NFIB overexpression cooperates with Rb/p53 deletion to promote small cell lung cancer

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ABSTRACT

Small cell lung cancer (SCLC) is a highly aggressive neuroendocrine tumor type that is typically metastatic upon diagnosis. We have a poor understanding of the factors that control SCLC progression and metastasis. The NFIB transcription factor is frequently amplified in mouse models of SCLC, but clear evidence that NFIB promotes SCLC in vivo is lacking. We report that in mouse models, Nfib amplifications are far more frequent in liver metastases over primary SCLC, suggesting roles in tumor progression/metastasis. Overexpression of Nfib in a sensitized mouse model led to acceleration of SCLC, indicating that Nfib functions as a bona fide oncogene. Suppression of Nfib expression in cell lines derived from the doxycycline-inducible Rb/p53/TET-Nfib model led to increased apoptosis and suppression of proliferation. Transcriptional analysis revealed that Nfib regulates the expression of genes related to axon guidance, focal adhesion and extracellular matrix-receptor interactions. These data indicate that Nfib is a potent oncogene in SCLC, and the enrichment of Nfib amplifications in liver metastases over primary SCLC points to Nfib as a candidate driver of SCLC metastasis.

INTRODUCTION

The most metastatic form of lung cancer is SCLC, a highly aggressive neuroendocrine cancer [1]. SCLC is typically metastatic at initial diagnosis, but little is known about the why this is so. The high frequency of metastasis at diagnosis raises the possibility that SCLC may acquire many of the genetic lesions needed for metastasis early, but there has been little work to compare primary SCLC to metastases using human samples. This is likely owing to the difficulty in accessing samples from a tumor type that rarely undergoes surgical resection. Thus, the timing of both genetic and non-genetic changes that promote SCLC progression and metastasis are very poorly understood.

Study of mouse models can overcome the limitations in accessing SCLC clinical samples. Human SCLCs almost universally exhibit RB and P53 inactivation [2, 3] and Rb/p53 deletion in a subset of cells in the murine lung leads to SCLC that arises with near complete penetrance [4, 5]. The resulting tumors resemble human SCLC, exhibiting strong neuroendocrine characteristics and frequent metastasis, especially to the liver. Tumors in the Rb/p53 mouse model undergo spontaneous secondary genetic alterations that also occur in human SCLC such as amplification of Mycl or deletion of Pten [6]. Moreover, overexpression of Mycl [7, 8] or deletion of Pten [6, 9] accelerates murine SCLC, showing that mouse models can be used to interrogate potential SCLC driver genes. We need a better understanding of which genetic changes occur very early in tumor initiation and which subsequent mutations contribute to tumor progression and metastasis.

Beyond Mycl amplification and Pten deletion, another genetic alteration that occurs in mouse models of SCLC is focal amplification of the Nfib gene [10]. These amplifications are high-level, and Nfib was found to be the only gene in the minimal region of amplification [10]. Nfib is one of four members of the Nfi family of transcription factors, a family that includes Nfia, Nfib, Nfic, and Nfix.
Genetic inactivation of \textit{Nfib} in mice revealed that \textit{Nfib} is important both for brain and lung development [11, 12]. In human SCLC cells, \textit{NFIB} can sometimes undergo high-level amplification [10]. An alternative mechanism through which \textit{NFIB} can be highly expressed is through the presence of atypically large transcriptional enhancers or super-enhancers [13, 14]. Super-enhancers, marked by high levels and long stretches of histone H3K27 acetylation (and/or other features associated with active enhancers) have been proposed to promote the expression of genes important for cell lineage and onco genesis [15-17]. Cell based studies support the notion that \textit{NFIB} exhibits oncogenic activity [10], but \textit{in vivo} analyses are required to rigorously assess \textit{Nfib} oncogenic function. In this study, we overexpressed \textit{Nfib} in a sensitized mouse model and also suppressed NFIB expression in SCLC cells; we report potent oncogenic activity of \textit{Nfib} in promoting SCLC \textit{in vivo}.

