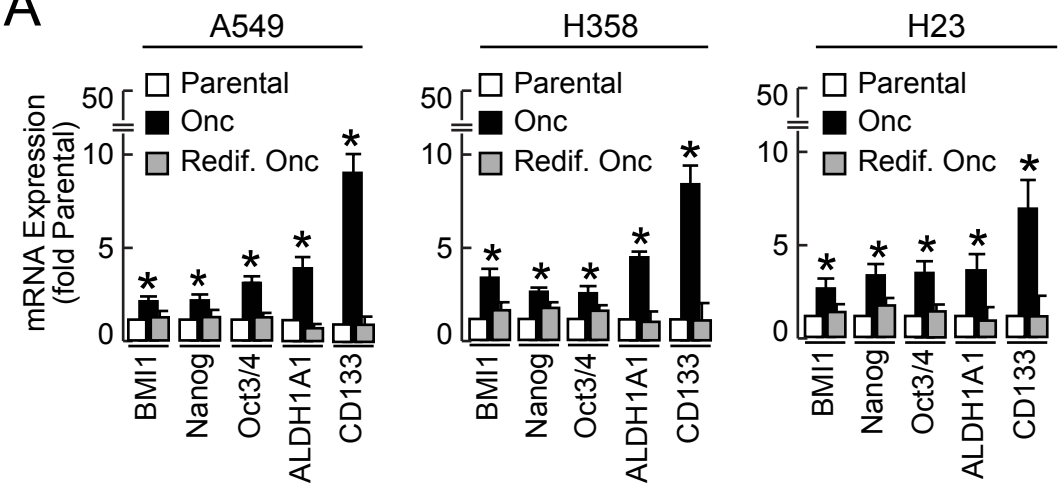
Supplemental Figures

A



B

Lung Orthotopic Tumor Take

Cells	$1x10^6$	$1x10^5$	$5x10^4$	$1x10^4$	$5x10^3$	$1x10^3$	100	10
Par	3/5	0/5	0/5	0/5	0/5	0/5	ND	ND
Oncos	5/5	5/5	5/5	5/5	5/5	4/4	5/5	0/5

C

Tumor Initiating Cell Frequency
(Conf. intervals; 1/TIC frequency)

Cells	Lower	Estimate	Upper
Parental	$4x10^6$	$1.4x10^6$	$4.5x10^5$
Oncosph	113	42	16

