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

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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 inflammatory signals such as NF-κB (4–6). Although concurrent MAPK–PI3K pathway inhibition is under clinical evaluation, multiple approaches are likely necessary to identify effective KRAS-targeted therapy.

Oncogenic RAS induces inflammatory cytokines that activate NF-κB and STAT3. For example, KRAS-driven non-small cell lung cancers (NSCLC; ref. 7) and pancreatic ductal adenocarcinomas (PDAC; ref. 8) engage cell autonomous interleukin (IL)-1 signaling. Similarly, oncogenic RAS-induced IL-6 promotes both oncogene-induced senescence (9) and cell transformation (10), and STAT3 is required for Kras-driven PDAC development in mice (11–13). RAS-associated IL-8, CXCL1, and CXCL2 expression also promotes senescence (14), tumor cell survival, and angiogenesis (15, 16). How oncogenic RAS activates these cytokines and their role in RAS-dependent cancers remains incompletely characterized.

Activation of RALA and RALB by RAL–GEF enhances cancer cell proliferation and survival (17). A specific RALB–SECS complex engages the innate immune signaling kinase Tank-binding kinase-1 (TBK1) to promote cell survival (18). TBK1 is required for transformation by oncogenic KRAS, sustains KRAS-dependent cancer cell viability, and regulates basal autophagy (18–22). The TBK1 homolog inhibitor of IκB kinase ε (IKKε; encoded by IKBKE) also promotes NF-κB 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 tumorigenesis.

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 tumorigenesis 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...
reduced expression of each cytokine/chemokine in
mentary Fig. S1B). Because TBK1 also regulates IL-6 (27),
eration and may contribute to
assessed conditioned medium from WT or
WT but not
medium lacked CCL5 and exhibited decreased CXCL10 lev-
secreted factor, we plated
MEFs (Fig. 1A). To assess the role of cell contact versus a
secreted factor, we plated
MEFs in the clonogenic assay
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
MEFs, revealing kinase-dependent regulation of this chemokine (Fig. 1E).
Because CCL5 and IL-6 induce prosurvival JAK–STAT sig-
Kras
IL-6 to TBK1-regulated survival, we supplemented media
with each factor and measured
MEF colony formation. CCL5 (10 ng/mL) rescued
MEFs colonies comparably with WT MEF conditioned medium, whereas
IL-6 had a modest effect and CXCL10 failed to rescue colony
(Fig. 1F and Supplementary Fig. S1E). Adding IL-6 or
CXCL10 to CCL5 did not increase
MEFs. Thus, autocrine CCL5 and IL-6 signaling promote TBK1-
regulated proliferation/survival.
Because CCL5 and IL-6 induce prosurvival JAK–STAT sig-

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**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 Tbki+/– MEFs. Tbki+/– 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 Tbki+/– MEFs (Fig. 1H and I). Supplementation of Tbki+/– MEFs 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 µmol/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 Gip1 (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 activation in a panel of human NSCLC lines. Because KRAS-driven lung cancer, we first measured TBK1 and STAT3 expression (Supplementary Fig. S3A). Although one group reported that A549 cells are KRAS-WT/independent cells (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 Y701 phosphorylation 24 hours after plating, and normalized values to total TBK1 or STAT3 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 = 228 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 measured on TBK1, except for H1819 cells (19, 20), which instead required I KKBE 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 IKKe 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 IKKe (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
Fig. S5D). Thus, interruption of CCL5 and IL-6 together required inhibition of TBK1/IKKe and JAK signaling.

We next assessed the effect of each inhibitor on this autocrine circuit in KRAS-dependent lung cancer cells. Treatment of A549 cells with 5 μmol/L MRT67307 transiently inhibited pSTAT3 levels, but within 2 hours it led to reactivation of STAT3 consistent with the feedback we observed in Tbk1−/− MEFs (Fig. 5A), as well as paradoxical induction of TBK1 S172 activation. 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 potentiates TBK1 and CCL5/IL-6 activation in A549 cells and promotes KRAS-dependent tumorgenicity (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 oncogenic KRAS and IL-1 signaling in primary human lung adenocarcinomas (P = 0.02, normalized mutual information statistic; refs. 19, 35) and IKKe overexpression in these tumors (Fig. 5B). In consonance with this finding, IL-1 also promoted IKKe 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 IKKe protein and mRNA expression (Fig. 5D and Supplementary Fig. S5F). MRT67307 treatment failed to suppress IL-1β–induced CCL5/IL-6 expression, and ruxolitinib

**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. Plotted 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 cell 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.
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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 IKKε-mediated rescue of TBK1 loss was dependent on its kinase activity, as IKKe 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. S6A). Instead, each clone expressed a TBK1/Ikkε-specific short hairpin RNA (shRNA) and measured cell viability. Although we were unable to overexpress TBK1 stably, IKBKE was the top-scoring ORF that rescued TBK1 suppression, whereas oncogenic KRAS enhanced dependency of A549 cells on TBK1 (Fig. 6A and Supplementary Table S1). IKKε-mediated rescue

partially reduced these levels of these cytokines, whereas CYT387 treatment strongly inhibited CCL5/IL-6 production (Fig. 5E and Supplementary Fig. S5G). These experiments confirmed that CYT387 targets TBK1/Ikkε and JAK signaling in lung cancer cells and disrupts this autocrine cytokine circuit, suppressing CCL5/IL-6 and inducible IKKε expression (Fig. 5F).

