Identification of a targetable KRAS-mutant epithelial population in non-small cell lung cancer

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Lung cancer is the leading cause of cancer deaths. Tumor heterogeneity, which hampers development of targeted therapies, was herein deconvoluted via single cell RNA sequencing in aggressive human adenocarcinomas (carrying Kras-mutations) and comparable murine model. We identified a tumor-specific, mutant-KRAS-associated subpopulation which is conserved in both human and murine lung cancer. We previously reported a key role for the oncogene BMI-1 in adenocarcinomas. We therefore investigated the effects of in vivo PTC596 treatment, which affects BMI-1 activity, in our murine model. Post-treatment, MRI analysis showed decreased tumor size, while single cell transcriptomics concomitantly detected near complete ablation of the mutant-KRAS-associated subpopulation, signifying the presence of a pharmacologically targetable, tumor-associated subpopulation. Our findings therefore hold promise for the development of a targeted therapy for KRAS-mutant adenocarcinomas.

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Non-small cell lung cancer (NSCLC), the most common epithelial tumor, comprising ~85% of pulmonary malignancies, is the leading cause of cancer-related deaths. Considerable heterogeneity exists among lung adenocarcinomas (ADCs). Among the genes implicated in their etiology, frequent activating mutations in KRAS have been identified in 10–30% of cases. In addition, loss-of-function mutations in p53 occur in ~50–70% of cases and co-occur with KRAS mutations in ~40% of cases. Besides direct covalent KRAS-G12C inhibition, no therapies have been approved for mutant-KRAS NSCLCs; therefore identification of tumorigenic subpopulations sustaining growth may contribute to improved targeted therapies.

Resolving the distinct subpopulations of healthy versus tumor-bearing lungs has been hampered by traditional ensemble-based methods such as bulk RNA sequencing, and gaps-in-knowledge on specific phenotypic markers. Recently, single-cell RNAseq (sc-RNAseq) has enabled analysis of complex tissues and characterization of cellular identity, by grouping cells based on their gene expression profiles, at an unprecedented high-resolution.

Pulmonary sc-RNAseq on tumor epithelial cells represents an underdeveloped field. A pioneering study on fluorescence-activated cell sorting-purified murine lungs distinguished healthy multi-potential, bipotential, and mature alveolar type II (ATII) epithelial cells. Subsequently, identification of markers for major normal body-wide lineages gave rise to the mouse cell atlas (MCA) with similar efforts currently underway for humans as part of the Human Cell Atlas. Pulmonary-associated immune cells in healthy, inflamed, or transformed lungs have been identified in both human and murine tissues, including our study comparing tumor-infiltrating myeloid subpopulations in both species NSCLCs.

Although tumor heterogeneity hampers major therapeutic advancements, little is known on how transformation events orchestrate molecular/cellular alterations within lung cancer. Our deconvolution of human NSCLCs leads to the identification of a distinct epithelial subpopulation, selectively detectable in ADCs carrying the aggressive mutant-KRAS oncogene.

We also comprehensively mapped pulmonary subpopulations in normal and tumor-bearing lungs, by adopting a model of ADC (Kras+/G12D;Trp53−/−, henceforth referred to as KP), which combines Kras activation with p53 ablation in pulmonary epithelium. Our data produced a unique cellular atlas of healthy lungs and KP ADCs, and found new cell subtypes that are distinctly associated with disease. Newly identified tumornenriched subpopulations were discovered, of which one represents a novel specific epithelial tumor cluster, matching a signature of markers that we also selectively identified in the human mutant-KRAS-specific subpopulation. Both murine and human mutant-KRAS-specific subpopulations are positive for the oncogene Bmi-1 (B-cell-specific Moloney murine leukemia virus integration site 1), a key component of the epigenetic complex polycomb repressive complex-1, which belongs to the 11-gene integration site 1), a key component of the epigenetic complex.

sc-RNAseq deconvolution of human NSCLCs unravels tumor heterogeneity between wild-type and mutant KRAS ADCs. To study the epithelial component constituting human NSCLCs, we performed sc-RNAseq analysis on freshly isolated biopsies from 12 patients (Supplementary Table 1). Once inter-sample and batch variability was accounted for, defined subpopulations were identified using SingleR, which used the annotated Human Primary Cell Atlas data set for reference cell signatures. Despite the typical high genomic variability of human NSCLCs, we were able to identify common subpopulations, which we visualized utilizing SPRING.

By splitting only the ADC samples according to their KRAS mutation status (mutants n = 8, wt = 2), we discovered the presence of a transcriptional epithelial cluster, C10, which was almost exclusively detected in mutant-KRAS ADCs alone (false discovery rate (FDR) = 2.07 × 10−118) (Fig. 1f, Supplementary Figures 1a–c, Supplementary Table 2, and Supplementary Data), urging us to ask whether it may represent a novel subpopulation capable of distinguishing the most aggressive and still untreatable mutant-KRAS ADCs from the KRAS WT ADCs.

sc-RNAseq of healthy and tumor-bearing lungs highlights the presence of a transformed epithelial tumor-specific cluster. Having identified a specific epithelial tumor subpopulation in human mutant-KRAS ADCs, this prompted us to ask whether we may detect its murine counterpart, and subsequently attempt its in vivo targeting, by employing KP mice that develop pulmonary ADCs.

By performing sc-RNAseq on normal lungs from control littermates and KP tumors, we identified which subpopulations were present in tumors versus healthy lungs. Algorithmically, 13 clusters (C1–C13) were identified (Fig. 2a). These clusters were transcriptionally distinct, as shown by the top 25 marker genes per cluster in both healthy (heatmaps and gene lists in Supplementary Figure 2a and Supplementary Table 3, respectively) and tumor tissues (Supplementary Figure 2b and Supplementary Table 4). By comparing lung tumors with healthy lungs by cell number quantification, we found that C3, C4, C7, and C11 were significantly underrepresented in tumors (FDR C3 = 0, C4 = 4.42 × 10−10, C7 = 1.47 × 10−288, C11 = 1.12 × 10−96), whereas clusters C1, C2, C5, C8, C10, C12, and C13 were enriched (FDR C1 = 2.46 × 10−3, C2 = 4.22 × 10−3, C5 = 4.39 × 10−15, C8 = 4.92 × 10−95, C10 = 1.36 × 10−192, C12 = 1.05 × 10−72, C13 = 1.22 × 10−289), as compared with control lungs (Fig. 2b and Supplementary Table 5). C9 instead was equally distributed in both tissues (FDR 6.58 × 10−1). Clusters C2, C10, and C13 were almost virtually exclusive to tumors, being barely detectable in healthy lungs, and therefore representing epithelial tumor-enriched clusters (TECs) (Figs. 2a and 2b, Supplementary Figure 2c).