\section*{RESULTS}

\textbf{Nfib} amplifications occur more frequently in liver metastases over primary SCLC

We used next generation sequencing to compare genetic alterations between primary SCLC and liver metastases that arise in murine models of SCLC. These models are initiated by intra-tracheal delivery of either broadly expressing Ad-CMV-Cre or Ad-Cre driven by a neuroendocrine promoter (Ad-CGRP-Cre). We collected primary tumors and liver metastases across a series of 17 mice derived from three models: \textit{Rb}^\textit{lox/lox};\textit{p53}\textit{lox/lox} [4] and \textit{Rb}^\textit{lox/lox};\textit{p53}\textit{lox/lox};\textit{Pten}\textit{lox/lox} [9] mice infected with Ad-CMV-Cre, and an \textit{Rb}^\textit{lox/lox};\textit{p53}\textit{lox/lox};\textit{Pten}\textit{lox/lox} model infected with Ad-CGRP-Cre [6, 7]. The use of neuroendocrine specific Ad-CGRP-Cre is necessary in the \textit{Rb}^\textit{lox/lox};\textit{p53}\textit{lox/lox};\textit{Pten}\textit{lox/lox} model, as this dramatically reduces the incidence of adenocarcinoma that otherwise occurs with Ad-CMV-Cre infection in these mice [6, 7, 9]. We analyzed one lung tumor and one liver metastasis from each of 17 SCLC-bearing mice (Supplemental Table 1). We performed copy number variation (CNV) analyses using low-coverage whole genome sequencing across each lung tumor and liver metastasis. Matched normal tail DNA was used as a control. We were particularly interested in recurrent changes enriched in metastatic tumors. We employed Segseq [18] and CNVseq [19] to identify CNV changes. We did not find recurrent examples of metastasis-specific enrichment or newly acquired genetic amplifications in \textit{Myc} or deletions of \textit{Pten} in the liver metastases (Supplemental Figure 1A, 2A and data not shown). However, we identified a region of focal amplification on chromosome 4 that was very frequently amplified in liver metastases over primary SCLC, this region harbored the \textit{Nfib} gene (Figure 1A, Supplemental Figure 2). In the mouse models tested, multiple primary tumors can arise in the lung and so the liver metastasis was not always clonally related to the paired lung tumor. However, across some of the pairs, obvious clonal relationships could be discerned where it was clear that the \textit{Nfib} amplification was present in the liver metastasis but undetectable in the bulk primary lung tumor. For example, Figure 1B shows a tumor from the \textit{Rb}/\textit{p53} model, H6904, with very similar genome-wide CNV profiles between the lung and liver metastasis. However, multiple chromosome 4 amplifications, including \textit{Nfib} (but not \textit{Mycl}) were present in the liver metastasis but not the matched lung tumor. Similarly, across another \textit{Rb}/\textit{p53} model pair that was clearly clonally related (H6210), \textit{Mycl} amplification was present in the lung SCLC (which lacked \textit{Nfib} amplification), and the liver metastasis exhibited \textit{Nfib} amplification but lacked \textit{Mycl} amplification (Supplemental Figure 1B). This example suggests different evolution of the lung tumor and liver metastasis by the time of sampling. We also compared \textit{Nfib} expression across a series of primary SCLC vs. metastases from the \textit{Rb}/\textit{p53} and \textit{Rb}/\textit{p53}/\textit{Pten}\textit{lox/lox} models using real time PCR. We found significantly increased expression of \textit{Nfib} in liver metastases over primary tumors (Figure 1C). We conclude that \textit{Nfib} amplifications are enriched in liver metastases over primary tumors in mouse SCLC models. Our data suggest that positive selection of SCLC cells harboring \textit{Nfib} amplification may contribute to SCLC progression.

