dependent cell lines (A549, HCC44, H23, H1792, H460, H2009, and H1944 cells) to KRAS-WT/independent cells (Fig. 3A and Supplementary Fig. S3B; ref. 19). We measured TBK1 activity by S172 phosphorylation and STAT3 activity by Y705 phosphorylation and STAT3 mRNA levels respectively. Although phosphorylated (p) TBK1 levels were low at baseline, KRAS-dependent NSCLC cells exhibited higher levels of both pTBK1 and pSTAT3 compared with KRAS-independent cells (Fig. 3A and Supplementary Fig. S3B). Activation of pTBK1 and pSTAT3 was more pronounced in KRAS-dependent NSCLC cells after 72 hours in culture (Fig. 3B). Suppression of KRAS in A549 cells reduced levels of both pTBK1 and pSTAT3 (Fig. 3C), consistent with direct engagement of RALB-TBK1 signaling by oncogenic KRAS (18).

These findings suggested that autocrine cytokine signaling downstream of oncogenic KRAS contributes to the proliferation of KRAS-dependent cells. Indeed, A549 and other KRAS-dependent cell lines secreted CCL5 and IL-6 into the media over time in culture, in contrast to KRAS-independent cells (Fig. 3D and E). Extracellular signals in the tumor microenvironment, such as IL-1 and EGF, further enhanced TBK1 activation as well as CCL5 and IL-6 production in A549 cells (Supplementary Fig. S3C and S3D). To study the functional consequences of this autocrine cytokine production, we assessed CCL5 and IL-6 neutralization in two-dimensional (2D) culture or in a microfluidic three-dimensional (3D) cell culture system in which A549 lung tumor spheroids are cocultured in collagen with human vascular endothelial cells (HUVEC) or growth factors such as EGF (Fig. 3F and Supplementary Fig. S3E; ref. 34). This 3D system more closely recapitulates cancer cell behavior in the extracellular matrix, captures features of the tumor microenvironment, and also enables inhibitor studies. Incubation of A549 cells with CCL5- and IL-6-neutralizing antibodies in 2D culture had a minor effect on proliferation (Supplementary Fig. S3F). In contrast, combined CCL5 and IL-6 blockade completely suppressed A549 cell proliferation in response to EGF in 3D culture, compared with neutralization of either cytokine alone (Fig. 3G). We also observed that KRAS-independent H1437 spheroids failed to disperse and proliferate in response to EGF even at 48 hours (Fig. 3H). Together, these findings demonstrate that TBK1 signaling and CCL5 and IL-6 production not only promote clonogenic proliferation of MEFs, but also KRAS-dependent lung cancer cell proliferation and migration.

**CYT387 Treatment Impairs KRAS-Dependent NSCLC Viability**

We next assessed the capacity of CYT387 to target this signaling pathway and impair the tumorigenic potential of KRAS-dependent lung cancer cells. Treatment of A549 cells with MRT67307 or CYT387 suppressed the clonogenic growth and induced cytotoxicity, in contrast to ruxolitinib (Fig. 4A and Supplementary Fig. S4A). Both MRT67307 and CYT387 preferentially impaired the viability of KRAS-dependent A549 and HCC44 cells (IC50 ~ 3 μmol/L) compared with KRAS-independent H1437 and H1568 cells, whereas ruxolitinib had a negligible effect (Fig. 4B and Supplementary Fig. S4B and S4C).

To extend these findings, we treated a panel of 12 NSCLC cell lines with 18 different kinase inhibitors targeting multiple pathways. Cell lines dependent on KRAS were also assessed on TBK1, with the exception of H1819 cells (19, 20), which instead required IKBKE expression for survival (Supplementary Figs. S3A and S4D). As expected, cell line sensitivity to MRT67307 and the related TBK1 inhibitor BX-795 was correlated, similar to the concordance between the MAPK/ERK kinase (MEK) inhibitors AZD6244 and CI-1040 (Fig. 4C). KRAS-dependent cell sensitivity to these TBK1 inhibitors was also comparable with MEK inhibitors. Cell line sensitivity to CYT387 significantly correlated with MRT67307, but not AZD6244 (Fig. 4C). Indeed, the nearest neighbor analysis showed correlation of CYT387 sensitivity with both BX-795 and MRT67307, despite different off-target effects (Fig. 4D). Similarly, CI-1040 was the nearest neighbor to AZD6244 and the AKT inhibitor MK-2206 was closely related to the phosphoinositide 3-kinase (PI3K) inhibitor GDC0941 (Supplementary Fig. S4E and S4F), confirming the specificity
of this analysis. Together, these findings demonstrated that CYT387 and other TBK1 inhibitors impair KRAS-dependent cell viability in a distinct fashion compared with other targeted inhibitors.