In addition, to classify the cell types within each cluster, a hybrid annotation strategy was employed wherein immunological
cells were identified using the Immgen data set\textsuperscript{35} via SingleR, and the remaining cells annotated using MCA transcriptional maps\textsuperscript{8} to leverage the strengths of each annotation data set. This approach enabled accurate detection of numerous epithelial cell types (Fig. 2c), namely alveolar bipotent progenitors, alveolar type I (ATI), ATII, basal epithelial, ciliated, club as well as endothelial, immune, and fibroblast subpopulations (Fig. 2d). Cell number quantification revealed that ATII-like cells are the most enriched within the tumor milieu expanding to 31.7%, as compared with 1.2% in healthy tissues (Supplementary Table 6).

By superimposing cell annotations (Fig. 2d) on the cluster distribution (Fig. 2a), our analysis revealed an unprecedented

**Fig. 1 Single-cell RNA sequencing deconvolution of human NSCLC unravels tumor heterogeneity between WT and mutant KRAS adenocarcinomas.**

\textbf{a} SPRING plot of the 15 human clusters identified in 12 NSCLCs. Each point represents one cell. Each color represents a defined transcriptomic cluster. 

\textbf{b} Epithelial cells (green); \textbf{c} immune cells (panel legend defines subpopulations); \textbf{d} endothelial cells (maroon), and \textbf{e} fibroblasts (blue) were identified. 

\textbf{f} SPRING plots of the 15 clusters in ten ADC patients (eight carrying KRAS mutations, two wildtypes for KRAS). Dotted squares highlight cluster 10 (C10) is unique to the KRAS-mutant samples.
definition of the healthy and tumor epithelial compartment, wherein we observed that healthy ATII-like cells are comprised of two distinct transcriptomic profiles, defined as C13 (0.3%) and the slightly more abundant C8 (2.8%) (Fig. 2d, and Supplementary Table 5). Interestingly, in the tumor, in addition to the enrichment of these two ATII-like clusters (TEC-C13 = 17.8% and TEC-C8 = 12.8%), an additional cluster (TEC-C2) can also be annotated as ATII-like. Furthermore, TEC-C10, while being annotated as positive for a generic "epithelial" cell signature (Fig. 2d, right panel and labeled by the *), did not match any normal pulmonary epithelial subpopulation, therefore representing a bona fide transformed TEC/cell subpopulation. Overall, our data identified KP tumor-enriched epithelial cells, which are mainly comprised of the tumor-specific subpopulation (C10), which does not match any normal epithelial signature, as well as ATII cells, which themselves consist of different clusters, as described above.

Having identified C10 as being virtually unique to tumor tissues and not ascribable to any common, defined epithelial cell type, we attempted to further characterize its differences over the other tumor epithelial clusters. Transcriptionally, the top 50 highest and lowest expressing genes are sufficient to show the unique transcriptional profile of C10, as compared with all other tumor epithelial clusters (Fig. 3a). The genes represented in the heatmap are listed in Supplementary Table 7. Gene expression profiling and gene set enrichment analysis (GSEA) of C10 cells versus all other tumor epithelial cells, showed enrichment of (i) stem cell signatures (Fig. 3b), (ii) stemness genes (stem cells, embryonic, mammary stem cells, liver cancer stem cells), cancer radiotherapy responsiveness (Supplementary Figure 2d), as well as target genes of the cancer stem cell gene BMI-1 (Fig.3b and Supplementary Figure 2d). Consistently, RNA velocity analysis, which attempts to elucidate cell’s transcriptional differentiation trajectory or “direction”36, showed that C10 contains a

Fig. 2 Single-cell RNA sequencing of healthy and tumor-bearing lungs highlights the presence of a transformed epithelial tumor-specific cluster. a SPRING plots of the 13 clusters identified in healthy (n = 2, left panel) and tumor tissues (n = 3, right panel). b Percentages of clusters distributions in healthy (blue) and tumor (red) tissues. c SPRING plots showing the epithelial compartment (green) in healthy and tumor tissues. d SPRING plots showing the annotated cell types (panel legend) in healthy and tumor tissues. * labels TEC-C10; the dotted boxes highlight ATII-like cells.
transcriptomic point of origin (short/no arrows; Fig. 3c) from which, the other tumor epithelial cells contained within C10 stem. These data, therefore, support the hypothesis that C10 potentially contains tumor-initiating cells.

Human and murine KP ADCs display a tumor cluster specifically associated with harboring KRAS mutations. After comparing differentially expressed genes between mutant-KRAS-associated C10 clusters, in both humans and mice, versus all other tumor epithelial clusters, we identified a common mutant-KRAS-associated signature of 21 upregulated (LogFC ≥ 1.5) and nine downregulated (LogFC ≤ −1.5) homologous markers (Fig. 4a), which is specific for C10. SPRING plots display the colocalization of the combined upregulated (Fig. 4b, Supplementary 3a) and downregulated (Fig. 4c, Supplementary 3a) markers comprising the signature, thereby showing conservation across species. Representative expressions of selected marker genes contained within the signature are depicted in Figs. 4d and 4e. In particular, they represent genes contained within the 50 highest/upregulated (Fig. 4d) or lowest/downregulated (Fig. 4e) genes, commonly present in both the human and murine data sets. Upregulated genes contained within the conserved top 50 genes whose expression is highly restricted to C10 (Fig. 4d), act as oncogenes associated with poor prognosis in multiple human malignancies (Human Protein Atlas, www.proteinatlas.org37 and38–41), supporting the aggressive nature of C10. In addition, among the conserved downregulated 50 genes C10 is more negatively labeled by SFTPC and HOPX (Fig. 4e), the major markers for ATII and ATI cells, respectively, as well as other relevant differentiation markers of secretory (SCGB3A1) and ATII (SFTPA1, NAPSA, SLC34A2, LPCAT1, LAMP3) epithelial cells42,43 (Fig. 4e). Overall, these data indicate that C10 represents a transformed subpopulation that does not match normal alveolar subtypes, thus representing a novel subpopulation capable of distinguishing mutant-KRAS from WT-KRAS ADCs. Of note, Ingenuity Pathway Analysis (IPA) identified EIF2, mTOR, eIF4/p706SK, and integrin signalings as the common enriched pathways within the top five (“cellular growth, proliferation, and development” category) in both murine and human C10 (Fig. 4f), which all act, according to the curated IPA software, downstream of activated Kras (Supplementary Figures 3b–e).

