Mutationally Activated PIK3CA<sup>H1047R</sup> Cooperates with BRAF<sup>V600E</sup> to Promote Lung Cancer Progression

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Abstract

Adenocarcinoma of the lung, a leading cause of cancer death, frequently displays mutational activation of the KRAS proto-oncogene but, unlike lung cancers expressing mutated EGFR, ROSI, or ALK, there is no pathway-targeted therapy for patients with KRAS-mutated lung cancer. In preclinical models, expression of oncogenic KRAS<sup>G12D</sup> in the lung epithelium of adult mice initiates development of lung adenocarcinoma through activation of downstream signaling pathways. In contrast, mutationally activated BRAF<sup>V600E</sup>, a KRAS effector, fails to initiate lung carcinogenesis despite highly efficient induction of benign lung tumorigenesis. To test if phosphoinositide 3-kinase (PI3K)-α (PIK3CA), another KRAS effector, might cooperate with oncogenic BRAF<sup>V600E</sup> to promote lung cancer progression, we used mice carrying a conditional allele of Pik3ca that allows conversion of the wild-type catalytic subunit of PIK3CA to mutationally activated PIK3CA<sup>H1047R</sup>. Although expression of PIK3CA<sup>H1047R</sup> in the lung epithelium, either alone or in combination with PTEN silencing, was without phenotype, concomitant expression of BRAF<sup>V600E</sup> and PIK3CA<sup>H1047R</sup> led to dramatically decreased tumor latency and increased tumor burden compared with BRAF<sup>V600E</sup> alone. Most notably, coexpression of BRAF<sup>V600E</sup> and PIK3CA<sup>H1047R</sup> elicited lung adenocarcinomas in a manner reminiscent of the effects of KRAS<sup>G12D</sup>. These data emphasize a role for PI3K signaling, not in lung tumor initiation per se, but in both the rate of tumor growth and the propensity of benign lung tumors to progress to a malignant phenotype. Finally, biologic and biochemical analysis of BRAF<sup>V600E/PIK3CA<sup>H1047R</sup></sup>-expressing mouse lung cancer cells revealed mechanistic clues about cooperative regulation of the cell-division cycle and apoptosis by these oncogenes. Cancer Res; 73(21); 6448–61. ©2013 AACR.

Introduction

Non–small cell lung carcinoma (NSCLC) is a leading cause of cancer-related mortality (1). Recently identified mutations in proto-oncogenes in NSCLC have provided strategies for the deployment of pathway-targeted therapies (2). However, despite the success of such agents in the treatment of genetically defined subsets of lung cancer, most patients with NSCLC do not have recourse to the use of such agents.

Of the proto-oncogenes mutated in NSCLC, KRAS remains the most common (>25%) and the most pharmacologically intractable (2). Consequently, attention has turned to inhibiting key downstream signaling pathways required for maintenance of KRAS-mutated NSCLC. Moreover, KRAS effectors such as BRAF, phosphoinositide 3-kinase (PI3K), and AKT are directly implicated as bona fide human lung cancer genes (2–5). However, a key outstanding question is, what KRAS<sup>G12D</sup>-regulated pathways are essential for lung carcinogenesis in GEM models? Although KRAS<sup>G12D</sup>-driven tumors are MAP–ERK kinase 1/2 (MEK1/2) dependent, expression of BRAF<sup>V600E</sup> in the lung epithelium elicits benign lung tumors that rarely progress to malignancy due to a senescence-like growth arrest (6–8). In contrast, although transgenic expression of mutationally activated PIK3CA<sup>H1047R</sup> in the lung epithelium was reported to promote lung tumorigenesis, combined inhibition of PI3K/mTorC had no effect on established KRAS<sup>G12D</sup>-initiated lung tumors (9). Consequently, we tested whether mutationaly activated BRAF<sup>V600E</sup> and PIK3CA<sup>H1047R</sup> might cooperate in promoting lung carcinogenesis using GEM models (6, 10).

Perhaps surprisingly, we could not substantiate reports that PIK3CA<sup>H1047R</sup> can initiate lung tumorigenesis. However, concomitant expression of both BRAF<sup>V600E</sup> and PIK3CA<sup>H1047R</sup> led...
to rapid onset of lung tumorigenesis with evidence of malignant progression observed 6 months after tumor initiation. The cooperative effects of PIK3CA<sup>H1047R</sup> in vivo were AKT dependent, and were modeled in vitro. Finally, we show that both BRAF<sup>V600E</sup> and PIK3CA signaling is necessary for proliferation and survival of BRAF<sup>V600E</sup>/PIK3CA<sup>H1047R</sup>-expressing lung cancer cells, and show that these pathways cooperatively regulate the cell-division cycle and apoptosis.

**Materials and Methods**

**Mice and adeno virus delivery**

Animal experiments were carried out in accordance with protocols approved by the University of California, San Francisco (San Francisco, CA) Institutional Animal Care and Use Committee (IACUC). Braf<sup>Cre</sup> (Braf<sup>tm1Mmcm</sup>), Trp53<sup>fl/fl</sup> (Trp53<sup>fl/fl</sup>U2B), PIK3CA<sup>lat-H1047R</sup>, and Pten<sup>fl/fl</sup> mice were bred and genotyped as previously described (6, 10–13). All mice were back-crossed onto the FVB/NJ genetic background for at least 10 generations. Experiments were carried out using littersmates as controls whenever possible. Stocks of adenovirus-encoding Cre recombinase were purchased from Virasource or the University of Iowa, Gene Transfer Vector Core (14). Adenoviruses with cell-specific expression of Cre recombinase were generously provided by Kate Sutherland and Anton Berns (NKI, Amsterdam) and purchased from the University of Iowa, Gene Transfer Vector Core (14). Intranasal instillation of adenovirus for infection of the mouse lung epithelium was carried out as previously described (15). Tumor-bearing mice were euthanized for analysis either at a predetermined time point or when their body conditioning score (BCS) was 2 or less (16).