To determine the sensitivity of cells in 3D culture, we next treated A549 spheroids with MRT67307, CYT387, or ruxolitinib. Only CYT387 prevented HUVEC-induced spheroid dispersal at 1 μmol/L concentration (Fig. 4E). Treatment with 1 μmol/L CYT387 also abrogated A549 spheroid proliferation and migration in response to EGF stimulation, in contrast to 1 μmol/L MRT67307 or ruxolitinib, whereas combination of MRT67307 and ruxolitinib inhibited spheroid dispersal (Fig. 4F and Supplementary Fig. S4G). These findings were consistent with the effects of CCL5 and IL-6 neutralization, suggesting a unique potential for CYT387 to disrupt these cytokines in lung cancer cells and suppress KRAS-driven tumorigenicity in vivo.

**CYT387 Suppresses an Autocrine Cytokine Circuit in KRAS-Dependent Lung Cancer Cells**

We noted that IKKε overexpression promoted CCL5 and IL-6 production even more potently than TBK1 (Supplementary Fig. SSA). IL6 mRNA expression also recovered during the culture of Tbk1−/− MEFs, coinciding with inducible expression of IKKε (Supplementary Fig. SSB and SSC). Because STAT3 induces IKKe (25), these findings suggested that disruption of a circuit involving IKKe and JAK signaling was required for effective suppression of IL6. Indeed, treatment of Tbk1−/− MEFs with ruxolitinib disrupted this circuit and sustained inhibition of CCL5 and IL6 expression (Supplementary...
activation in A549 cells and promotes pTBK1 expression in KRAS-dependent lung cancer cells. Treatment with dimethyl sulfoxide (DMSO), 2.5 μmol/L MRT67307, CYT387, or ruxolitinib, inhibited pSTAT3 and induced pTBK1 to a lesser degree compared with 17 other targeted inhibitors at 5 μmol/L. Nearest neighbor analysis by Pearson correlation, *, compared with MRT67307 or ruxolitinib (top), or CYT387/MRT67307 and A549 spheroids cocultured with HUVECs and treated with DMSO, MRT67307, CYT387, or ruxolitinib (1 μmol/L CYT387 for 36 hours. Mean number and SD of dispersed cells per spheroid from triplicate devices shown. # Dispersed cells/spheroid

**Figure 4.** Inhibition of lung cancer cell proliferation and viability by CYT387. A, crystal violet stain of A549 cell clonogenic assay after 10-day treatment with dimethyl sulfoxide (DMSO), 2.5 μmol/L MRT67307, CYT387, or ruxolitinib. B, dose-response to MRT67307 or CYT387 treatment at 72 hours normalized to DMSO control, mean ± SEM of quadruplicate samples shown. Blue, KRAS-mutant/dependent cell lines; red, KRAS-WT/ independent cell lines. C, cell viability of 12 KRAS-dependent (blue) or independent (red) lung cancer lines (purple, KRAS-WT/dependent) treated with 5 μmol/L inhibitors for 72 hours normalized to DMSO control. Potted are relative cell line sensitivities for MRT67307/BX795 and AZD6244/C1-1040 (top), or CYT387/MRT67307 and CYT387/AZD6244 [bottom]. *, *P < 0.05, Pearson correlation. D, normalized relative CYT387 sensitivity in the same NSCLC lines compared with 17 other targeted inhibitors at 5 μmol/L. Nearest neighbor analysis by Pearson correlation, *, *P < 0.05. E, phase-contrast images (×20) of A549 spheroids cocultured with HUVECs and treated with DMSO, MRT67307, CYT387, or ruxolitinib (1 μmol/L) for 18 or 36 hours. Mean number and SD of dispersed cells per spheroid from triplicate devices shown. F, phase-contrast images (×20) of A549 spheroids stimulated with EGF and treated with DMSO or 1 μmol/L CYT387 for 36 hours. Mean number and SD of dispersed cells per spheroid from triplicate devices shown.