\textbf{Nfib overexpression accelerates SCLC in a mouse model}

We next used a novel TET-regulated \textit{Nfib} transgenic strain (TRE-\textit{Nfib}) to investigate the role of \textit{Nfib} in SCLC initiation (see Methods for details on transgenic strain generation). By combining this allele with the \textit{Rosa26}^\textit{lox-stop-lox}\textit{rtTA} allele, \textit{rtTA}, and consequently \textit{Nfib}, is overexpressed upon Cre-mediated recombination (Figure 2A). Intra-tracheal AdenoCre delivery leads to Cre expression in lung epithelial cells. We used this inducible system to test oncogenic roles for \textit{Nfib} in the sensitized \textit{Rb}^\textit{lox/lox};\textit{P53}\textit{lox/lox} AdenoCre SCLC model [4]. We compared tumorigenesis in \textit{Rb}^\textit{lox/lox};\textit{P53}\textit{lox/lox};\textit{Rosa26}^{\textit{SE-rtTA/SE-rtTA}};\textit{TRE-Nfib} (herein referred to as \textit{Rb}/\textit{p53}/TET-\textit{Nfib}) vs. \textit{Rb}^\textit{lox/lox};\textit{P53}\textit{lox/lox};\textit{Rosa26}^{\textit{SE-rtTA/SE-rtTA}} mice (herein referred to as \textit{Rb}/\textit{p53}). The expression of \textit{Nfib} is activated by addition of doxycycline to the feed starting one week after Ad-CMV-Cre infection. We followed mice until respiratory changes from tumor burden necessitated euthanasia. Western blot analysis of lung tumors collected from \textit{Rb}/\textit{p53}/TET-\textit{Nfib} mice showed increased expression of NFIB compared to lung tumors from \textit{Rb}/\textit{p53} controls (Figure 2B). As shown in Figure 2C, we found that \textit{Nfib} overexpression rapidly accelerated time to SCLC with a median time to morbidity of 256 days in the \textit{Rb}/\textit{p53}/TET-\textit{Nfib} model vs. 324 days.
Figure 1: *Nfib* is amplified in liver metastases over primary tumors in mouse models of SCLC. A. Integrated genome viewer (IGV) plot showing *Nfib* copy number in a series of 17 primary murine SCLCs (left) and liver metastases (right). Log2 copy number ratios of tumor DNA relative to normal tail DNA is shown, with scale to the right. B. Copy number variation (CNV) analysis showing matched pair of primary SCLC and liver metastases, analyzed using CNVSeq. The majority of copy number alterations are similar between the primary and metastasis. Chromosome 4 harbors focal amplifications including *Nfib* amplification (arrow) exclusively in the metastasis. C. Real-time PCR analysis of *Nfib* expression (bottom) across a panel of primary SCLC and liver metastases. Data are normalized to *Gapdh*. Note the clear upregulation in *Nfib* expression in a large proportion of the liver metastases over matched primary lung tumors. p-value from Student’s T-test is indicated.
in the Rb/p53 controls (p = 0.04, log-rank test). Resulting tumors in the Nfib overexpressing model exhibited histology typical of the Rb/p53 model [20] and exhibited neuroendocrine features, as shown by Calcitonin Gene Related Peptide (CGRP) immunostaining (Figure 2D). While Rb/p53 deletion in this system leads to predominant SCLC, cooperating mutations can lead to a change in tumor spectrum, as in Cui et al [9], where Pten inactivation promoted lung adenocarcinoma. We found no instances of adenocarcinoma in the Rb/p53/TET-Nfib group. Given Nfib amplification enrichment in liver metastases (Figure 1), we were interested in whether Nfib-overexpressing SCLC exhibited higher rates of liver metastasis. Upon necropsy, gross liver metastases were found in 6/14 (43%) of lung tumor bearing mice from the Rb/p53/TET-Nfib compared to 7/15 (47%) of lung tumor bearing mice from the Rb/p53 group. Liver metastases were found at an average +/- s.d. of 299 +/- 53 days in the Rb/p53/TET-Nfib model and 318 +/- 29 days in the Rb/p53 model, a difference that was not statistically significant. Our data indicate that Nfib functions as a potent lung cancer oncogene in the Rb/p53 model and this ability of Nfib to function as an oncogene was not specific to SCLC metastasis.

Suppressing NFIB levels reduces proliferation

We established cell lines from Rb/p53/TET-Nfib mice that had Nfib overexpressed in a doxycycline-dependent fashion. By washing out doxycycline from the media after cell line establishment, we could interrogate the cellular consequences of suppressing NFIB expression in tumors initiated with high levels of NFIB. Upon removal of doxycycline from the media, we found suppression of proliferation in two of three cell lines (Figure 3A) and sharply decreased NFIB protein levels (Figure 3B). In the responsive cell lines, removal of doxycycline led to increased apoptosis. We observed an increased sub-G1 population in FACS analysis for DNA content (Figure 3C, 3D and not shown). Increased cleaved caspase 3 was consistent with higher levels of apoptosis in responsive cells upon doxycycline removal.

