Gene Regulation:

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J. Biol. Chem. published online August 20, 2013

JBC Papers in Press. Published on August 20, 2013 as Manuscript M113.481523
The latest version is at http://www.jbc.org/cgi/doi/10.1074/jbc.M113.481523

Loss of Tumor Suppressor PTEN Induces Leptin Mediated Leptin Gene Expression: A Feed-Forward Loop Operating in the Lung

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***Running Title:** *PTEN* loss induces leptin expression and adipokine signaling

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Key Words: *PTEN*; Leptin signaling; obesity; lung diseases; lung cancer

Background: Leptin expression is induced in lung diseases and lung cancer but the mechanism of leptin gene expression remains elusive.
Result: Leptin mediates leptin and leptin receptor expression, setting-up a feed-forward loop.
Conclusion: DNA elements and intracellular signals activating leptin gene expression were identified.
Significance: Mechanism of leptin/leptin receptor gene regulation will aid in targeting leptin signaling in lung pathologies.

ABSTRACT

Elevated levels of systemic and pulmonary leptin are associated with diseases related to lung injury and lung cancer. However, the role of leptin in lung biology and pathology, including the mechanism of leptin gene expression in the pathogenesis of lung diseases, including lung cancer remains elusive. Herein, using conditional deletion of tumor suppressor gene *Pten* in the lung epithelium *in vivo* in transgenic mice and human *PTEN*-null lung epithelial cells, we identify leptin driven feed-forward signaling loop in the lung epithelial cells. Leptin mediated leptin/leptin-receptor gene expression, likely amplifying leptin signaling that may contribute to the pathogenesis and severity of lung diseases, resulting in poor clinical outcomes. Loss of *Pten* in the lung epithelial cells *in vivo*, activated adipokine signaling, and induced leptin synthesis as ascertained by genome-wide mRNA profiling and pathway analysis. Leptin gene transcription was mediated by binding of transcription factors NRF-1 and C/EBP-δ to the proximal and STAT3 to the distal promoter regions as revealed by leptin promoter-mutation, chromatin immuno-precipitation (ChIP) and gain- and loss-of-function studies in lung epithelial cells. Leptin treatment induced expression of leptin/leptin-receptor in the lung epithelial cells via activation of MEK/ERK, PI3K/AKT/mTOR and JAK2/STAT3 signaling pathways. Expression of constitutively active MEK-1, AKT and STAT3 proteins increased, while treatment with MEK, PI3K, AKT and mTOR inhibitors decreased LEP expression, indicating that leptin via MAPK/ERK1/2, PI3K/AKT/mTOR and JAK2/STAT3 pathways, in turn, further induces its own gene expression. Thus, targeted inhibition of the leptin mediated feed-forward loop provides a novel rationale for pharmacotherapy of disease associated with lung injury and remodeling, including lung cancer.

INTRODUCTION

Leptin (LEP) is a 16 kDa pleotropic hormone and a pro-inflammatory adipokine/cytokine. LEP binds to the leptin receptor (LEPR) and activates multiple intracellular signaling pathways(1,2). Elevated levels of LEP in the lung and serum are associated with, and potentially exacerbate severity and progression

of lung diseases including acute lung injury (ALI), acute respiratory distress syndrome (ARDS), chronic obstructive pulmonary disease (COPD), airway remodeling associated with asthma and lung cancer(3-10). In patients, circulating and airway LEP concentrations negatively correlate with lung function(11). Increased LEP expression and secretion following lung injury promotes fibroproliferation, contributing to pulmonary fibrosis(12), particularly in the setting of hyperoxia-induced ALI(13,14). Pulmonary LEP is also increased in asymptomatic smokers and in mice exposed to cigarette smoke where it modulates innate and adaptive immune cell recruitment(5,8). In contrast, resistance to the effects of LEP attenuates lung disease pathology, while reduction in LEP levels is a strong predictive factor in the improvement of lung function(15-17). Thus, accumulating evidence indicates that LEP is causally linked to the pathogenesis of many lung diseases associated with injury as well as lung cancer.

ALI/ARDS and COPD cause considerable morbidity and mortality(18), while close to 160,000 people die of lung cancer every year in the U.S alone, imposing a major healthcare burden(19). Although clinical interventions do improve alveolar functions marginally in some patients, deterioration of lung function cannot be prevented in these diseases, leading to respiratory failure and death(20). Despite advancements in understanding the pathophysiology of ALI, ARDS, COPD, asthma and lung cancer, how LEP is induced and contributes to severity and progression of these lung diseases remains poorly understood(21). LEP and adiponectin (ADIPOQ) and their respective receptors are expressed by human lung bronchiolar and type II epithelial cells(7). Airway LEP concentrations are high in COPD patients, while increased LEP is associated with greater airway inflammation and disease severity in asthma patients; however, these data remain conflicting(7,11).

LEP signals are pro-angiogenic, pro-inflammatory and mitogenic, mediated via multiple cross-regulatory pathways involving oncogenes, cytokines and growth factors, driving growth of solid tumors(22,23). LEP activates JAK2/STAT3, MAPK/ERK1/2 and PI3K/AKT signaling pathways(2,24). As a pro-angiogenic factor, LEP up-regulates VEGF and its receptor VEGFR2 via activation of the IL-1 signaling pathway(1,25,26). However, despite the importance well-established LEP signaling pathways outside the adipose tissue and their roles in disease pathology, mechanisms of regulation and induction of *Leptin (LEP)* gene expression largely remains limited to adipocytes. While the *LEP* gene proximal promoter was defined(27,28), its role in transcriptional regulation of LEP expression remains limited to adipocytes, including the roles of transcription factors SP1, GR, CREB, PPARγ, C/EBPα, AP-2 and SREBP1c (29-34). Given the emerging role of LEP in the structural and functional maintenance of the normal and injured lung as well as in the progression of lung cancer(6,7), it is imperative that a transcriptional regulatory mechanism, especially induction of *LEP* gene expression in lung epithelial cells be elucidated.

In the present work, we demonstrate that loss of *Pten* in the lung epithelium *in vivo* in transgenic mice and in *PTEN*-null human lung epithelial cells induced LEP signaling in lung epithelial cells. LEP mediated transcription of *LEP* and *LEPR* was mediated by binding of transcription factors NRF-1 and C/EBP-δ to the proximal and STAT3 to the distal *LEP* gene promoter in lung epithelial cells. Increased LEP expression in $Pten^{\Delta\Delta}$ respiratory epithelial cells elicited an autocrine feed-forward loop via up-regulation of LEPR on the lung epithelial cells. LEP/LEPR signaling loop was driven by activation of PI3K/AKT/mTOR, MEK/ERK and JAK/STAT3 pathways. These three signaling pathways activated expression of both, LEP and LEPR, setting up a positive feed-forward LEP/LEPR signaling loop in the lung epithelium. Taken together, aberrant amplification of the LEP mediated LEP signaling loop potentially deregulates modulatory role of LEP, likely exacerbating the severity of lung diseases, including cancer, leading to poor clinical outcomes.

MATERIALS AND METHODS

Generation of Transgenic Mouse Lines- Compound-transgenic mice harboring *Pten* gene with loxP-flanked exon-V (*Pten*$^{flox/flox}$), SP-C–rtTA$^{tg/–;}$ and TetO-Cre$^{tg/-}$ were generated and genotyped as described previously(35)with mice harboring *SPC-rtTA/Pten*$^{flox/flox}$, *TetO-Cre*$^{tg/-}$*/Pten*$^{flox/flox}$, *SPC-rtTA*, or *TetO-Cre*$^{tg/-}$ as controls. Likewise, Dox treatment (1 month) in 4 week old mice induced tumors in *CCSP-rtTA/TetO-Cre*$^{tg/-}$*/LSL-Kras*^{G12D}*/Pten*$^{\Delta/\Delta}$ mice between 10-12 weeks of age. Mice expressing *rtTA*, or bearing *TetO*-Cre$^{tg/-}$ alone, were normal controls. Animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of University of South Florida. Mice were housed in humidity- and temperature-controlled rooms on a 12-hour light/12-hour dark cycle with food and water ad libitum. There was no serologic or histologic evidence of either pulmonary pathogens or infections in sentinel mouse colonies. Gestation was dated E0.5 by vaginal plug. Mice were killed by injection of anesthetic to obtain lung tissue at approximately 12 weeks when tachypnea associated with lethargy was observed.

RNA microarray analysis– Lung cRNA was hybridized to the murine genome MOE430 chips (Affymetrix) according to the manufacturer's protocol. Affymetrix Microarray Suite 5.0 was used to scan and quantitate the gene chips under default settings. Normalization was performed using the robust multichip average model (36,37). Data were analyzed using Genespring 7.2 (Silicon Genetics). A volcano plot was used to identify significance (negative log of *P* values from Welch's approximate *t* test on *y* axis) and magnitude of change (log2 of fold change on the *x* axis) in the expression of a set of genes between *Pten*$^{\Delta/\Delta}$ mice and control littermates(38). The selection criteria included a *P* value of 0.05 or less by 2-tailed Student's *t* test, false discovery rate(39,40) (FDR) of no more than 10% (41), and fold change of at least 1.5. Differentially expressed genes were subjected to an additional filter and classified according to Gene Ontology classification on Biological Process using the publicly available web-based tool David (42). The Fisher exact test was used to calculate the probability of each gene ontology category that was overrepresented in the selected list, using the entire MOE430 mouse genome as a reference data set. Differentially expressed genes ($P < 0.05$, 2-tailed Student's *t* test; fold change, >1.5) were compared, and correlations of transcript changes among 3 microarray experiments were measured.

Bioinformatic analyses of differentially regulated genes– The differentially regulated genes were enriched into different functional clusters using the DAVID Bioinformatic resources 6.7 and quantitatively measured by statistical methods, including X^2, Fisher's exact test, Binomial probability and Hypergeometric distribution. Pathway analysis was performed on the enriched clusters using DAVID pathway viewer, GeneGo and Ingenuity software suites and top scoring pathways were considered for biological interpretation. Analysis of the promoter regions of the top 20 *PTEN* responsive genes (up and down regulated) was performed using the MatInspector tool (default settings) of the Genomatix software suite.

Cell Culture, Transfection, and Reporter Gene Assays– H1650 cells (ATCC# CRL-5883), a gift from Dr. Chellappan (H. Lee Moffitt Cancer Center and Research Institute, University of South Florida, Tampa FL) were cultured in RPMI medium (Invitrogen) with 10% fetal bovine serum and 5% mixture of penicillin G, streptomycin and Plasmocin (Invitrogen) in a 5% CO_2 incubator at 37 °C. A series of *lep* promoter-luciferase constructs were used in transient transfection assays using the PEI method(43). Briefly, 6-well plates at 30–50% confluence were transfected with a fixed amount of *lep* promoter-luciferase plasmid and various amounts of CMV-based cDNA expressing transactivator plasmids. Total DNA was normalized with corresponding CMV-empty vectors, and transfection efficiency was normalized to β-galactosidase activity using 100 ng/well of pCMV β-galactosidase. Two days after transfection, luciferase and β-galactosidase assays were performed using 50 µl of the supernatant. The light units were assayed by

luminometry (MLX, Microtiter Luminometer, DYNEX). Data obtained represent the average of three transfection experiments, each carried out in triplicate and depicted as means ± S.E. unless stated otherwise. Primer sequence for *lep* promoter-luciferase constructs: BGL-HU-LEP-B1-R: cggaacagatcttgcaaccgctggcgctg; 800MluHuLEP-F3:gcgagcacgcgttgacaaaaa cgtg gctacatctggg; 620MluHuLep-F4:gcgagcacgcgtg aggcttggaactcgattctccg; 399MluHuLep-F5: gcga gcacgcgtcggagcccctcacagcca; 150MluHuLep-F6:gcgagcacgcgtcggcacgtcgctaccctgag; 89MluHuLep-F7: gcga gcacgcgtcggggcagttgcgc aagt and 52MluHuLep-F8: gcgagcacgcgtagt tgtgatcgggccgctataagag.

