HIF-2 deletion promotes Kras-driven lung tumor development
HIF-2α deletion promotes Kras-driven lung tumor development

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Non-small cell lung cancer (NSCLC) is the leading cause of lung cancer deaths worldwide. The oxygen-sensitive hypoxia inducible factor (HIF) transcriptional regulators HIF-1α and HIF-2α are overexpressed in many human NSCLCs, and constitutive HIF-2α activity can promote murine lung tumor progression, suggesting that HIF proteins may be effective NSCLC therapeutic targets. To investigate the consequences of inhibiting HIF activity in lung cancers, we deleted Hif-1α or Hif-2α in an established KrasG12D-driven murine NSCLC model. Deletion of Hif-1α had no obvious effect on tumor growth, whereas Hif-2α deletion resulted in an unexpected increase in tumor burden that correlated with reduced expression of the candidate tumor suppressor gene Scgb3a1 (HIN-1). Here, we identify Scgb3a1 as a direct HIF-2α target gene and demonstrate that HIF-2α regulates Scgb3a1 expression and tumor formation in human KrasG12D-driven NSCLC cells. AKT pathway activity, reported to be repressed by Scgb3a1, was enhanced in HIF-2α-deficient human NSCLC cells and xenografts. Finally, a direct correlation between HIF-2α and Scgb3a1 expression was observed in approximately 70% of human NSCLC samples analyzed. These data suggest that, whereas HIF-2α overexpression can contribute to NSCLC progression, therapeutic inhibition of HIF-2α below a critical threshold may paradoxically promote tumor growth by reducing expression of tumor suppressor genes, including Scgb3a1.

hypoxia | inducible mouse model | non-small cell lung cancer | tumor suppressor

Despite the access of normal pulmonary epithelia to atmospheric O2 levels, tumor hypoxia has been observed in human non-small cell lung cancer (NSCLC) and correlates with poor prognosis and decreased disease-free survival (1, 2). Cells can overcome hypoxic stress through multiple mechanisms, including the stabilization of hypoxia inducible factor (HIF) transcriptional regulators. HIFs consist of an oxygen labile α-subunit and a constitutively expressed β-subunit (also called ARNT), which together bind hypoxia response elements (HREs) to activate a large number of target genes encoding proteins that mediate adaptive responses to hypoxic stress (3, 4). Two distinct subunits, HIF-1 and HIF-2, have been demonstrated to regulate partly overlapping sets of target genes in hypoxic cells, although each protein also fulfills unique essential functions (5–7).

Many HIF target genes encode proteins important for tumor growth and progression, including glycolytic enzymes, VEGF, matrix metalloproteinase-2 (MMP2), transforming growth factors α and β (TGF-α and TGF-β), and numerous others (4, 8). Collectively, these factors modulate cancer cell metabolism and can promote angiogenesis, invasion, and metastasis, suggesting that HIF proteins may represent suitable targets for antitumor therapies (9–11). It is interesting to note that both HIF-1α and HIF-2α proteins are overexpressed in approximately 50% of human NSCLCs (12), further suggesting they may contribute directly to NSCLC progression.

In a recent report, ectopic expression of a nondegradable HIF-2α promoted growth of KrasG12D-driven murine lung tumors (13). Relative to KrasG12D controls, KrasG12D lung tumors expressing stabilized HIF-2α were larger, displayed evidence of enhanced angiogenesis and epithelial-mesenchymal transition (EMT), and resulted in decreased survival (13). These phenotypes were correlated with increased expression of multiple HIF-2α target genes including VEGFR2, Snail, and others (13). Collectively, these results demonstrated that increased HIF-2α activity can drive NSCLC growth and underscore the potential utility of developing targeted therapies to inhibit HIF proteins in general, and HIF-2α in particular, for NSCLC and renal cell carcinomas (RCC).

To determine the effects of eliminating HIF-1α and HIF-2α expression in NSCLC, we used conditional “floxed” alleles to delete HIF-1α or HIF-2α in lung tumors using the murine KrasG12D NSCLC model (13). Deletion of HIF-1α in these tumor cells had no detectable effect on tumor growth but, surprisingly, deletion of HIF-2α actually increased tumor number and size. This phenotype correlated with reduced expression of Scgb3a1 (14) also known as high in normal-1 (HIN-1), a candidate tumor suppressor gene implicated in lung, breast, pancreatic, and other cancers (15–17). We demonstrate here that Scgb3a1 is a direct HIF-2α target gene and that HIF-2α deficient lung tumor xenografts are characterized by enhanced AKT signaling, consistent with previous observations that Scgb3a1 suppresses AKT activity in human breast cancer cells (18). We further demonstrate that ectopic expression of Scgb3a1 suppresses the growth of HIF-2α–deficient lung tumor xenografts, concomitant with reduced AKT signaling. Finally, a direct correlation between HIF-2α and Scgb3a1 expression was observed in human NSCLC cells and primary NSCLC tumors. These data suggest that, although HIF-2α overexpression can contribute to NSCLC progression, therapeutic inhibition of HIF-2α below a critical threshold may actually promote tumor growth by repressing Scgb3a1 and other HIF-2α target genes.