Figure 2: Nfib overexpression accelerates SCLC in vivo. A. Cartoon showing doxycycline inducible Nfib expression and Rb/p53 deletion driven by Adeno Cre expression, delivered using intratracheal delivery to the lung epithelium. B. Western blot showing Nfib overexpression in lung tumors from the Rb/p53/TET-Nfib model, as compared to Rb/p53 model controls. As a positive control, two liver metastases from tumors harboring spontaneous genomic amplification of Nfib in the Rb/p53 model are also shown (NFIB AMP). C. Kaplan-Meier curve showing acceleration of SCLC in Rb/p53/TET-Nfib compared to the Rb/p53 model. Median survival for each model is indicated and p-value from log-rank test is shown. D. Hematoxylin and eosin stain of representative SCLC arising in the Rb/p53/TET-Nfib model, black scale bar, 50mm. Inset: CGRP immunostaining of Rb/p53/TET-Nfib tumor, white scale bar, 10mm.
Figure 3: Suppression of NFIB expression reduces cell viability and migration. A. Cell TiterGlo assay showing proliferation in Rb/p53/TET-Nfib cell lines upon removal of doxycycline (DOX). Mean +/- s.d. of triplicate wells shown and data are representative of 3 independent experiments. *p < 0.001, Student’s T-test. B. Western blot showing NFIB, cleaved Caspase 3 (CC3) and p27 protein levels across three Rb/p53/TET-Nfib cell lines in the presence of DOX or 7 days post washout of DOX. Beta actin is used as a loading control. C. FACS analysis for DNA content shows increased subG1 population, indicative of apoptosis, and decreased G1 and S-phase populations upon doxycycline removal in NfibCL2 cell line from Rb/p53/TET-Nfib lung tumor. D. Quantification of FACS data. Data representative of 2 independent experiments, each done in triplicate. *p < 0.001, Student’s T-test. E. Western blot showing NFIB expression in NCI-H446 cell line that underwent lentiviral CRISPR-mediated NFIB deletion. Three NFIB-deleted sub-lines, derived from single cells, are shown along with two control sublines with guide RNAs targeting GFP, or a non-targeting control guide RNA. Two different sgRNA sequences were used, with one clone using sgRNA sequence NFIB#1 and two clones using sgRNA sequence NFIB#2 (see methods). F. CellTiter Glo experiment showing reduced proliferation in the NFIB-deleted H446 cells. G. Transwell migration assay in which NFIB-deleted and control H446 cells were transferred to transwells and allowed to migrate over 17 hours. Crystal violet stained migrated cells were counted. Each cell line was plated in triplicate transwells and results show mean +/- s.d. * p < 0.001, # p < 0.005, Student’s T-test.
we were interested in identifying NFIB-regulated expression. Using RNAseq and EdgeR analysis, we identified 372 differentially expressed genes (Figure 4B). Considering all three comparison (Figure 4A). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of this 372-gene set revealed highly significant NFIB-dependent pathways (focal adhesions $p = 5\times10^{-9}$, extracellular matrix-receptor interaction $p = 5\times10^{-8}$, axon guidance, $p = 5\times10^{-8}$ and pathways in cancer $p = 5\times10^{-9}$) shown in Figure 4B. Considering all three differential expression comparisons, i.e. liver metastases with high vs. low Nfib expression, DOX-inducible Rb/p53/TET-Nfib vs. Rb/p53 lung tumors, and TET-Nfib cell lines with or without DOX, we found 66 genes to be commonly changed depending on Nfib status (Figure 4C, Supplemental Table 5). 62 of these genes changed in the same direction, 43 were up-regulated when Nfib expression was increased and 19 down-regulated. KEGG pathway analysis of these consistently Nfib-regulated 62 genes, showed the highest enrichment in axon guidance genes ($p = 0.00004$, Benjamani-Hochberg FDR $q = 0.001$). Axon guidance related genes including Epha5, Epha8, Slit1 and Unc5d were upregulated and PlexinA1 and Robo1 downregulated upon Nfib overexpression (Figure 4D, Supplemental Figure 3).