**Histology and quantification of lung tumor burden**

Lungs were removed, fixed in zinc-buffered formalin (ZBS), and stored in 70% (v/v) ethanol before paraffin embedding. Then, 6 µm sections were stained with hematoxylin and eosin (H&E) on slides, and scanned with an Aperio ScanScope scanner. Quantification of tumor number, size, and overall burden of individual lung sections was conducted using Aperio Spectrum ImageScope software. Overall tumor burden was calculated as (area of lung section occupied by tumor)/(total area of section) in micron per square meter.

**Treatment of mice with pathway-targeted therapeutics**

PD325901 (Hansun Trading Co.) was formulated in 0.5% (w/v) hydroxy-propyl-methylcellulose (HPMT; Sigma) and administered every day by oral gavage (orally) at a dose of 12.5 mg/kg. MK-2206 (Merck) was formulated in 30% (w/v) hydroxy-propyl-methylcellulose (HPMT; Sigma) and dosed orally at 120 mg/kg every day. LGX-818 and BKM-120 (Novartis) were formulated in 0.5% carboxymethylcellulose (Sigma) plus 0.5% Tween 80 (Sigma) and dosed orally every day at doses of 30 and 60 mg/kg, respectively. Mice were dosed with drug for predetermined time points or until their BCS was 2 or less, at which point they were euthanized for analysis (16).

**Immunostaining of mouse lung tissue and immunoblotting**

ZBS-fixed sections of mouse lungs were subject to citrate-mediated antigen retrieval and then probed with anti-pS473-AKT, anti-vimentin, anti-PTEN (Cell Signaling Technology), anti-NKX2.1, anti-Ki67, anti-SP-C, and anti-p63 (Santa Cruz Biotechnology). Immunoblotting was done on 50 µg of extracted protein and probed against various phospho- or backbone-specific antisera as described. Immunoblots were quantified using an Odyssey FC system and Image Studio software.

**Lung tumor cell isolation, culture, and analysis**

Single-cell suspensions were generated from BRAF<sup>V600E</sup>/TP53<sup>Null</sup> or BRAF<sup>V600E</sup>/PIK3CA<sup>H1047R</sup>/TP53<sup>Null</sup>-induced lung cancers and cultured in Hams-F12/Glutamax media with 10% (v/v) FBS. Following outgrowth, single cell-derived clones were isolated. Recombination of Braf<sup>Cre</sup>, Trp53<sup>fl/fl</sup>, and Pik3ca<sup>fl/fl</sup> alleles was verified by PCR (10). Expression of oncogenic BRAF<sup>V600E</sup> was determined by immunoblotting with a mAb (VE1) specific to BRAF<sup>V600E</sup> (17).

Cell proliferation was assessed in triplicate cultures of cells treated for 72 hours with dimethyl sulfoxide (DMSO) control, MEK1/2 inhibitor (PD325901; 15.6 nmol/L to 1 µmol/L), extracellular signal–regulated kinase 1/2 (ERK1/2) inhibitor (SCH772964; 7.8 nmol/L to 5 µmol/L), AKT-3 inhibitor (MK-2206; 80 nmol/L to 5 µmol/L), or class 1 PI3K inhibitor (GDC-0941; 80 nmol/L to 5 µmol/L) alone or in combination as indicated using a CellTiter-Glo assay (Promega). Cell-cycle status was assessed in fixed floating and adherent cells stained with anti-bromodeoxyuridine (BrdUrd)–fluorescein isothiocyanate (FITC; Becton-Dickinson) and propidium iodide (Sigma). Sorting of mCherry encoding ecotropic retroviruses was carried out using a FACSCalibur system (Becton Dickinson; refs. 18, 19). Anchorage-independent colony formation was done by plating 5 × 10<sup>3</sup> cells in 1.4% (w/v) Sea-Plaque low-melting temperature agarose (Cambrex) and culturing them for 3 weeks, at which time colony formation was enumerated using a dissecting microscope.

**Reverse phase protein array analysis**

Reverse phase protein array (RPPA) analysis (Functional Proteomics Core Facility at MD Anderson Cancer Center, Houston, TX) was conducted as previously described (20, 21) and detailed in the Supplementary Data.

**Results**

**Expression of PIK3CA<sup>H1047R</sup> combined with PTEN silencing fails to induce lung tumors in mice**

To test whether PIK3CA<sup>H1047R</sup> could initiate lung tumorigenesis, we used Pik3ca<sup>lat-H1047R</sup> mice (Pik3ca<sup>lat</sup>) carrying a modified Pik3ca allele that expresses normal PIK3CA before Cre-mediated recombination, after which PIK3CA<sup>H1047R</sup> is expressed under control of the gene’s chromosomal regulatory elements (9, 10). Cre-mediated genetic alterations were initiated using an adenovirus encoding Cre recombinase (Ad-Cre; ref. 6). Because expression of PIK3CA<sup>H1047R</sup> from a single allele might be insufficient for lung tumorigenesis, we generated...
mice with every possible heterozygous or homozygous combination of Pik3calat in combination with a conditional null allele of Pten (Pten<sup>lox/lox</sup>; ref. 11). The lungs of adult mice of the various Pik3calat/Pten<sup>lox/lox</sup> genotypes were infected with Ad-Cre and euthanized for analysis 3, 6, or 12 months or later postinfection. As a control for successful tumor induction, BRAF<sup>V600E</sup> expression was initiated in the lung epithelium of BRaf<sup>CA</sup> mice, which developed benign lung adenomas at high multiplicity requiring euthanasia approximately 12 weeks postinitiation (Fig. 1A, right). In contrast, we failed to detect lung tumors in mice carrying conditional alleles of Pik3ca or Pten, either alone or in combination, at 3 or 6 months after Ad-Cre infection (Fig. 1A, left and middle). That PTEN silencing was insufficient for lung tumorigenesis was consistent with previous observations, but the lack of lung tumorigenesis in the compound Pik3calat/Pten<sup>lox/lox</sup> mice was surprising (22). At 12 months or more, we detected benign adenomas in Pik3ca<sup>/lat</sup>; Pten<sup>lox/lox</sup> mice; however, these were rare (< one tumor per mouse). Most of these tumors stained positive for PTEN expression and negative for phospho (p)-AKT, suggesting that they may be spontaneously arising lung tumors unrelated to mouse genotype. However, we also detected very rare lung tumors in Pik3ca<sup>/lat</sup>; Pten<sup>lox/lox</sup> mice that stained brightly for pAKT and may, therefore, be due to this combination of genetic modifications (Fig. 1T). Immunofluorescence analysis of Ad-Cre infected Pik3ca<sup>lat</sup> or Pik3ca<sup>/lat</sup>; Pten<sup>lox/lox</sup> mice failed to detect evidence of cells with pAKT or Ki67 expression above that detected in normal mouse lung (Fig. IF–MI). However, in Pik3ca<sup>lat</sup> mice carrying either one or two Pten<sup>lox/lox</sup> alleles, small numbers of airway epithelial hyperplasias were detected (Fig. IN–U). Most prevalent in Pik3ca<sup>lat/lat</sup>; Pten<sup>lox/lox</sup> mice, these lesions comprised small numbers of cells that stained negative for PTEN and positive for pAKT, with the strongest pAKT signal detected in lesions in Pik3ca<sup>/lat/lat</sup>; Pten<sup>lox/lox</sup> mice (Fig. 1R). These lesions were 50 μm or less in diameter and not proliferative (Fig. 1S). Therefore, in contrast