Results

HIF-2α Deletion Promotes KrasG12D-Induced Lung Tumor Growth. The inducible LSL-KrasG12D murine genetic model generates lung tumors that faithfully model human lung adenocarcinoma initia-
ation and progression (19). To evaluate the effect of HIF-2α loss-of-function in lung tumor progression, we crossed mice carrying conditional floxed Hif-2αfl or null Hif-2αα mice (20) to mice carrying the LSL-KrasG12D allele. The resulting experimental KrasG12D/Hif-2αfl/+ and control KrasG12D/Hif-2αΔα animals were treated with adenovirus encoding the Cre recombinase by intranasal instillation. Cre-mediated deletion of the Lox-Stop-Lox (LSL) gene cassette in the LSL-KrasG12D allele produced KrasG12D-driven lung tumors as previously described (19) and in this case also deleted the conditional Hif-2αfl allele. As the control KrasG12D/ Hif-2αΔα animals harbor a wild-type Hif-2α allele, HIF-2α function was consequently retained in tumor tissue (Fig. S1). Southern blot analysis confirmed efficient deletion of the Hif-2αfl allele specifically in tumors but not surrounding lung tissue (Fig. S1B).

To evaluate the effect of HIF-2α deletion on lung tumor progression, animals were analyzed 12 wk and 24 wk after adenoviral Cre delivery (Fig. S1C). Based on the recent report that HIF-2α gain-of-function promotes lung tumor progression and correlates with poor survival in this model (12, 13), we predicted that deletion of HIF-2α would reduce tumor burden and progression. Surprisingly, KrasG12D/Hif-2αfl/+ mutant mice displayed a significant increase in tumor number and size at 12 wk (Fig. 1, A, C, and D) and 24 wk (Fig. 1, B, E, and F) postinfection, as compared with their control littermates. Notably, deletion of a floxed Hif-1αfl allele (21) in parallel experiments had no detectable effect on tumor number or volume in KrasG12D lung tumors (Fig. S2 A–F).

HIF-2α Deletion Promotes Tumor Progression. Lung tumors generated in LSL-KrasG12D mice display distinct types of progressive lesions including epithelial hyperplasia, adenomas, and adenocarcinomas (19). HIF-2α deficient KrasG12D lung tumors displayed a significant increase in hyperplastic lesions at 24 wk (Fig. 2A), and a similar trend at 12 wk. Similarly, KrasG12D/Hif-2αfl/+ animals displayed significantly increased numbers of adenomas (Fig. 2B) at 24 wk compared with control animals. A similar trend toward increased numbers of adenocarcinomas was observed at 24 wk but did not achieve statistical significance (Fig. 2C). Collectively, these data indicate that loss of HIF-2α accelerates tumor progression in this context. Interestingly, deletion of HIF-1α in parallel experiments did not alter disease kinetics (Fig. S2 G–I), underscoring the differential effects of HIF-1α and HIF-2α in these tumors.

The increased size of HIF-2α-deficient tumors correlated with elevated cell proliferation (Fig. 2D and E) and decreased apoptosis (Fig. 2F and G), which was not observed in HIF-1α-deficient tumors (Fig. S2 J and K). We also noted increased numbers of infiltrating leukocytes in HIF-2α-deleted tumors (Fig. S3 A and B), and granulocytes in particular (Fig. S3 C and D), suggesting that inflammatory responses may differ between KrasG12D/Hif-2αΔα, and KrasG12D/Hif-2αΔα lung tumors.

Identification of Sgcb3a1 as a HIF-2α Target. To investigate molecular mechanisms underlying HIF-2α’s tumor suppressive effects, we conducted global gene expression profiling on individual tumors from KrasG12D/Hif-2αΔα or control KrasG12D/Hif-2αΔα...
mice. Specifically, RNA was isolated from tumors from experimental and control animals (n = 7 for each group) 28 wk after infection and analyzed independently. Comparisons between the genotypes identified a small set of genes (Table S1) whose expression was significantly and reproducibly reduced in HIF-2α-deficient tumors, whereas expression levels of most genes were unchanged. Subsequent quantitative RT-PCR (qRT-PCR) analyses on the same tumor RNAs revealed dramatic down-regulation of multiple transcripts, including those encoding Scgb3a1, lactotransferrin, aquaporin 4, and ceruloplasmin (Fig. 3A). In contrast, transcript levels of the HIF-1α target gene acominase (Aco1) were unaffected by Hif-2α deletion (Fig. 3A). The reduced expression of Scgb3a1 was particularly intriguing as (i) Scgb3a1 is expressed primarily in epithelial organs including lung, mammary gland, trachea, prostate, pancreas, and salivary gland (22); (ii) Scgb3a1 expression is silenced in a variety of human cancers including lung, breast, pancreas, and prostate (15, 16); and (iii) down-regulation of Scgb3a1 is a significant independent predictor of poor clinical outcome in early stage NSCLC (17).