p-value=$1.9x10^{-49}$

D

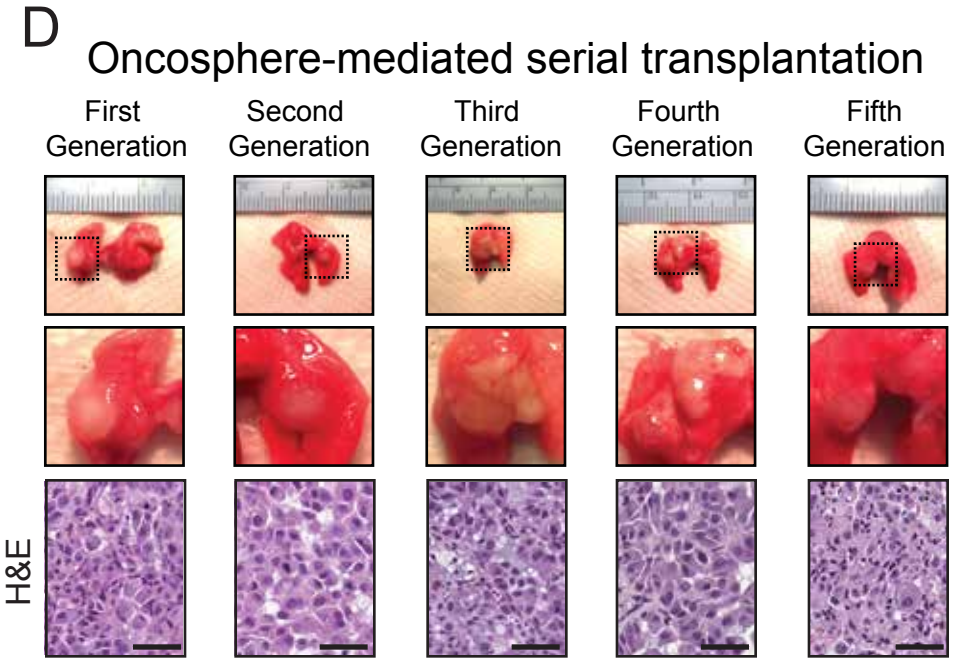


Figure S1. Expression of Stem-related genes, Extreme Limiting Dilution Assay (ELDA) and serial transplantation analysis of LADC oncospheres. Related to Figure 1. (A) QPCR analysis of parental, oncosphere and re-differentiated oncosphere cells from A549, H358 and H23 cells for BMI, NANOG, OCT3/4, ALDH1A1 and CD133. Results are expressed as fold parental cells +/-SEM, n=5, *$p<0.05$ compared to parental cells. (B) Orthotopic tumor take data for A549 parental and oncosphere cells. N.D.= not determined. (C) Tumor initiating cell frequency calculated by extreme limiting dilution analysis (Hu and Smyth, 2009). Chart indicates upper and lower confidence intervals and an estimated TIC frequency; p-value indicates a statistically significant difference in TIC frequency between parental and oncospheres. (D) Oncospheres were serially transplanted through five successive generations of mice as described previously (Justilien et al., 2014), demonstrating the persistence of TIC activity. H&E staining demonstrates that successive generations of oncosphere-derived tumors exhibit similar histology. Scale bars, 50 μm.