In contrast, treatment with ruxolitinib inhibited pSTAT3 but had no effect on pTBK1 levels (Fig. 5A). Uniquely, treatment of A549 cells with CYT387 inhibited pSTAT3 and induced pTBK1 to a lesser degree than MRT673037 (Fig. 5A). These findings were consistent with our observations in macrophages and suggested dual targeting of JAK and TBK1 signaling by CYT387 in KRAS-dependent lung cancer cells.

Because IL-1 signaling potentiated TBK1 and CCL5/IL-6 expression in A549 cells and promotes KRAS-dependent tumorigenicity (7, 8), we focused on the consequences of inhibitor treatment in this setting. Using single sample gene set enrichment analysis (ssGSEA) of KRAS and IL-1β signatures, we confirmed coactivation of oncopgenic KRAS and IL-1 signaling in primary human lung adenocarcinomas (P = 0.02, normalized mutual information statistic; refs. 19, 35) and IKBKE overexpression in these tumors (Fig. 5B). In consonance with this finding, IL-1 also promoted IKBKE mRNA and protein expression in A549 cells (Fig. 5C).

Following short-term stimulation with IL-1 or IL-6 as a control, CYT387 treatment targeted both pTBK1 and pSTAT3, in contrast to MRT67307 or ruxolitinib (Supplementary Fig. S5E). CYT387 and MRT67307 treatment but not ruxolitinib also inhibited basal and IL-1β–induced pCYLD levels, whereas CYT387 uniquely prevented IL-1β–induced IKKα protein and mRNA expression (Fig. 5D and Supplementary Fig. S5F). MRT67307 treatment failed to suppress IL-1β–induced CCL5/IL-6 expression, and ruxolitinib...
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Suppressing CCL5/IL-6 and inducible IKK

We expressed an arrayed library of 600 kinase ORFs in A549 cells (36), then suppressed KRAS expression and IKKE gene expression in a panel of human lung adenocarcinomas. KRAS mutation status indicated by tick marks, scale reflects ssGSEA normalized enrichment scores and IKKε gene expression. NMI, normalized mutual information statistic relative to KRAS signature.

Figure 5. CYT387 treatment disrupts a TBK1/IKKε and JAK-dependent autocrine cytokine circuit. A, immunoblot of Y705 pSTAT3. STAT3, S172 pTBK1, TBK1, and β-actin levels in A549 cells treated with 5 μmol/L MRT67307, CYT387, or ruxolitinib. B, heatmap of KRAS/IL-1β signature expression and IKKε gene expression in a panel of human lung adenocarcinomas. KRAS mutation status indicated by tick marks, scale reflects ssGSEA normalized enrichment scores and IKKε gene expression. NMI, normalized mutual information statistic relative to KRAS signature. C, IKKε mRNA levels and immunoblot of IKKε and β-actin levels in IL-1β-stimulated A549 cells. D, immunoblot of IKKε, S418 pCYLD, CYLD, or β-actin levels in IL-1β-stimulated A549 cells following dimethyl sulfoxide (DMSO), MRT67307, CYT387, or ruxolitinib (5 μmol/L) treatment. E, ELISA of CCL5 and IL-6 levels in A549 cells stimulated for 4 hours with IL-1β following pretreatment for 1 hour with DMSO, MRT67307, CYT387, or ruxolitinib (5 μmol/L). Mean and SD of duplicate samples shown. F, model of autocrine cytokine circuit involving TBK1/IKKε, CCL5/IL-6, and JAK–STAT activation.