In addition, GSEA showed that murine C10-upregulated genes were significantly enriched within three different murine-curated data sets derived from samples displaying Kras upregulation (Supplementary Figure 3f). A similar result is shown by comparing human C10-upregulated genes to two curated data sets displaying upregulated KRAS signaling as well (Supplementary Figure 3g). Conversely, a comparison between the human C10 with both a data set containing genes overexpressed in NSCLCs genetically defined by copy number amplification and a Reactome data set of EGFR signaling (another main lung cancer driver) did not show any significant enrichment (Supplementary Figure 3h). Overall, these data confirm C10 is specifically associated with Kras signaling.

![Fig. 3 The transformed epithelial tumor-specific cluster shows a defined signature. a Heatmap showing the 50 highest and lowest expressed genes in C10. b GSEA shows enrichment of stem cell module genes (FDR = 0.0) and Bmi-1 target genes (FDR = 0.0) in C10, as compared with all other tumor epithelial clusters. Normalized enrichment score (NES) is indicated in each panel. c RNA velocity analysis recapitulates dynamics of tumor epithelial cells (zoomed in the dotted box) differentiation. Within the dotted box short/no arrow-containing cells indicate the point of origin from which other C10 (orange) tumor cells are originated.](https://doi.org/10.1038/s42003-021-01897-6)
**Fig. 4** Human and murine KP ADCs display a tumor cluster specifically associated with harboring KRAS mutations. 

**a** Heatmap of the common differentially expressed genes in both murine (upper panel) and human (lower panel) KRAS-mutated ADCs, as defined by differential gene expression analysis. Row-scaled z scores of the log2-normalized expression values per gene are shown.

**b-c** SPRING plots showing the common signature enrichment score for C10 clusters, calculated for each cell equivalent to the number of detected genes from the common signature shown in **a**. For the positive/upregulated (4b) and the negative/downregulated signature (4c) the more genes detected per cell, the stronger the enrichment score, represented as a scale from 0 (gray) to 0.5 (blue) to 1 (red), where an enrichment score of 1 signifies detected expression of all marker genes within that cell. SPRING plots showing a visual representation of the log2-normalized gene expression for the selected **d**, upregulated, and **e** downregulated genes of interest in both murine (left panels) and human (right panels) C10 clusters.

**f** IPA analysis showing common top three enriched pathways in KP and Human C10s.
In addition, by utilizing the Enrichr enrichment suite, in both species, the molecular pathway downstream of the nuclear factor-kB subunit RelA/p65, which is required for Kras-induced lung tumorigenesis\textsuperscript{19} are found to be enriched in C10, as compared with the other epithelial clusters (FDR = 0.01 and 0.03, in mouse and human data set, respectively) (Supplementary Table 8). LGALS3, SI00A6, AGR2, and TFF1, which are contained within the mutant-KRAS-associated signature, are among the common RelA/p65-downstream effectors. IPA software also detected that both murine and human C10 display active Integrin, epidermal growth factor, insulin-like growth factor-1, and insulin receptor pathways (Supplementary Figure 3i), extracellular signal-regulated kinase/mitogen-activated protein kinase, and insulin receptor pathways (Supplementary Figure 3i), similarly found through proteogenomic network analysis on kinase, and insulin receptor pathways (Supplementary Figure 3i), extracellular signal-regulated kinase/mitogen-activated protein kinase, and insulin receptor pathways (Supplementary Figure 3i).

Fig. 5 C10-specific signature is conserved in multiple tissue ADCs and it is associated with lower overall survival. a Boxplots for distributions of the signature scores for combining the positive and negative signature genes over KRAS-mutant samples versus WT samples cross four adenocarcinomas (colon, COAD, N = 100 mutant KRAS and N = 211 WT; pancreas, PAAD, N = 127 mutant KRAS and N = 31 WT; stomach, STAD, N = 18 mutant KRAS and N = 294 WT). P values are indicated. b Survival analysis between C10 signature high and low groups (p value = 1.96 × 10^{-6}, hazard ratio = 2.37). C10 low group: n = 223, and C10 high group: n = 222.

PTC596 treatment of the mutant-KRAS A549 and SKLU1 cell lines affects their cell cycle progression. Having discovered that BMI-1 is expressed in both human and murine mutant-KRAS-associated clusters C10, we initially investigated the efficiency of its pharmacologically driven downregulation in mutant-KRAS ADC cells. By collaborating with PTC Therapeutics, we utilized two of their compounds, PTC596 (Supplementary Figure 4a) and its analog PTC028 (Supplementary Figure 4b). PTC596, identified by its ability to kill BMI-1\textsuperscript{+} cancer cells\textsuperscript{32}, is currently being tested in clinical trials for solid malignancies, as an orally bioavailable drug, which displays a long-circulating half-life, and lacks the multidrug transporter P-glycoprotein substrate activity\textsuperscript{47}. PTC596 and PTC028 reportedly result in hyperphosphorylated BMI-1\textsuperscript{32,48} associated with cell cycle arrest in G2-M\textsuperscript{31}. By treating A549 and SKLU1 cells with PTC596 (1 μM), PTC028 (1 μM) or vehicle control (0.5% dimethyl sulfoxide (DMSO)) for 24 h, 48 h, and 72 h, we confirmed by western blot presence of a band higher than 40–42 kDa at 24 h of drug treatment (Fig. 6a, b, Supplementary Figures 4c, d), corresponding to the hyperphosphorylated BMI-1 form\textsuperscript{32,48}. Concurrent cell cycle analysis carried out at 24 h (n = 3), when the major effect on BMI-1 hyper-phosphorylation is observed, concomitantly reveals a significantly higher number of cells in G2-M upon PTC596 (80.5% ± 3.7 p = 4.52 × 10^{-4} in A549; 69.9% ± 2.8 p = 4.58 × 10^{-4} in SKLU1) and PTC028 (80.2% ± 1.9 p = 6.05 × 10^{-6} in A549; 74.9% ± 3.1 p = 6.02 × 10^{-4} in SKLU1) treatment, as compared with DMSO-treated cells (15.2% ± 1.2 in A549; 35% ± 1.3 in SKLU1) (Fig. 6c–f).

BMI-1 acts as a major component of the chromatin remodeling complex PRC1, which ubiquitinates Histone H2A at lysine 119 (H2AK119ub). To determine whether PTC596 treatment affected BMI-1 activity, immunofluorescence staining for H2AK119ub, as readout for BMI-1 activity\textsuperscript{49} was performed. A549 cells treated with the clinical-grade compound PTC596, which was adopted in the subsequent murine in vivo studies, show almost undetectable H2AK119ub nuclear staining, as compared with vehicle-treated cells (n = 32 each, mean±standard error fluorescence for PTC596-treated cells 170 ± 26 and vehicle-treated 2800 ± 110; p = 2.90 × 10^{-24}), validating our pharmacological approach (Figs. 6g and 6h).