To extend our studies to human NSCLC, we introduced a retroviral shRNA gene construct targeting human Hif-2α mRNA (23) into human A549 lung adenocarcinoma cells, which harbor an activating Kras mutation (24). Despite extensive screening, only partial HIF-2α knockdown was observed in multiple cell clones, as revealed by Western blot and qRT-PCR analyses (Fig. 3B and C). Nevertheless, pooled HIF-2α knockdown cells (Fig. 3D), as well as independent knockdown cell clones HIF-2α KD C1 and HIF-2α KD C2 (Fig. 3E), exhibited a significant reduction in Scgb3a1 transcript levels (approximately 2.5-fold), consistent with our previous observations. A similar reduction was observed for transcripts encoding aquaporin 4 (Aqp4), ceruloplasmin (CP), and VEGF (Fig. 3D and Fig. S4A and B) but not the HIF-1α specific target PGKI (Fig. S4B). As a control for functional specificity, we introduced a murine cDNA encoding HIF-2α into the human HIF-2α KD C1 knockdown cells (designated HIF-2α KD C1R, Fig. 3C), which in turn restored Scgb3a1 transcript levels (Fig. 3E). Interestingly, HIF-1α depletion in A549 cells (Fig. 3B) did not alter transcript levels of

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Scgb3a1 or other genes identified in the microarray experiment (Fig. 3D and Fig. S4C).

Our results indicate that HIF-2α regulates Scgb3a1 expression in lung adenocarcinoma cells in a cell autonomous manner and that this activity is not shared by HIF-1α. We next investigated the possibility that HIF-2α regulates Scgb3a1 directly. Analysis of human and murine Scgb3a1 gene sequences revealed multiple putative HREs spanning the upstream promoter and enhancer regions (Fig. S4D). ChIP assays revealed HIF-2α occupancy of two HREs (H4 and H5) in the Scgb3a1 promoter in AS49 cells, which increased (4- to 7-fold) under hypoxic conditions (Fig. 3F). Not all HREs in the Scgb3a1 promoter appear functional, as H6 fails to bind HIF-2α (Fig. 3F). Moreover, HIF-1α failed to bind H4 and H5 (Fig. S4E). Collectively, these data demonstrate that Scgb3a1 is a direct HIF-2α target gene.

To test the effects of HIF-2α knockdown in tumor formation by the AS49 cells, we implanted 5 × 10^6 HIF-2α KD C1 or HIF-2α KD C2 cells s.c. in immunocompromised mice to generate xenograft tumors. Consistent with our results from the autochthonous HIF-2α deficient lung tumors, xenograft tumors from HIF-2α KD C1 cells displayed a modest but significant growth advantage over AS49 control tumors and a similar trend was also observed for HIF-2α KD C2 cells (Fig. S5 A and B). As the recipient mice had to be killed between 25 and 35 d due to the large size of these xenografts, the experiment was repeated using fewer (1 × 10^6) HIF-2α KD C1 cells (Fig. 4A). In this experiment, HIF-2α KD C1 xenografts displayed a clear and significant growth advantage over the vector control xenografts, and restored HIF-2α expression (HIF-2α KD C1R cells) suppressed tumor growth to levels essentially indistinguishable from the vector control tumors (Fig. 4 A and B). Importantly, ectopic expression of human Scgb3a1 in the HIF-2α KD C1 cells (Fig. S5F) also effectively suppressed tumor growth, implicating Scgb3a1 as a critical mediator of HIF-2α activity in this setting (Fig. 4B).

**HIF-2α Deficiency Reduces AKT Signaling.** Enforced Scgb3a1 expression was shown to inhibit AKT pathway signaling associated with decreased AKT phosphorylation at Ser^Thr^183 in human breast cancer cells (18). We therefore hypothesized that HIF-2α knockdown in AS49 cells would increase AKT pathway activity. Exposure of HIF-2α KD C1 and HIF-2α KD C2 cells to hypoxia significantly increased levels of phosphorylated AKT at Ser^Thr^473 (Fig. 4C). Moreover, levels of phosphorylated AKT downstream effectors including pGSK-3β and 4E-BP-1 were higher compared with control cells (Fig. 4C). Notably, restoring either HIF-2α or Scgb3a1 expression in the HIF-2α KD C1 cells reduced AKT, GSK-3β, and 4E-BP-1 phosphorylation to nearly control levels (Fig. 4C). A similar trend was observed in the xenograft tumors, as Western blot analysis revealed that HIF-2α KD C1 tumors displayed increased pAKT and pGSK3β levels compared with control tumors (Fig. 4D).

