Nuclear Factor I/B is an Oncogene in Small Cell Lung Cancer

Alison L. Dooley¹, Monte M. Winslow¹, Derek Y. Chiang²,³,⁴, Shantanu Banerji²,³, Nicolas Stransky², Talya L. Dayton¹, Eric L. Snyder¹, Stephanie Senna¹, Charles A. Whittaker¹, Roderick T. Bronson⁵, Denise Crowley¹, Jordi Barretina²,³, Levi Garraway²,³, Matthew Meyerson²,³, Tyler Jacks¹,⁶

¹David H. Koch Institute for Integrative Cancer Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA
²The Broad Institute, Cancer Program, Cambridge, Massachusetts, USA
³Dana-Farber Cancer Institute, Department of Medical Oncology and Center for Cancer Genome Discovery, Boston, Massachusetts, USA
⁴Current address: Lineberger Comprehensive Cancer Center, 450 West Drive, CB #7295, Chapel Hill, North Carolina, USA
⁵Department of Pathology, Tufts University School of Medicine and Veterinary Medicine, North Grafton, Massachusetts, USA
⁶Howard Hughes Medical Institute, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA

Supplemental Information

Supplemental Figure Legends

Supplemental Figure 1. Tumor progression and metastasis in a mouse model of small cell lung cancer. (A) Schematic of the mouse model. Exon 19 and exons 2-10 are flanked by loxP sites (floxed) in the Rb1 and Trp53 genes (p53fl/fl;Rbfl/fl), respectively. Following Cre recombinase-mediated recombination, the exons are deleted. (B) An in situ small cell lung lesion 8 months after infection. (C) High magnification of an invasive tumor. At later stages, tumors disseminate within the lung to the (D) blood vessels and (E) lymphatic vessels, and metastasize locally to the (F) lymph nodes, and distantly to the (G) liver, (H) adrenal glands and (I) bone. (J) Detection of a bone metastasis using luciferase imaging of a p53fl/fl;Rbfl/fl;Rosa26LSL-Luciferase/LSL-Luciferase mouse. (K) Micro-
computed tomography imaging of the bone metastasis. The bone metastasis is osteolytic, consistent with metastatic human SCLC. (left panel: right knee with bone metastasis, right panel: normal left knee.) (L) Kaplan-Meier survival curve of mice infected with two different titers of adenovirus expressing Cre recombinase. (squares: $2.5 \times 10^7$ PFU, median latency: 475 days; diamonds: $1 \times 10^8$ PFU, median latency: 350 days). (B-I): Hematoxylin and eosin stain.

**Supplemental Figure 2.** Nfib is located at the peak of the amplification in mSCLC. (A) Focally amplified peak containing Nuclear Factor I/B is shown in two tumors. (B) Focally amplified peak containing Nuclear Factor I/B is shown in two cell lines. Copy number data is plotted as the tumor to somatic copy number ratio. T: primary lung tumor, Liv: liver metastasis, LN: lymph node metastasis, gray labels: tumor samples, black labels: cell lines.

**Supplemental Figure 3.** Amplification of $L$-myc in murine small cell lung tumors and cell lines. (A) Amplification of $L$-myc ($Myc1$) in four cell lines analyzed using Illumina sequencing-based DNA copy number analysis. T: primary lung tumor, Liv: liver metastasis, LN: lymph node metastasis. (B) Real time PCR on genomic DNA for amplification of the $L$-myc locus indicates that $L$-myc is amplified in tumor- and metastasis-derived cell lines. A DNA copy number ratio greater than 1.2 was considered an amplification, as determined by real time PCR on genomic DNA. (C) Real time PCR indicates that cell lines that express high levels of Nfib also express high levels of $L$-myc. (D) IGV display of copy number from 120-8-124.8 Mb on chromosome 4. The
samples are organized from highest $L$-myc amplification to lowest. Scale bar indicates the log$_2$ copy number ratio of tumor to somatic reference sample. Dotted line indicates the boundaries of the minimally conserved region. T: primary lung tumor, Liv: liver metastasis, LN: lymph node metastasis, gray labels: tumor samples, black labels: cell lines.