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 TBK1-regulated proliferation/survival in A549 cells. We expressed an arrayed library of 600 kinase ORFs in A549 cells (36), then suppressed TBK1 using a TBK1-specific short hairpin RNA (shRNA) and measured cell viability. Although we were unable to overexpress TBK1 stably, IKBKE was the top-scoring ORF that rescued TBK1 suppression, whereas oncogenic KRAS enhanced dependency of A549 cells on TBK1 (Fig. 6A and Supplementary Table S1). IKKε-mediated rescue of TBK1 loss was dependent on its kinase activity, as IKKe 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. S6A). Instead, each clone expressed a TBK1/Ikkε-specific short hairpin RNA (shRNA) and measured cell viability. Although we were unable to overexpress TBK1 stably, IKBKE was the top-scoring ORF that rescued TBK1 suppression, whereas oncogenic KRAS enhanced dependency of A549 cells on TBK1 (Fig. 6A and Supplementary Table S1). IKKε-mediated rescue of TBK1 loss was dependent on its kinase activity, as IKKe 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. S6A). Instead, each clone expressed a TBK1/Ikkε-specific short hairpin RNA (shRNA) and measured cell viability. Although we were unable to overexpress TBK1 stably, IKBKE was the top-scoring ORF that rescued TBK1 suppression, whereas oncogenic KRAS enhanced dependency of A549 cells on TBK1 (Fig. 6A and Supplementary Table S1). IKKε-mediated rescue

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Figure 6. IKKe-induced CCL5 and IL-6 promote CYT387 resistance. A, kinase ORF rescue screen of TBK1 suppression in A549 cells. Dotted lines mark 2 SD above and below the mean. Highlighted are IKKe and KRASG12V. B, immunoblot of IKKe, TBK1, and β-actin levels in A549 cells 3 days following TBK1 suppression and IKKe expression. Relative viability after 6 days was normalized to control LACZ expression and suppression. Mean ± SD of 48 replicate samples shown. C, schematic of resistant cell line generation over 2 months of CYT387 treatment. D, immunoblot shows IKKe and β-actin levels in parental A549 cells, A549-C1, and A549-C2. E, crystal violet stain of A549 or A549-C1 cells stably infected with control shEGFP, shIKKe-1, or shIKKe-2 and treated with dimethyl sulfoxide (DMSO) or CYT387 for 5 days. Immunoblot shows IKKe and β-actin levels. F, model of CYT387 binding to IKKe or JAK2. *, Homologous IKKe Y88 and JAK2 Y931 residues. G, ELISA of CCL5 and IL-6 levels in A549 or A549-IKKε-Y88C cells following 24 hours of treatment with DMSO or CYT387. Mean ± SD of duplicate samples shown. H, phase-contrast images (×20) of A549-IKKε-Y88C spheroids stimulated with EGF and treated with DMSO or 1 μmol/L CYT387 ± CCL5/IL-6 neutralization for 36 hours. Number of cells dispersed per spheroid from triplicate devices shown (mean ± SD).

homologous orientation of JAK2 Y931 and IKKe Y88 (Fig. 6F), suggesting that substitutions involving this residue would block CYT387 binding to IKKe. Indeed, IKKe 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 IKKe Y88C, IKKe WT, IKKe KD, or enhanced GFP (EGFP) in A549 cells and cultured cells in 5 μmol/L CYT387. In contrast to EGFP or IKKe KD, both IKKe Y88C and to a lesser extent IKKe WT accelerated resistance (Supplementary Fig. S6C). Sequencing of IKKBE 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 IKKe-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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Furthermore, 2 of the docetaxel-treated mice died by 4 weeks and tumor regression was durable even at 16 weeks (Fig. 7B). The combination at 2 weeks. Two mice treated with the combination were imaged at 3 weeks, following CYT387/AZD6244 therapy. Arrows indicate baseline lung tumor burden.

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 of KrasG12D-driven lung cancer (−23.4% compared with vehicle or docetaxel alone (Fig. 7C and D). TBK1 and p–actin in tumors from vehicle or two different CYT387-treated mice. E, mRNA levels of Ccl5, I6, and Ikbk in tumors from vehicle or two different CYT387-treated mice. F, Percentage change in tumor burden of KrasG12D-driven lung cancer treated with vehicle, AZD6244, CYT387, or the combination at 2 weeks. Two mice treated with the combination were imaged at 3 weeks. *, P < 0.001, t test. G, baseline and 8-week MRI images 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. B, baseline and 16-week MRI images following CYT387 treatment. Arrows indicate baseline lung tumor burden. H, heart. C, Immunohistochemistry for Y705 p–STAT3, STAT3, S418 p–CYLD, CYLD, S172 p–TBK1, TBK1, and β–actin in tumors from vehicle or two different CYT387-treated mice. D, mRNA levels of Ccl5, I6, and Ikbk in tumors from vehicle or two different CYT387-treated mice. E, Percentage change in tumor burden of KrasLSL-G12D/WT-induced lung cancer treated with vehicle, AZD6244, CYT387, or the combination at 2 weeks. Two mice treated with the combination were imaged at 3 weeks. *, P < 0.001, t test; n.s., not significant. B, baseline and 16-week MRI images following CYT387 treatment. Arrows indicate baseline lung tumor burden.