**DISCUSSION**

The most metastatic form of lung cancer is small cell lung carcinoma (SCLC). Unfortunately, there is little known about the signaling pathways that control SCLC metastasis. Human metastatic SCLC is rarely resected, hindering our understanding of genetic determinants of SCLC metastasis. We compared genetic differences between primary SCLC and liver metastases from genetically engineered mouse models of SCLC. We identified Nfib as a gene that is frequently amplified in distant liver metastases (Figure 1). Nfib was previously found amplified in SCLC from the Rb/p53 model [10], but in vivo evidence that Nfib functions as an oncogene was needed. Thus, we interrogated Nfib oncogenic function using a sensitized mouse model of SCLC driven by inactivation of Rb and p53, genes inactivated in almost all human SCLCs. Our observation of acceleration of SCLC upon Nfib overexpression (Figure 2) provides definitive evidence that Nfib functions as an SCLC oncogene.

The mechanisms through which Nfib promotes SCLC are as yet unclear. We found that deletion of Nfib in a human SCLC cell line and Nfib suppression in cell lines derived from the Rb/p53/TET-Nfib SCLC mouse model led to reduced numbers of viable cells, with increased apoptosis and reduced proliferation (Figure 3). Our gene expression analysis showed that Nfib overexpression leads to activation of genes involved in axon guidance/neuronal function, which is consistent with roles for Nfib in brain development revealed through gene knockout studies [11]. Nfib also regulated genes involved in focal adhesions and extracellular matrix-receptor interactions (Figure 4). We also found reduced migration upon Nfib deletion in a human cell line harboring Nfib amplification. Our identification of Nfib-dependent gene expression differences in SCLC cells provides a starting point for functional interrogation of Nfib-regulated genes and pathways that promote SCLC.

By comparing primary SCLC to liver metastases from mouse SCLC models we found clear enrichment of Nfib amplifications in liver metastases (Figure 1). In matched pairs of lung tumors and liver metastases we...
Figure 4: NFIB controlled transcriptional programs in SCLC. A. Top: Venn diagram showing overlapping differential expressed genes across two comparisons, including liver metastases with high vs. low Nfib expression and lung SCLCs from the Rb/p53 vs. Rb/p53/TET-Nfib models. 475 overlapping genes were differentially expressed in both liver metastasis and DOX-inducible lung tumor comparisons. Bottom: Top KEGG pathways enriched in this gene set are indicated with p-value shown on X-axis. B. KEGG pathways enriched in 372 genes differentially expressed in an RNAseq comparison of 4 Rb/p53/TET-Nfib SCLC cell lines, comparing cells on doxycycline vs. 7 days after doxycycline washout. C. Venn diagram showing 66 genes were commonly differentially expressed across all three comparisons. D. Heat map showing the expression of the commonly altered 66 genes in the comparison of lung SCLCs from the Rb/p53 vs. Rb/p53/TET-Nfib models.
observed \( Nfib \) amplifications in the metastasis but not in the bulk primary tumor. However, surprisingly, our mouse study did not reveal a higher rate of liver metastasis when \( NFIB \) was overexpressed at tumor initiation in the \( Rb/p53 \) mutant model. It may be that the acceleration of primary tumor development by \( Nfib \) overexpression limited our ability to detect a role for \( Nfib \) in driving liver metastasis, since the \( Nfib \) overexpressing mice died earlier with less time for metastasis to develop. Transplant experiments will help us address whether NFIB overexpressing tumor cells derived from the \( Rb/p53/TET-Nfib \) model exhibit increased propensity to metastasis. Also using the novel \( Rb/p53/TET-Nfib \) mouse model described, it will be interesting to activate \( Nfib \) expression not at tumor initiation but later in tumor progression. Following detection of lung tumor burden by magnetic resonance imaging, \( Nfib \) expression could be induced and the effect of \( Nfib \) overexpression in tumor progression discerned. For example, numbers of circulating tumor cells and influence of \( Nfib \) overexpression on rate of liver metastasis in this context could be assessed. It will also be interesting to remove doxycycline from \( Nfib \)-driven SCLC and assess the requirement for \( Nfib \) in the maintenance of lung tumors and liver metastases.