**HIF-2α Levels Correlate with SCGB3A1 in Human NSCLC.** The role of HIF-2α in regulating Scgb3a1 led us to investigate whether HIF-2α and SCGB3A1 transcript levels correlate in human NSCLC. The qRT-PCR analysis of 15 independent human adenocarcinoma biopsies revealed that in 73% (11/15), HIF-2α mRNA levels were significantly reduced relative to normal tissue, which correlated directly with reduced SCGB3A1 mRNA expression (Fig. 5A). In contrast, tumors that retained normal HIF-2α mRNA levels did not display statistically significant changes in SCGB3A1 mRNA levels. To further investigate the connection between HIF-2α and SCGB3A1 expression, we evaluated lung cancer datasets available through the Oncomine dataset repository (www.oncomine.org). Filtering specifically for NSCLC tumor datasets containing HIF-2α and SCGB3A1 probes, we found significant correlation between HIF-2α and SCGB3A1 expression in three of four datasets (Fig. 5B).

Discussion

Many human cancers display a direct correlation between HIF-α overexpression and poor prognosis (4, 8), consistent with the idea that HIF target genes positively regulate critical features of tumor progression, including cancer cell metabolism, survival, movement, and local angiogenesis. This correlation has spurred the development of HIF inhibitors as therapeutic treatments for cancer, as well as other diseases (11). The specific activities of HIF-1α and HIF-2α in tumor progression are quite complex, however, and appear to vary depending on cell type and oncogenic background. For example, although HIF-1α overexpression drives murine mammary tumor progression and metastasis in a murine model (25), it also inhibits the growth of certain of Vippel-Lindau (VHL)-deficient RCCs (26, 27), in part by restricting the activity of the c-Myc oncoprotein (28, 29). In contrast, HIF-2α expression potentiates c-Myc signaling in RCCs and promotes tumor growth (29). Furthermore, a recent report demonstrated that expression of a stabilized (gain-of-function) HIF-2α allele similarly promotes tumor progression in the LSL-Kras^{G12D} murine NSCLC model (13). These and other data suggest that inhibiting either HIF-1α or HIF-2α (or both) may be of therapeutic value in specific disease settings.

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The data presented here indicate that HIF-2α has an unexpected tumor suppressive role in Kras(G12D)-driven lung tumors and that reduced expression of the HIF-2α target Sgcb3a1 contributes to this effect. This interpretation is supported by the observation that restored Sgcb3a1 expression inhibited the growth of HIF-2α-deficient A549 xenograft tumors. Moreover, elevated AKT signaling was observed in HIF-2α-deficient A549 cells and was inhibited by restored expression of either HIF-2α or Sgcb3a1. These observations are consistent with previous reports that enforced Sgcb3a1 expression inhibits AKT activity in human breast cancer cells (18).

Sgcb3a1 was originally identified as a candidate tumor suppressor through gene expression studies comparing human mammary carcinomas to normal mammary epithelial cells (14). It was subsequently shown that Sgcb3a1 expression is reduced in human lung, prostate, pancreatic, and nasopharyngeal cancers, and corresponding hypermethylation of the Sgcb3a1 promoter was reported for multiple malignancies (15–17, 30). Moreover, decreased Sgcb3a1 expression was identified as a primary independent predictor of poor clinical outcome in early stage human NSCLCs (17). Our data indicate that Sgcb3a1 expression is directly regulated by HIF-2α in human lung adenocarcinoma cells: this relationship is underscored by the direct correlation between HIF-2α and Sgcb3a1 mRNA levels observed in approximately 70% of human lung adenocarcinomas analyzed and in three of four NSCLC datasets obtained from the Oncomine database.

Interestingly, our results contrast directly with those from a recent report in which HIF-2α inhibition in A549 cells, as well as in HCT116 colon carcinoma and U87MG glioblastoma cells, actually reduced xenograft tumor growth (31). The nature of these disparate results is not clear, as identical shRNA sequences were used; however, our data are supported by two important controls. First, the phenotypes observed in our HIF-2α-deficient A549 cells are reversed by enforced expression of a murine HIF-2α mRNA that escapes shRNA inhibition, confirming the HIF-2α dependent nature of these phenotypes. Second, the HIF-2α deficient A549 xenograft tumors phenocopy directly the behavior of autochthonous tumors generated by Hif-2α genetic deletion in the LSL-Kras(G12D) model.