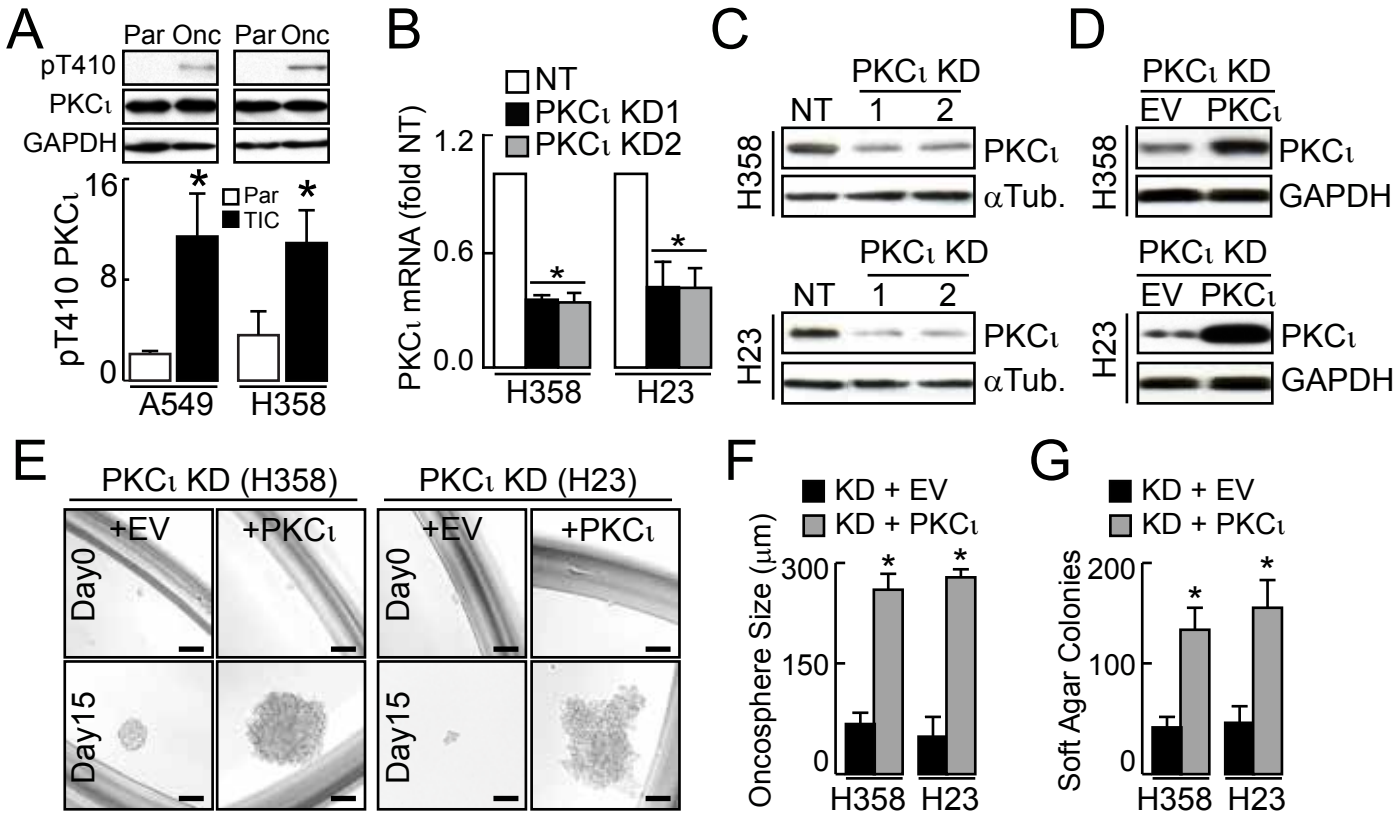
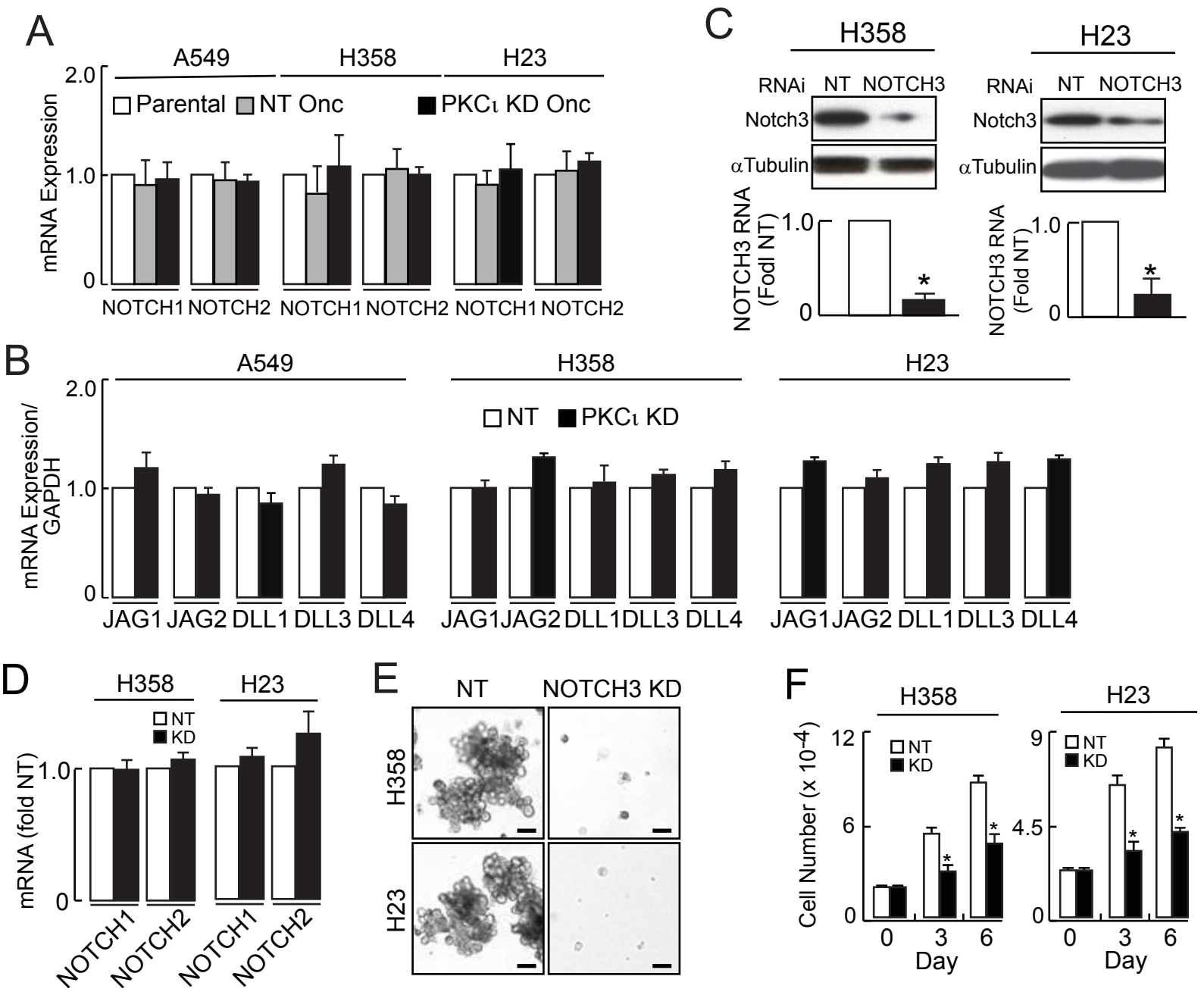