PTC596 in vivo treatment affects the growth of KRAS-mutant ADCs. As PTC596 and PTC208 treatments affect A549 cell cycle progression in culture, we tested their efficacy in in vivo tumor growth of human mutant-KRAS cells, by generating xenograft models of A549 cells in immunocompromised NSG mice. After subcutaneously injecting 2 × 10\textsuperscript{6} cells per flank, once subcutaneous tumors reached ~80–90 mm\textsuperscript{3}, we started bi-weekly treatment with PTC596 (n = 15), PTC028 (n = 7), or vehicle (n = 20) for 3 weeks. Figure 7a depicts the size of individually treated tumors at treatment termination, normalized to day 0. PTC596- and PTC028-treated xenografts showed a significant

\[ \text{P}(0.05) = 2.83 \times 10^{-7} \]
Fig. 6 PTC596 treatment of the KRAS mutant A549 and SKLU1 cell lines affects their cell cycle progression. Western blot analyses of human A549 and SKLU1 cell lines treated for 24, 48, and 72 hours with DMSO vehicle as control, and PTC596 or PTC028. Protein lysates were immunoblotted with an anti-BMI-1 antibody. Loading was assessed with an anti-β-actin antibody. The expected size is shown in kDa. The slower migrating hyperphosphorylated BMI-1 band is indicated by the arrowhead. Cell cycle analysis of the A549 and SKLU1 cell lines after treatment for 24 hours with DMSO (red), PTC596 (blue), or PTC028 (black). The bar charts represent the distribution of cells in G0/G1, S, and G2-M phases. P values are indicated. Error bars represent standard deviation (SD). Percentages of cells in each cell cycle phase are indicated. Fluorescence nuclear imaging of DNA and H2AK119ub in A549 cell line by confocal microscopy. Upper panels show DMSO-treated cells; lower panels show cells treated with PTC596 for 24 hours. Left panels show DNA staining by Hoechst 33342 (blue acquisition channel). Right panels show H2AK119ub staining by immunofluorescence (scale bar 10 μm). Dot plots of H2AK119ub average nuclear fluorescence for DMSO— (red) and PTC596-treated (blue) A549 cells. Error bars represent SD of the average values. Data are expressed in fluorescence counts which are proportional to H2AK119ub concentration. P value is indicated.
Fig. 7 PTC596 in vivo treatment affects the growth of KRAS-mutant ADCs. 

a) A549 xenografts tumor volumes at treatment termination (vehicle $n = 20$, red; PTC596 $n = 15$, blue; PTC208 $n = 7$, black) normalized to the tumor volume measured at the beginning of treatment. The difference in tumor size at day 21 was statistically significant ($p$ values are indicated).

b) SPRING plots showing epithelial compartments (green) in vehicle- ($n = 3$, left panels) versus PTC596-treated tumors ($n = 2$, right panels).

c) Pie charts summarizing the epithelial cell proportions in vehicle- (left panels) and PTC596-treated tumors (right panels).

d) SPRING plots of the 13 clusters in vehicle- (left panel) and PTC596- (right panel) treated tumor samples.

e) Pie charts summarizing epithelial sub-cluster compositions in vehicle- (left panels) and PTC596- (right panels) treated tumors.

f) The graph shows the percentage of change in tumor volume measured by MRI at the indicated time points, between PTC596 ($n = 7$, blue) and vehicle-treated ($n = 8$, red) groups. Error bars represent SD. $P$ value is indicated.
increase in average tumor size by 78.37% ($p = 9.52 \times 10^{-5}$) and 78.04% ($p = 9.70 \times 10^{-5}$) respectively, as compared to vehicle-treated mice.

Having proved these pharmacological treatments significantly affect tumor growth of human mutant-KRAS cells in vivo, we tested the clinical-grade PTC596 efficacy in the KP model of lung cancer. Here, we adopted a combined strategy by both profiling lung tumor response to PTC596 through sc-RNAseq and, concomitantly, monitoring in vivo tumor growth by bi-weekly MRI. When tumors reached a comparable size (~1–2 mm diameter), we enrolled the mice for PTC596 or vehicle treatment and followed their response for up to one month. At treatment completion, tumors were isolated and profiled by sc-RNAseq. SPRING plots displayed major differences in the epithelial subpopulations of PTC596- versus vehicle-treated mice (Fig. 7b).

Specifically, epithelial cells represent 47.9% of the vehicle-treated tumor cells, whereas they display an approximately twofold decrease (FDR = $5.75 \times 10^{-5}$), reaching a total of 22.2% overall cells, upon PTC596 treatment (Fig. 7c). SPRING plots in Fig. 7d depict cluster distribution in the vehicle- and PTC596-treated tumors. In particular, ATII-like clusters TEC-C8 and TEC-C13 showed a 5.6- and 2.7-fold reduction, respectively, decreasing their total cell number from 12.8% and 17.8% in vehicle-treatment to 2.3% and 6.6% in PTC596-treated tumors, respectively (TEC-C8 FDR = $3.28 \times 10^{-7}$ and TEC-C13 FDR = $2.76 \times 10^{-5}$) (Fig. 7e). TEC-C10, the epithelial tumor-specific cluster, showed a 5.4-fold reduction (from 11.8% to 2.2%, FDR = $3.96 \times 10^{-6}$) (Fig. 7e).

Consistently, MRI showed a concomitant significant decrease in tumor volume (Fig. 7f). Changes in lung tumor burden over the course of treatment were calculated as a percentage change in volume over tumor volume at day 1 (set at 100%). In PTC596-treated mice, tumor size started being significantly impaired by day 7, with notable tumor size regression after day 10. On day 28 of PTC596 treatment, tumors shrank by 83.7%, as compared to day 1. In addition, although tumor volume increased up to 156.1% by day 28 in the vehicle-treated group, drug-treated tumors shrank to 83.7%, resulting in a significant 46.3% reduction in tumor size (FDR = $2.36 \times 10^{-5}$), as also shown in the representative MRI images of tumors at day 1 and 28 of both vehicle and PTC596 treatment (Supplementary Figure 5a).

Overall, we show that PTC596 significantly decreases tumor size by MRI, in accordance with the major decrease detected in epithelial clusters, as assessed by sc-RNAseq. We also asked whether adopting selumetinib (an allosteric MEK1/2 inhibitor) in combination with PTC596 may show improved antitumor response. Remarkably, MRI data show that PTC596 alone achieves better results than selumetinib alone, and that their combined inhibition is not synergistic, in that adding selumetinib does not significantly improve the effects achieved by PTC596 alone (Supplementary Figure 5b).