The tumor suppressive activity of HIF-2α we observed was surprising, as expression of a stabilized HIF-2α in the identical LSL-Kras(G12D) model promoted tumor growth, and HIF-2α overexpression similarly promotes growth of RCCs (29). In particular, it raises the question of how HIF-2α can behave as both a tumor suppressor and an oncogene in the same NSCLC model. It appears that entirely different sets of HIF-2α target genes mediate these effects in a manner perhaps analogous to the proproliferative and proapoptotic activities of c-Myc (32). Whereas increased HIF-2α function elevates the expression of genes associated with angiogenesis and epithelial-to-mesenchymal transition (EMT) in LSL-Kras(G12D) NSCLCs (13), HIF-2α deletion in the same model diminishes the expression of a putative tumor suppressor Sgcb3a1, among other genes.

Collectively, these results strongly suggest that inhibition of overexpressed HIF-2α in NSCLCs (and perhaps other carcinomas) might have beneficial effects but that reducing HIF-2α function below a critical threshold might also drive tumor progression by inhibiting the expression of specific tumor suppressor genes. This model (Fig. 5C) assumes that HIF-2α overexpression either fails to increase Sgcb3a1 function above normal levels or that increased Sgcb3a1 activity is offset by elevated expression of other HIF-2α targets. It is also interesting to note that the time at which HIF-2α function affects tumor progression differs between the two models. In a previous study (13), expression of stabilized HIF-2α was initiated at the earliest stage in lung tumorigenesis (i.e., Ad-Cre infection). In contrast, HIF-2α protein stabilization in the lung tumors of our control mice probably occurs subsequent to Kras activation, either as a result of tumor hypoxia or additional oncogenic events. The importance of this temporal difference in expression is not yet clear. In general, however, our results caution that inhibition of HIF complexes in cancer treatment may require a narrower therapeutic window than initially imagined.

Our results also raise a number of interesting questions for subsequent work, including (i) to what degree does the suppression of other putative HIF-2α target genes (lactotransferrin, ceruloplasmin, etc.) contribute to the phenotypes we observe; (ii) are there non-cell-autonomous effects of HIF-2α deletion that regulate LSL-Kras(G12D) NSCLC tumor progression (for example, the increase in granulocyte infiltration); and (iii) is the relation between HIF-2α and Sgcb3a1 observed only in the context of activating Kras mutations or does it extend to other oncogenic backgrounds? Importantly, how does Sgcb3a1 regulate AKT activity and are additional signaling pathways involved in its tumor suppressive effects? To what degree does elevated AKT activity, in concert with other direct and indirect HIF-2α targets, contribute to the proliferative and apoptotic phenotypes we observe in Kras(G12D)-driven, HIF-2α deficient lung tumors?

In summary, we used both autochthonous and xenograft genetic tumors to reveal a tumor suppressive effect of HIF-2α in NSCLC and identify its direct target gene Sgcb3a1 as a downstream effector. This may be a more general phenomenon: for...
example, a previous report demonstrated that either HIF-1α or HIF-2α expression can limit tumor growth in genetically manipulated GS9L glioma and murine embryonic stem cells by altering angiogenesis and tumor cell apoptosis (33). Determining the cellular conditions and molecular mechanisms that distinguish the tumor promoting, or tumor suppressive, activities of HIF-1α and HIF-2α proteins will clearly require additional investigation.

Materials and Methods

Generation of and Characterization of Inducible LSL-KrasG12D, Conditional HIF-αΔMice, and Lung Tumors. Mice carrying the previously described inducible (LSL-KrasG12D) knock-in allele (19), the conditional floxed Hif-2α allele, and the related null Hif-2α allele (20) were intercrossed to generate experimental and control animals (SI Materials and Methods). Lung morphology, histology, and immunohistochemistry were performed as described in SI Materials and Methods.

Cell Culture and Infection. A549 cells (ATCC) were grown in DMEM with 10% FBS (Gemiini), amino acids, and antibiotics (SI Materials and Methods); A549 cells were infected with retroviruses encoding shRNAs targeting HIF-1α or HIF-2α, respectively, or with the retroviral backbone as the negative viral control (VC).

Western Blot Analysis. Protein extracts were prepared and analyzed as described in SI Materials and Methods. For hypoxia analysis, cells were cultured under 0.5% O2 and 5% CO2 for 16–20 h in an InVivox 400 hypoxia workstation with O2 control (Ruskinn Technology Ltd.). Western blots were probed with antibodies against HIF-1α (Cayman for mouse protein, R&D for human protein), HIF-2α (Novus Biologicals), Scgb3a1 (Abcam), AKT, pAKT, GSK-3β, pGSK-3β, p4EBP1, and p-akt (Cell Signaling).