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/IκKε signaling in vivo, we measured several markers of activity in tumors from treated mice. CYT387 or CYT387/docetaxel treatment specifically inhibited tumor p–STAT3 levels compared with vehicle or docetaxel alone (Fig. 7C and D). TBK1 signaling was active in these tumors as measured by elevated p–CYLD and p–TBK1 levels, and, in consonance with our findings in vitro, CYT387 treatment blocked CYLD phosphorylation.
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 vitro 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 (33). Inhibition of either MRT67307 or CYT387 rapidly induced feedback phosphorylation as expected (Supplementary Fig. S7B). Treatment with ruxolitinib or with AZD6244, which inhibited MEK–ERK signaling, in an inappropriate 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 protumorigenic 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/IKKε-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 inversely 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/IKKε 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/IKKε 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 IKKe-driven resistance confirms the involvement of this autocrine signaling loop in KRAS-dependent lung cancer proliferation.

TBK1/IKKε 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

MEF clonogenic proliferation by CCL5 or conditioned media was incomplete, suggesting that cell-intrinsic defects in AKT, NF-kB signaling, or autophagy (22) also contribute to TBK1-regulated cell survival. Indeed, MRT67307 treatment impaired cell viability at higher concentrations, suggesting that TBK1 likely counterbalances oncogenic stress by additional means. Given the emerging link between autophagy and RAS-driven tumorigenesis (47), it will be interesting to elucidate the interface between TBK1 signaling and this particular stress-response pathway in KRAS-dependent cancer cells.

Implications for Therapy for KRAS-Driven Cancer

CYT387 treatment of murine Kras-driven lung cancers was well tolerated, and suppression of tumor growth was more durable than standard docetaxel chemotherapy. Combined CYT387 and AZD6244 treatment exhibited significant therapeutic activity in KP-induced murine lung cancers, a treatment-refractory model. Because CYT387 and MEK inhibitors are in advanced stages of clinical development, these observations provide a rationale for testing this combination in human KRAS-driven cancers.

Although kinase inhibitor selectivity is desirable, the complexity of solid tumors and cancer cell signal transduction necessitates concomitant inhibition of several signaling pathways (48). For example, multitargeted RTK inhibitors are clinically effective against a variety of tumors. Clinical trials of CYT387 in myelofibrosis have also demonstrated safety and efficacy uniquely among JAK inhibitors in improving transfusion dependence (49). Thus, multitargeted kinase inhibition need not correlate with enhanced toxicity. Finally, because RALB-TBK1 signaling is distinct from MAPK and PI3K, the observation that CYT387 treatment synergizes with MEK inhibition suggests that further combination strategies may be effective in treating KRAS-driven cancers and overcoming resistance.

METHODS

Detailed protocols for all sections are described in the Supplementary 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 gamma NO(−) cells and HUVECs were from ATCC or the DFCI-84 collection, and cell identity was additionally validated by sequencing (34). Murine RAW 264.7 and wild-type (WT) MEFs were kindly provided by K. Fitzgerald. Murine MEFs were cultured at 37°C in a humidified incubator with 5% CO2.Murine RAW 264.7 gamma NO(−) cells and HUVECs were from ATCC or the DFCI-84 collection, and cell identity was additionally validated by sequencing (34).

Gene Transduction

Cells nucleofection/transfection (38) or introduction of shRNAs/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.

In Vitro Enzyme Kinetic Assays

Recombinant enzymes were incubated with ATP and IKKε peptide substrate (ADDDYDSDL/DWADDK) as a specific TBK1/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). The sequences of the primers used for RT-PCR are listed in Supplementary Table S3.

Animal Studies

Mouse experiments were conducted in agreement with a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Dana-Farber Cancer Institute. Lung tumors in Kras(loxp-G12D/WT or Kras(loxp-G12D/Flox/Flox) mice were induced by inhalation of adenoviral Cre recombinase and monitored by serial MRI scanning as described previously (40).

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, Amgen, and AstraZeneca and is a consultant/advisory board member of Novartis, Sanofi-Aventis, Genentech, GlaxoSmithKline, Cell Signaling Technology, Agios, Janssen, and Endo. K.-K. Wong has received commercial research support from AstraZeneca, Infinity, and Millennium. W.C. Hahn has received a commercial research grant from Novartis and is a consultant/advisory board member of Novartis and Blueprint Medicines. D.A. Barbie is a consultant/advisory board member of N-of-One Therapeutics. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions


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