![Figure 1. PI3K pathway activation is insufficient to initiate tumorigenesis in the mouse lung. A, lungs of mice of the indicated genotypes were infected with Ad-Cre and monitored for 3 or 12 months or more as indicated, at which time they were euthanized and their lungs processed for H&E staining. Representative H&E-stained tissue sections from Pik3ca<sup>lat/lat</sup> (left) or Pik3ca<sup>/lat/lat</sup>; Pten<sup>lox/lox</sup> (middle) animals euthanized 6 months after infection. BRaf<sup>CA</sup> mice analyzed 12 weeks following Ad-Cre initiation are presented as a positive control (right). B–U, mouse lung sections from control (uninfected) or mice of the indicated genotypes treated as described in A were stained with 4', 6-diamidino-2-phenylindole (DAPI), antisera against phospho-(p)-AKT, or antisera against phospho-ki67-AKT, or Ki67 as indicated. Insets indicate higher magnification of regions of airway hyperplasia.](image-url)
to the effects of KRAS<sup>G12D</sup> or BRAF<sup>V600E</sup>, there was no combination of Pik3ca<sup>mut</sup> and Pten<sup>lox/lox</sup> that elicited lung tumors in mice within 6 months after Ad-Cre (6, 9, 12).

PIK3CA<sup>H1047R</sup> dramatically accelerates BRAF<sup>V600E</sup>-driven lung tumorigenesis

To determine if PIK3CA<sup>H1047R</sup> cooperates with BRAF<sup>V600E</sup> in lung tumorigenesis, we infected adult BrAf<sup>CA</sup> or compound BrAf<sup>CA</sup>; Pik3ca<sup>mut</sup> mice with either 5 × 10<sup>6</sup> or 10<sup>7</sup> pfu Ad-Cre. Kaplan–Meier survival analysis of mice infected with 5 × 10<sup>6</sup> pfu Ad-Cre indicated that all BrAf<sup>CA</sup>; Pik3ca<sup>mut</sup> mice reached end-stage by approximately 50 days based on their BCS, a time at which all BrAf<sup>CA</sup> mice remained healthy (Fig. 2A; ref. 16). Taken together, BrAf<sup>CA</sup> mice lived twice as long as BrAf<sup>CA</sup>; Pik3ca<sup>mut</sup> littermates after initiation (median survival, 100 vs. 39 days, P = 1.25 × 10<sup>−5</sup>; Fig. 2B). Furthermore, 3 weeks after infection with 10<sup>7</sup> pfu Ad-Cre, many BrAf<sup>CA</sup>; Pik3ca<sup>mut</sup> mice displayed labored breathing and reduced body weight, indicating onset of lethal lung tumorigenesis, at which time representative mice were euthanized for necropsy (Fig. 2B).

Compared with control BrAf<sup>CA</sup> mice, BrAf<sup>CA</sup>; Pik3ca<sup>mut</sup> mice presented with more tumors (96 vs. 54, P = 0.025) and...
BRAFV600E/PIK3CAH1047R-expressing tumors were larger than their BRAFV600E-expressing counterparts (52,516 vs. 6,782.5 μm², P = 0.004; Fig. 2B and C). Three weeks postinitiation, BRAFV600E/PIK3CAH1047R-expressing lung lesions manifested as large adenomas, whereas BRafCA mice displayed only alveolar and/or airway hyperplasias (Fig. 2B and C). Taken together, BRafCA; Pik3calat mice displayed a 14-fold higher tumor burden at 3 weeks postinitiation compared with BRafCA mice (16.8% vs. 1.2%, P = 0.01; Fig. 2C).

PTEN silencing enhances BRAFV600E-induced lung tumorigenesis

To complement analysis of the effects of PIK3CAH1047R on BRAFV600E-induced lung tumorigenesis, we tested if deregulation of PI3K signaling through PTEN silencing might have similar effects. Indeed, this is a combination of alterations commonly found in human melanoma and that cooperate in GEM models of the disease (5, 23). We infected BRafCA mice with PTENnull mice infected with 10⁶ pfu Ad-Cre with mice euthanized 4 weeks after initiation. Compared with control BRafCA mice, BRafCA; Pik3calat and BRafCA/+/Ptenlox/lox mice displayed a 10-fold or more and a 4-fold increase in overall tumor burden, respectively (2.8% vs. 31.2% vs. 10.8%, Fig. 3A and B). Although most lung tumor cells arising in BRafCA mice displayed readily detectable PTEN expression, the vast majority of tumor cells in BRafCA; Ptenlox/lox mice were PTEN negative (Fig. 3C). These data indicate that the statistically significant difference between lung tumorigenesis in BRafC2; Pik3calat vs. BRafC2/++; Ptenlox/lox mice is not due to inefficient PTEN silencing. Taken together, these data support the hypothesis that activation of PI3K signaling, either by coexpression of PIK3CAH1047R or PTEN silencing, cooperates with BRAFV600E in lung tumorigenesis (22).