RNA Purification, Reverse Transcription, and Real-Time PCR Amplification. RNA was extracted with TRIzol (Invitrogen) or RecoverAll Total nucleic Acid Isolation kit (Ambion) and purified on RNaseasy spin columns (Qiagen) as described in SI Materials and Methods; cDNA was synthesized using a high fidelity reverse transcriptase (ReverTra-α; Toyama Chemicals) primed with oligo(dT)18 or appropriate primers (siRNA transfection). Transcript levels were normalized to 18S rRNA using an Applied Biosystems 7900HT analyzer and represented as fold change 2–△△CT (normoxia)/ 2–△△CT (hypoxia); mean normal

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SI Materials and Methods

Generation of Inducible LSL-KrasG12D and Conditional Hif-αt Mice. To generate experimental LSL-KrasG12D; Hif-2αf/ and control LSL-KrasG12D; Hif-2αf/ cohorts, mice heterozygous for the previously described inducible (LSL–KrasG12D) knock-in allele (1) were crossed to animals carrying the conditional floxed Hif-2α allele, or the related null Hif-2α allele (2). Lung tumorigenesis was initiated by infecting 6-wk-old mice with 2.5 × 107 pfu of recombinant adenovirus expressing the Cre recombinase (University of Iowa Gene Transfer Vector Core) by intranasal instillation. Southern blot analysis of NcoI-digested genomic DNA from tumors 24 w.p.i. revealed efficient deletion of the conditional Hif-2α allele in tumors but not surrounding lung tissue (2) (Fig. S1). A corresponding strategy was used to generate experimental LSL–KrasG12D; Hif-1α f/ f and control LSL–KrasG12D; Hif-1α f/ f cohorts. Mice were maintained on a mixed 129/B6 background, and analysis was largely restricted to littermates. Occasionally, multiple litters of the same age (within approximately 1 wk) were used to provide sufficient numbers of each genotype; however, these multiple litters were typically the offspring of sisters mated to a single male. All mice were maintained in microisolator cages and treated in accordance with the National Institutes of Health and American Association of Laboratory Animal Care Standards and consistent with the animal care and use regulations of the University of Pennsylvania and Massachusetts Institute of Technology.

Lung Morphometry, Histology, and Immunohistochemistry. Lungs from mice 12 or 24 w.p.i. were fixed in 3.6% paraformaldehyde and all lobes embedded in paraffin. Sections (5 μm) were cut from three distinct zones (top, middle, bottom) of each paraffin block, stained with H&E and assessed for tumor type (hyperplasia, adenoma, adenocarcinoma), numbers, and areas using AxioVision digital image processing software as per manufacturer’s protocol. Briefly, the desired region of quantification was imaged, total region area measured (μm2), pixels matching tumor cells selected, and a binary mask created to compute tumor numbers and area. The measurements were independently confirmed by manual grading of tumor lesions. Tumor lesions were graded as described previously (3). Immunohistochemistry was performed using the MOM kit (Vector Labs) as per manufacturer’s protocol with minor modifications. Ki67 (Novocastra) and Gr.1 (ebiosciences) were used at a 1:100 dilution, and incubated with tissue sections overnight (O/N) at 4 °C. Secondary of anti-dig incubation time was increased to 1 h (RT) and sections were counterstained with hematoxylin. Cell death was detected by TUNEL staining (Millipore), as per manufacturer’s protocol.

Cell Culture and Infection. The pcDNA3.0-HIF-2α gene construct was used to restore HIF-2α expression in HIF-2α KD C1R cells that have been described previously (4). To establish HIF-2α KD C1R cells expressing Sgb3a1 (HIF-2α KD C1 Sgb3a1), human Sgb3a1 cDNA (MHS 1010-7430241; Open Biosystems) was subcloned into pLK0-1 Neo lentiviral plasmid (13425; Addgene) using AgeI and EcoR1 restriction sites. The empty lentiviral backbone served as a negative control.

Western Blot Analysis. Whole cell extracts were made using RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% TritonX-100, 1% Sodium deoxycholate, 0.1% SDS, 5 mg/mL Aprotinin, 5 mg/mL Leupeptin, 1 mM PMSF). Xenograft tumor samples were homogenized (Euro Turrax T20b; Ika Labortechnik) in RIPA buffer. For detection of HIF-α protein knockdown following retrovirus infection, cells were treated with the hypoxia mimetic iron chelator deferoxamine (200 μM) for 5 h at 37 °C.