Taken together, our data show that BMI-1 inhibition is better than MEK inhibition alone and that PTC596 is able to significantly affect murine mutant KRAS tumorigenesis in vivo, and almost completely abrogates (5.4-fold reduction, decreasing the subpopulation from 11.8% to 2.2%, FDR = $3.96 \times 10^{-6}$), the murine C10 subpopulation, which shares a common signature with the human mutant-KRAS-specific C10 subpopulation, as demonstrated by high-resolution transcriptomics.

Discussion
Considerable heterogeneity among ADCs of the lung exists, which hampers efficient therapeutic targeting. There remains much to be explored with respect to the physiological significance of molecular and cellular heterogeneity, in an attempt to design improved therapeutic treatments. Therefore, to study tumor milieus at the single-cell transcriptional level, we adopted the inDrop single-cell capture30, coupled with our newly designed annotation approach, which combines different annotation data sets, therefore allowing for more accurate and robust cell identification. By deconvoluting tumor milieus in both human NSCLCs and a murine model of Kras-driven lung cancer, we classified, in both species, clusters/subpopulations comprising the tumor microenvironment. Specifically, we identified endothelial cells, immune infiltrating cells, fibroblasts, and the epithelial component, which is the primary focus of our investigation, as it is a frequent target of transformation51. We herein report exceptional parallelism, detected at single-cell level, between human and murine mutant-KRAS-driven pulmonary ADCs, which are associated with poor prognosis and aggressiveness. Despite the increased intra-tumor heterogeneity of human ADC samples over clonally bred mice, we discovered a novel tumor epithelial subpopulation (referred to as C10 in the text), which was specifically found in human mutant-KRAS ADCs and KP tumors and virtually undetectable in human WT-KRAS ADCs and in murine healthy lungs. This subpopulation expresses a set of genes that we identified as a mutant-KRAS-associated signature. Of note, we observed that the mutant-KRAS-associated signature identified in pulmonary ADCs, is also predictive of KRAS mutation status in other ADCs (colon, pancreas, and stomach). These data suggest the signature contains oncogenic KRAS-signaling components that act independently of the specific cancer contexts, and that aggressive KRAS-mutant-driven malignancies may be similarly targeted.

Despite the current lack of unique markers to identify aggressive pulmonary subpopulations, we discovered a unique molecular signature associated with malignant C10 mutant-KRAS cells, which show downregulation of SGC3A1 (marker of airway secretory cells), SFTPA1, SFTPC, NAPSA, SLC34A2, LPCAT1, and LAMP3 (markers of ATII cells), as well as HOPX (the main marker of ATII cells)42,43, therefore confirming that such population does not match any of the other common epithelial cell type signatures. Furthermore, genes upregulated in the mutant-KRAS-specific signature contained oncopogenes involved in many human cancers, whose expression is often associated with poor prognosis (Human Protein Atlas, www.proteinatlas.org37 and38–41) and/or resistance to therapy, such as the membrane protein Plac8. Noteworthy, its upregulation causes NSCLC resistance to the tyrosine-kinase inhibitor Osimertinib52 and its silencing in renal cell carcinomas significantly increases their sensitivity to Cisplatin53.

We also observed that human C10, which is specific to KRAS mutant patients, is present in both TP53 mutant and TP53 wt patients, suggesting the mutant-KRAS-associated signature is independent of TP53-status. Besides having identified a mutant KRAS-specific signature, our results show at the single-cell level a model in which KP ADCs are enriched in ATII-like cells, supporting a model in which ATII cells are the cells-of-origin of pulmonary ADCs, and in murine healthy lungs. This subpopulation expresses a set of genes that we identified as a mutant-KRAS-associated signature. Of note, we observed that the mutant-KRAS-associated signature identified in pulmonary ADCs, is also predictive of KRAS mutation status in other ADCs (colon, pancreas, and stomach). These data suggest the signature contains oncogenic KRAS-signaling components that act independently of the specific cancer contexts, and that aggressive KRAS-mutant-driven malignancies may be similarly targeted.
GSEA of murine C10 cells versus all other tumor epithelial cells demonstrated enrichment of stem cell signatures and target genes of the cancer stem cell gene BMI-1. Consistently, RNA velocity analysis, a bioinformatic tool adopted to elucidate the transcriptional dynamics of cells' evolution, showed that C10 contains a point of origin able to give rise to the other tumor epithelial cells contained within C10, thus corroborating the hypothesis that C10 might contain tumor-initiating cells.

These observations make a significant contribution to the already existing wealth of data proving the relevance of murine models as valid preclinical tools. In this study, we demonstrated their applicability in testing actionable therapies. Overall, our data suggest that large-scale single-cell transcriptomics will eventually impact the development and implementation of enrollment criteria for clinical trials and the evaluation of therapeutic response at the molecular level.

Taken together, our data emerge as a prototype cancer-focused study in which we first identified tumor-specific subpopulations and concomitantly assessed their transcriptional rewiring during target therapy, while also following tumor growth by MRI. To date, besides the new KRAS-G12C inhibitors, trials addressing NSCLC patients harboring KRAS mutations have unfortunately not led to major positive therapies. It is noteworthy that PTC596, which is undergoing clinical trials for solid tumors, achieved the therapeutic outcome, we reported with undetectable/noticeable side effects. BMI-1 inhibition alone was able to achieve better results than MEK inhibition, and additional pathways will have to be further tested to identify more efficient combinations. Overall, our data strongly suggest that PTC596 might be a promising drug to be tested in the ~30% NSCLC patients carrying highly aggressive KRAS mutations.

**Methods**

**Cell culture.** The human lung ADC cell lines A549 and SKLU1 were purchased from ATCC. Cells were cultured in Roswell Park Memorial Institute Medium (RPMI) 1640 medium containing 10% fetal bovine serum (BSA, Sigma) and grown at 37 °C in a humidified incubator with 5% CO₂. These cells were authenticated via DNA fingerprinting and tested negative for mycoplasma.

Cells line were treated with PTC596 (1 μM), PTC208 (1 μM), or vehicle (0.5% DMSO) for different time points (24, 48, and 72 hours) and the corresponding lysates subjected to Western Blotting to assess BMI-1 protein levels.