Pharmacologic blockade of MEK or AKT prevents the growth of BRAFV600E/PIK3CAH1047R lung tumors

To assess the role of downstream signaling components on the cooperation between BRAFV600E and PIK3CAH1047R, we used pharmacologic inhibitors of either MEK1/2 (PD325901) or AKT.
or AKT (MK-2206; refs. 24, 25). These agents are nonsubstrate competitive, allosteric inhibitors with high specificity and selectivity. BRafV600E or BRafG12C;Pika3calat mice were infected with 10^6 Ad-Cre and then separated into two groups for treatment with either vehicle control or MK-2206 (120 mg/kg, every day, orally) to inhibit AKT for additional 4 to 5 weeks, at which time mice were euthanized.

MK-2206 treatment had no effect on BRAFV600E-initiated lung tumor burden (vehicle, 25% vs. MK-2206, 22%; P = 0.63; Supplementary Fig. S1A). In contrast, consistent with previous results, MEK1/2 inhibition largely abolished BRAFV600E-induced lung tumorigenesis (not shown; refs. 6, 7). These data strongly suggest that BRAFV600E-induced lung tumors are AKT independent for their initial growth.

In contrast to BRAFV600E-driven tumors, MK-2206 had a significant effect on BRAFV600E/PIK3CAH1047R-driven lung tumorigenesis (Fig. 4A and B). Although 4 weeks of MK-2206 dosing had no effect on overall tumor number (vehicle, 31.8 vs. MK-2206, 28.7; P = 0.76), both tumor size (vehicle, 121,413 mm^2 vs. MK-2206, 30,083 mm^2; P < 0.0001) and overall tumor burden (vehicle, 20.7%; MK-2206, 5%; P = 0.0004) were significantly reduced following AKT inhibition (Fig. 4B). Indeed, tumor sizes and overall tumor burden that developed in BRafG12C;Pika3calat mice in the presence of MK-2206 were roughly equivalent to that observed in BRafG12C mice. These data support the hypothesis that AKT activity is required for the cooperation between BRAFV600E and PIK3CAH1047R in lung tumorigenesis but largely dispensable for the growth of BRAFV600E-induced lung tumors. Inhibition of pS473-AKT in BRAFV600E-driven tumors was confirmed by immunoblot analysis of lysates of BRAFV600E or BRAFV600E/PIK3CAH1047R-expressing lung tumors treated with either vehicle or MK-2206 2 hours before euthanasia (Supplementary Fig. S1B and S1C, respectively).

Previous studies have indicated the exquisite sensitivity of BRAFV600E-driven lung tumors to MEK1/2 inhibition with PD325901 (6, 7). However, analysis of lung, pancreas, and colon cancer cell lines suggests that PI3K signaling, either by mutation of PIK3CA or PTEN silencing, can render cells less sensitive to MEK inhibition possibly by sustaining the expression of D-type cyclins (26, 27). Therefore, we sought to determine if BRAFV600E/PIK3CAH1047R lung tumors remained sensitive to MEK1/2 inhibition in vivo. Lung tumors were initiated in BRafG12C;Pika3calat mice, and 2 weeks later mice were dosed with vehicle or PD325901 (12.5 mg/kg, every day, orally) for a further 4 weeks, at which time mice were euthanized (Fig. 4A and B). Compared with controls, MEK1/2 inhibitor-treated mice displayed a 15-fold reduction in tumor number (31.8 vs. 2.0, P = 0.005). Lesions detected in PD325901-treated BRafG12C;Pika3calat mice were largely small alveolar or airway hyperplasias, and no fully formed adenomas were present in these mice. This resulted in an approximately 12-fold reduction in average tumor size (vehicle, 121,413 mm^2 vs. PD325901, 10,914 mm^2 for drug-treated mice, P < 0.0001) and a more than 20,000-fold reduction in overall tumor burden (vehicle, 20.7% vs. PD325901, 0.001%; P = 0.0004) compared with controls (Fig. 4B).

Consequently, these data indicate that BRAFV600E/PIK3CAH1047R lung tumors remain sensitive to the antitumor effects of MEK1/2 inhibition and highlight the central importance of BRAFV600E->MEK->ERK signaling in the growth of BRAFV600E/PIK3CAH1047R-induced lung tumors.

Enhanced therapeutic benefit of combined BRAFV600E plus PIK3CAH1047R inhibition against BRAFV600E/PIK3CAH1047R-expressing lung tumors

To examine the requirement for sustained signaling in maintenance of BRAFV600E/PIK3CAH1047R-expressing lung tumors, we initiated tumorigenesis in 34 BRafG12C;Pika3calat mice and monitored them for 5 weeks until they were close to end-stage. At this time, two mice were euthanized with the remaining mice divided into four cohorts for the following interventions: (i) no treatment; (ii) class 1 PI3K inhibitor (BKM-120, 60 mg/kg, every day, orally); (iii) BRAFV600E inhibitor (LGX-818, 30 mg/kg, every day, orally); or (iv) combination of BKM-120 and LGX-818 (60 and 30 mg/kg, respectively, every day, orally; refs. 28, 29). Drug benefit was assessed by Kaplan–Meier analysis with mice euthanized when their BCS was 2 or less (Fig. 4C; ref. 16). In addition, we monitored mouse body weight, which correlates with changes in tumor burden (Fig. 4D).

There was no statistical difference in survival between untreated (n = 6) or BKM-120-treated (n = 8) mice, all of which lost weight requiring euthanasia within 5 days after dosing (Fig. 4C and D). In contrast, mice treated with LGX-818 (n = 8) or combined LGX-818 plus BKM-120 (n = 8) displayed increased survival (Fig. 4C) such that no mice in the combination arm required euthanasia. Although LGX-818-treated mice continued to lose weight, this was at reduced rate and only one-eighth mice developed end-stage disease. Finally, mice treated with combined LGX-818 plus BKM-120 displayed immediate signs of improvement in their BCS that continued throughout the treatment period (Fig. 4D).

Analysis of mouse lungs from each group indicated that untreated and BKM-120–treated mice displayed a substantial tumor burden (average of ~28% and 23%) with no difference between them, consistent with the lack of benefit afforded by BKM-120 (Fig. 4E and F). In contrast, LGX-818–treated mice displayed reduced tumor burden (~10%) compared with control or BKM-120–treated mice. The therapeutic benefit of LGX-818–treated mice was further enhanced by combination with BKM-120 (~3% tumor burden) consistent with the survival and health benefits afforded these mice. Taken together, combined blockade of BRAFV600E and PIK3CAH1047R signaling in BRAFV600E/PIK3CAH1047R-expressing lung tumors was superior to blockade of BRAFV600E or PIK3CAH1047R alone.