A
Par Onc Par Onc
pT410
PKCι
GAPDH
pT410 PKCι
16
8
0
Par
TIC
*
*
A549
H358
B
NT
PKCι KD1
PKCι KD2
PKCι mRNA (fold NT)
1.2
0.6
0.0
*
*
H358
H23
C
PKCι KD
NT 1 2
H358
PKCι
αTub.
PKCι KD
NT 1 2
H23
PKCι
αTub.
D
PKCι KD
EV PKCι
H358
PKCι
GAPDH
PKCι KD
EV PKCι
H23
PKCι
GAPDH
E
PKCι KD (H358)
PKCι KD (H23)
+EV
+PKCι
+EV
+PKCι
Day0
Day15
Day0
Day15
F
KD + EV
KD + PKCι
Oncosphere Size (μm)
300
150
0
*
*
H358
H23
G
KD + EV
KD + PKCι
Soft Agar Colonies
200
100
0
*
*
H358
H23

Figure S2. PKCι activity and function in KRAS LADC oncospheres. Related to Figure 2. (A) Immunoblot of A549 and H358 oncosphere cells for phospho-T410 PKCι levels compared to parental cells (top panel). Quantitative analysis of pT410-PKCι levels (bottom panel). Results expressed as mean band intensity +/-S.E.M. n=3. *p<0.05 compared to parental cells. (B) PKCι KD using two lentiviral constructs (PKCι KD1 and KD2). QPCR results expressed as fold NT control +/-SEM, n=5. *p<0.05. (C) Immunoblot for PKCι in PKCι KD1 and KD2 oncospheres. α-tubulin is a loading control. (D) Immunoblot for PKCι in PKCι KD1 H358 and H23 oncospheres expressing empty vector (EV) or exogenous PKCι (PKCι). GAPDH is a loading control. (E) Effect of exogenous PKCι expression on oncosphere formation in PKCι KD H358 and PKCι KD H23 cells. Scale bars, 50 μm. (F) Analysis of oncosphere size expressed as diameter +/-SEM. Number of oncospheres assessed: H358: KD+EV (7), KD+PKCι (26); H23: KD+EV (11), KD+PKCι (35). *p<0.05. (G) Anchorage-independent growth expressed as soft agar colonies +/-SEM. n=3. *p<0.05 compared to KD+EV.


A
A549
H358
H23
2.0
1.0
0
mRNA Expression
Parental
NT Onc
PKCι KD Onc
NOTCH1 NOTCH2 NOTCH1 NOTCH2 NOTCH1 NOTCH2
C
H358
H23
RNAi NT NOTCH3
Notch3
αTubulin
NOTCH3 RNA (Fodl NT)
1.0
0
*
B
A549
H358
H23
mRNA Expression/ GAPDH
NT
PKCι KD
JAG1 JAG2 DLL1 DLL3 DLL4
D
H358
H23
NT
KD
mRNA (fold NT)
NOTCH1 NOTCH2 NOTCH1 NOTCH2
E
NT
NOTCH3 KD
H358
H23
F
H358
H23
Cell Number (x 10-4)
12
6
0
9
4.5
NT
KD
0 3 6
Day