**Supplemental Figure 4.** mSCLC samples contain focal amplifications and deletions. (A) IGV plot of DNA copy number changes in all samples across the genome. Samples are plotted as the log$_2$ ratio of tumor to somatic copy number. Scale bar indicates the log$_2$ copy number ratio of tumor to somatic reference sample. The dotted line indicates the boundaries of the minimally conserved region. T: primary lung tumor, Liv: liver metastasis, LN: lymph node metastasis, gray labels: tumor samples, black labels: cell lines.

**Supplemental Figure 5.** Co-amplification of $L$-myc and $Nfib$ in SCLC. (A) Number of mSCLC cell lines with amplifications of $Nfib$, $L$-myc or both $Nfib$ and $L$-myc. A DNA copy number ratio greater than 1.2 was considered amplified, as determined by real time PCR on genomic DNA. (B) Number of human SCLC cell lines with amplifications of NFIB and/or L-MYC. A DNA copy number ratio greater than 1.2 was considered amplified, as determined by the copy number profiling.

**Supplemental Figure 6.** Nfib protein is expressed in lung neuroendocrine cells. (A) Percent of neuroendocrine bodies that contain Nfib-expressing cells. Lung
neuroendocrine cells express Nfib as detected by double IHC for a neuroendocrine marker, calcitonin gene-related peptide (CGRP, red, cytoplasmic) and Nfib (brown, nuclear). (B) Representative image of a neuroendocrine body that lacks Nfib-expressing cells (CGRP\(^{-}\)Nfib\(^{-}\)). (C) Representative image of a neuroendocrine body that contains Nfib-expressing cells (CGRP\(^{+}\)Nfib\(^{+}\)). (B, C: 40X objective)

**Supplemental Figure 7.** Nfib protein is expressed in mSCLC. (A) Nfib can be detected by IHC in an in situ SCLC lesion. (B) Nfib is expressed in an invasive small cell lung tumor. (C) Nfib is expressed in a lymph node metastasis. (D) Nfib is expressed in a liver metastasis. T: liver metastasis, Liv: normal liver. (E) Nfib is not expressed in adenomas that arise in the mouse model. A: adenoma, L: normal lung. (A, C: 20X objective, scale bar is 100 µm; B, D: 10X objective, scale bar is 200 µm, E: 20X objective) (F) Percent of lesions positive (dark gray), weak (light gray), and negative (white) for Nfib expression in lung tumors that are in situ, invasive (Inv.), disseminated (Dis.) as well as lymph node (LN) and liver metastases.

**Supplemental Figure 8.** NFIB is amplified in human SCLC cell lines. (A) NFIB amplifications were confirmed by real time PCR on genomic DNA. All cell lines are NCI cell lines, except where otherwise noted.

**Supplemental Figure 9.** NFIB is expressed in human SCLC samples. (A) Representative images of human SCLC samples with negative, weak/moderate, or high
NFIB expression as detected by IHC. NFIB protein expression was graded on a scale of 0 (not expressed) to 3 (highly expressed). The scale bar is 200 µm.

**Supplemental Figure 10.** NFIB knockdown in human SCLC cell lines. (A) Two hairpins knocked down NFIB in an adherent cell line, NCI-H446. (B) Western blot demonstrating NFIB knockdown in NCI-H196 using the same two hairpins as in (A). (C) NFIB knockdown is detected by Western blotting in NCI-H82. (D) NFIB knockdown reduces proliferation in NCI-H82, as assessed by BrdU incorporation detected by FACS. Values represent the mean +/- standard deviation of duplicate wells of a representative experiment. (Cont.: control GFP shRNA, Uninf.: uninfected cell line)