PIK3CAH1047R promotes malignant progression of BRAFV600E-induced tumors

KRASG12D-induced lung tumorigenesis in GEM models depends on RAF—MEK—ERK, PI3K, RAC1 and NF-κB signaling (7, 9, 30–33). However, to date, activation of single nodes downstream of KRAS.GTP fails to recapitulate the malignant progression displayed by KRASG12D-initiated lung tumors. Although BRAFV600E induces benign lung tumors, such lesions are low grade and fail to progress to lung cancer (6, 7). As shown here, although PIK3CAH1047R is unable to initiate lung tumorigenesis, it promotes the growth BRAFV600E-initiated lung...
tumors. This prompted us to evaluate whether BRAF\textsuperscript{V600E} and PIK3CA\textsuperscript{H1047R} might cooperate for malignant lung cancer progression. To that end, tumors were initiated in either BRaf\textsuperscript{CA} or BRaf\textsuperscript{CA}; Pik3ca\textsuperscript{lat} mice (5 \times 10\textsuperscript{5} pfu Ad-Cre) with mice euthanized 25 weeks later for evaluation (7, 34).

In contrast with early time points, overall tumor number was not different between BRaf\textsuperscript{CA} and BRaf\textsuperscript{CA}; Pik3ca\textsuperscript{lat} mice with an average of 30 tumors per lobe recorded in both genotypes (Fig. 5A and B). However, individual lung tumors in BRaf\textsuperscript{CA}; Pik3ca\textsuperscript{lat} mice were larger than those in BRaf\textsuperscript{CA} mice (35,000 vs. 5,000 \mu m\textsuperscript{2}; P = 0.0005; Fig. 5B). Differences in tumor size were reflected by overall increased tumor burden in BRaf\textsuperscript{CA}; Pik3ca\textsuperscript{lat} mice (24% vs. 6%; P = 0.0001; Fig. 5B).

These data suggest that PIK3CA\textsuperscript{H1047R} does not influence the
frequency of BRAF<sup>V600E</sup>-initiated cells in the lung epithelium, but decreases the latency period before tumorigenesis and also increases their overall proliferative potential.

One possible explanation for the larger tumors observed in BRaf<sup>CA; Pik3calat</sup> mice is that each tumor arises in a polyclonal manner from more than one initiated cell. To test the clonality of BRAF<sup>V600E</sup>/PIK3CA<sup>H1047R</sup> lung tumors, we generated BRaf<sup>CA;</sup> Pik3calat<sup>-</sup>; R26Confetti mice containing the "Confetti" reporter (Gt(ROSA)<sup>26Sortm1(CAG-Brainbow2.1)Cle</sup>, R26Confetti hereafter; Supplementary Fig. S2; ref. 35). The action of Cre on the R26<sup>Confetti</sup> reporter elicits a recombination event that stochastically places one of four different fluorescent proteins (FP) downstream of the CAG promoter. Therefore, if BRAF<sup>V600E</sup>/PIK3CA<sup>H1047R</sup>-induced lung tumors are clonally derived, each tumor should express a single FP. However, if tumors are polyclonal in origin, they will contain a mixture of cells with different FP expression. BRaf<sup>-</sup>; Pik3calat<sup>−/−</sup>; R26<sup>Confetti</sup> mice were infected with 10<sup>7</sup> pfu Ad-Cre, with lung tumors analyzed 9 weeks later. Individual BRAF<sup>V600E</sup>/PIK3CA<sup>H1047R</sup> lung tumors were uniformly positive for a single FP, indicating that each tumor is clonally derived from a single initiated cell (Supplementary Fig. S2).

To assess malignant progression, we used a grading scheme based on the classification of lung lesions in GEM models to assess the grade of more than 250 tumors from mice of each genotype.
genotype (Fig. 5C and Supplementary Fig. S3A and S3B; refs. 12, 34). Hyperplasia was designated grade 1, benign adenomas as grade 2, larger adenomas as grade 3, and adenocarcinomas as grade 4. BRAFV600E-induced lung lesions were predominantly grade 1 or 2 with rare examples of grade 3 detected. In contrast, BRAFV600E/PIK3CAH1047R-induced lesions were generally grade 2 or 3 adenomas but approximately 3% of tumors were malignant grade 4 adenocarcinomas or adenocarcinoma lesions. These data indicate that BRAFV600E cooperates with PIK3CAH1047R to both accelerate early-stage lung tumorigenesis and promote late-stage malignant progression (12).

To determine if the cell of origin might influence the spectrum of lung tumors initiated in these mice, we used adenovirus vectors with lung cell-specific expression of Cre recombinase (14). Ad-SPC-Cre, Ad-CCS-P-Cre, and Ad-CGRP-Cre direct Cre expression to alveolar type II pneumocytes, Clara, or neuroendocrine cells, respectively. Of these, we noted that Ad-SPC-Cre was the most potent inducer of lung tumorigenesis in either BrafCA or BrafCA; Pik3cafl/fl mice, and was equivalent in potency to Ad-CMV-Cre. Moreover, tumors emerging in mice initiated with either Ad-CCS-P-Cre or Ad-CGRP-Cre, which were less potent in inducing lung tumorigenesis, displayed the cuboidal morphology and expression of SP-C characteristic of AT2 cells (data not shown).

In these experiments we assessed tumor grade at 22 weeks in groups of BrafCA or BrafCA; Pik3cafl/fl mice initiated with Ad-SPC-Cre to confirm the ability of PIK3CAH1047R to promote malignant progression of BRAFV600E-initiated lung tumors (Supplementary Fig. S4). As before, the majority of BRAFV600E-expressing lung tumors were small, grade 2 adenomas. In contrast, more than 50% of lung tumors in the BrafCA; Pik3cafl/fl mice were high-grade adenomas (grade 3) and 8.5% were either grade 4 adenocarcinomas or adenocarcinomas, confirming observations made with Ad-CMV-Cre (36).