RNA isolation and Real Time PCR Assays – Total RNA was isolated from 60-80% confluent H1650 cells grown in RPMI medium using TRIZOL reagent (Ambion) as per manufacturer's instructions. Total RNA was treated with RQ1 RNase-Free DNase (Promega) and purified using the RNeasy MinElute Cleanup Kit (Qiagen). Purified RNA was converted into cDNA using the SuperScript® III Reverse Transcriptase kit (Invitrogen) and used for Real Time PCR assays. cDNA samples were mixed with 10 µl of 2× Fast SYBR Green Real-Time PCR Master Mix containing gene specific primers. The reaction mixture was denatured at 95 °C for 3 min, followed by 40 cycles of PCR reactions with the following settings: 95 °C for 15 s, 60 °C for 15 s and 72 °C for 20 s. The PCR reaction was monitored by the ABI StepOnePlus™ Real-Time PCR System (ABI PRISM 7700; Applied Biosystems, Foster City, CA, USA), and the results were analyzed with the ABI StepOnePlus™ Real-Time PCR v2.0 software (ABI PRISM 7700). Sequences for primers used are as follows: *Lep*-R: caccaaaaccctcatcaagaca; *Lep*-F: gatagaggcccagg catttttta; *LEPR*-F: tagagaaggccagcacgtgaa, *LEPR*-R: acaccactctctctcttttgattga, *GAPDH*-F: tgttgccatcaatgacccctt; *GAPDH*-R: ctccacgacgtac tcagcg, *NRF-1* F: ccgaggacacctcttacgatg, *NRF-1* R: tacatgaggccgtttccgttt.

RNA Interference Assay – Short hairpin RNAs (shRNAs) specific to human NRF1 (Hu-SH-29, 29mer shRNA constructs in retroviral GFP vector) were purchased from OriGene Technologies, Inc. (Rockville, MD). A non-effective 29-mer scrambled shRNA cassette in pGFP-V-RS was used as control. NRF1 shRNA and scrambled control (3 µg/ml) was transfected into H1650 cells and RNA was isolated from cells after 48 hours. The efficiency of shRNA-based interference of NRF1 was monitored via Real time PCR analysis and gene-specific NRF1 primers.

Site-directed mutagenesis– The C/EBP-δ and NRF-1 mutant plasmids were generated using site directed mutagenesis (QuikChange Lightening Site-Directed Mutagenesis Kit, Agilent Technologies). Briefly, Lep150 was used as a template with oligos containing mutations in the CEBP-δ and NRF-1 sites respectively (listed below) to generate PCR products. An annealing temperature of 51 deg Celsius was employed for 18 cycles with an extension time of 3 minutes at 68 deg Celsius. This was followed by DpnI digestion of the parental DNA and transformation in XL10-Gold Ultracompetent cells using beta-mercaptoethanol provided in the kit. This was followed by routine plating and colony culture procedures. The mutants were confirmed by sequencing. Sequences for primers used are as follows: C/EBP-delta (-60/-53): Forward primer: 5'-ggcagttcagta cgttgtgatcg-3'; Reverse primer: 5'-acaacgt actgaactgcccg-3'; NRF-1 (-81/-78): Forward primer: 5'-tagaaatacaccgggggcctg-3'; Reverse primer: 5'-caggccccggtgtatttcta-3'.

Chromatin Immunoprecipitation (ChIP) Assay– ChIP lysates were made using the ChIP-IT Express Magnetic Chromatin Immunoprecipitation kits (Active Motif, Carlsbad, CA). H1650 cells that were 90% confluents were treated with formaldehyde solution and the chromatin isolated, digested and immunoprecipitated as per manufacturer's instructions. The sheared chromatin was incubated with antibody directed against NRF-1, C/EBPδ and STAT-3, and the antibody-bound protein/DNA complexes were precipitated using

magnetic Protein G-coupled beads. The captured chromatin was eluted, the uncross-linked, and the DNA was recovered ChIP DNA were subjected to RT-PCR using specific primers flanking DNA binding sites for NRF-1, C/EBPδ and STAT3. Sequences for primers used are as follows:*NRF-1/CEBP-D*-ChIP-R: cggaacagatctt gcaaccgctggcgctg; *NRF-1/CEBP-D*-ChIP-F: gcgagcacgcgtcggcacgtcgctaccctgag; *STAT3*-ChIP-R: tcctctctttgtactctctctctttatttctcagc; *STAT3*-ChIP-F: ccagatgcagtggctcatgcttgta: *GAPDH*-ChIP-R: tactagcggttttacgggcg, *GAPDH*-ChIP-F: tcgaacaggaggagcagagagcga.

Immunohistochemistry– Lungs from experimental mice $Pten^{\Delta/\Delta}$ (n = 10 total) and control littermates (n =8 total) were inflation-fixed by gravity (25 cm of water pressure) with 4% paraformaldehyde in PBS, removed from the chest, and immersed in fixative overnight at 4°C. The tissue samples were rinsed in PBS, dehydrated, and then embedded in paraffin blocks. Sections were cut at 5-µm intervals and antigen retrieval was done using pepsin. DAB was used as a substrate and sections were counter stained with Mayer's hematoxylin (BioGenex, Fremont, CA, U.S.A.) to assess histologic changes**.**

LEP treatments and protein analysis– PTEN deficient H1650 lung cancer cell lines were plated at $1x10^6$ cells per well of a 6-well plate and allowed to attach overnight. Cells then were serum-deprived for 24 hours followed by treatment with 100 ng/mL of human recombinant LEP (R&D Systems, Minneapolis, MN) for 48 and 72 hours in serum free medium. For measurements of ERK/MAPK activity after LEP treatment cells were treated with 100ng/ml of human recombinant LEP (R&D Systems, Minneapolis, MN) for 15 and 30 minutes. Proteins were isolated post LEP treatment and separated by SDS-PAGE on 10% and electroblotted to nitrocellulose membranes (0.1 µm; Invitrogen). Blots were blocked with 5% nonfat dry milk in TBST (10 mM Tris, pH 8, 150 mM NaCl, 0.1% Tween 20) and incubated with 1:1000 diluted specific primary antibodies to P-mTOR (#2971), P-AKT (#9271) and Phospho-p44/42 Erk1/2 (Thr202/Tyr204, #4370) from Cell Signaling Technology. While P-STAT3 (Y-705, #2236-1) was from EPITOMICS, ERK1/2 (Abcam; #AB17942) and β-actin (A5060; Sigma-Aldrich) Peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG secondary antibody (Jackson ImmunoResearch Laboratories, INC.) was used at 1:10,000 concentration. Blots were developed by chemiluminescence (Pierce Biotechnology) and autoradiographed.

Cell Proliferation Assay– Cell proliferation assays were performed using Cell Counting Kit-8 (Fluka,Biochemika). Cells were plated in 96-well plates at increasing density ranging from 5×10^3 to 1.2×10^4 cells/well and cultured in RPMI growth medium as described above. The cells were transferred to serum free medium for 16 hours and replenished with serum free medium containing increasing concentrations (50-200ng/ml) of human recombinant LEP (R&D Systems Minneapolis, MN). At the indicated time points, the cell numbers in triplicate wells were measured as a function of absorbance (450 nm) of reduced WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt).

Inhibitor studies– Dose response studies for pathway specific inhibitors of PI3K/mTOR (BEZ-235, LY294002), MEK1/2 (U0126) and AKT 1/2/3 (MK-2206) (Selleck Chemicals) were carried out on 30-40% confluent H1650 cells grown in RPMI medium for 24 hours in the presence of *LEP* promoter fragment (150bp). Post transfection, luciferase and β-galactosidase assays were performed using 50 µl of the supernatant on MLX, Microtiter Luminometer, (DYNEX). Data obtained represent the average of three transfection experiments, each carried out in triplicate and depicted as mean ± S.E. unless stated otherwise.

Electric Cell Substrate Impedance Sensing Wounding (Migration) Assay– H1650 cells were grown on electric cell substrate impedance sensing (ECIS) 8-well plate arrays (8W1E; Applied Biophysics, Troy, NY) in growth media with serum until fully confluent, after which the media were replaced with serum-free media for 24 h. Serum-deprived cells were treated with

100 ng/mL of human recombinant LEP (R&D Systems, Minneapolis, MN) for 2 hours prior to wounding. Cells were wounded using an elevated field pulse of 1,400 mA at 32,000 Hz applied for 20 seconds, producing a uniform circular lesion of 250 mm in size, and wounds were tracked over a period of 24 hours. The impedance (Z) was measured at 4,000 Hz, normalized to its value at the initiation of data acquisition, and plotted as a function of time. Assays were performed in triplicates and reported as mean ± S.E. unless stated otherwise (P Value≤ 0.05).

RESULTS

Loss of Pten induces LEP signaling in the lung epithelial cells in vivo and in vitro– Triple transgenic mice harboring a conditional *Pten* allele were developed (Figure 1A) and *Pten* was conditionally deleted *in vivo* from the lung epithelial cells using the doxycycline dependent Cre/LoxP approach (Figure 1Ai) as described previously(44). *Pten* was selectively deleted in the respiratory epithelial cells after administration of doxycycline (Dox) to the dam (Figure 1Aii). At birth, the transmission of all of the genes followed Mendelian inheritance as confirmed by genotyping (Figure 1Aiii). Selective deletion of *Pten* gene in the lung resulted in epithelial hyperplasia at 20 weeks as compared to control littermates, the bronchial epithelium in $Pten^{\Delta/\Delta}$ mice was hyper-cellular (denoted by red arrow) (Figure 1Aiv). Microarray analysis of lung RNA isolated from *Pten*-deleted lung epithelial cells ($Pten^{\Delta/\Delta}$) and control mice revealed that expression of 1389 genes was altered significantly (≥2-fold change, P-Value ≤ 0.05) (Figure 1B). Network enrichment analysis of the *Pten* responsive genes using the MetaCore software suite(45,46) (MetaCore from GeneGo Inc. NY, USA) revealed that LEP signal transduction is among the most significantly perturbed networks (Figure 2C). This was independently confirmed by the disease enrichment analysis using the Ingenuity Pathway Analysis suite(47) (IPA CA, USA), which identified LEP signaling in obesity as one of the most significantly altered pathways (Figure 2D). Loss of *Pten* in the lung epithelial cells up regulated the adipocyte signaling pathway including a number of genes involved in the LEP pathway as assessed by KEGG signaling pathway database(48) (Figure 2A). Robust expression of LEP expression was observed after deletion of *Pten* ($Pten^{\Delta/\Delta}$) in lung adenocarcinomas (Figure 2B) developed in mice. In order to identify a PTEN null cancer cell line, we compared endogenous *PTEN* protein expression in A549, H292 and H1650 human lung epithelial cell lines (Figure 3A). Since H1650 did not show any expression of PTEN as opposed to the other 2 cell lines, we used it for all further experiments. Significant expression of *LEP* and *LEPR* mRNAs (Figure 3B) and LEP/LEPR proteins (Figure 3A and 3C) was observed in *PTEN* deficient human lung cancer cells (NCI-H1650), consistent with the concept that LEP/LEPR signaling pathway is operational and may be up-regulated following loss of PTEN in lung epithelial cells, including in lung cancer.