**Supplemental Figure 11.** Nfib is an oncogene in mSCLC cell lines. (A) Stable expression of Nfib and/or L-myc in a mouse primary SCLC cell line (3151T4) with low endogenous expression of both Nfib and L-myc. (B) Crystal violet staining of soft agar colonies in the uninfected cell line or cell line stably expressing Nfib and/or L-myc. Images are representative of two separate experiments and visualized using crystal violet staining. (C) Quantification of the number of soft agar colonies in the uninfected cell line or cell line stably expressing Nfib, L-myc or L-myc & Nfib. The values represent the mean +/- standard deviation of triplicate plates. (D) Growth curve of a primary tumor cell line, 3151T4, either uninfected or stably expressing Nfib, L-myc or L-myc & Nfib. (E) Growth curve of a primary tumor cell line, 3583T3, either uninfected or stably expressing Nfib, L-myc or L-myc & Nfib. (B-E: Uninfected and Nfib results are also shown in Figure 5B) (D, E) The slope decreases from day 3 to 4 because the cells were
reaching confluency after three days. Values represent the mean +/- standard error of the mean of two separate experiments, each performed in triplicate. (*: p value < 0.05, **: p value < 0.005).

**Supplemental Figure 12.** Significant curated gene sets from the Gene Set Enrichment Analysis (GSEA). Curated gene sets of interest following expression of Nfib in mouse SCLC cell lines. A description of each of the gene sets is included. The values in the table represent the normalized enrichment score (NES). A FDR q-value less than 0.05 was required to be significant.

**Supplemental Figure 13.** Nfib is an oncogene in mouse embryonic fibroblasts. (A) Western blot demonstrating stable expression of Nfib and/or L-myc in wild type and p53-/- MEFs. (B) Crystal violet staining and quantification of low density colonies in wild type MEFs or wild type MEFs stably expressing Nfib and/or L-myc. Images are representative of two different MEF preparations and visualized using crystal violet staining. Values represent the mean +/- SEM of the total number of colonies in triplicate plates utilizing two different MEF preparations. (C) Crystal violet staining and quantification of low density colonies in p53-/- MEFs stably expressing Nfib and/or L-myc. Images are representative of two different MEF preparations and visualized using crystal violet staining. Values represent the mean +/- SEM of the total number of colonies in triplicate plates utilizing two different MEF preparations. (D) MEFs either uninfected or infected with Nfib and/or L-myc expressing viruses were plated at a high density and assayed for three-dimensional colony formation. Colonies were visualized
using crystal violet and quantified. Values represent the mean +/- SEM of the total number of foci in triplicate plates utilizing two different MEF preparations. (E)

Quantification of the number of soft agar colonies in the uninfected MEFs or MEFs stably expressing Nfib, L-myc or L-myc & Nfib. The values represent the mean +/- SEM of triplicate plates. (*: p value < 0.05, **: p value < 0.005, ***: p value < 0.0005).

**Supplemental Table 1.** Copy number alterations in mSCLC tumor and cell line samples. An amplification has a somatic to reference copy number ratio greater than or equal to 1.3 and a deletion has a copy number ratio of less than or equal to 0.7. In addition, the X chromosome (Chr. 20) appears to be amplified (copy ratio of 2) if the tumor sample was derived from a female mouse because the reference genome is male. We have removed all amplifications occurring on Chr. 20 if this is the case. We have flagged all amplifications that occurred due to strain differences in the somatic and reference genomes (Germline CNV = “Y”).

**Supplemental Table 2.** Read depth of Illumina sequencing of each mSCLC tumor and cell line sample. Samples correspond to those shown in Figure 1, where T: primary lung tumor, Liv: liver metastasis, LN: lymph node metastasis, gray labels: tumor samples, black labels: cell lines. Total reads and confidently aligned reads (mapping quality greater than 30) are shown.
Supplemental Methods

Mouse imaging. Bioluminescence imaging was performed according to manufacturer’s instructions (Xenogen). Micro-computed tomography (GE Healthcare) was performed on fixed specimens as previously described (Meylan et al. 2009) with a 27 µM voxel size.