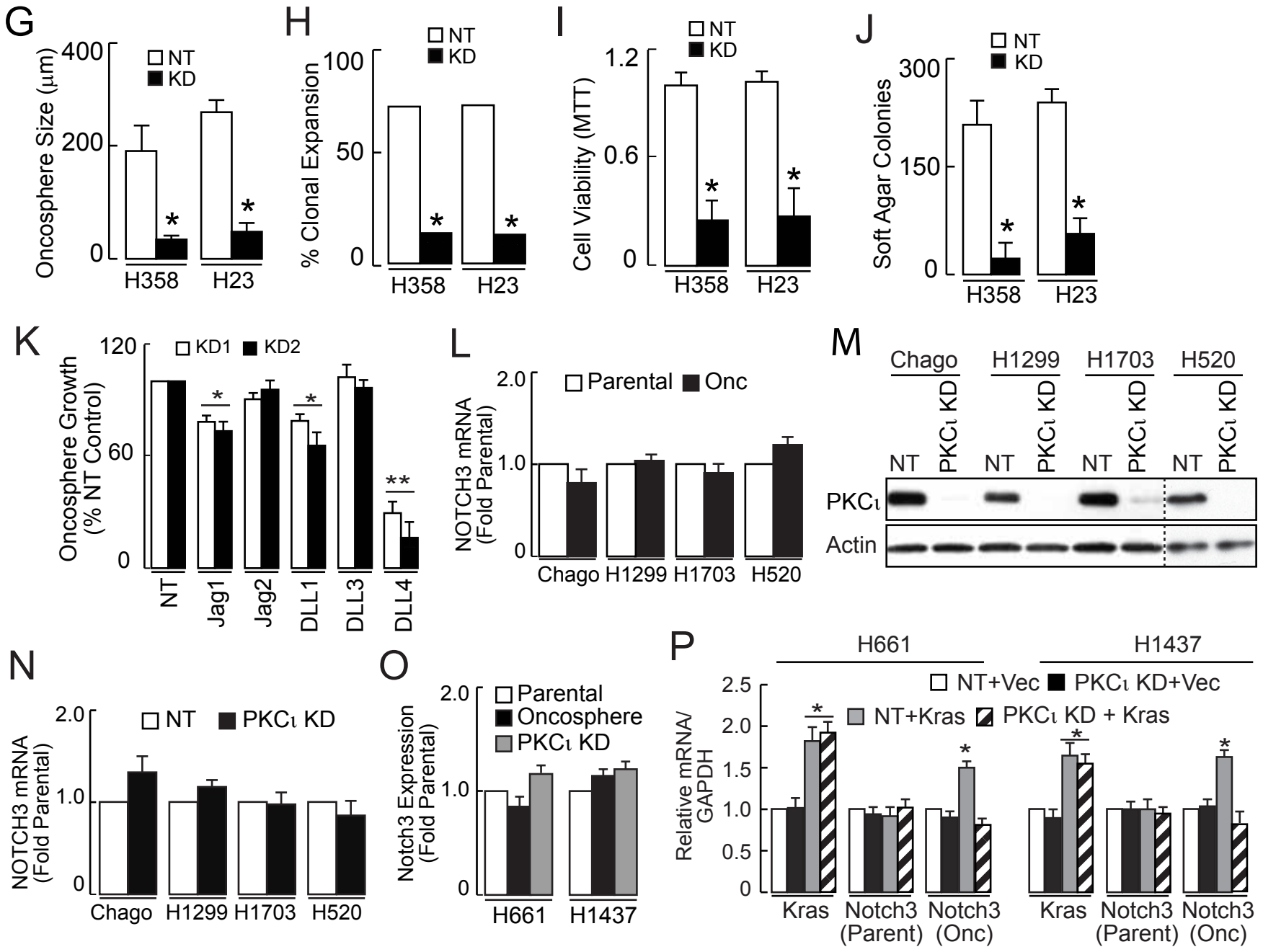

G
Oncosphere Size (μm)
400
200
0
NT
KD
H358
H23
H
% Clonal Expansion
100
50
0
I
Cell Viability (MTT)
1.2
0.6
0
J
Soft Agar Colonies
300
150
0
K
Oncosphere Growth (% NT Control)
120
60
KD1
KD2
NT
Jag1
Jag2
DLL1
DLL3
DLL4
L
NOTCH3 mRNA (Fold Parental)
2.0
1.0
Parental
Onc
Chago
H1299
H1703
H520
M
PKCι
Actin
PKCι KD
N
PKCι KD
O
Notch3 Expression (Fold Parental)
Oncosphere
H661
H1437
P
Relative mRNA/ GAPDH
2.5
2.0
1.5
1.0
0.5
NT+Vec
PKCι KD+Vec
NT+Kras
PKCι KD + Kras
Kras
Notch3 (Parent)
Notch3 (Onc)