**Western blot.** A549 and SKLU1 cells were collected and then lysed with TritonX 1% and Complete ethylenediaminetetraacetic acid-free Protein Cocktail (Roche #1187358001) for protease inhibitors and PhosSTOP (Roche #4906845001) as phosphatase inhibitor cocktail. Lysates were centrifuged at 12,000 x g for 15 min at 4 °C, the supernatant was snap frozen in Liquid N2 and stored at −80 °C. In all, 15 μg of total protein were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and transferred to nitrocellulose membrane using the TransBlot Transfer System (BioRad). Membranes were blocked in tri-buffered saline and tween 20 containing 5% non-fat dry milk and incubated with primary antibodies anti-BMI-1 (1:1000 Cell Signaling Technologies #9694 S), anti-BMI-1 (1:1000 Cell Signaling Technologies #9694 S), and anti-BMI-1 mouse monoclonal antibody (Millipore, #05637; 1/100 dilution). Membranes were then stripped with Restore Western Blotting Buffer Solution (Thermo Fisher #21059) for 15 minutes at room temperature (RT) and incubated overnight (O/N) with an anti-β-actin mouse antibody (Santa Cruz #81178) at a 1:1000 dilution to assess equal loading. Blots were incubated with specific HRP-conjugated secondary antibodies, anti-rabbit IgG-HRP (Santa Cruz #SC2054) or anti-mouse IgG-HRP (Santa Cruz #SC2031). An enhanced chemiluminescence blotting analysis system (Pierce, Thermo Scientific #32106) was used for antigen-antibody detection. The density of western blot bands was quantified by Image software (Version 1.51i9, National Institutes of Health, Bethesda, MD, USA).

**Cell cycle analysis.** A combination of Vybrant DyeCycle Violet and Pyronin Y was used for the differential staining of cellular DNA and RNA. A549 and SKLU1 cells treated for 24 hours with PTC596, PTC208, and DMSO, were permeabilized in phosphate-citrate buffer solution (pH 4.8), washed in phosphate-buffered saline (PBS) 1x, and then resuspended in a solution of 5 μM Vybrant DyeCycle Violet (Thermo Fisher Scientific) and 4 μg/ml pyronin Y (Polysciences). Cycle status was then evaluated by flow cytometry on CytoFLEX Flow Cytometer (Beckman Coulter, Inc.).

**Immunofluorescence analyses on A549 cells.** A549 cells were washed with phosphate buffer saline 1x (PBS, three times) and then fixed with paraformaldehyde (2% in PBS) for 15 min. After washing with PBS, PTC596 was permeabilized with 0.1% Triton-X-100 made in PBS, for 15 min. Cells were then washed with PBS (three times), 0.5% bovine serum albumin in PBS (PBBS) four times, and exposed for 40 min to 2% BSA in PBS (BSA 2%). After washing with PBBS (four times), cells were incubated with rabbit anti-human ubiquityl-histone H2A monoclonal antibody (Cell Signaling Technologies #8240 S; 1:1600 dilution in PBBS) for 1 h at RT and additional 1.5 hours at 4 °C. Cells were washed with PBBS (four times), and incubated with the secondary antibody (donkey anti-Rabbit IgG AlexaFluor 647 from Jackson ImmunoResearch #711-605-152 at 1:250 dilution in PBBS for 1 h at RT in dark. Next, cells were washed with PBBS (four times), stained with Hoechst 33342 (1 mg/100 ml in water) for 30 s, and washed with PBS (three times). Cells were then maintained in PBS at 4 °C before imaging, no longer than 7 days.

The negative control was obtained by means of the same procedure but incubating the cells with PBBS only instead of a primary antibody solution in PBBS.

**Murine models.** Mice were housed in a sterile-barrier facility, and all experiments were approved by the Institutional Animal Care and Use Committee at the Beth Israel Deaconess Medical Center.

**Xenografts and drug treatment.** To study the in vivo effect of PTC596 or its analog PTC208 on the ADC cell line A549, NOD-SCID IL2r(null) mice (non-obese diabetic/severe combined immunodeficient interleukin-2 receptor y null, NSG mice, Jackson Laboratories) were injected subcutaneously in flanks on both sides with 2 × 10⁶ cells with 50 μl Matrigel (BD Basement Membrane Matrix Phenol-red free #356237). Once tumors became measurable (~80–90 mm³), mice were randomized to receive PTC596 (n = 15; 12 mg/kg), PTC208 (n = 7; 15 mg/kg), or vehicle (0.5% hydroxypropyl methylcellulose−0.2% Tween 80 in distilled water) by oral gavage twice a week. In order to determine tumor volume by caliper measurement, the greatest longitudinal diameter (length) and the greatest transverse diameter (width) were determined. Tumor volume was calculated by the modified ellipsoidal formula (tumor volume = ½ × Length² × Width), as previously reported. Treatment was started when tumor volume was measured with a caliper as being at least 0.06 cm³, and tumor growth was followed up to 21 days.

**Transgenic mice, drug treatments, and MRI.** To generate K-RasG12D/Pt53 null mice (Lox-stop-lox/LSL x K-RasG12D, Pt53 flox/flrex58) they received intranasal administration of Cre-expressing adenovirus (2.5 × 10⁶ viral particles per mouse) at 8 weeks of age, to achieve recombination in airway cells. Sibling mice, receiving the same amount of Adeno-empty virus, have been used as negative controls to study healthy tissues. Tumor growth was assessed by MRI at the BIDMC Imaging Facility after 5–6 weeks of induction, and were then monitored every 1–2 weeks to detect baseline tumor volume and recruited into treatment groups (PTC596, or vehicle) when tumor size reached 1–2.0 mm diameter.

Mice were treated with PTC596 (n = 7; 12 mg/kg in 0.5% HPMC and 0.2% Tween, oral gavage twice per week), selumetinib (n = 12; daily oral gavage, 8 mg/kg in 0.5% HPMC and 0.2% Tween, oral gavage daily), combination of PTC596-selumetinib (n = 6) or vehicle control (n = 5; 0.5% HPMC and 0.2% Tween, same regimen as PTC596). Negative control mice received vehicle treatment. All mice were killed 1 month after treatment when the tumor burden of vehicle-treated mice became too large.

Mice were then scanned by MRI twice/week to capture the effects of drug treatment on tumor size over a month period. Processing and quantification techniques of tumor burden were based on manual segmentation/volume calculation of diffuse lung tumors. Changes in lung tumor volumes over the course of treatment were calculated as a percentage change in volume over volume of tumor at day 1 of treatment, which was set at 100%. MRI images of mouse lungs were captured with a Bruker Biospec 94/20.94 Tesla scanner and the primary imaging sequence used was RARE (rapid acquisition with refocused echoes), with TR/TE = 1200 ms/17.5 ms.