Identification of germline amplifications. Germline copy number variants were identified by first identifying regions with similar start and end positions with respect to the mouse reference genome, within statistical sampling error. At an average sampling density of ~4.4 million aligned reads per sample, an average distance between reads of ~600 bp is expected. Thus, a window size of 12 kb (~20 adjacent reads) was used as the expected resolution for the boundaries of copy number variants. We also expected that germline copy number variants would occur in at least 25% of samples (at least 4 samples). A simple filter was applied to identify putative germline copy number variants. We sorted the left coordinates for all predicted copy number segments. Clusters of left boundaries from 4 different tumors within 12 kb were flagged, and adjacent clusters within 100 kb were merged. The same procedure was performed for the right breakpoints. Segments with both left and right breakpoints meeting these criteria were flagged as potential germline copy number variants (denoted in column “B” in Supplementary Table 1). With these filtering criteria, 76 of the 686 predicted copy number segments corresponded to 14 potential germline copy number variants.
RNA purification, reverse transcription, and real time PCR. RNA was isolated following manufacturer’s instructions for TRIzol (Invitrogen). 1.5 µg of RNA was reverse transcribed following manufacturer’s instructions for High-Capacity cDNA Reverse Transcription (Applied Biosystems). cDNA was then diluted 1:10 for real time PCR reactions. Real time PCR reactions on genomic DNA were performed using 100 ng (mouse cell lines) or 200 ng (human cell lines) of DNA. All real time PCR reactions were performed using SYBR green (Applied Biosystems). Reactions were performed in triplicate and normalized to the levels of an internal control and analyzed using the comparative Ct method.

Genomic DNA primers:

| Organism | Name              | Sequence                        |
|----------|-------------------|---------------------------------|
| Mouse    | Nfib F            | 5’ ATTGCTTCACCCGGCGTTCTGTIT    |
| Mouse    | Nfib R            | 5’ AGGGTGCCAAGACAGGTGTGAAT     |
| Mouse    | L-myc F           | 5’ AGACTCAGGCCTGCTC            |
| Mouse    | L-myc R           | 5’ GATTTCAAAACAGCGGTAGATAG     |
| Mouse    | Chromosome 5 control F | 5’ GAAGAAATTAGAGGGCATGCTTC |
| Mouse    | Chromosome 5 control R | 5’ CTTCTCCAGTGACCTTTATGTA   |
| Human    | NFIB F            | 5’ TTATTCCTCAGGCAGCCATAACCCA  |
| Human    | NFIB R            | 5’ GGTGGAGAGAGAGAGGAGACTCTGA  |
| Human    | L-MYC F           | 5’ ACTGCACTCCAGGCTCAGA         |
| Human    | L-MYC R           | 5’ TGAGAAGAGAGCAATGCTGACCT    |
| Human    | NUCLEOLIN F       | 5’ AAACCTTTTGCGACGCGTGAC      |
| Human    | NUCLEOLIN R       | 5’ GGGACTCCGACTAGGGCC         |

cDNA primers:

| Organism | Name     | Sequence                        |
|----------|----------|---------------------------------|
| Mouse    | Nfib F   | 5’ GGGACTAAGCCCAAGAGACC        |
| Mouse    | Nfib R   | 5’ GTCCAGTCACAATCCTCAGC        |
| Mouse    | L-myc F  | 5’ ACTCCTAGTCTGAAGCCACTAGGGCC |
| Species | Gene | Primer 1 | Primer 2 |
|---------|------|----------|----------|
| Mouse   | L-myc R | 5' ACGGTCAACCACGTCAATCTCTTCA | |
| Mouse   | Gapdh F | 5' AGCTTGTCAACTCAACGGAAG | |
| Mouse   | Gapdh R | 5' TTTGATGGATGGGGGTCTCG | |
| Human   | NFIB F | 5' GCCAAATGATCTCTGCAAGAA | |
| Human   | NFIB R | 5' GGTGGAGAAGACAGACCTCTGA | |
| Human   | L-MYC F | 5' ACTGCACTCCAGCCTCGA | |
| Human   | L-MYC R | 5' TGATGGGACCGGCTTCGAATAC | |
| Human   | GAPDH F | 5' AGCCACATCGCTCAGACAC | |
| Human   | GAPDH R | 5' GCCCAATACGACCAATCC | |