In human SCLC, NFIB expression can be deregulated through multiple mechanisms. It was previously shown that 16/46 human SCLC cell lines harbored \( NFIB \) copy number gains, including occasional focal high-level amplifications [10]. NFIB can also be highly expressed in SCLC cells through the presence of atypically large enhancers or super-enhancers [13, 14] and NFIB is a target gene of ASCL1, a master regulator of neuroendocrine cell fate [14]. As our mouse study shows that NFIB clearly exhibits oncogenic function in SCLC, it will be critical to compare human primary tumors and metastases to determine whether human SCLC also exhibits increased \( NFIB \) expression in metastases. Moreover, the extent to which genetic and non-genetic changes in human SCLC controls \( NFIB \) expression needs to be determined.

While this work was under review, two studies were published with data also supporting roles for NFIB in promoting SCLC and driving metastasis [21, 22]. Using a different transgenic allele, the Berns group found that \( NFIB \) overexpression also promoted small cell lung cancer in an \( Rb/p53 \)-deleted background [22]. One difference compared to our work is the authors observed an increased rate of liver metastasis upon \( Nfib \) overexpression. It is possible that differences in the level of \( Nfib \) overexpression between the models could have an impact on metastasis rate. Overall, findings that \( Nfib \) amplifications were enriched in liver metastases over lung tumors (our study, Figure 1), along with the increased metastasis observed with NFIB overexpression in [22] and promotion of metastasis in transplant studies [21] all support NFIB as an oncogene in SCLC with roles in metastasis. NFIB was found to remodel chromatin and increase DNA accessibility in SCLC cells to promote metastasis [21]. It is now critical to identify the relevant NFIB target genes and mechanisms through which NFIB promotes advanced and metastatic SCLC.

Our data reveal that \( NFIB \) functions as a potent oncogene in SCLC. Patients with SCLC face a dismal clinical prognosis. It is essential that we make inroads to understand the pathways through which \( NFIB \) and other SCLC driver genes control the steps between tumor initiation and metastatic progression.

**MATERIALS AND METHODS**

**Mice**

SCLC models bearing \( Rb/p53 \) and \( Rb/p53/Pten \) inactivation were previously described [4, 7, 9]. To test the effect of \( Nfib \) overexpression in vivo, the \( Rb/p53 \) floxed model was bred to mice with a \( Rosa26^{GFP-STOP-Lox} \) reverse tetracycline controlled transactivator (\( rtTA \)) allele (Jackson Laboratories) and to a novel mouse strain that we generated harboring an inducible Tetracycline Responsive Element (TRE)-\( Nfib \) allele. Mice were maintained on a mixed genetic background. Adenoviral Cre driven by a CMV promoter was used to infect the lungs of mice via intratracheal delivery. A week after Cre delivery, mice were switched to doxycycline-containing chow (625mg/kg, Harlan) and were maintained on this feed through the duration of the experiment. Mice were euthanized when they exhibited signs of altered breathing patterns, which was typically caused by lung tumor burden.

**Construction of TET-\( Nfib \) allele**

To generate the doxycycline inducible \( Nfib \) transgene, the mouse \( Nfib \) cDNA containing an amino terminal hemagglutinin (HA) tag was cloned under control of the (TetO),CMV minimal promoter and the 3' untranslated sequence and polyadenylation signal from the bovine growth hormone gene was added to generate a stable mRNA as described previously [23]. The HA-tagged \( Nfib \) cDNA was subcloned from pCHNFI-B2 [24] as a NotI- BamHI (blunted with T4 polymerase) fragment into pCDNA3.1Teto-CMV-zeo [25] cut with NotI and Xba1 (blunted with T4 polymerase). The final construct, pCMVtetoNFIB2, was verified by sequencing. This plasmid was digested with Spe1/Sph1 and the 2kb fragment containing (TetO),CMV promoter driven HA-tagged \( Nfib \) cDNA was purified and microinjected into F/ VBN mouse oocytes by the transgenic core at Cincinnati Children’s Hospital Medical Center.
Next generation sequencing

**RNAseq:** Total RNA was isolated in Trizol (Invitrogen) and RNaseq libraries generated from oligo dT purified mRNA. We used the Truseq RNA kit (Illumina) for library generation. A HiSeq 2500 was used for sequencing, generating 50 base pair single end reads. After splicing aware alignment using Tophat [26] we used EdgeR [27] to identify differentially expressed transcripts. An FDR of 0.05 was used as a cutoff for identifying differentially expressed transcripts and the DAVID Bioinformatics Resources 6.7 online tool [28] (http://david.abcc.ncifcrf.gov/) was used to identify significant differentially expressed pathways, focusing on KEGG pathway analysis. We also generated FPKM values for each RNAseq comparison using Tophat/Cuffdiff (Supplemental Tables 6,7,8) [26].