LEP directly influences lung epithelial cell physiology by inducing cell proliferation and wound healing– Pathophysiological alterations in the lung after *Pten* loss in the respiratory epithelium have been characterized in great detail (35,49-52). We hypothesized that increase in LEP signaling will likely alter lung epithelial cell physiology and behavior, and as a hallmark of LEP signaling, treatment with LEP should enhance proliferation of H1650 cells. To test this premise, we treated exponentially growing H1650 cells with increasing concentration of LEP. There was approximately 3-fold increase in cell proliferation in a dose dependent manner (Figure 3D), indicating a physiological response to LEP in H1650 lung epithelial cells, consistent with studies on other cancer cells (53). Since leptin signaling pathway was up regulated in H1650 *PTEN* null cells, we sought to determine the physiological role of leptin on wound healing using Electric Cell-substrate Impedance Sensing (ECIS) method(54-57). For the wound-healing assays, confluent H1650 cells were serum starved for 24 hours on ECIS 8W1E plates were treated with 100ng/ml of leptin and impedance values were measured. Real time measurements of impedance values prior to wounding clearly indicated that cells treated with leptin reached confluence earlier than the

ones without treatment (Figure 3E). As shown in Figure 3E, the application of the high-field pulse led to a drastic drop of cell impedance. Post wounding (as represented by red dotted line), control H1650 cells (no leptin treatment) had a lower impedance value as compared to leptin treated H1650 cells (Figure. 3E), indicating that *Pten*-null H1650 cells showed a physiological response to leptin in the medium. Taken together, leptin treatment increased cell proliferation (Figure 3D) and accelerated wound healing due to increased cell migration (Figure 3E). Consequently, it is highly likely that pathological conditions associated with increased LEP synthesis and secretion may accompany LEP binding to LEP receptor on lung epithelial cells, activating its own synthesis and setting up a self-sustaining feed-forward auto-regulatory loop that may further drive pathogenic events in the lung following injury or in lung cancer. Therefore, we sought to define the molecular mechanisms underlying LEP-mediated LEP gene expression.

LEP gene expression is regulated by a proximal enhancer via transcription factors NRF-1 and C/EBP-δ– To define potential proximal enhancer elements that drive LEP gene expression in *PTEN-null* the lung epithelial cells, luciferase reporter plasmids comprising various lengths of the 5'-upstream regulatory regions of the *LEP* gene promoter were sub-cloned. Transcription activity of each promoter-deletion plasmid was evaluated in transient transfection assays using H1650 *PTEN*-null lung epithelial cells (Figure 3F). The promoter region spanning -149 to +21 (*Luc-150*) was found to be transcriptionally most active, while further deletion of the promoter up to -52 bps (*Luc-50*) decreased the activity to basal levels, indicating the presence of a proximal enhancer element in the region between –149 to –50 bps (Figure 3F). Consensus sites for NRF-I (pink solid box) and C/EBP (green solid box) were identified in the region spanning –83 to –78 bps and –60 to –53 bps from the transcription start site respectively (Figure 3G) using MatInspector 7.0(58), a transcription factor binding site identification software derived from the Genomatix Suite (Genomatix Software GmbH, Munich, Germany).

To ascertain the role of transcription factors NRF-1 and C/EBP in transcriptional regulation of *LEP* gene via the proximal enhancer, constitutively active NRF-1 (CA-NRF-1) was co-transfected with *Luc-150* in H1650 cells. Dose dependent expression of CA-NRF-1 increased the transcriptional activity from *Luc-150* (Figure 4A, lanes 3-5). In contrast, co-transfection of *Luc-150* with increasing amount of dominant negative NRF-1 (DN-NRF-1) in H1650 cells decreased *Luc-150* activity (Figure 4B, lanes 3-5), indicating that NRF-1 is able to transcriptionally activate the *LEP* gene via the proximal enhancer that contains an NRF-1 binding element. Role of C/EBP site within the proximal enhancer (–60/–53 bps) was examined by co-transfection of *Luc-150* together with either C/EBP-α, C/EBP-β or C/EBP-δ expressing plasmids since all three forms bind similar DNA consensus sites and are highly expressed in lung epithelial cells(59,60). Plasmid expressing C/EBP-δ selectively up regulated *Luc-150* activity by approximately 20 fold (Figure 4C, lane 5) as opposed to C/EBP-α and C/EBP-β, which showed 4 to 5 fold activation (Figure 3C, lanes 3 and 4), indicating that C/EBP-δ likely plays a major regulatory role in transcriptional activation of *LEP* gene. Indeed, dose dependent co-transfection of C/EBP-δ and fixed amount of *Luc-150* activated (Figure 4D, Lanes 3-5), while co-transfection of dominant negative C/EBP-δ (DN-C/EBP-δ) abrogated *Luc-150* activity (Figure 4D, Lanes 6-7).

Since NRF-1 and C/EBP-δ both up-regulated *LEP* gene promoter activity, we tested the possibility that NRF-1 and C/EBP-δ might have potential synergistic or additive effect on the proximal enhancer in the induction of LEP transcription. Co-transfection of *Luc-150* in combination with CA-NRF-1 and C/EBP-δ expressing plasmids showed approximately 8 fold increase in Luc-*150* activity (Figure 4E, lane 5) as compared to 4 fold increase after expression of CA-NRF-1 (Figure 4E, lane 3) and 2.5 fold increase after expression of C/EBP-δ alone (Figure 4E, lane 4), indicating additive effect of the two transcription factors. Since canonical LEP signaling is mediated via binding of the transcription factor STAT3 to various

gene promoter elements(61,62) we tested the hypothesis whether STAT3 itself may play a critical role in transcriptional activation of *Luc-150*. However, co-transfection of *Luc-150* with plasmids expressing constitutively active STAT3 (CA-STAT3) did not activate transcription from *Luc-150*, suggesting that the proximal *LEP* gene enhancer functions independently of STAT3 and that LEP transcription may be regulated by STAT3 binding elements present further upstream in the *LEP* gene promoter. Together, NRF-1 and C/EBP-δ activated *LEP* gene transcription utilizing the *LEP* gene proximal enhancer element.

LEP gene proximal enhancer binds transcription factors NRF-1 and C/EBP-δ– The NRF-1 and C/EBP-δ DNA-binding elements that were identified by MatInspector were matched with true consensus elements defined by JASPAR database(58,63). While C/EBP site showed complete conservation (5'-TTGCGCAAC-3'), NRF-1 site deviated by one nucleotide at position 2 (5'-AAATGCGCN-3'). Site-directed mutagenesis of the NRF-1 and C/EBP-δ binding-sites within the proximal enhancer abrogated transcription activity, indicating that both these transcription factors directly bind to the enhancer element and transcriptionally regulates LEP gene expression (Figure 5A, lane 3 and 5B, lane3). Indeed, chromatin immuno-precipitation (ChIP) of NRF-1 and C/EBP-δ using the DNA primers spanning the 5′-regulatory region containing the proximal LEP gene enhancer (Figure 5C) readily detected bound form of NRF-1 and C/EBP-δ in the chromatin in vivo in H1650 cells (Figure 5D, lane 4 and 5E, lane 4). In contrast, NRF-1 and C/EBP-δ failed to bind GAPDH proximal promoter (Figure 5D lane 3 and 5E, lane 3), implicating NRF-1 and C/EBP-δ as direct transcriptional activators of LEP gene. Taken together, these experiments confirm that LEP gene transcription is mediated by binding of NRF-1 and C/EBP-δ to the LEP gene proximal enhancer in the chromatin context in lung epithelial cells.

PI3K/AKT and MEK pathways regulate LEP gene transcription via a proximal enhancer– When LEP binds to its receptor LEPR, it triggers the activation of PI3K/AKT, MEK/ERK and JAK2/STAT3 pathways in many cell types(24). However, whether LEP expression is controlled and induced by its own signaling via these three signaling pathways remains unknown. It is plausible that in order to maintain continuous LEP signaling, mainly in an autocrine loop, LEP itself may regulate its own expression via up-regulating LEPR and its downstream signaling pathways. To test this hypothesis, we transfected H1650 cells with Luc-150 and subsequently subjected these cells to PI3K/AKT and MEK pathway specific inhibitors. MK-2206, an AKT inhibitor, U0126 a MEK inhibitor, LY-294002 a pan-PI3K inhibitor and BEZ-235 a dual-PI3K/mTOR kinase inhibitor reduced LEP promoter activity in a dose dependent manner respectively (lanes 3-5 in Figure 6A, 6B, 6C and lanes 3-4 in Figure 6D). A combination of PI3K/AKT, PI3K/MEK and MEK/mTOR pathway inhibitors further reduced LEP promoter activity, indicating that signals from these pathways independently regulate LEP gene promoter (lane 5 in Figure 6E, 6F and 6G). In all these experiments, using Trypan blue staining, we made sure that cell-viability was not compromised. The highest concentrations used in our experiments were consistent or lower than previously reported studies (64-67) without any observable toxicity.

To further support the role of PI3K/AKT/mTOR and MEK/ERK signaling pathways we performed co-transfection of Luc-150 with plasmids expressing constitutively active AKT (CA-AKT) and constitutively active MEK (CA-MEK) in lung epithelial cells H1650. Indeed, expression of CA-AKT and CA-MEK induced LEP gene promoter activation in H1650 cells (Figure 9C). In summary, the *LEP* gene proximal promoter was regulated by PI3K/AKT/mTOR and MEK/ERK signaling pathways in lung epithelial cells. These observations raise the possibility that LEP via PI3K/AKT/mTOR and MEK/ERK signaling likely mediates its own transcription followed by increased LEP synthesis in lung epithelial cells

that are exposed to extracellular LEP, reinforcing an auto-regulatory LEP signaling loop.

NRF-1, C/EBP-δ and STAT3 up-regulates endogenous LEP and LEP receptor gene transcripts– Although the potential role of NRF-1 and C/EBP-δ in the regulation of LEP gene transcription was established in transient transfection assays using the *Luc-150* as a reporter, whether NRF-1 and C/EBP-δ can directly activate endogenous *LEP* gene transcription in the native chromatin context in H1650 lung epithelial cells was not determined. Therefore, following expression of CA-NRF-1 and CA-C/EBP-δ in H1650 cells, induction of LEP mRNA transcription was evaluated by quantitative real-time PCR (qRT-PCR). Indeed, expression of CA-NRF-1 and C/EBP-δ significantly increased *LEP* and its receptor *LEPR* mRNA expression (Figure 7A and 7B, lanes 3-4 and lanes 2-3 of insets). To further confirm that endogenous NRF-1 does activate *LEP* and its receptor *LEPR* transcription in lung epithelial cells, plasmid vectors expressing small hairpin RNA (shRNA) targeting the NRF-1 mRNA were expressed in H1650 cells. NRF-1 shRNA expression vectors 1A and 1B resulted in ~60–80% decrease in levels of NRF-1 transcripts as measured by RT-qPCR (Figure 7C, lanes 2 and 3 and inset lanes 3 and 4). Expression of shRNA-1B in H1650 cells significantly down-regulated endogenous *LEP* and *LEPR* gene mRNA transcripts as measured by RT-qPCR analysis (Figure 7D and 7E), thereby validating the role of NRF-1 in the transcriptional regulation of LEP and LEP receptor expression in lung epithelial cells.

LEP mediated LEP and LEPR gene expression– LEP as a cytokine and a paracrine factor activates JAK2/STAT3, PI3K/AKT/mTOR and MEK/ERK signaling pathways that are directly involved in cancer progression(24). LEP also activates expression of several gene targets that participate in cancer progression, including pro-inflammatory cytokines and factors promoting angiogenesis(68). However, whether LEP signaling modulates the transcription of its own gene *(LEP)* and receptor *(LEPR)*, amplifying its function has not been studied. When H1650 cells were treated with recombinant LEP for 48 and 72 hours, JAK2/STAT3 and

PI3K/AKT/mTOR signaling pathways were activated as assessed by increase in phosphorylation of STAT3 AKT and mTOR, (Figure 8 A, B and C). Within 15 minutes after treatment with leptin, increase in phosphorylation of ERK was observed (Figure 8D) Likewise, 30 minutes of treatment with leptin regulated P38 and JNK signaling pathways as assessed by increase in p-P38, p-P54 SNP/JNK and p-P46 SNP/JNK (Figure 8 E F and G). These results clearly indicate that the LEP/LEPR signaling pathway was operational in the lung epithelial cells. Furthermore, treatment of LEP significantly increased *LEP* and *LEP* receptor gene expression as assessed by measurement of their mRNA transcripts by RT-qPCR (Figure 9A and 9B). Taken together, LEP via LEP receptor up-regulated the expression of *LEP* and *LEPR* genes in lung epithelial cells, likely driving a feed-forward LEP-signaling loop, that may potentially be required for sustained LEP signaling as observed in chronic lung injury diseases and lung cancer.