RNA Purification, Reverse Transcription, and Real-Time PCR Amplification. RNA was extracted with TRIzol (Invitrogen) and purified on RNeasy spin columns (Qiagen). Fresh frozen xenograft and NSCLC tissue was mechanically disrupted in TRIzol using either a Euro Turrax T20b (Ika Labortechnik) homogenizer or a tissue grinding pestle (Kontes Glass Company). RNA from formaldehyde- or paraformaldehyde-fixed, paraffin embedded (FFPE) sections was harvested using RecoverAll Total nucleic Acid Isolation kit (Ambion).

cDNA Microarray Analysis. Dye-coupled cDNA was hybridized to MOE430A microarray gene chips (Affymetrix) (n = 7 in each group). Quality analysis on each individual chip was performed using affyPLM, available through Bioconductor (5). The normalized unscaled standard error (NUSE) boxplots were computed, and GCRMA software package used to obtain gene expression values (6). A principal component analysis (PCA) identified one outlier, which was removed for subsequent analysis. Cyber-T analysis (7) was used to identify differentially regulated genes based on a preselected false discovery rate (FDR) of 10%. This generated a list of 677 genes; P values were computed using the MAS5 algorithm. Genes that displayed significant (P < 0.05) change between the samples in each group were selected for further validation (Table S1).

Oncomine Analysis. The Oncomine database (www.oncomine.org) was searched for Hif-2α and Sgb3a1 genes. This generated 48 lung cancer datasets for Hif-2α and 16 datasets for Sgb3a1. We further filtered for datasets derived from tumors, and identified four datasets (namely Bilm lung, Ding lung, Kim lung, and Bittner lung) with expression values for both Hif-2α and Sgb3a1 genes. All of the datasets have Affymetrix U133 Plus 2.0 microarray platform and have three probes corresponding to Hif-2α: 200878_at, 200879_at, and 241055_at, and one probe corresponding to Sgb3a1: 230378_at.

For each dataset the following was performed: the complete data were downloaded from the National Center for Biotechnology Information’s Gene Expression Omnibus (GEO) repository and processed to obtain the expression value matrix of the samples and the probe sets. Only those samples corresponding to the NSCLC categories were selected and the signal values of the probe sets corresponding to the two genes were further selected. The data were log2 transformed to approximate data symmetry. For Hif-2α, boxplots of the probe sets were generated and the probe set with the highest median intensity (200878_at) was used for further analysis.

ChIP Analysis. Nuclear lysate was harvested and sonicated (Sonic Dismembrator Model 500; Fisher Scientific) to obtain 500-bp to 1,000-bp DNA fragments. Chromatin-protein complexes were cleared by centrifugation at 14,000 rpm for 15 min. The soluble chromatin was immunoprecipitated with polyclonal HIF-2α and monoclonal HIF-1α antibodies (Novus Biologicals). DNA cross-links were reversed by heating at 65 °C for 16 h with NaCl, treated with RNase A and protease K, and analyzed by qRT-PCR.

Fig S1. (A) Breeding scheme to generate Kras\textsuperscript{G12D}/Hif-2alpha\textsuperscript{+/Δ} and Kras\textsuperscript{G12D}/Hif-2alpha\textsuperscript{fl/Δ} mice. Mice carrying either a Hif-2alpha floxed allele (Hif-2alpha\textsuperscript{fl/Δ}) or Hif-2alpha null (Hif-2alpha\textsuperscript{Δ+}) allele were crossed with LSL-Kras\textsuperscript{G12D} animals to yield Kras\textsuperscript{G12D}/Hif-2alpha\textsuperscript{+/Δ} (mutant) and Hif-2alpha\textsuperscript{fl/Δ} (control) mice. # indicates concomitant activation of Kras\textsuperscript{G12D} allele and Hif-2alpha deletion upon exposure to Cre recombinase. (B) Efficient recombination of Hif-2alpha indicated by the presence of 1loxP product in two independent test tumors (designated T1 and T2) but not in adjacent uninfected lung tissue or ear as detected by Southern blot analysis. (C) A binary mask (Right) of an H&E stained section (Left) generated using the imaging software Axiovision 4 correctly identifies the tumor lesions (arrowheads).
Fig. S2. (A–F) Kras<sup>G12D</sup>, Hif-1α<sup>+/Δ</sup> (designated +/Δ) or Kras<sup>G12D</sup>, Hif-1α<sup>fl/Δ</sup> (designated fl/Δ) mice were infected with Adeno-Cre virus and killed 12 (A) or 24 (B) w.p.i, and tumor sections were treated with H&E stain. (C–F) Tumor numbers and volumes in each cohort (n = 5) were measured as described in Materials and Methods. (G–I) Tumors from mutant and control mice were graded for hyperplastic lesions (G), adenomas (H), and adenocarcinomas (I). Tumors were also assessed for Ki67 (J) and TUNEL (K) staining as measures of proliferation and apoptosis, respectively. Error bars (C–I) represent SEM. *P < 0.05.