**Histopathological analyses.** Mice were killed by CO₂ euthanasia. Lungs and xenograft subcutaneous tumors were fixed in 10% formalin (formalin solution neutral buffered 10%, Sigma-Aldrich) O/N. Fixed specimens were embedded in paraffin and sectioned at 5-μm thickness. IHC were performed on paraffin sections with an anti-BMI-1 mouse monoclonal antibody (Millipore, #05637; 1:100 dilution) on mouse tissues, and a rabbit anti-human BMI-1 (Cell Signaling #9694, 1:200 dilution) for the xenografts. Fresh tumor tissue was collected from patients undergoing surgical resection of NSCLC and placed in RPMI prior to being fixed in 10% formalin. Subsequently, they were stained as mentioned above. In brief, tissue sections were deparaffinized with xylene and hydrated in graded ethanol. Antigen retrieval was performed in 10 mM citrate buffer (pH 6.0) on a 2100 Retriever for 40 min. To prevent non-specific binding we applied as protein blocking solution 7% horse serum in PBS for 30 minutes at RT. Primary antibody was incubated at
4 °C O/N. Next, we applied peroxidase blocking solution for 10 minutes at RT and subsequently we performed secondary antibody incubation for one hour at RT. Secondary antibodies were used: horseradish-peroxidase anti-mouse BA1001 at 1/1200 dilution, and goat anti-rabbit BA1000 at 1/1000 from Vector Laboratories, Inc. CA. ABC-HRP standard kit (Vector Labs, CA PK-6100) was adopted and incubated for 30 minutes and the signal was revealed with DAB (Vector Labs, CA SK-4100). Tissue sections were counterstained with hematoxylin–eosin and mounted with Cytoseal 60 (Electron Microscopy Science), for pathology analysis.

Mouse lung and tumor dissociation into single cells. Murine pulmonary tissue (healthy and tumor lung) were dissociated into single-cell for further RNA sequencing downstream applications using the Tumor Dissociation kit by Miltenyi Biotec (# 130-096-730). In brief, the tissue was placed in a petri dish on ice and cut into small pieces of 2–4 mm. The pieces were infused with RPMI/enzyme mix (Miltenyi Biotec), transferred to a gentleMACS C tube containing RPMI/enzyme mix, attached to the sleeve of the gentleMACS Octo Dissociator and run using a “37C_m_TDK1” protocol. After termination of the program, the cells were spun down at 300 x g for 10 min at 4 °C, resuspended in RPMI-2% FBS, passed through a 70 μm strainer and centrifuge was repeated. The cell pellet was treated with 1 ml of ACK solution for 7 min at RT, and the lysis stopped with 4 ml of RPMI-2% FBS. After centrifugation, the cells were suspended in 1 ml RPMI-2% FBS and passed through a cell strainer (70 μm) to obtain a single-cell suspension. Immediately before transcriptome barcoding using the InDrop platform, cells were manually counted on a hemocytometer and diluted to 60,000 cells/ml. The final cell suspension included 15% v/v Optiprep (Sigma-Aldrich, Cat. No. D1556).

Patient description and preparation. Twelve NSCLC (ten ADCs and two squamous carcinomas) patient samples were analyzed in this study. Fresh samples were obtained from patients undergoing surgical resection. Patients were selected when having tumors ≥1.6 cm that were treatment-naive. Analysis related to mutant versus wt-KRAS mutation status was only performed on ADC samples (n = 10). This study was conducted with approval of the Dana-Farber Brigham and Women’s Cancer Center IRB and written informed consent from subjects. The protocol allows the collection of discarded tissue samples. De-identified genomic information is deposited in protected public repositories for subjects explicitly allowing it on the consent form. Human tissue samples were de-identified and analysis is not considered human subject research under the US Department of Human and Health Services regulations and related guidance (45 CFR part 46).

Dissociation kit Human from Miltenyi (Cat. #130-095-929), similar to the protocol described for murine tumors, except the “37C_h_TDK2” program was used.

Genomic DNA was extracted from four 10 μm sections of paraflin-embedded tissues per sample utilizing the QIAamp DNA FFPE Tissue Kit (#56404), according to the manufacturer’s instructions.

All clinical samples were sequenced using the Oncopanel platform.11 Samples 18, 21, 36, and 37 were sequenced using Ion S5 targeted sequencing (Ion Torrent; Applied Biosystem, Calbad, CA, USA). KRAS mutation status was confirmed by digital droplet PCR (ddPCR) using the ddPCR Mutation Assay (BioRad, Hercules, CA) as per manufacturer instructions. A fluorescence intensity threshold of 3000 was set as a cutoff point. A mutation was called when at least one droplet was above the threshold level. Mutations values were reported as mutant allele frequency (MAF), defined as the proportion of mutant to wild-type PCR products in the ddPCR readout. The analyzed mutations were KRAS codons 12 and 13. Patients have been divided into KRAS mutated and KRAS wt (Supplementary Table 1) based on the presence of activating mutations (see Supplementary Data). Some tumors displayed KRAS amplification or loss. However, no functional analyses were conducted to determine the effects of these additional mutations.

sc-RNAseq data processing, quality control, filtering, and cell type identification. Raw fastq files were obtained from a NextSeq 500. Next, transcripts were mapped to the mouse transcriptome (GRCm38.81) following an established bioinformatics pipeline for InDrop experiments to create raw gene-cell counts matrices (available on GEO: GSE136246)50, with the slight modification of adding an additional filtering step to remove lower quality base calls using Trimmomatic (v0.36,63) with the parameters “LEADING=24 SLIDINGWINDOW:4:26 MIN-
LEN:15” after the “identifying abundant barcodes” step. Human samples were mapped to GRCh38.91 using a similar approach. Subsequent analysis was performed in R (v3.6.2) and was based on a previously published Bioconductor workflow with minor modifications51, and published guidelines for sc-RNAseq analysis. First, cells with low total raw counts were removed if they had fewer than a calculated number of counts. This trim threshold was based on the mode of the total counts for a given sample and was calculated as follows (eq. 1).

\[
\text{If mode estimate } < 100, \text{ trim threshold } = \text{mode estimate } + 5.5 \\
\text{If 100 } \leq \text{ mode estimate } \leq 450, \text{ trim threshold } = (-0.01 \times \text{mode estimate}) + 6.5 \\
\text{If mode estimate } > 450, \text{ trim threshold } = \text{mode estimate } + 2
\]

Next, cells with library sizes more than three median absolute deviations (MADs) below the median library or six MADs above the median were filtered out. Cells with a total percent of expressed genes (≥1 read) more than two MADs below the median total number of expressed genes or five MADs above the median total number of expressed genes were filtered out. Cells with a total percent of expressed genes originating from mitochondrial DNA more than six MADs above the median were filtered out. A doublet score was computed to estimate the percentage of barcodes for two or more cells66, and cells with a doublet score of 0.99 and greater were excluded. The expression of each cell was normalized by a size factor approach67. Principle component analysis, Uniform Manifold Approximation and Projection for Dimension Reduction and t-distributed stochastic neighbor embedding visualizations revealed no significant batch effects to be regressed out for the mouse samples. For the human samples, monCorrect() from the batchelor package68 was utilized for batch correcting samples.