IKKε Reactivation Promotes CYT387 Resistance

In parallel to these studies, we conducted a kinase open reading frame (ORF) rescue screen to identify genetic modifiers of TBKI-regulated proliferation/survival in A549 cells. We expressed an arrayed library of 600 kinase ORFs in A549 cells (36), then suppressed TBKI using a TBKI-specific short hairpin RNA (shRNA) and measured cell viability. Although we were unable to overexpress TBKI stably, IKKε was the top-scoring ORF that rescued TBKI suppression, whereas oncogenic KRAS enhanced dependency of A549 cells on TBKI (Fig. 6A and Supplementary Table S1). IKKε-mediated rescue of TBKI loss was dependent on its kinase activity, as IKKε WT but not a kinase-dead allele restored A549 cell viability (Fig. 6B).

We also treated A549 cells with escalating doses of CYT387 for 2 months and identified two different clones (A549-C1 and A549-C2) resistant to CYT387 at concentrations as high as 5 μmol/L (Fig. 6C). CYT387 or ruxolitinib treatment inhibited pSTAT3 in these clones, making it unlikely that drug efflux or mutations in JAK were responsible for resistance (Supplementary Fig. 5A). Instead, each clone expressed a kinase domain from the TBK1 structure (38). We noted substantially increased levels of IKKε and β-actin levels in IL-1β-stimulated A549 cells, whereas TBKI-resistant A549-C1 cells were resistant to CYT387 treatment after IKKε suppression (Fig. 6E), suggesting that IKKε reactivation contributes to CYT387 resistance.

To further assess the role of IKKε kinase signaling as a resistance mechanism, we mapped the JAK2 inhibitor resistance alleles Y931C and G935R (37) onto a model of the IKKε kinase domain from the TBKI structure (38). We noted...
homologous orientation of JAK2 Y931 and IKKε Y88 (Fig. 6F), suggesting that substitutions involving this residue would block CYT387 binding to IKKε. Indeed, IKKε Y88C specifically retained kinase activity and rescued CCL5 and IL-6 production following expression and CYT387 treatment in 293T cells (Supplementary Fig. S6B). We then stably expressed IKKε Y88C, IKKε WT, IKKε KD, or enhanced GFP (EGFP) in A549 cells and cultured cells in 5 μmol/L CYT387. In contrast to EGFP or IKKε KD, both IKKε Y88C and to a lesser extent IKKε WT accelerated resistance (Supplementary Fig. S6C). Sequencing of IKKβ KE in resistant A549-IKKε-Y88C cells that emerged confirmed dominant expression of the Y88C allele (Supplementary Fig. S6D). A549-IKKε-Y88C cells produced high levels of CCL5 and IL-6 insensitive to CYT387 treatment (Fig. 6G). When cultured as spheroids in 3D culture, A549-IKKε-Y88C cells dispersed despite 1 μmol/L CYT387 treatment (Fig. 6H). Addition of CCL5- and IL-6-neutralizing antibodies completely suppressed proliferation, confirming that IKKε-mediated CCL5 and IL-6 production was responsible for CYT387 resistance (Fig. 6H). Together, these findings reveal that inhibition of this autocrine cytokine circuit contributes directly to CYT387 activity in KRAS-dependent lung cancer cells.

**Therapeutic Effect of CYT387 in Murine Kras-Driven Lung Cancers**

CYT387 inhibits disease progression of JAK-dependent murine myeloproliferative neoplasms and suppresses pSTAT3 in vivo (29). Using the daily dose of CYT387 in these studies as a starting point, we examined CYT387 treatment in a murine...
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2 of the docetaxel-treated mice died by 4 weeks following CYT387/AZD6244 therapy. Arrows indicate baseline lung tumor burden. Figure 7. Therapeutic efficacy of CYT387 in Kras-driven murine lung cancer. A, percentage change in MRI tumor volume of KrasLSL-G12D/WT- induced lung cancer 2 and 4 weeks following vehicle, docetaxel, CYT387, or CYT387 + docetaxel treatment. *, F < 0.01, t test; n.s., not significant. B, baseline and 16-week MRI images following CYT387 treatment. Arrows indicate baseline lung tumor burden. H, heart. C, immunohistochemistry for Y705 pSTAT3 in tumors from vehicle-, docetaxel-, CYT387-, or CYT387 + docetaxel–treated mice compared with hematoxylin and eosin (H&E) stain. D, immunoblot of Y705 pSTAT3, STAT3, S418 pCYLD, CYLD, S172 pTBK1, TBK1, and β-actin in tumors from vehicle or two different CYT387–treated mice. E, mRNA levels of Ccl5, Il6, and Ikbke in tumors from docetaxel-, CYT387–, or CYT387 + docetaxel–treated mice, normalized to vehicle control. Mean ± SEM of triplicate samples shown. F, percentage change in tumor burden of KrasLSL-G12D/WT; p53fl ox/fl ox lung cancer treated with vehicle, AZD6244, CYT387, or the combination at 2 weeks. Two mice treated with the combination were imaged at 3 weeks. **, 0 < 0.001, t test. G, baseline and 8-week MRI images following CYT387/AZD6244 therapy. Arrows indicate baseline lung tumor burden.