**Immunohistochemistry.** Slides used for the single Nfib IHC were deparaffinized, boiled in 10mM sodium citrate buffer pH 6 in a pressure cooker, washed in H20, and incubated in 3% H2O2 for 10 minutes. Slides were blocked for avidin and biotin (Vector Laboratories), blocked in 10% goat serum, and incubated overnight in 1:500 anti-NFIB antibody (Abcam). Slides were incubated in biotinylated secondary antibody and then in ABC reagent (ABC kit, Vectastain), signal was detected with a DAB peroxidase substrate kit (Vector Laboratories), and counterstained with hematoxylin. Double IHC was performed sequentially using the Autostainer 360 (Thermo Scientific). Antigen retrieval was performed using the Thermo Pre-treatment module. Anti-Nfib staining (Abcam, 1:1000) was performed first and signal was detected using DAB and was followed by anti-CGRP staining (Sigma, 1:5000), which was detected using Fast Red.

**Fluorescence In Situ Hybridization.** Human tissue arrays (LC2001 and LC2082) were purchased from US Biomax, Inc. Human BAC clones RP11-355C15 and RP11-1107G7 (spanning NFIB in 9p22-23), and mouse BAC clones RP23-386C23 and RP23-132D24 (for Nfib in 4qC3), together with proximal MMU4 reference clone RP23-458D19 were utilized in FISH analyses (BACPAC resources). BAC DNA was isolated by standard
alkaline lysis and labeled with Red dUTP (NFIB, Nfib) or Green dUTP (reference probe) (Abbott Molecular, Inc., Des Plaines, IL) by nick translation. Human cosmid clone cCMP9.27, containing chromosome-specific satellite 3 repeats in 9q12, was used as reference on the human TMAs. Mouse reference BAC RP23-59B17 was part of a 1Mb clone set kindly proved by YJ Chung (Chung et al. 2004). The samples were imaged with an Applied Precision deconvolution microscope, using a 60X, NA 1.3 oil objective, and Softworx software. The acquired z-stack images (5 microns thick sample, 0.2 micron/z section) were deconvolved, and a maximum projection was generated for each z-stack (Koch Institute Microscopy Core Facility).

**Immunoblotting.** Anti-NFIB antibody (Active Motif, 1: 2000), anti-MYCL1 (Santa Cruz, clone C-20, 1:200), anti-Hsp90 (BD Transduction Laboratories, 1:20000) were used for western blotting following standard methods.

**Human SCLC cell line functional experiments.** Human SCLC cell lines were obtained from ATCC. Individual clones from the TRC-Hs1.0 library were used for NFIB knockdown in human SCLC cell lines (shRNA #1: TRCN0000014680, CCGTGCTGTGTCTTATCCAAT, shRNA #2: TRCN0000014681, GCACGAAAGAGATCAAGATAT, Control (GFP shRNA): GCAAGCTGACCCTGAAGTTCA). All human cell lines were maintained in RPMI supplemented with 10% FBS, 1% L-glutamine and 50 units/mL Penicillin, 50 µg/mL Streptomycin. Cells were infected with NFIB or control vectors, and after two days were selected using puromycin (Sigma). Cleaved caspase 3 (CC3) staining was performed
according to manufacturer’s instructions for flow cytometry staining (Cell Signaling). For cell cycle analysis, cells were incubated for 1 hour with BrdU prior to collection and stained for cell cycle analysis according to manufacturer’s instructions for the BrdU Flow Kit (BD Pharmingen). p values were determined using Student’s T-tests.

**Senescence-associated β-galactosidase staining.** SA-β-gal staining was performed as previously described (Dimri et al. 1995; Itahana et al. 2007) except the citric acid/sodium phosphate buffer was used at pH 5.5 and staining was performed overnight. The percent of SA-β-gal+ cells was determined by counting the number of SA-β-gal+ cells in at least 500 cells. p values were determined using Student’s T-tests.

**Nf1b and L-myc overexpression.** Nf1b and L-myc were stably expressed in mouse cell lines using MSCV-Nfib-PGK-Neo and MSCV-L-myc-PGK-Puro, and selected with G418 (Invitrogen) or Puromycin (Sigma), respectively. Mouse embryonic fibroblasts (MEFs) were infected with the overexpression vectors, but did not undergo selection. The mouse Nfib cDNA was FLAG tagged, and a Kozak sequence was added before the ATG in both the L-myc and Nfib expression vectors.