TP53 constrains malignant progression of BRAFV600E/PIK3CAH1047R lung tumors

TP53 mutations frequently coexist with mutations in KRAS, BRAF, or PIK3CA mutations in human lung adenocarcinomas and TP53 mutation or silencing promotes malignant progression of either KRASG12D or BRAFV600E-driven lung tumors (6, 12). Consequently, we determined if TP53 loss would enhance lung tumorigenesis initiated by the combined expression of BRAFV600E and PIK3CAH1047R. Tumorigenesis was initiated in a BrafCA/c; Pik3cafl/fl, Trp53slox/lox mouse with a BrafCA; Trp53slox/lox mouse as a littermate control. Within 4 weeks, the former mouse displayed end-stage disease requiring euthanasia. Although the BrafCA; Trp53slox/lox mouse had a tumor burden of 0.2% made up of mainly small hyperplasias with a solitary benign adenoma detected (Supplementary Fig. S5A), the BrafCA/c;c, Pik3cafl/fl, Trp53slox/lox mouse had a tumor burden of 41% made up of high-grade, anaplastic adenocarcinoma. Moreover, these high-grade tumors stained positive for vimentin, a marker of epithelial–mesenchymal transition, lacked TTF-1/NKX2-1 expression, a marker of the distal lung epithelium, and displayed a high proliferative index (Ki67; Supplementary Fig. S5B). Similar results were obtained using a second BrafCA/c; Pik3cafl/fl, Trp53slox/lox mouse.

PIK3CAH1047R promotes BRAFV600E-induced oncogenic transformation in vitro

An advantage of GEM models of cancer is the ability to engineer defined genetic alterations and then generate cell lines for in vitro analysis. To that end, we generated BRAFV600E/TP53Null and BRAFV600E/PIK3CAH1047R/TP53Null mouse lung cancer-derived cell lines (BT and BPT cells hereafter) from suitably manipulated mice (7).

Because BT cells displayed a limited capacity for anchorage-independent growth, we engineered them to express either mCherry (control), PIK3CAH1047R or a myristoylated, constitutively activated AKT1 (M AKT1) to test whether activation of PI3K signaling might cooperate with BRAFV600E to influence the behavior of these cells (Fig. 6). Cell extracts were analyzed by immunoblotting for the activation status of key nodes in the PI3K signaling pathway (Fig. 6A; Supplementary Fig. S8A). Compared with control, cells expressing either PIK3CAH1047R or M AKT1 displayed elevated pAKT, p4E-BP1, pFOXO1, and pGSK3β. PIK3CAH1047R and M AKT1 cells also showed a decrease in p27KIP1 expression. Consistent with the ability of PIK3CAH1047R to cooperate with BRAFV600E in lung tumorigenesis in vivo, expression of either PIK3CAH1047R or M AKT1 led to increased anchorage-independent colony formation in vitro (Fig. 6B). Indeed, the parental and mCherry control BT lung cancer cells failed to form colonies in agarose even when cultured for approximately 8 weeks. In contrast, the PIK3CAH1047R or M – AKT1 expressing cells cloned with an efficiency of 1% and 1.4%, respectively, after 3 weeks. These data indicate that PI3K AKT signaling cooperates with BRAFV600E in oncogenic transformation of cultured lung cancer cell lines.

The ability of BPT lung cancer cells to form colonies in agarose was assessed in the presence of various pathway-targeted inhibitors. Unlike BT cells, BPT cells are capable of anchorage-independent colony formation (vehicle; Fig. 6C). However, when cultured in the presence of an MEK inhibitor, a class 1 PI3K inhibitor, or an AKT inhibitor, colony formation was abolished even when cultured for 8 weeks (Fig. 6C; ref. 37). These data emphasize the ability of PI3K AKT signaling to cooperate with BRAFV600E in promoting the transformed phenotype on lung cancer cells.

BRAFV600E/PIK3CAH1047R/TP53Null lung cancer cell lines are sensitive to combined pharmacologic inhibition of RAF or PI3K pathway inhibition

To assess the mechanisms of cooperation between BRAFV600E and PIK3CAH1047R on the behavior of BPT cells, they were treated with various pathway-targeted inhibitors of BRAFV600E or PIK3CAH1047R signaling, either alone or in combination, at which time effects on cell viability or the cell-division cycle were assessed (Fig. 7A–C).

As expected, single-agent inhibition of MEK1/2 or class 1 PI3Ks effectively decreased cell proliferation. Moreover, the combination of these agents had the strongest antiproliferative...
Mutationally Activated PIK3CA Cooperates with BRAF^{V600E}

Figure 6. Ectopic expression of either activated PIK3CA or AKT1 promotes anchorage-independent growth to BRAF^{V600E}/TP53null lung cancer-derived cells. A, BRAF^{V600E}/TP53null lung cancer-derived cells were infected with retroviruses as described earlier. Cell extracts were analyzed by immunoblotting as indicated. Quantification of blots is included as Supplementary Fig. S6A, B, anchorage-independent growth of BRAF^{V600E}/TP53null lung cancer cells engineered to express mCherry, PIK3CA^{H1047R}, or M^1AKT1 was quantified over 3 weeks. C, BRAF^{V600E}/ PIK3CA^{H1047R}/TP53null lung cancer cells were plated in soft agar in the absence (vehicle) or presence of inhibitors of MEK1/2 (1 \mu mol/L PD325901), class 1 PI3Ks (5 \mu mol/L GDC-0941), or AKT (5 \mu mol/L MK-2206). Anchorage-independent growth was assessed over 3 weeks.

To assess the effects of the various agents on the cell-division cycle, BPT cells were incubated with the various agents either alone or in combination and then labeled with BrdUrd to assess S-phase progression (Fig. 7C; see Supplementary Table S1 for drug concentrations). Vehicle-treated cells displayed a high proliferative index with more than 75% of cells incorporating BrdUrd. As expected, single-agent blockade of MEK1/2, ERK1/2, PI3K, or AKT led to decreased BrdUrd incorporation and increased the G_1 population with the PI3K inhibitor most potent, the MEK and ERK inhibitors roughly equipotent, and the AKT inhibitor least potent. Cells treated with combined MEK1/2 plus PI3K or ERK1/2 plus AKT inhibitors displayed the most profound effects on the cell-division cycle, in addition to displaying evidence of cell death as assessed by the number of nonadherent cells and cells with a sub-G_1 DNA content (Fig. 7C and Supplementary Table S2). Taken together, these data indicate that BRAF^{V600E} and PIK3CA^{H1047R} cooperatively regulate the cell-division cycle and cell death in vitro.