A model of KrasG12D–driven lung cancer (39). We identified mice with established lung tumors by MRI (40) and treated them with 100 mg/kg CYT387 daily by oral gavage, docetaxel 16 mg/kg every other day by intraperitoneal injection, or combination of CYT387 and docetaxel.

Single-agent CYT387 treatment reduced mean tumor volume on MRI as effectively as docetaxel at 2 weeks (~23.4% CYT387 vs. ~13.0% docetaxel), whereas vehicle–treated animals progressed similar to previous studies (Fig. 7A; ref. 40). At 4 weeks, CYT387 treatment resulted in statistically significant and durable reduction in mean tumor burden (~30.5%, P < 0.01 vs. vehicle, t test) in contrast to docetaxel (+25.8%, P = 0.626 vs. vehicle, t test; Fig. 7A). CYT387–induced lung tumor regression was durable even at 16 weeks (Fig. 7B). Furthermore, 2 of the docetaxel–treated mice died by 4 weeks from toxicity, whereas CYT387–treated mice exhibited no overt signs of toxicity. We observed potent synergy between CYT387 and docetaxel (mean tumor volume reduction ~62.8% and ~52.8% at 2 and 4 weeks, respectively, for the combination; Fig. 7A), although this combination resulted in weight loss, which was not observed in mice treated with CYT387 alone (Supplementary Fig. S7A).

To determine whether CYT387 suppressed JAK and TBK1/IKKe signaling in vivo, we measured several markers of activity in tumors from treated mice. CYT387 or CYT387/docetaxel treatment specifically inhibited tumor pSTAT3 levels compared with vehicle or docetaxel alone (Fig. 7C and D). TBK1 signaling was active in these tumors as measured by elevated pCYLD and pTBK1 levels, and, in consonance with our findings in vitro, CYT387 treatment blocked CYLD phosphorylation levels in tumors from vehicle–, docetaxel–, CYT387–, or CYT387 + docetaxel–treated mice, normalized to vehicle control. Mean ± SEM of triplicate samples shown. F, percentage change in tumor burden of KrasLSL-G12D/WT; p53fl ox/fl ox lung cancer treated with vehicle, AZD6244, CYT387, or the combination at 2 weeks. Two mice treated with the combination were imaged at 3 weeks. **, 0 < 0.001, t test. G, baseline and 8-week MRI images following CYT387/AZD6244 therapy. Arrows indicate baseline lung tumor burden.
and paradoxically increased pTBK1 (Fig. 7D). As a consequence, levels of Cils, Il6, and Ikkbe were all preferentially reduced in CYT387 and CYT387/docetaxel-treated animals (Fig. 7E). Thus, response to CYT387 therapy correlated with effective disruption of this cytokine circuit in vivo.

Mitogen-activated protein kinase (MAPK) pathway inhibition is a central focus of current therapeutic strategies targeting KRAS-driven cancers. Using a phosho-MAPK antibody array, we confirmed that CYT387 treatment in vivo failed to suppress MAPK pathway activation in KRAS-dependent A549 cells, and instead increased pERK1/2 levels compared with ruxolitinib or with AZD6244, which inhibited MEK–ERK signaling as expected (Supplementary Fig. S7B). Treatment with either MRT67307 or CYT387 rapidly induced feedback phosphoxylated extracellular signal–regulated kinase (pERK) activation in A549 cells (Supplementary Fig. S7C), also reported phosporylated extracellular signal–regulated kinase (pERK) activation in A549 cells (Supplementary Fig. S7C), also reported phosporylated extracellular signal–regulated kinase (pERK) activation in A549 cells (Supplementary Fig. S7C), also reported phosporylated extracellular signal–regulated kinase (pERK) activation in A549 cells (Supplementary Fig. S7C) in a context- and cell type–dependent manner.