STAT3 binds to a distal enhancer and activates LEP gene transcription –When CA-STAT3 was expressed in H1650 cells, transcription of *LEP* and its receptor *LEPR* mRNA were significantly induced (Figure 9D and 9E) as assessed by RT-qPCR. This result, indicated that while STAT3 did not function via the proximal enhancer, it certainly regulated *LEP* and *LEP* gene transcription from the endogenous promoter in H1650 cells, likely via an upstream STAT3 responsive element located distally. To ascertain whether distal enhancer element comprising STAT3 binding sites is present within the *LEP* gene promoter, we scanned up to -2Kb of the human *LEP* gene 5'-upstream regulatory promoter sequence. The STAT3 binding sites were identified by homology search using softwares such as JASPAR, GENOMATIX and published STAT3 consensus DNA-binding sequence(69). While STAT3 site was not completely conserved at every nucleotide as per JASPAR, it did show the classical TT-N_{3-6}-GG STAT3 binding sequence(69). Chromatin

immuno-precipitation (ChIP) of STAT3 using DNA primers spanning the 5′-regulatory region (-1610 to -1493 bps) containing the STAT3 site (Figure 9F) readily detected bound form of STAT3 on the chromatin *in vivo* in H1650 cells (Figure 9G lane 5). In contrast, STAT3 antibodies failed to bind GAPDH proximal promoter (Figure 9G lane 4), implicating STAT3 as a direct transcriptional activator of *LEP* gene expression. Taken together, these experiments confirm that transcriptional regulation of *LEP* and its receptor *LEPR* gene expression is mediated by STAT3 in lung epithelial cells.

DISCUSSION

LEP and LEP receptor are synthesized by several non-adipose tissues, wherein, LEP functions as a pleiotropic cytokine, modulating a variety of physiological and pathological functions(70). Increased pulmonary and circulating LEP levels are observed with several lung diseases associated with injury/repair and remodeling, including lung cancer(6,71-73). LEP is also involved in fetal lung development and pulmonary homeostasis (74,75). Emerging evidence indicates that LEP as a pro-inflammatory and pro-angiogenic cytokine may play critical roles in exacerbating acute and chronic pulmonary pathologies and drive lung cancer as an inflammatory molecule(7,12,76). However, the molecular mechanism of LEP gene expression in lung diseases and lung cancer remains elusive.

In the present study, we demonstrate that Cre/LoxP mediated conditional deletion of *Pten* (*Pten*$^{\Delta\Delta}$) activated adipocyte signaling in the respiratory epithelium that was associated with increased expression of LEP and its receptor. Using *PTEN*-null lung epithelial cells, we show that *LEP* gene was transcriptionally activated by a proximal enhancer element via binding of NRF-1 and C/EBP-δ transcription factors, while STAT3 bound a distal promoter element in the *LEP* gene and activated its expression. Transcription of the active form of the LEP receptor (LEPR), was also induced by NRF-1, C/EBP-δ and STAT3, suggesting that these three factors concertedly activate LEP/LEPR signaling pathway in the lung epithelial cells. C/EBP-δ and STAT3 play critical roles during inflammatory responses in the lung(59,77,78). Since lung epithelial cells, particularly type II alveolar epithelial cells (Type II AECs) have high lipid metabolic activity and turnover that is required for surfactant synthesis(79), it is plausible that LEP may play a critical role in surfactant homeostasis following lung epithelial injury. Indeed, several studies have demonstrated that LEP directly stimulates proliferation of Type II AECs(80) up-regulating type IV collagen synthesis, that reinforce the alveolar walls(81,82).

Extra lipid accumulation in Type II pneumocytes may lead to lipotoxicity as observed in many non-adipose tissues(83,84). Since LEP stimulates fatty acid oxidation via activation of AMP-activated protein kinase (AMPK)(85), it participates in reducing lipid stores, thereby reducing lipotoxicity(85-87). Consistent with this concept, sustained LEP signaling, as seen in many inflammatory conditions, causes chronic activation of AMPK(85), which activates the transcription factor NRF-1(88). Activated NRF-1 binds gene promoters involved in enhancing oxidative capacity and mitochondrial biogenesis, including LEP as demonstrated in the present work, increasing energy metabolism via known LEP signaling pathways(88,89). Thus, our results presented here are consistent with the role of NRF-1 in *PTEN*-null H1650 lung epithelial cells. In addition, loss of *PTEN* in lung epithelial cells drives rapid cell proliferation that would indeed be associated with increased mitochondriogenesis(35). Supporting these observations, our *LEP* promoter-reporter deletion analysis, site-directed mutagenesis, ChIP and NRF-1 over-expression studies demonstrated increased activity of NRF-1 on the *LEP* promoter itself, indicating that LEP mediated activation of NRF-1 contributes to transcriptional activation and expression of the *LEP* gene in lung epithelial cells.

In adipocytes, LEP gene transcription is regulated by C/EBP-α(32,90), however, transcriptional regulation of *LEP* gene and the roles of C/EBP isoforms, including C/EBP-β and C/EBP-δ in non-adipocyte tissues remain

unclear. The present study revealed that C/EBP-δ, but not C/EBP-α or C/EBP-β, strongly activated LEP gene transcription in lung epithelial cells, suggesting a critical and selective role for C/EBP-δ in LEP gene regulation. Given the important role of LEP in inflammatory processes, it is highly likely that concerted modulation of pro-inflammatory genes including LEP is under the control of C/EBP-δ in the lung epithelium(60). Recent studies demonstrate that C/EBP-β and C/EBP-δ participate in inflammatory responses following lung injury and infection (91), consistent with the identified role of C/EBP-δ in the transcriptional activation of LEP gene expression in the lung epithelial cells.

LEP signaling is transduced via the activation of canonical PI3K/AKT, MEK/ERK and JAK2/STAT3 pathways in many cell types(24). However, whether LEP itself is activated by these three signaling pathways and regulates its own expression has not been explored.

Tumors maintain continuous LEP signaling that facilitate cancer progression and metastasis, while inhibition of LEP-signaling results in efficient anti-tumor activity(92); therefore, it is likely that LEP itself may regulate its own gene expression When H1650 *PTEN*-null lung epithelial cells were treated with LEP, activation of PI3K/AKT/mTOR, MEK/ERK and JAK2/STAT3, P38 and JNK signaling pathways was detected by immune-blotting for p-mTOR, p-AKT, p-STAT, p-ERK, p38 MAPK as well as the active and inactive forms of JNK (p54 JNK and p46 JNK). This is consistent with previous findings where leptin mediated activation of canonical (PI3K, ERK) and non-canonical (p38 MAPK, JNK, and PKC) signaling pathways has been observed(93,94) Together, our results support the concept that loss of *PTEN* in lung adenocarcinoma would activate PI3K/AKT/mTOR, MEK/ERK and the p38/JNK MAPK signaling pathways, which would in turn, contribute to the induction of *LEP* gene expression and subsequent secretion.

To test this hypothesis in vivo, we developed a mouse model with conditional deletion of PTEN in an oncogenic K-RAS background (95). Exuberant LEP secretion was indeed detected in tumors generated after PTEN loss in oncogenic KRAS background (Figure 2B), indicating an important role for LEP signaling in lung cancer progression. Since treatment of H1650 cells with inhibitors of PI3K, AKT, mTOR, and MEK abrogated LEP gene transcription while constitutive expression of AKT and MEK activated LEP gene promoter activity, we proposed in our conceptual model that LEP mediated LEP gene expression is directly influenced by PI3K/AKT/mTOR, MEK/ERK and P38 JNK signaling pathways (Figure 10). The major hallmarks of LEP signaling are activation of STAT3 via JAK2 phosphorylation, and increased cell proliferation. Over-expression of constitutively active STAT3 induced LEP gene expression while treatment with LEP enhanced proliferation and wound healing of H1650 cells, consistent with promotion of invasion and migration in cancer cells(96). Taken together, our findings demonstrate that LEP itself regulate its own expression via LEP receptor mediated downstream signaling in lung epithelial cells. The present study supports the concept that therapies that can abrogate LEP signaling in lung pathologies, may reduce disease morbidity.

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Foot Note: R. R. P. A.G, W. Q. and P. M. performed the experiments, A. P. and D.A.G. designed the ECIS experiments. The project was conceived and designed by V. D. and written by R. R. P. and V. D.

List of abbreviations:

PTEN: Phosphatase and tensin homolog, LEP: Leptin, LEPR: Leptin receptor, MEK: Mitogen Activated Protein Kinase, ERK: Extracellular signal-regulated kinase, PI3K: Phosphatidylinositide 3-kinase,AKT: Protein Kinase B, mTOR: mammalian target of rapamycin,STAT3: Signal transducer and activator of transcription 3,C/EBP: CCAAT-enhancer-binding proteins, ChIP: Chromatin Immunoprecipitation, JAK2: Janus kinase 2,VEGF: Vascular endothelial growth factor,VEGFR2: Vascular endothelial growth factor receptor 2, KEGG: Kyoto Encyclopedia of Genes and Genomes, Dox: Doxycycline, PEI: Polyethylenimine, JNK: c-Jun N-terminal kinases

Acknowledgements

We thank Dr. Todd Gulick, M.D, Sanford-Burnham Medical Research Institute, Lake Nano Orlando for the NRF-1 plasmids, Dr. Steven McKnight, Ph.D., UT-Southwestern Medical Center , Dallas for C/EBP- δ plasmid and Dr. Jim Darnell, The Rockefeller University, New York for CA-STAT3 expression plasmid. We would also like to thank Dr. Jeffrey Whitsett MD (Cincinnati Children's Hospital, Cincinnati OH) for the use of microarray core facility. This work was supported by American Heart Association Scientist Development Grant SDG-0830101N (V. D.) and NIH-Lung SPORE Career Development Grant, Moffitt Cancer Center (V. D.).

FIGURE LEGENDS

Figure 1. **Generation of *Pten*$^{\Delta/\Delta}$ mice and microarray profiling of *Pten*$^{\Delta/\Delta}$ lung mRNA identifies 1389 *Pten* responsive genes.** **(A)** Generation of the *Pten*$^{\Delta/\Delta}$ mice. Triple-transgenic mice containing 3 alleles: loxP-flanked exon V (*Pten*$^{flox/flox}$); SP-C–rtTA$^{tg/-}$; TetO-Cre$^{tg/-}$ (orange oval) were produced (i, and ii) and selected by genotyping (iii). Mice harboring *SPC-rtTA*$^{tg/-}$*/Pten*$^{flox/flox}$, TetO-*Cre*$^{tg/-}$*/Pten*$^{flox/flox}$, or *Pten*$^{flox/flox}$ were used as controls. Lungs were harvested from 20-week old mice. Hematoxylin/eosin staining of lung sections from *Pten*$^{\Delta/\Delta}$ mice demonstrated normal branching morphogenesis (indicated by blue arrows) and postnatal lung formation with increased hyperplasia (indicated by red arrows) (iv). **(B)** Microarray analysis of lung RNA isolated from *Pten*-deleted lung epithelial cells (*Pten*$^{\Delta/\Delta}$) and control mice revealed that expression of 1389 genes was altered significantly (≥2-fold change, P-Value ≤ 0.05).

Figure 2. **Activation of the adipocytokine signaling pathway in *PTEN*$^{\Delta/\Delta}$ mouse lungand expression of LEP and LEP receptor in lung epithelial cells.** **(A)** KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis of *PTEN* responsive genes identified adipocytokine signaling as being significantly up-regulated. LEP, LEPR and adiponectin (ADIPQ) were up-regulated in the *PTEN*$^{\Delta/\Delta}$ mice and overlapped (red asterisks) on the canonical KEGG adipocyte signaling pathway, suggesting that loss of *PTEN* activated adipokine synthesis and signaling in the lung epithelium. (**B**) Immuno-staining with LEP antibodies confirmed exuberant induction and secretion of LEP in *K-RAS*^{G12D}(95) driven *Pten*$^{\Delta/\Delta}$lung tumors in mice. (**C**) Enrichment for gene networks in *PTEN*$^{\Delta/\Delta}$ mice. Biological networks were enriched from the *PTEN* responsive gene set using standard software tools from MetaCore^{TM}. LEP signaling was identified as a significant metabolic pathway activated in the *PTEN*$^{\Delta/\Delta}$ enriched list (P value = 0.007). **(D)** Likewise, application of Ingenuity Systems software tool called Intelligent Pathway Analysis (IPA$^{TM)}$) independently enriched LEP signaling pathway (P value = 0.04). The linear plot shows enhanced threshold calculated using the right-tailed Fisher Exact Test for different diseases. Diseases that fall below the threshold are not statistically significant. Enrichment score (y axis) is reported as the minus log transformation on the geometric mean of P-values from the enriched annotation terms associating with one or more of the gene group members. The genes are clustered into significantly enriched groups for specific disease pathways.