**Primers:**
Mouse *Nfib* cDNA F: 5’ CTAGAGTTAACATGATGGATTACAAAGACGATGACGATAAATATTCTCCCATCTGTCTC,
Mouse *Nfib* cDNA R: 5’ TGACAAGATCTTCAGTTGCTTGTCTCCGCTT,
Mouse *L-myc* cDNA F: 5’ ATGATCTCGAGCCACCATGGACTTCGACTCGTAT,
Mouse *L-myc* cDNA R: 5’ TAGCACGGAATTCTTAGTAGCCACTGAGGTACGCGA
**Mouse SCLC cell line functional experiments.** Following stable expression of Nfib, L-myc or both L-myc & Nfib, cells were used in two assays. For soft agar colony formation assays, $1.5 \times 10^4$ cells were plated in 3 cm plates in 0.4% agarose on top of a layer of 0.8% agarose (Seaplaque). Soft agar colony formation assays were done in triplicate and performed at least twice. Cultures were fed weekly and after two weeks, colonies were stained with 0.2% crystal violet and the number of colonies in at least 9 camera views per plate using a 10X objective was quantified. p values were determined using Student’s T-tests. Growth curve assays were performed in triplicate and performed twice. $10^5$ cells were plated in triplicate wells in 6 well plates and the number of cells was counted each day for 4 days (Coulter Counter, Beckman). Graphs represent the mean number of cells +/- standard error of the mean (SEM) of the two experiments.

**MEF Transformation assays.** The mouse embryonic fibroblast (MEF) experiments were performed using wild type and p53$^{+/−}$ MEFs each from two different embryos, in triplicate, and before passage 6. Following infection with L-myc and/or Nfib expression vectors, cells were used in three different assays. The low-density plating experiments were performed essentially as previously described (Sage et al. 2000), except colonies were allowed to grow for three weeks and colonies were stained with 0.5% crystal violet in 25% methanol. In the foci formation assay, $3.8 \times 10^5$ MEFs were plated in 6 cm plates and were performed as previously described (Sage et al. 2000) except the foci were stained using 0.5% crystal violet in 25% methanol after two weeks. All MEFs were grown in DME, 10% FBS, 1% L-glutamine and 50 units/mL Penicillin, 50 µg/mL.
Streptomycin, 1 mM sodium pyruvate (Sigma), non-essential amino acids (Sigma), 0.1 mM ß-mercaptoethanol (Sigma). For soft agar colony formation assays, 1.5x10^4 cells were plated in 3 cm plates in 0.4% agarose on top of a layer of 0.8% agarose (Seaplaque). Cultures were fed weekly and after two weeks, colonies were stained with 0.2% crystal violet and the number of colonies in at least 9 camera views per plate using a 10X objective was quantified. p values were determined using Student’s T-tests.

**Gene expression analysis.**

**Microarray analysis.** RNA was isolated with TRIzol (Invitrogen), labeled and hybridized to Affymetrix 430A_v2 chips according to manufacturer’s instructions. Affymetrix data analysis was done using statistical tools provided by the r/Bioconductor projects (http://cran.r-project.org/; http://www.bioconductor.org/). Data import and quality control assessment was done using the packages Affy and AffyPLM and data was summarized and normalized using gcRMA (Bolstad et al. 2005a; Bolstad et al. 2005b). Differential expression analysis was carried out using limma (Smyth 2005). Prior to differential expression testing, the biological replicates of the different treatments of the 3583T3 and 3151T4 cell lines were averaged and the mean values from these replicates were used in limma and GSEA analysis (see below). Gene expression data was deposited in Gene Expression Omnibus (GSE29533).

**Gene Set Enrichment Analysis (GSEA).** GSEA (http://www.broad.mit.edu/gsea/) was used to examine the distribution of groups of manually curated gene sets (http://www.broadinstitute.org/gsea/msigdb/genesets.jsp?collection=C2) within genes rank-ordered based on differential expression between the treatment classes. All the
files required for these analyses and the results are available here (http://luria.mit.edu/caw_web/Dooley_Supplemental/MsgDB_v3/).
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