DISCUSSION

In addition to their key roles in innate immunity, TBK1 and IKKe have also been implicated in malignant transformation (42), basal autophagy (22), and obesity (43). These observations suggest that TBK1 and IKKe likely have multiple substrates whose phosphorylation regulates specific functions in a context- and cell type–dependent manner.

Oncogenic KRAS co-opts TBK1/IKKe signaling by activating RALB (18) and inducing IKKe expression (23). Yet, the mechanism by which TBK1 and IKKe regulate transformation and cancer cell survival remains incompletely characterized. Here, we found that CCL5 and IL-6, key TBK1/IKKe–associated cytokines during innate immunity, are also aberrantly activated in KRAS-driven lung cancers. These cytokines, in turn, act in an autocrine manner to activate JAK–STAT signaling, which amplifies IL-6 production, promotes IKKe expression, and sustains growth factor–activated lung cancer cell proliferation and migration. We identified CYT387, a clinical-stage JAK inhibitor, as a potent TBK1/IKKe inhibitor that disrupts this autocrine loop and causes regression of established murine Kras-driven lung tumors in vivo. These observations elucidate a novel KRAS effector pathway emanating from RAL–GEF through RALB and TBK1 that activates cytokine production in an inappropriately context to promote tumor maintenance. Because genetic and pharmacologic perturbation of these kinases extinguishes this cytokine signaling network and inhibits KRAS-driven tumorigenicity, targeting this effector pathway represents a promising new strategy for these treatment-refractory cancers.

Contributions of Inflammatory Signaling to KRAS-Induced Tumorigenesis

Oncogenic KRAS constitutively activates multiple effectors, including MAPK, PI3K, and RAL–GEF. Although most cancers that harbor oncogenic KRAS mutations also depend on KRAS signaling, KRAS mutation and dependency are not strictly correlated (19, 31), indicating that RAS signaling is active and required other contexts as well. The finding that TBK1 links RALB to the generation of specific protumor cytokines provides mechanistic insights into the factors that drive KRAS-induced cell transformation. CCL5–CCR5 signaling promotes chemotaxis and enhances metastasis of RAS-driven breast cancer cells (44). Multiple studies have established that IL-6– and STAT3-induced survival signaling are required for RAS-mediated tumorigenesis (10–13). TBK1/IKKe–regulated CCL5 and IL-6 influence the local inflammatory microenvironment and may also support tumorigenesis in a non–cell-autonomous fashion (45). Activation of this pathway is conversely influenced by factors in the tumor microenvironment and thus may occur in other contexts, although our observations demonstrate that TBK1 is activated and required by lung cancer cells that depend on KRAS. KRAS-independent cancers that exhibit little activation of TBK1 and cytokine signaling may maintain tumorigenesis via alternative means.

These studies identified an autocrine cytokine circuit involving TBK1/IKKe and JAK activity that sustained CCL5 and IL-6 production in cancer cells, similar to IFN-β signaling (26). Like IFN-β, both CCL5 and IL-6 activate JAK–STAT signaling, inducing IKKe expression (25) and triggering further CCL5 and IL-6 production (Fig. 5F). TBK1 inactivation and JAK inhibition together were required to ablate CCL5 and IL-6 production in MEFs, and inhibition of TBK1/IKKe and JAK by CYT387 was necessary to inhibit induction of IKKe, CCL5, and IL-6 in KRAS-dependent lung cancer cells. IKKe overexpression rescued TBK1 suppression, CYT387-resistant clones overexpressed IKKe, and an inhibitor-resistant IKKe allele conferred resistance to CYT387, suggesting that these closely related kinases are key targets of CYT387. Although CYT387 inhibits IKKe kinase activity, IKKe may have a scaffolding function or require maximal ATP-site occupancy for full inhibition, which is more difficult in the presence of excess kinase and has been observed for other enzymatic drug targets such as dihydrofolate reductase (DHFR; ref. 46). The requirement of CCL5 and IL-6 for IKK-driven resistance confirms the involvement of this autocrine signaling loop in KRAS-dependent lung cancer proliferation.