BRAF^{V600E} and PIK3CA^{H1047R} likely regulate many biochemical processes in the cell to influence cell proliferation in vitro and tumorigenesis in vivo. To assess the molecular mechanisms of such oncogenic cooperation, we used RPPA analysis to quantify protein expression/modification in BPT cells treated with inhibitors (Fig. 7D). Triplicate cultures of BPT cells were treated with inhibitors of MEK1/2 (PD), ERK1/2 (SCH), class 1 PI3Ks (GDC), or AKT1-3 (MK) either alone or in combination as indicated (Fig. 7D). Before RPPA analysis, we confirmed that each of the agents had the anticipated biochemical effects (Supplementary Fig. S6B). As expected, inhibition of MEK1/2 or ERK1/2 led to strongly diminished pERK1/2 and promoted a modest increase in pAKT. Inhibition of PI3Ks or AKT led to strongly diminished pAKT with no obvious effects on pERK. Finally, the combinations of inhibitors potently suppressed both pERK1/2 and pAKT.

The triplicate samples were subject to RPPA analysis using antisera against more than 150 proteins or protein modifications (20, 21). Unsupervised hierarchical clustering of the data indicated that samples clustered on the basis of the pathway inhibited such that MEKi- or ERKi-treated samples clustered together as did PI3Ki- and AKTi-treated samples (Fig. 7D and Supplementary Fig. S7). Samples from cells treated with combined pathway inhibitors clustered separately from samples from cells treated with the various single agents with the...
Cell viability (fold relative to vehicle)

<table>
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<tr>
<th>µmol/L PD:</th>
<th>0</th>
<th>0.015</th>
<th>0.03</th>
<th>0.06</th>
<th>0.125</th>
<th>0.25</th>
<th>0.5</th>
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<td>µmol/L GDC:</td>
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<td>0.15</td>
<td>0.3</td>
<td>0.6</td>
<td>1.25</td>
<td>2.5</td>
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Percentage of cells in the cell cycle

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<th>G2</th>
<th>S</th>
<th>G1</th>
<th>Sub-G1</th>
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<tr>
<td>0%</td>
<td>20%</td>
<td>40%</td>
<td>60%</td>
<td>80%</td>
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Cell-cycle progression and prevention of apoptosis

- KRAS<sup>G12D</sup>
- PIK3CA<sup>H1047R</sup>
- BRAF<sup>V600E</sup>

<table>
<thead>
<tr>
<th>Genes</th>
<th>pAKT pS473</th>
<th>tAKT</th>
<th>pERK1/2 pT202/Y204</th>
<th>tERK 1/2</th>
<th>BIM</th>
<th>c-MYC</th>
<th>CC3</th>
<th>CC7</th>
<th>CDK4</th>
<th>p27&lt;sup&gt;Kip1&lt;/sup&gt; pS293/p15</th>
<th>p4E-BP1 pS65</th>
<th>pPRAS40 pT246</th>
<th>pRP-S6 pS240/244</th>
<th>Cyclin D1</th>
<th>Cyclin A</th>
<th>β-Actin</th>
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<td>PD+GDC</td>
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Genes and pathways of interest:

- KRAS<sup>G12D</sup>
- PIK3CA<sup>H1047R</sup>
- BRAF<sup>V600E</sup>
- No lung pathology

Prevention of apoptosis and cell-cycle progression

<table>
<thead>
<tr>
<th>Gene</th>
<th>Prevention of apoptosis</th>
<th>Cell-cycle progression</th>
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<tbody>
<tr>
<td>KRAS&lt;sup&gt;G12D&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BRAF&lt;sup&gt;V600E&lt;/sup&gt;</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PIK3CA&lt;sup&gt;H1047R&lt;/sup&gt;</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>PIK3CA&lt;sup&gt;H1047R&lt;/sup&gt;</td>
<td>BA</td>
<td>NSCLC</td>
</tr>
</tbody>
</table>

Time

- ?
- +
- +

Graphical representations and diagrams showing changes in cell viability, cell-cycle progression, and protein expression levels under different conditions.
samples from cells treated with combined MEK1/2 plus class PI3K showing the greatest separation.

RPPA analysis generated predictions with regard to the consequences of inhibition of BRAFV600E or PIK3CAH1047R signaling on regulators of cell proliferation, some of which were then validated in an independent sample set by immunoblotting (Fig. 7E and Supplementary Fig. S8). As predicted by RPPA analysis, inhibition of MEK1/2—ERK1/2 signaling led to increased expression of the proapoptotic BCL-2 family protein BIM and decreased expression of c-MYC as well as cyclins D1 and A. In addition, inhibition of PIK3CAH1047R, but not AKT, promoted BIM expression and inhibited c-MYC and cyclins D1 and A, and led to elevated p27kip1 expression and suppression of p70S6. Consistent with the lack of the effect of MK-2206 against the proliferation of cultured BPT cell, we noted that inhibition of AKT had no single-agent effect on BIM, c-MYC, cyclins D1 or A, p-H-IPI3 or p-H-S6, but largely extinguished phosphorylation of PRAS40, a known AKT substrate. Finally, combined inhibition of both MEK and PI3Ks displayed additive effects on expression of c-MYC, cyclins D1 and A and BIM, the latter correlating with increased cleaved caspases-3 (CC3) and -7 (CC7) that were also predicted by RPPA analysis. These analyses indicate the complexity with which these pathways cooperatively regulate apoptosis and the cell division cycle, which in turn influences tumor latency, burden, and malignant progression. Although these experiments do not identify a single mechanism of BRAFV600E–PIK3CAH1047R cooperation in lung carcinogenesis, they suggest numerous areas of future investigation.