Figure 3. H1650 human lung adenocarcinoma cells are PTEN deficient and express high leptin and leptin receptor mRNA. **(A)** Comparison of different lung cancer cell lines (A549, H292 and H1650) reveals H1650 cells are PTEN deficient and **(B)** express highest levels of *LEP* and its receptor *LEPR* mRNA **(C)** Leptin and leptin receptor protein levels as a result of *PTEN* deletion as assessed in H1650 cells, suggesting the likely presence of a functional LEP signaling pathway in lung epithelial cells. **(D)** Dose dependent increase in H1650 cell proliferation (~3 fold) was observed when cells were treated for 48 hours with increasing concentration (50, 100 and 200ng/ml; lane 2-4) of human recombinant LEP. **(E)** Continuous impedance sensing measurements identifies greater wound closure efficiency in cells treated with 100ng/ml leptin prior to wounding as compared to control cells without leptin. **(F)** Identification of the *LEP* gene core promoter region in H1650 lung cancer cell line revealed a proximal enhancer containing NRF-1 and C/EBP binding sites. Sub-confluent cultures were transiently transfected with various promoter-reporter deletion constructs derived from 5'-upstream regulatory sequence of *LEP* gene. Luciferase activity was expressed relative to the base-line luciferase activity of a promoter-less luciferase reporter construct (pGL3-Basic) set to unity. Data are represented from 4 independent experiments performed in triplicate (± S.E ; P-value ≤ 0.05*). **(G)** Diagrammatic representation of the most active promoter region -149 to +21bp of the *LEP* gene (*Luc-150*), comprising proximal enhancer depicting the positions of NRF-1 and C/EBP sites.

Figure 4. **NRF-1 and CEBP-δ activates *LEP* gene proximal promoter. (A)** NRF-1 increased *LEP* promoter activity as assessed after co-transfection of a fixed amount of *Luc-150* (0.1 μg) together with increasing amounts of CA-NRF-1 expression plasmid (1, 2, and 4 μgs/per well). All wells were normalized with empty pCDNA control vector as empty vector had no significant activity **(B)** Expression of dominant negative NRF-1 (DN-NRF-1) repressed *LEP* gene promoter activit as assessed after co-transfection of a fixed amount of *Luc-150* (0.1 μg), with increasing amounts of DN-NRF-1 expression plasmid (1, 2, and 4 μgs/per well). **(C)** CEBP-δ selectively activated *LEP* gene proximal promoter after co-transfection of a fixed amount of *Luc-150* (0.1 μg), and 4 μgs of CEBP-α, -β and -δ expression plasmids. **(D)** Expression of increasing amounts of CEBP-δ (0.25, 0.5, and 1μg/per well) stimulated (lane 3,4 and 5), while dominant negative form (0.5 and 1μg) of CEBP-δ (DN-CEBP-δ) repressed *LEP* gene promoter activity (lane 6 and 7) as assessed after co-transfection of a fixed amount of *Luc-150* (0.1 μg). **(E)** Additive effect of NRF-1 and CEBP-δ expression on the proximal enhancer as assessed on *Luc-150*. Independent expression of CA-NRF-1 and CEBP-δ (1μg/per well) stimulated LEP gene promoter activity (lane 3 and 4) as assessed after co-transfection with a fixed amount of *Luc-150* (0.1 μg) per well in 6-well plates containing H1650 cells, while co-expression of CA-NRF-1 and CEBP-δ (1μgs each/per well) showed an additive effect. **(F)** Expression of CA-STAT3 did not affect *Luc-150* activity. Co-transfection of *Luc-150* (1 μg) with CA-STAT3 expression plasmid (1 μg per well) in 6-well plates containing H1650 cells was performed. All transfections were done in H1650 lung epithelial cells in 6-well plates. Values represent three independent experiments carried out in triplicate ± S.E (P-value ≤ 0.001**).

Figure 5. Site-directed mutations to the NRF-1 and CEBP-δ consensus binding sites abrogate *LEP* gene proximal promoter activity. **(A)** As compared to wild–type *LEP* promoter (*Luc-150*), mutation in the NRF-1 site (*Luc-150-NRF-1-Δ)* luciferase promoter-reporter plasmid (1ug each) decreased the activity by >50% as assessed after transfection in H1650 cells. Values represent three independent experiments carried out in triplicate ± S.E (P-value ≤ 0.001**). **(B)** Site-directed mutagenesis of CEBP-δ binding site (*Luc-150-CEBP-δ-Δ*) abrogated *LEP* gene proximal promoter activity following transient transfection in H1650 cells. Values represent three independent experiments carried out in triplicate ± S.E (P-value ≤ 0.001**). **(C)** Primer locations that spanned the proximal enhancer containing NRF-1 and CEBP-δ sites for identification of PCR product following ChIP **(D)** ChIP analysis reveals NRF-1 occupancy on the LEP proximal promoter (lane 4). ChIP was performed with antibodies to NRF-1. IgG was used as a control (lane 2). Primers flanking the NRF-1 site were used for PCR analyses, while GAPDH primers used as an internal control did not show amplification of specific product (lane 3). **(E)** ChIP analysis reveals C/EBP-δ occupancy on the LEP proximal promoter (lane 4). ChIP was performed with antibodies to C/EBP-δ. IgG was used as a control (lane 2). Primers flanking the C/EBP-δ site were used for PCR analyses, while GAPDH primers used as an internal control did not show amplification of specific product (lane 3) as compared to lane 4 and DNA marker M showing 100 and 200 bps reference DNA sizes (lane 5).

Figure 6. ***LEP* gene transcription is regulated by PI3K/AKT/mTOR and MEK/ERK signaling pathways.** Effect of inhibitors of AKT (MK-2206), MEK (U0126), PI3K (LY294) and PI3 Kinase/mTOR dual inhibitor (BEZ-235) on *LEP* promoter activity. H1650 cells transfected with *Luc-150* (1ug) was either treated with increasing concentrations of (**A)** AKT inhibitor MK-2206 (50, 100 and 250nM), **(B)** MEK-inhibitor U0126 (50, 100 and 250nM), **(C)** PI3K/mTOR dual inhibitor BEZ-235 (50, 75 and 250nM) and **(D)** a potent PI3Kinase inhibitor LY294002 (5, and 10nM), a dose dependent decrease in luciferase activity was observed at 16 hours after transfection (lanes 3, 4 and 5 for A, B and C, and lanes 3 and 4 for D). Combination of**(E)** MK-2206 (50 nM) and LY294002 (10 nM), **(F)** U0126 (50 nM) and LY294002 (10 nM) or **(G)** BEZ-235 (50 nM) and LY294002 (10 nM) completely abrogated *Luc-150* luciferase activity, indicating that

PI3K/AKT/mTOR and MEK/ERK pathways independently modulate the *LEP* proximal promoter activity, likely via posttranslational modification of NRF-1 and CEBP-δ, influencing their transcription activity. Throughout, values represent three independent experiments carried out in triplicate. Data are presented as ± S.E. (P-value ≤ 0.05*).

Figure 7. ***LEP* and *LEPR* mRNA levels are up-regulated by NRF-1 and CEBP-δ.** **(A)** When H1650 lung epithelial cells growing in 10 cm plates were transfected with expression plasmids (8μgs) containing CA-NRF-1 or CEBP-δ, endogenous expression of *LEP* mRNA was increased by 8 and 4 fold respectively after 48 hours (lane 3 and 4, and inset lane 2 and 3). mRNA expression was quantitated by real time qPCR using *LEP* exon-specific primers as described in methods section. **(B)** Likewise, transfection of H1650 cells with CA-NRF-1 and CEBP-δ increased endogenous *LEPR* mRNA by approximately 3.5 and 2.5 fold respectively after 48 hours, suggesting a direct role for CA-NRF-1 and CEBP- δ in modulating *LEP* and *LEPR* gene expression. **(C)** Robust expression of endogenous NRF-1 mRNA was detected in H1650 cells. Two distinct shRNAs abrogated NRF-1 mRNA, shRNA-1B being more effective (lane 3). **(D)** Over-expression of shNRF-1B significantly decreased *LEP* and **(E)** *LEPR* mRNA expression in H1650, confirming a critical transcriptional role for NRF-1 in *LEP* and *LEPR* expression in lung epithelial cells. Throughout, values represent three independent experiments carried out in triplicate. Data are presented as ± S.E. (P-value ≤ 0.05*).

Figure 8. Leptin mediated activation of AKT/mTOR, MEK/ERK and JAK2/STAT3 signaling pathways. H1650 cells were serum starved for 16 h and then replenished with either 10% FCS, or 10% FCS with LEP (100 ng/ml). After 48 and 72 hours, total protein was isolated and immuno-blotted to detect activation of PI3K/AKT/mTOR, MEK/ERK and JAK2/STAT3 pathways via phosphorylation of AKT, mTOR and STAT3. Increase in **(A)** P-STAT3, **(B)** P-AKT and **(C)** P-mTOR and was readily observed at 48 and 72 hours after treatment with LEP (lane 2 and 4), suggesting an active LEP signaling pathway in the lung epithelial cells that operates via downstream activation of AKT, MEK and STAT3 pathways. β-actin was used as a biological and loading control. All data are representative of at least 3 independent experiments. Likewise, following serum starvation, when H1650 cells were treated with LEP (100 ng/ml), activation of **(D)** P-ERK **(E)** P-P38 **(F)** P-P54 SNP/JNK and **(G)** P-P46 SNP/JNK was transient but detectable at 15 min, for P-ERK and at 30 min, for P-P38,P-P54 SNP/JNK and P-P46SNP/JNK respectively . Throughout, values represent three independent experiments carried out in triplicate. Data are presented as ± S.E. (P-value ≤ 0.05*).

Figure 9. **LEP treatment increases endogenous *LEP* and *LEPR* mRNA levels.** **(A)** H1650 cells were serum starved for 16 h and then replenished with either 10% fetal calf serum (FCS), or 10% FCS containing LEP (100 ng/ml) for 24 and 72 hours (lane 2 and 3). Endogenous levels of *LEP* mRNA were measured using RT-qPCR analysis as described in the methods section. **(B)** When LEP treatment was performed on H1650 cells and expression of *LEPR* mRNA was analyzed by RT qPCR a modest but significant increase in *LEPR* mRNA was detected. Exon-specific primers for *LEP* and *LEPR* were used as described in Materials and Methods. Values represent three independent experiments carried out in triplicate. Data are presented as ± S.E. (P-value ≤ 0.05*). **(C)** Co-transfection of *Luc-150* (1 μg) together with expression plasmids CA-AKT (6ug) or CA-MEK (6 μg) in 6-well plates containing H1650 cells stimulated LEP promoter activity by approximately 3 and 4 fold respectively (lane 3 and 4), indicating a direct role for AKT and MEK pathways in the modulation of *LEP* proximal promoter activity. Values represent three independent experiments carried out in triplicate. Data are presented as ± S.E. (P-value ≤ 0.001**). **(D)** When H1650 cells growing in 10 cm plates were transfected with plasmid (8 μg) expressing constitutively active STAT3 (CA-STAT3), *LEP* and **(E)** *LEPR* mRNA expression was induced by approximately 2.5 fold as measured by RT-QPCR using gene-specific primers. Values represent three

independent experiments carried out in triplicate. Data are presented as ± S.E. (P-value ≤ 0.05*). **(F)** Location of primers spanning the distal promoter region containing STAT3 consensus site for identification of PCR product following ChIP using STAT3 antibodies. **(G)** ChIP analysis reveals STAT3 occupancy on the LEP distal promoter (lane 5). ChIP was performed with antibodies to STAT3. IgG was used as a control (lane 3). Primers flanking the STAT3 site were used for PCR analyses, while GAPDH primers used as an internal control did not show amplification of specific product (lane 4) as compared to lane 5 and DNA marker M showing 100 and 200 bps reference DNA sizes (lane1).