Fig. S3. (A and B) Mutant tumors displayed significantly increased association with tumor infiltrating CD45 cells (n = 6), and in particular granulocytes (n = 5–6 for each group) (C and D). Error bars (B and D) represent SEM. *P < 0.05.
Fig. S4. (A) Hypoxic induction of \( \text{Scgb3a1} \), \( \text{aquaporin} \), and \( \text{ceruloplasmin} \) transcripts was reduced in HIF-2\( \alpha \) KD C1 and HIF-2\( \alpha \) KD C2 clones. (B) Expression of the HIF-1\( \alpha \) specific target gene \( \text{Pgk1} \) was unaffected by HIF-2\( \alpha \) expression levels in HIF-2\( \alpha \) KD cells. In contrast, hypoxic induction of the previously characterized HIF target gene \( \text{Vegf} \) (HIF-1\( \alpha \) and HIF-2\( \alpha \) shared target) was significantly reduced in HIF-2\( \alpha \) KD C1 cells but restored in HIF-2\( \alpha \) KD C1R cells. (C) Hypoxic induction of \( \text{Scgb3a1} \), \( \text{aquaporin} \), and \( \text{ceruloplasmin} \) transcripts is not significantly affected in HIF-1\( \alpha \) KD C1 and HIF-1\( \alpha \) KD C2 clones. (\( n = 3 \)). *\( P < 0.05 \). Error bars represent SD. (D) Schematic representation of the human \( \text{Scgb3a1} \) promoter region (2,000-bp 5\' region) demonstrating the presence of multiple putative HREs. (E) Nuclear extracts were isolated from A549 cells cultured under hypoxia (0.5\% \( \text{O}_2 \), 16h), sonicated, and immunoprecipitated with HIF-1\( \alpha \) antibody. DNA was amplified using primers for human \( \text{Scgb3a1} \) HRE regions 4 and 5 (designated H4 and H5), and normalized to mouse IgG antibody. Human \( \text{Pgk1} \) HRE 1 and 2 served as a positive control (human \( \text{Pgk1} \) 5\' 18-bp region containing HREs 1 and 2 have been previously described) (8). Error bars represent SD. *\( P < 0.05 \).
Fig. S5. (A) Nude (Nu/Nu) mice were injected s.c. with 5 x 10^6 HIF-2α KD C1 and C2 cells. A549 cells injected in contralateral flank served as controls, and individual tumor volumes were measured (mm³). HIF-2α KD C1 cells (n = 6) showed a modest but significant increase in tumor size compared with control tumors. **P < 0.005. HIF-2α KD C2 tumors did not reach statistical significance but showed a similar trend. (B) SCGB3a1 transcript levels in HIF-2α KD C1 cells engineered to stably express a human SCGB3a1 cDNA. Error bars represent SD. *P < 0.05, **P < 0.005.

Table S1. Microarray detection of misregulated genes in HIF-2αΔfl lung tumors

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Description</th>
<th>Fold difference</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ltf</td>
<td>Lactotransferrin</td>
<td>−6.51</td>
<td>0.018</td>
</tr>
<tr>
<td>Abp1</td>
<td>Amiloride binding protein 1</td>
<td>−3.74</td>
<td>0.032</td>
</tr>
<tr>
<td>Aqp 4</td>
<td>Aquaporin 4</td>
<td>−3.53</td>
<td>0.039</td>
</tr>
<tr>
<td>Scgb3a1</td>
<td>Secretoglobin, family 3a, member 1</td>
<td>−3.25</td>
<td>0.047</td>
</tr>
<tr>
<td>Gabrp</td>
<td>Gamma-aminobutyric acid (GABA-A) receptor, pi</td>
<td>−2.83</td>
<td>0.042</td>
</tr>
<tr>
<td>Slco 1a5</td>
<td>Solute carrier organic anion transporter family, member 1a5</td>
<td>−2.82</td>
<td>0.041</td>
</tr>
<tr>
<td>Slc27a2</td>
<td>Solute carrier family 27 (fatty acid transporter), member 2</td>
<td>−2.52</td>
<td>0.041</td>
</tr>
<tr>
<td>LOC672450</td>
<td>V (kappa) gene product</td>
<td>−2.03</td>
<td>0.045</td>
</tr>
<tr>
<td>Fbxo36</td>
<td>F-box protein 36</td>
<td>−2</td>
<td>0.033</td>
</tr>
<tr>
<td>Cp</td>
<td>Ceruloplasmin</td>
<td>−1.61</td>
<td>0.42</td>
</tr>
<tr>
<td>Mocs1</td>
<td>Molybdenum cofactor synthesis 1</td>
<td>1.59</td>
<td>0.05</td>
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<tr>
<td>Aco1</td>
<td>Aconitase 1</td>
<td>0.72</td>
<td>0.204</td>
</tr>
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</table>

n = 7 (HIF-2α+/Δ tumors), n = 7 (HIF-2α fl/Δ tumors).