**CNV analysis:** Genomic DNA was isolated from tumors and matched tail samples. Sequencing libraries were prepared using the NEBNext DNA library preparation kit (New England Biolabs). We used an Illumina Hiseq 2500, generating 50 base pair single end reads. The Burrows Wheeler Aligner (BWA) was used to align reads to the mm9 genome. Mapped reads were placed into 100kb bins and a read depth normalized ratio of tumor to normal was visualized using IGV. We also employed CNVseq to identify regions of copy number alteration [19].

Real-time RT-PCR

Total RNA from tumors were isolated using TRIzol (Invitrogen) following manufacturer’s instructions. cDNA was synthesized using SuperScript II reverse transcriptase (ThermoFisher Scientific). Quantitative PCR was performed with SYBR Green Real-Time PCR Master mix (ThermoFisher Scientific). Quantitative expression data were acquired and analyzed with a 7900 Real-time PCR System (Applied Biosystems). Primers were designed to detect endogenous Nifb and Gapdh (as endogenous control).

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CellTiter-Glo cell proliferation assay

Experiments were performed according to the manufacturer’s instructions (Promega). Briefly, 100μl of 5000 cells were seeded in white-walled 96-well plates. Plate was equilibrated at room temperature for 30 min before added CellTiter-Glo reagent at a 1:1 ratio to the cell culture volume. The plate was then mixed for 2 minutes on an orbital shaker and incubated at room temperature for another 10 minutes to stabilize the luminescent signal. Luminescence was recorded on plate reader (BioTek) at 1 second per well integration time.

FACS analysis

Cells were resuspended in 0.6 ml PBS and fixed with 1.4 ml ice-cold 100% ethanol. Cells were treated with 0.1mg/ml RNase and 25μg/ml propidium iodide and analyzed on a flow cytometer. FACS data was analyzed with FlowJo Software.

Cell migration assay

Briefly, 800μl of DMEM containing 10% FBS was placed in the lower chamber of 24-well 8 micron Transwells (Falcon). 200μl of 20,000 cells were placed in the upper chamber and allowed to migrate for 17 hours at 37 °C. At the end of the migration assay, the filter side of the upper chamber was cleaned with a cotton swab. The filters were then fixed in 100% methanol for 5 min, stained with crystal violet staining solution (0.05% crystal violet, 1% formaldehyde, 1% methanol in PBS) for 10 minutes and rinsed with water. The filters were air-dried and the
cells that have migrated through filter pores were counted. For each migration condition, the number of cells that migrated across the filters in 4X high-power fields per insert was counted. Triplicate transwells were employed for each condition.

**Histology and immunohistochemistry**

We fixed lungs in formalin for 24 h before paraffin embedding. Paraffin blocks were sectioned at four-microns and stained with hematoxylin and eosin. Paraffin sections were cut and processed from xylene through a graded ethanol series (100%, 95% and 70%) to PBS. Unmasking was performed using microwave heating in sodium citrate buffer (0.01M at pH6.0). Endogenous peroxidases were blocked with 3.5% H$_2$O$_2$, and immunohistochemistry was performed with an overnight incubation with anti-CGRP antibody (Sigma C8198, 1:1000). Biotin-conjugated secondary antibodies (Vector Laboratories) were used at a dilution of 1/200 in blocking solution. After secondary antibody binding, detection was performed via a biotin-peroxidase complex (Vectastain ABC, Vector Laboratories) with DAB substrate (Vector Laboratories). Haematoxylin was used to counterstain.

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**CONFLICTS OF INTERESTS**

We have no potential conflicts of interest to disclose.

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