Discussion

The mechanisms by which KRASG12D promotes lung carcinogenesis in humans and mice remain incompletely understood. However, here we show that two well-credentialed KRAS/GTP effectors, BRAF and PI3KCA, cooperate to mimic the effects of oncogenic KRASG12D in GEM models of lung cancer. Although BRAFV600E expression elicits benign lung tumorigenesis, such tumors rarely progress to lung cancer, which contrasts with KRASG12D-initiated lung tumors (Fig. 7F). Unexpectedly, we noted that PIK3CAH1047R failed to initiate lung tumorigenesis, even when combined with PTEN silencing, a result at odds with a previous report (Fig. 7F; ref. 9). Indeed, no combination of heterozygous or homozygous Pik3cafl and Ptenfl initiated lung tumorigenesis, contrasting with the effects of a tetracycline-inducible PIK3CAH1047R transgene. It remains possible that strength of pathway activation, genetic background, or the promoter system may influence these disparate results, as suggested by similar observations in pancreatic cancer (31, 39). However, the lack of PIK3CAH1047R effects in lung is consistent with the effects of PIK3CAH1047R in ovarian epithelial cells (10). Furthermore, others have shown that PI3K activation through PTEN silencing failed to elicit lung tumorigenesis (22). It is unclear whether the inability of PIK3CAH1047R to initiate lung tumorigenesis will be predictive of antitumor effects of pharmacologic inhibition targeting of PI3K in the clinic (9).

Despite its inability to initiate tumorigenesis, PIK3CAH1047R cooperated with BRAFV600E in dramatic fashion to promote lethal lung tumorigenesis, which occurred with short latency and complete penetrance (Fig. 7F). That PTEN silencing also cooperated with BRAFV600E, albeit less strikingly, confirmed these observations, indicating an important role for PI3-lipid signaling. Although at early time points, BRAFV600E/PIK3CAH1047R–induced tumors were benign, 6 months postinitiation there was evidence of cancer progression in a manner reminiscent of KRASG12D. Finally, TP53 silencing, in conjunction with combined BRAFV600E/PIK3CAH1047R expression, led to rapid lung cancer development, which showed signs of dedifferentiation and epithelial-to-mesenchymal transition, hallmarks of advanced disease (40–42).

In conjunction with previous reports of the MEK1/2 and PI3K dependency of KRASG12D–induced lung tumors (43), the simplest explanation is that combined activation of RAF—MEK—ERK and PI3K—AKT signaling cooperates to accelerate benign tumorigenesis and to promote malignant lung cancer progression. Although activation of BRAF and PI3K signaling accounts for much of the phenotype associated with KRASG12D expression, a role for other KRASG12D effectors remains possible because approximately 10% of KRASG12D-initiated tumors display malignant progression, whereas only approximately 3% to 8% of BRAFV600E/PIK3CAH1047R tumors do so (7). Indeed, because approximately 10% or less of KRASG12D– or BRAFV600E/PIK3CAH1047R–initiated lung tumors display malignant progression, there must be additional stochastic genetic/epigenetic changes in benign tumor cells that promote malignant progression as emphasized by the effects of TP53 silencing on the progression of KRASG12D– or BRAFV600E/PIK3CAH1047R–initiated lung tumors.

The absence of malignant progression of BRAFV600E–initiated lung tumors has been ascribed to a senescence-like...
proliferative arrest, which is due to feedback inhibition of P38 lipid production (44). This hypothesis is supported by our data because PIK3CA<sup>H1047R</sup> expression enhanced the proliferative capacity of BRAF<sup>V600E</sup>-initiated lung tumors. However, it remains unclear how PI3K signaling sustains the proliferation of these tumors. Previous data indicate cooperative regulation of cyclins and cyclin-dependent kinase inhibitor expression by these pathways (45). Analysis of BPT cells suggests that the cell-division cycle is, indeed, under the coordinate control of both BRAF<sup>V600E</sup> and PIK3CA<sup>H1047R</sup>. However, future experiments are required to determine which of the nodes of cell-cycle regulation are essential to the observed cooperation.

BPT cells were sensitive to combined inhibition of BRAF<sup>V600E</sup> and PIK3CA<sup>H1047R</sup> with pathway-targeted agents. In general, single-agent inhibition of either pathway had inhibitory effects on the cell-division cycle without promoting apoptosis. In contrast, combined pathway inhibition had stronger inhibitory effects on the cell cycle and also promoted cell death in vitro and in vivo. That AKT inhibition was without effect against lung tumors initiated by BRAF<sup>V600E</sup> but potently suppressed the cooperation between BRAF<sup>V600E</sup> and PIK3CA<sup>H1047R</sup> suggests a genotype–drug response phenotype with regard to AKT inhibition in GEM models. Whether these data will translate into the clinical use of single or combined pathway-targeted interventions in humans with BRAF-mutated lung cancer remains an open question. Although KRAS mutation is frequent in lung cancer, expression of BRAF<sup>V600E</sup> or PIK3CA<sup>H1047R</sup> accounts for a smaller percentage of patients with NSCLC (2). Because mutated KRAS has proved to be an intractable pharmacologic target, it will be interesting to determine whether combined pathway-targeted inhibition of RAF and PI3K signaling will promote regression of KRAS-mutated lung cancers.

Disclosure of Potential Conflicts of Interest

M. McMahon has commercial research grant from Novartis. No potential conflicts of interest were disclosed by the other authors.

References


Authors' Contributions

Conception and design: C.L. Trejo, M. McMahon

Development of methodology: C.L. Trejo, E.A. Collisson, M. McMahon

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.L. Trejo, S. Green, Y. Marsh, E.A. Collisson, W.A. Phillips

Analysis and interpretation of data (e.g., statistical analysis, biosistics, computational analysis): C.L. Trejo, S. Green, Y. Marsh, M. McMahon

Writing, review, and/or revision of the manuscript: C.L. Trejo, S. Green, V. Marsh, E.A. Collisson, G. Iezza, W.A. Phillips, M. McMahon

Study supervision: C.L. Trejo, M. McMahon

Other (first author): C.L. Trejo

Other (hlistologic analysis and interpretation): G. Iezza

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Mutationally Activated PIK3CA<sup>H1047R</sup> Cooperates with BRAF<sup>V600E</sup> to Promote Lung Cancer Progression

Christy L. Trejo, Shon Green, Victoria Marsh, et al.


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