TBK1/IKKe also activate AKT (20, 21) and regulate NF-κB signaling components such as CYLD (19, 30). Rescue of Tbk1−/−
Targeting Oncogenic KRAS via Cytokine Suppression

Malignant transformation of lung cancer cells is associated with elevated cytokine production and activation of innate immune responses, suggesting that inhibition of these pathways could prevent tumor cell proliferation. Indeed, murine KRAS mutant cells demonstrated increased tumor burden in the absence of cytokines (34). The addition of cytokines to these cultures inhibited tumor cell proliferation, suggesting that cytokine inhibition may have therapeutic potential. However, the mechanisms by which cytokines promote tumor growth are not well understood, and the effectiveness of cytokine inhibition in patients with KRAS-mutant lung cancer is unknown.

**Methods**

**Cell Culture**

Cells were cultured at 37°C in a humidified incubator with 5% CO₂. NSCLC cell lines were obtained from the American Type Culture Collection (ATCC) or the DFCI-84 collection, and cell identity by KRAS mutation status was previously validated by sequencing (19). Murine RAW 264.7 macrophage-like cells and HUVECs were obtained from ATCC. Tbk1−/− and WT MEFs were kindly provided by K. Fitzgerald. Tumor spheroids were generated from A549 or NCI-H1437 cells by lentiviral transduction as described previously (34).

**Gene Transduction**

Cells were transduced with lentiviral particles to overexpress ORFs by lentiviral transduction was performed as described previously (19, 36). shRNA sequences are listed in Supplementary Table S2.

**Immunoblotting, Antibodies, and ELISA**

Immunoblotting was performed as described previously (19). Cytokines, ELISA kits, and cytokine antibody arrays were purchased from R&D Systems.

**Enzyme Kinetic Assays**

Recombinant enzymes were incubated with ATP and IKKε tide (ADDDDYDSDL) to measure the specific IKKε peptide substrate as described previously (30).

**Quantitative Real-Time PCR**

Real-time PCR (RT-PCR) was performed using LightCycler 480 SYBR Green I Master (Roche) and primers that target the cytokines were designed using Primer3 software. RT-PCR (RT-PCR) was performed using LightCycler 480 SYBR Green I Master (Roche). The sequences of the primers used for RT-PCR are listed in Supplementary Table S3.

**Animal Studies**

Mouse experiments were conducted in agreement with the Institutional Animal Care and Use Committee (IACUC) of Dana-Farber Cancer Institute. Lung tumors in KRAS mutant mice were transduced with MRT67307, and mice were monitored for lung tumor burden over time.

**Immunohistochemical Analysis**

Immunohistochemical staining was performed on formalin-fixed and paraffin-embedded tissues from treated mice, sectioned at 5-μm thickness.

**Disclosure of Potential Conflicts of Interest**

S.E. Moody is employed as a consultant at N-of-One Therapeutics. R.R. Shen is employed as a scientist at Agensys, Inc. P. Tamayo is employed as a consultant at Oracle Corporation. S. Gaudet is a consultant/advisory board member of Merrimack Pharmaceuticals. J.A. Engelman has received commercial research grants from Novartis, Sanofi-Aventis, and AstraZeneca. J. Mesirov is a scientist at Agensys, Inc. P. Tamayo is employed as a consultant at Oracle Corporation. S. Gaudet is a consultant/advisory board member of Merrimack Pharmaceuticals. J.A. Engelman has received commercial research grants from Novartis, Sanofi-Aventis, and AstraZeneca. J. Mesirov is a scientist at Agensys, Inc. P. Tamayo is employed as a consultant at Oracle Corporation. S. Gaudet is a consultant/advisory board member of Merrimack Pharmaceuticals. J.A. Engelman has received commercial research grants from Novartis, Sanofi-Aventis, and AstraZeneca.
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Targeting Oncogenic KRAS via Cytokine Suppression


Inhibition of KRAS-Driven Tumorigenicity by Interruption of an Autocrine Cytokine Circuit


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