For expression data visualization, SPRING was used. In brief, a graph of cells connected to their nearest neighbors in gene expression space was determined and this was then projected into two dimensions using a force-directed graph layout. For cell annotations for the murine samples, a custom hybrid annotation strategy was performed.

Supplementary Table 9 shows a cross-table of (a) predominant cell populations present in each murine and/or human cluster and (b) cell types across both species.

For the patient samples, a total of 23 tumor nodules were sequenced with the number of nodules from each patient as shown in Table 1.

Throughout the paper, we refer to deconvolution that was performed considering solely the ADC samples contained within the NSCLC set and the mutant-KRAS-associated cluster was annotated as Cluster 10 (C10). For validation purposes, we also deconvoluted only the ADC samples, and in such case, the mutant-KRAS-associated cluster was annotated as Cluster 3 (C3), as shown in Supplementary Figures 1b, c and Supplementary Figure 3a.

Differential gene expression analysis, marker gene identification, and figure generation. Differential gene expression was calculated using genewise negative binomial generalized linear models (glmFit) from the edgeR package.2,7 In brief, binomial dispersion was estimated using the estimateDisp(), followed by model fitting using glmFit() and finally, likelihood ratios for differential expression were calculated using glmLRT(). Differential gene expression results are provided in Supplementary Tables including Log2 fold change (Log2FC), p value and Benjamini–Hochberg adjusted p values (FDR)73.

For the heatmaps in Supplementary Figure 1, heatmaps were generated as follows. To identify marker genes per expression cluster, findMarkers() from the scan package was utilized with the parameter (direction = “up” or “any”).

Table 1 Patients’ sample replicates.

| Patient sample | Number of replicates |
|----------------|----------------------|
| NSC004         | 4                    |
| NSC009         | 2                    |
| NSC010         | 2                    |
| NSC016         | 3                    |
| NSC018         | 2                    |
| NSC019         | 2                    |
| NSC020         | 2                    |
| NSC021         | 2                    |
| NSC025         | 2                    |
| NSC030         | 1                    |
| NSC037         | 1                    |
| NSC040         | 1                    |
depending on the heatmap created. From the 25 highest expressed genes per cluster in tissue samples (25 genes, 13 cluster, totaling 325 genes), only unique genes were selected (i.e., genes identified as a marker in only 1 cluster) and shown. This resulted in a final count of 235 genes for murine healthy samples and 222 for murine tumor tissue samples. Heatmaps were generated using the heatmap R package (Kolde R, heatmap: Pretty Heatmaps. R package version 10.12 2019) with normalized expression matrices being row (gene) scaled prior to plotting. Genes (rows) were hierarchically clustered in an unsupervised manner based on Euclidean distances between genes by toggling the flag “cluster_rows=TRUE”. The genes corresponding to the rows of the heatmaps are listed in Tables S3 and S4, with genes in the same order as the heatmap.

Ingenuity pathway analysis. Analysis was conducted using IPA (Ingenuity Systems, www.ingenuity.com). Gene lists containing mean expression values of sc-RNAseq data of murine and human C10, filtered for p value ≤ 10−10, were submitted to IPA. Core analysis of the uploaded data identified canonical pathways, diseases and functions, and gene networks that are affected. Heatmaps were used to show comparison analysis between murine and human data.

Gene set enrichment analysis. Differential expression gene sets were filtered with an FDR < 0.01 and then ranked based on logFC. GSEA was then performed by comparisons with curated expression grp lists downloaded from the Broad Institute using the GSEA tool also from the Broad. Specifically, we used murine data sets M190977, M91187, M879759, M199978, and M94739 and human data sets M5953, M2897, M27039 M45270, M253441, M70799, M216062, M1695461, and M231616. For the Bmi-1 targets, we used themurine data set GSE56937. The GSEA data set database used was the full version of MsigDB V7.2.

Survival analysis and mutation-level association analysis. For survival analysis of C10 signature, lung adenocarcinoma samples were taken from TCGA (https://tcga-data.nci.nih.gov/tcga/), and the RNAseq raw counts data and clinical data of C10 signature, lung adenocarcinoma samples were used to segregate the samples into C10 signature high and low groups. Survival analysis was performed between these two groups (C10 signature high—higher than the overall score median and C10 signature low—lower than the overall score median) based on the Kaplan–Meier method using overall survival data. TCGA ADC cohorts (colon, COAD; pancreas, PAAD; stomach, STAD) were scanned with the C10 to score its enrichment in KRAS-mutant versus WT cancers.

EnrichR analysis. Following differential gene expression of C10 versus other epithelial clusters in both murine and human data sets, gene lists, filtered logFC ≥1.5, FDR ≤0.05, were submitted to the EnrichR enrichment suite.

Method for scoring the signature genes. TCGA RNAseq samples from several ADCs (colon, rectum, pancreatic, stomach) were downloaded for gene expression analysis. After mapping with STAR software, mapped counts were employed to generate the raw expression counts using the FeatureCounts. The raw expression counts were then further normalized using the Cross-Correlation method. From the mutation status (mutant or wild type) across all samples was evaluated based on the called DNA files. For each signature gene in a sample, z score transform of normalized expression was performed over all samples in each ADC type. The z score transformed values of all signature genes in a sample were averaged to obtain the signature score of the sample. For a combination of the positive and negative Kras-mutant signature genes, negative equal weights were given for all negative signature genes and the same positive equal weights for all positive signature genes prior to averaging. Student t statistics were used to assess the significance between the two means of the signature scores in Kras-mutant and WT sample groups.

Data availability. The data sets generated during and/or analyzed during the current study are available in the GEO repository: GSE136246. All other data are available from the corresponding author on reasonable request.

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E.L., D.G.T., A.A., G.M., I.K., and M.A.B. designed the study; E.L., A.A., G.M., I.K., M.A.B., J.C., D.S.B., A.G., M.D.R., R.B., R.S.W., and J.G.C. performed and planned research; E.L., D.G.T., A.A., G.M., I.K., M.A.B., V.S., R.Z., B.S., J.C., R.P., J.L., D.S.B., M.W., A.B., A.G., R.C., M.D.R., R.D., R.B., H.Y., O.K., M.C.M., R.S.W., A.M.K., R.B., and J.G.C. analyzed data; F.C.W., V.S., R.Z., V.A.M., N.P., E.C., J.Z., C.E.G., S.F., R.D.L., C.V.M., P.J.T., K.Y., A.D.R., S.C., and R.C. performed research; and E.L., D.G.T., A.A., G.M., I.K., and M.A.B. wrote the paper.

Competing interests
The authors declare no competing interests.

Additional information
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