Figure 10. Proposed model of LEP mediated LEP gene expression. Proposed model of LEP mediated LEP gene expression indicates that loss of PTEN in the lung epithelial cells activates AKT, which in turn induces *LEP* expression and LEP secretion into the extracellular space. Such AKT mediated increases in extracellular LEP (LEP) concentration may occur in lung cancer and many lung diseases associated with increased PI3K activity. Since lung epithelial cells express LEP receptor (LEPR) that readily bind to LEP, multiple kinase pathways are activated, including AKT/mTOR, GSK3, JAK2/STAT3 and MEK/ERK that influence NRF-1, CEBP-δ and STAT3 binding, activating LEP and LEPR expression. Thus, a continuous LEP/LEPR feed-forward loop is set-up, further deteriorating lung function. Activation of these anti-apoptotic pathways also provides survival benefit to lung tumor cells, contributing to chemo- radio- and targeted therapy resistance, leading to poor clinical outcomes.

Figure 1

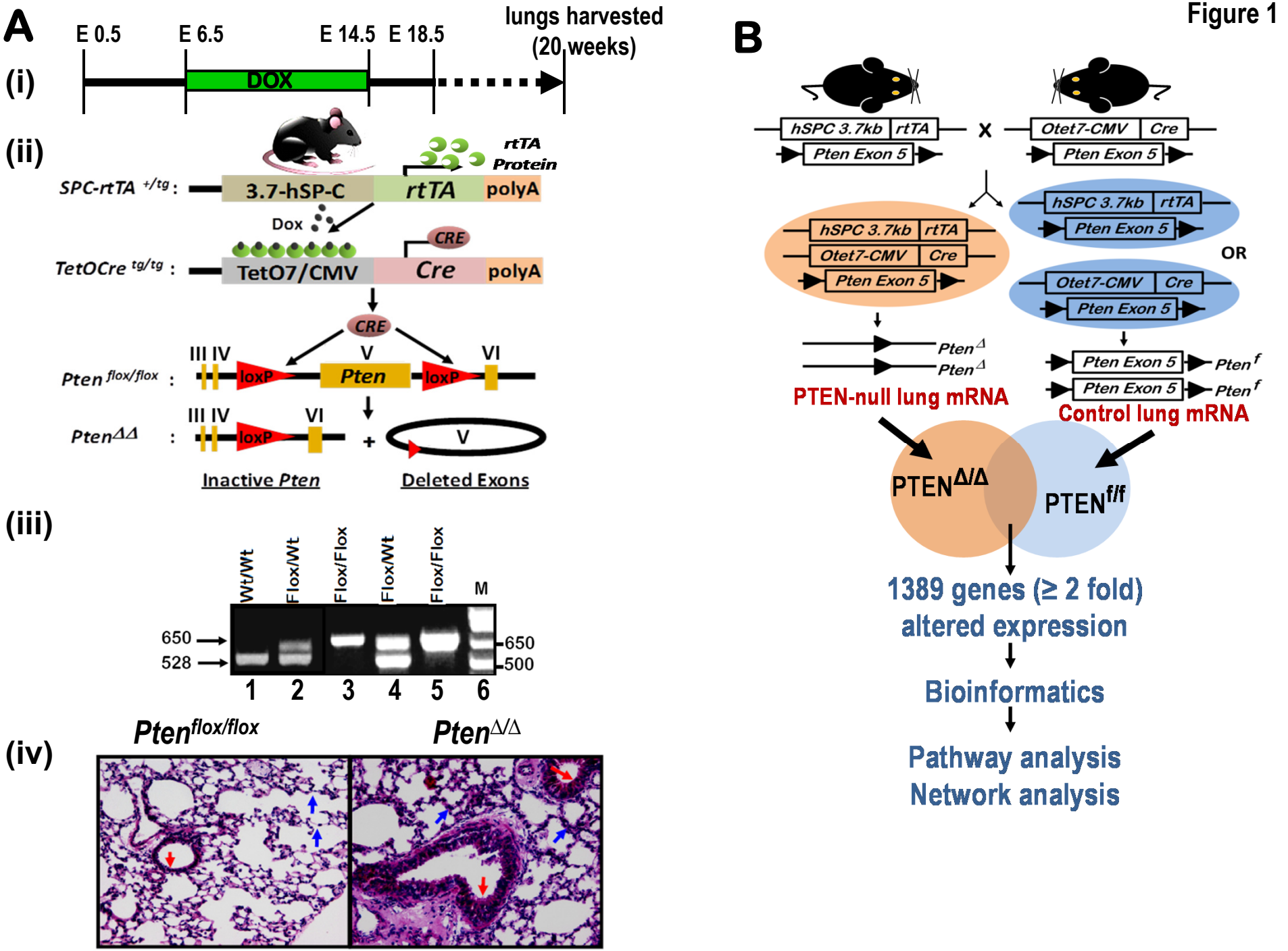


Figure 2

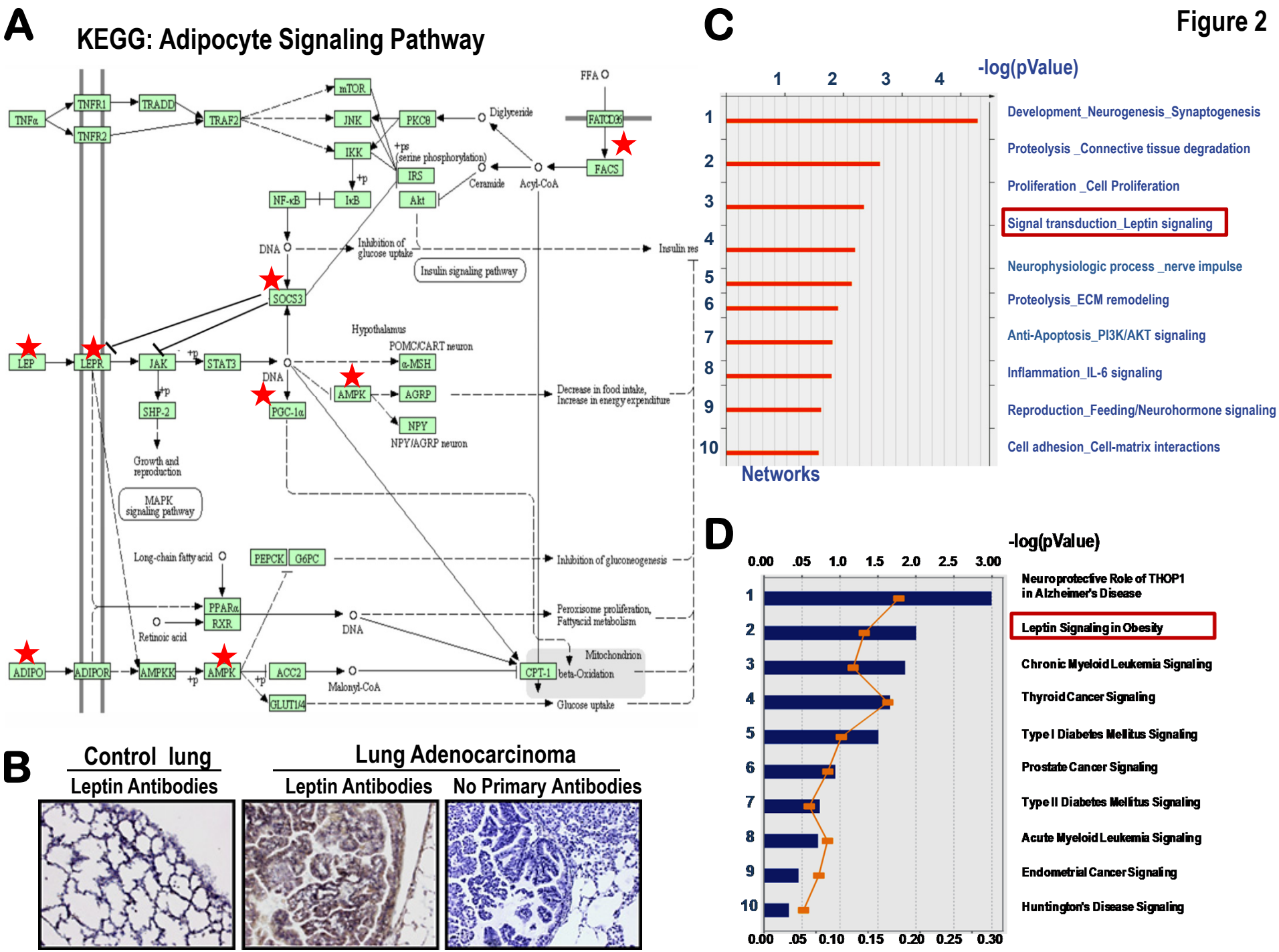


Figure 3

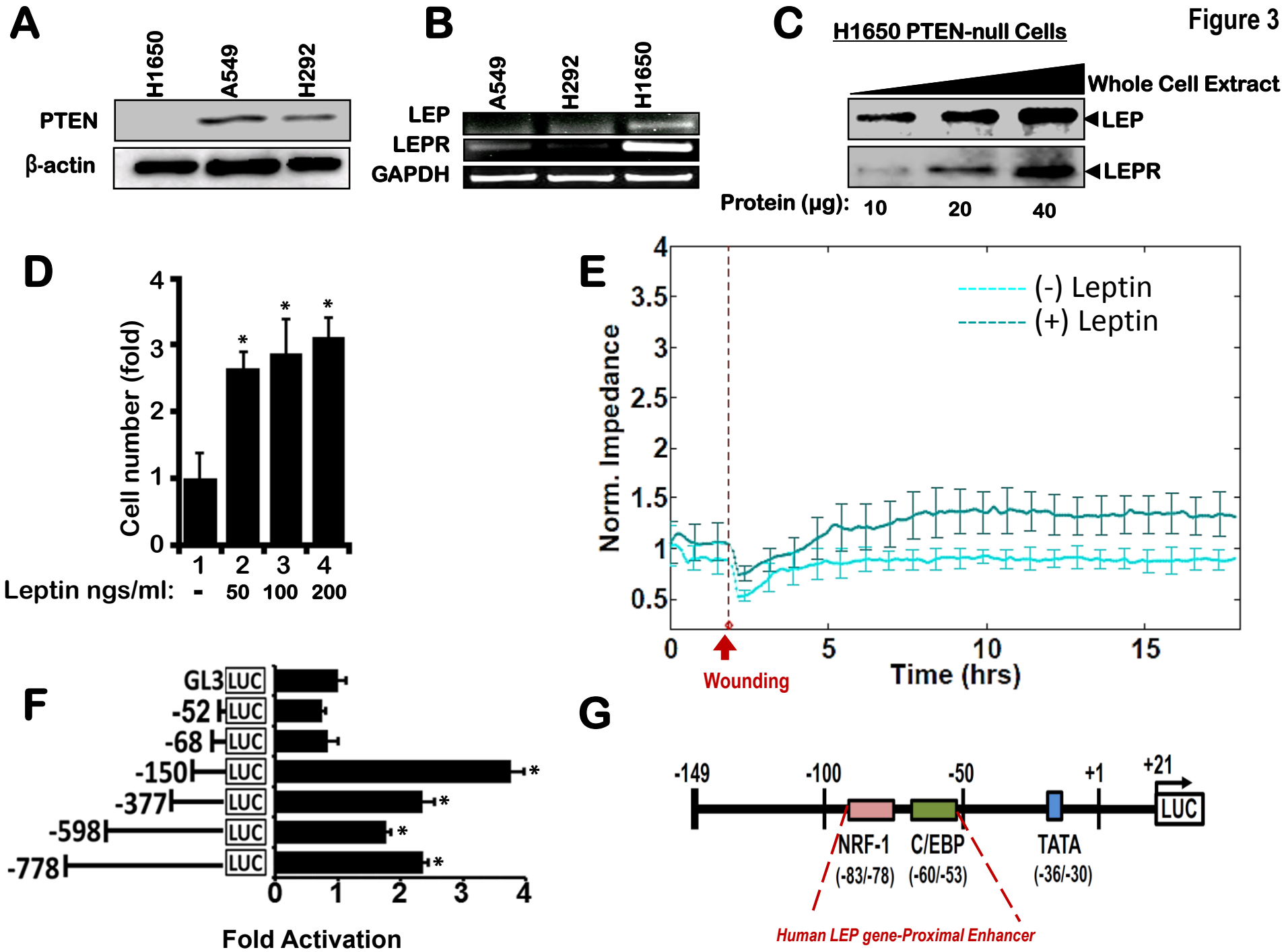


Figure 4

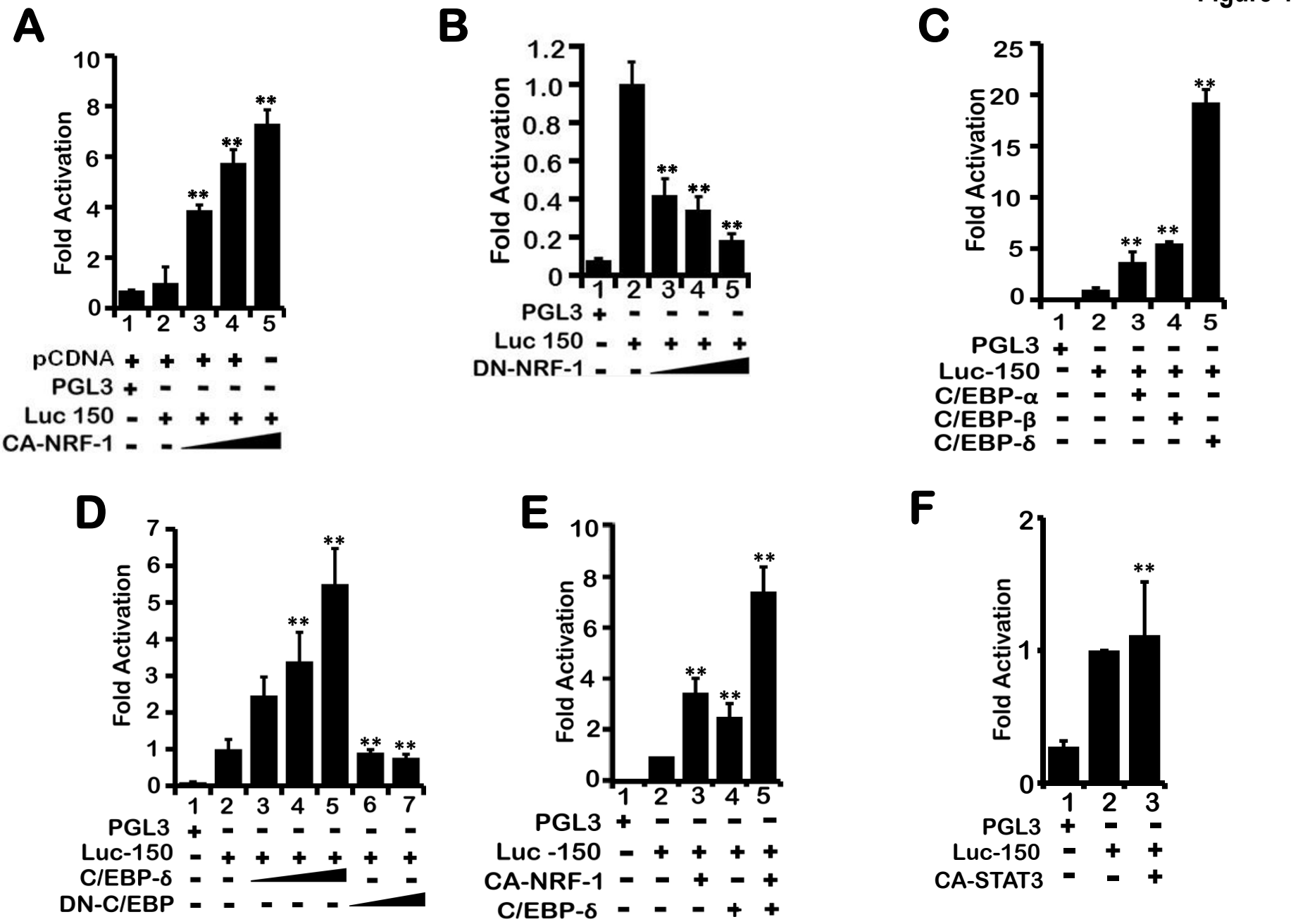


Figure 5

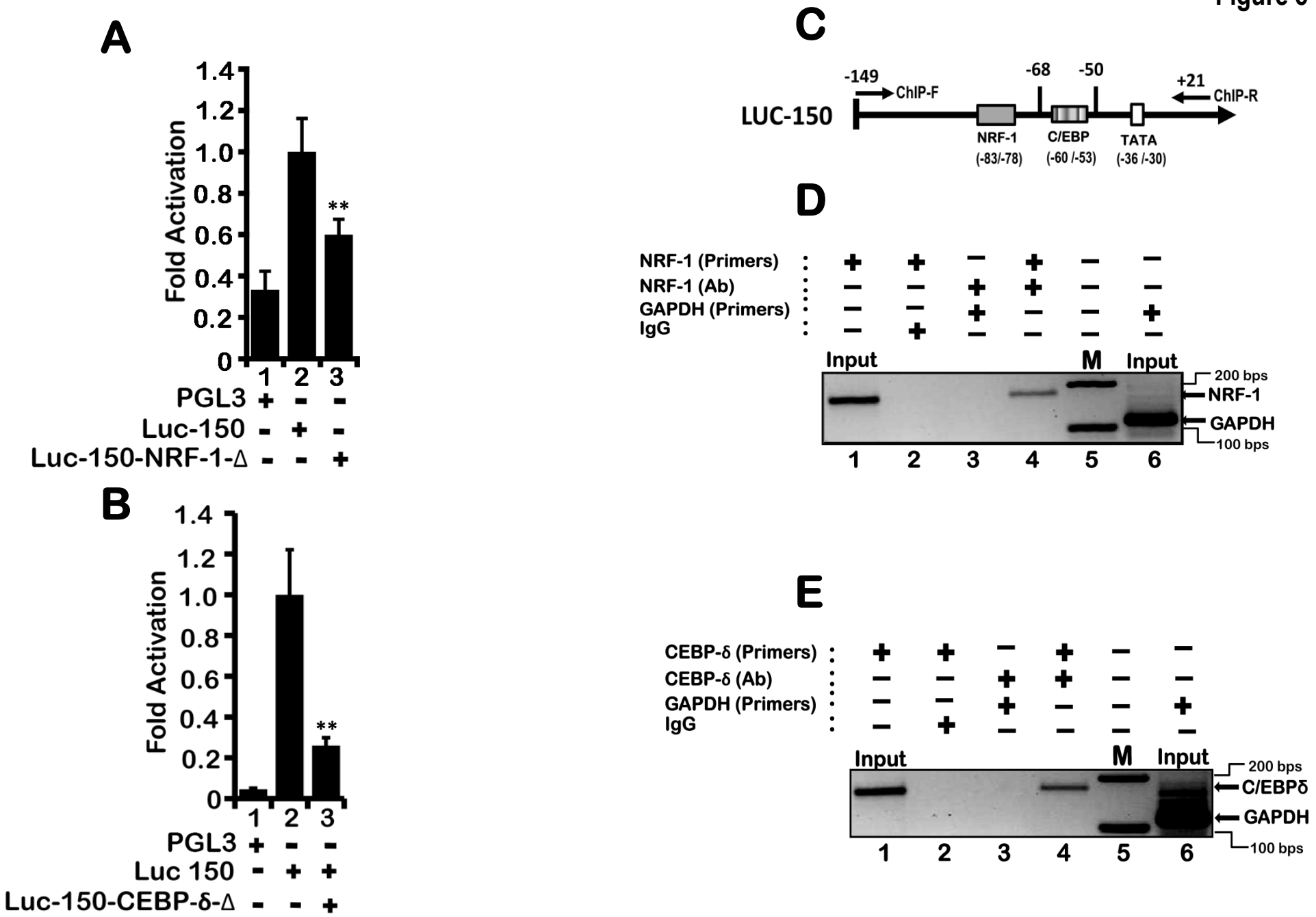


Figure 6

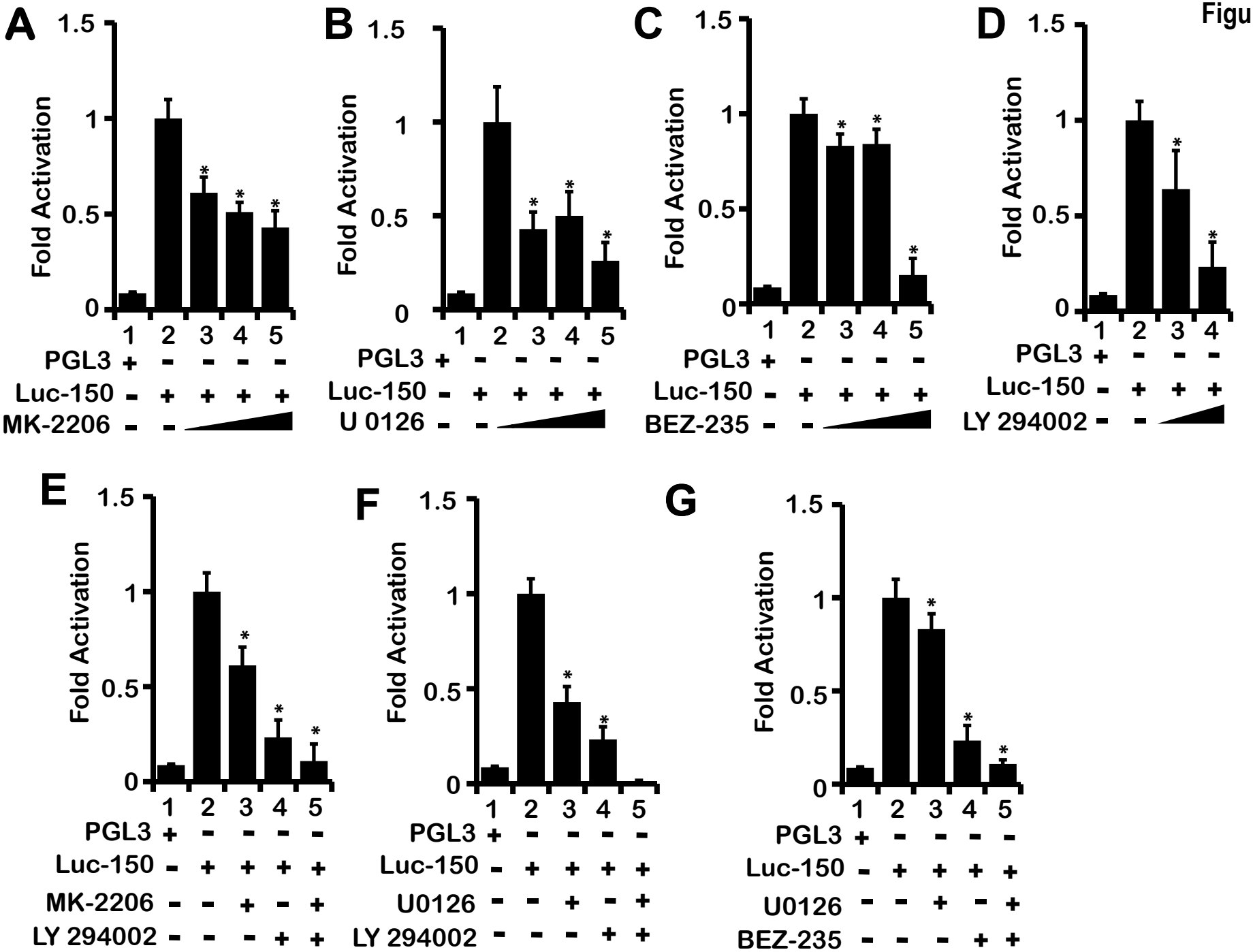
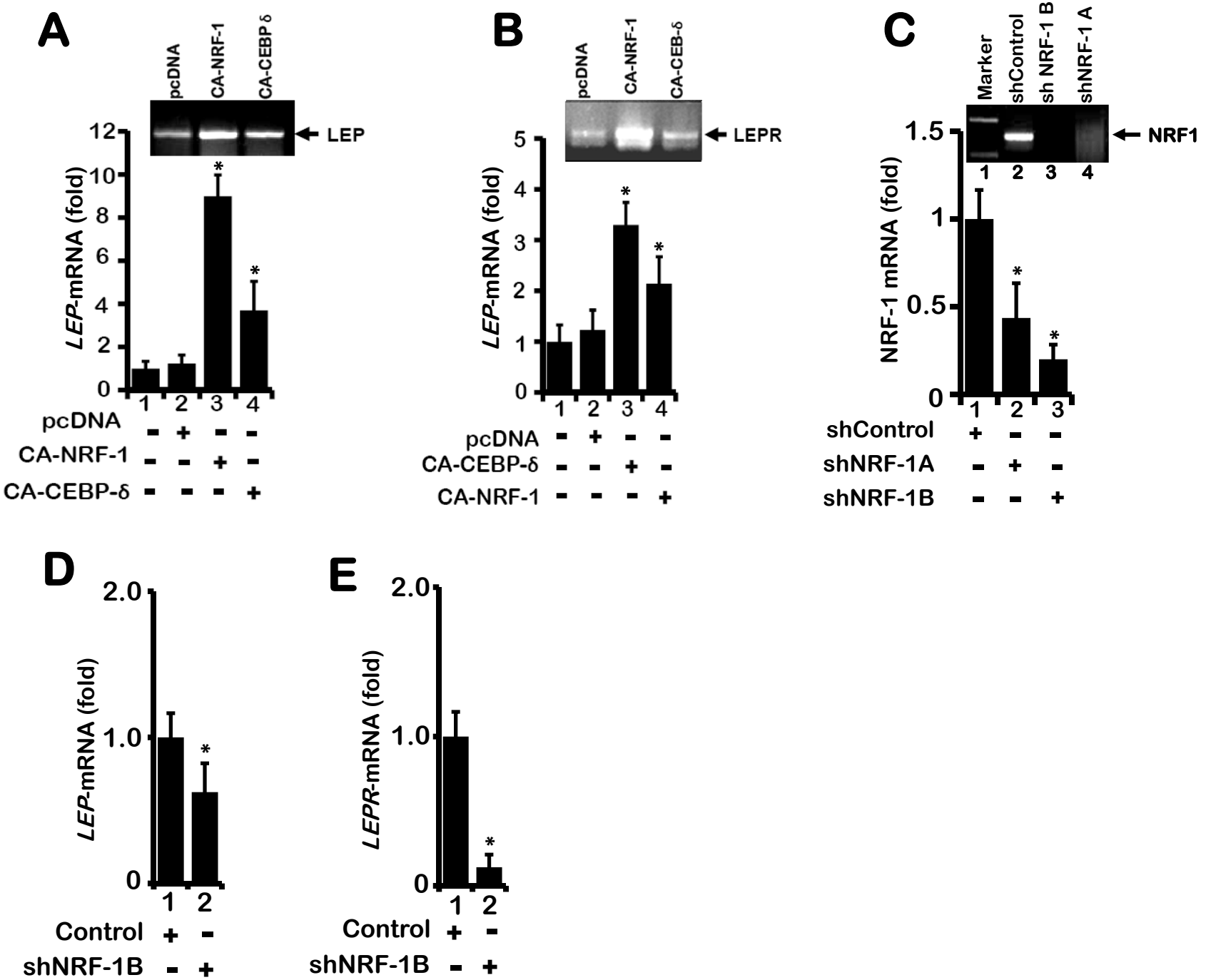


Figure 7



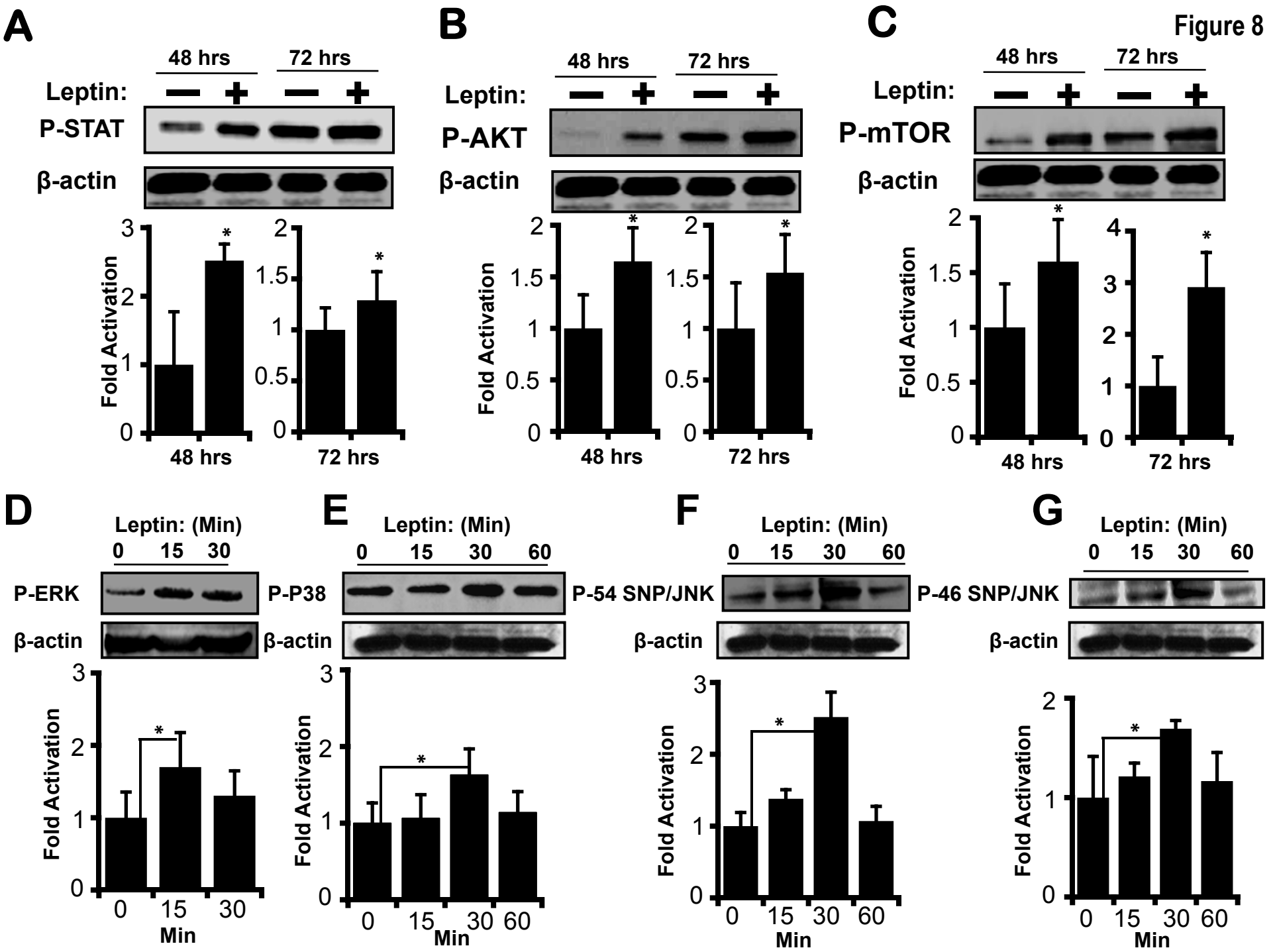


Figure 8

Figure 9

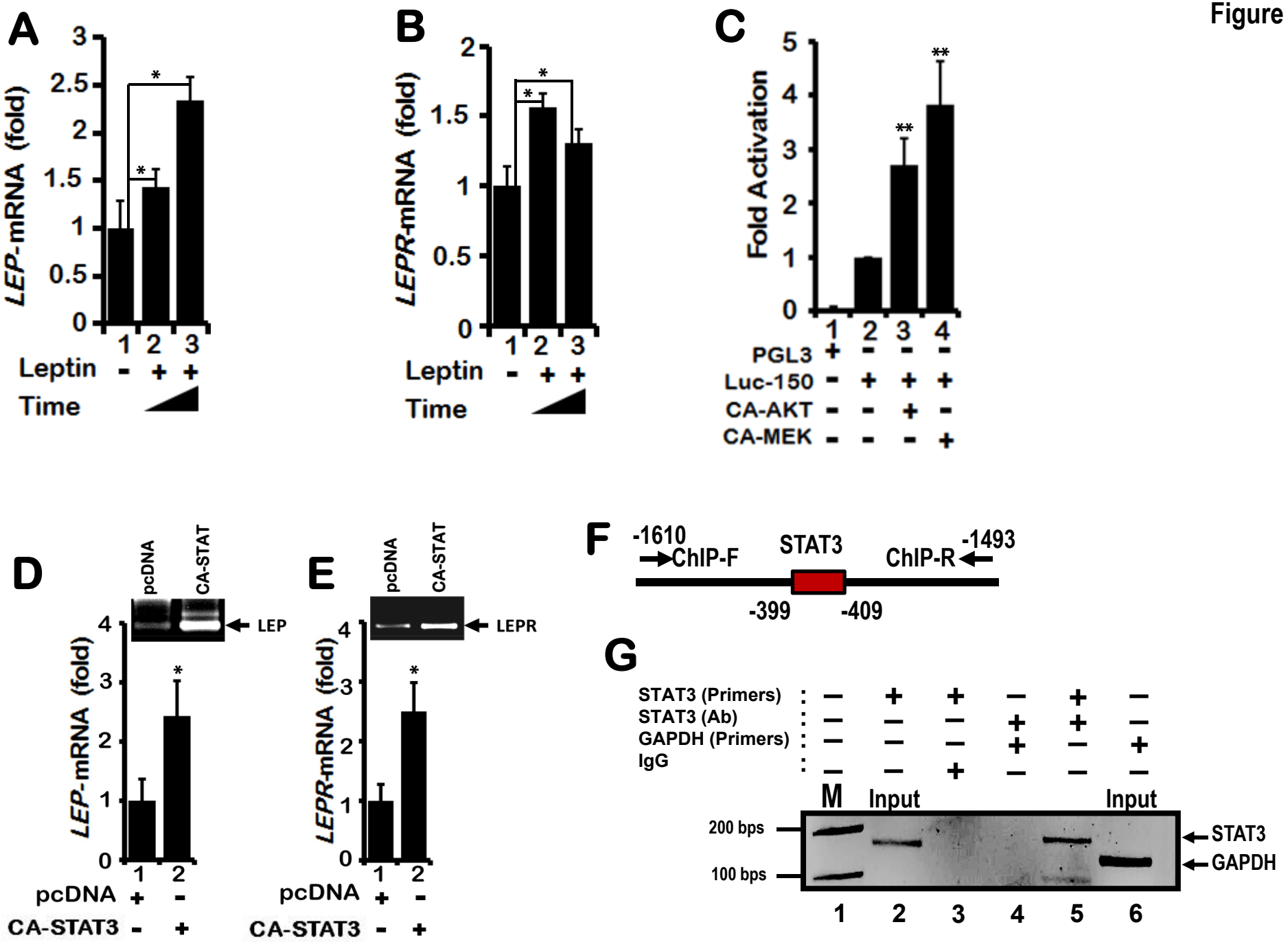


Figure 10