Figure S3. Effect of NOTCH3 knockdown on LADC TIC behaviors. Related to Figure 3. (A) QPCR of NOTCH1 and 2 in parental cells, and NT and PKCι KD oncospheres from A549, H358 and H23 cells. QPCR results presented as fold parental cells +/-SEM. n=3. (B) QPCR of the NOTCH ligands JAG1, JAG2, DLL1, DLL3 and DLL4 in parental cells, and NT and PKCι KD oncospheres from A549, H358 and H23 cells. QPCR results presented as fold parental cells +/-SEM. n=3. (C) Immunoblot (upper panels) and QPCR (lower panels) of NOTCH3 protein and mRNA respectively, in H358 and H23 oncospheres. α-tubulin is included as a loading control. QPCR results are expressed as fold NT control +/-SEM. n=3. *p<0.05. (D) QPCR analysis of NOTCH1 or NOTCH2 expression in H358 and H23 NT and NOTCH3 KD oncospheres. Data expressed as fold NT control +/-SEM. N=5. *p<0.05. (E) Representative micrographs of H358 and H23 NT and NOTCH3 KD oncosphere growth. Scale bars 50 μm. (F) Quantitative analysis of oncosphere growth expressed as cell number (x 10^{-4}) +/-SEM. n=4. *p<0.05 compared to NT. Effect of NOTCH3 KD on (G) oncosphere size (μm), n=3,*p<0.05 compared to NT. (H) Individual oncosphere cells were assessed for the ability to clonally expand and results plotted as % expanded. Number of cells expanded/total cells scored: H358: NT (33/43), KD (4/36); H23: NT (30/41), KD (4/34). *p≤0.001 based on Fisher's exact test. (I) Cell viability (expressed as MTT reduction), and (J) Anchorage-independent growth (expressed as soft agar colonies) in H358 and H23 NT and NOTCH3 KD oncospheres. *p<0.05. (K) Effect of RNAi-mediated knock down of NOTCH ligands on oncosphere growth. The NOTCH ligands JAG1, JAG2, DLL1, DLL3 and DLL4 were knocked down (>70% KD for all constructs) using two independent lentiviral shRNA constructs (KD1 and KD2). Oncosphere growth is shown expressed as % NT control +/-SEM. n=3. *p<0.05; **p<1.5 x 10^{-8}. (L) QPCR for NOTCH3 in parental and oncosphere cultures of Chago, H1299, H1703 and H520 LSCC cells. Results expressed as fold parental +/-SEM, n=5. (M) Immunoblot analysis of PKCι KD in Chago, H1299, H1703 and H520 LSCC cells. (N) QPCR for NOTCH3 in NT and PKCι KD oncospheres from Chago, H1299, H1703 and H520 LSCC cells. Results expressed as fold parental +/-SEM, n=5. (O) QPCR for NOTCH3 in parental cells, NT TICs, and PKCι KD oncospheres from H661 and H1437 LADC cells harboring wild-type *KRAS*. Results expressed as fold parental +/-SEM, n=5. (P) QPCR for Kras and NOTCH3 in H661 and H1437 parental (Parent) and oncosphere (Onc) cells expressing NT or PKCι KD and either an empty vector (Vec) or oncogenic $KRAS^{G12V}$ (*Kras*). Results expressed as fold parental NT+Vec +/-SEM, n=5. *p<0.05.

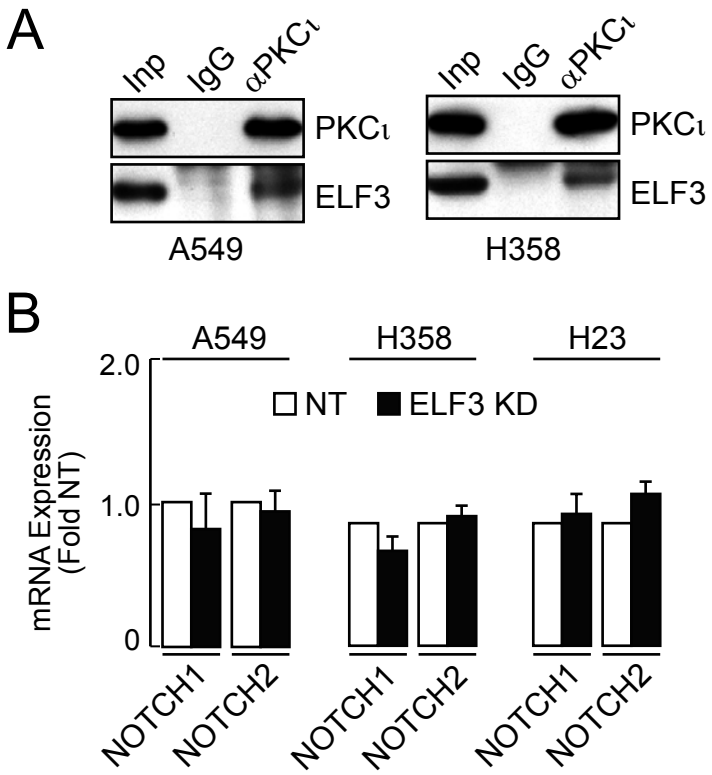

A
Inp
IgG
αPKCι
PKCι
ELF3
A549
H358
B
A549
H358
H23
NT
ELF3 KD
mRNA Expression
(Fold NT)
2.0
1.0
0
NOTCH1
NOTCH2

Figure S4. Effect of ELF3 knockdown on expression of NOTCH1 and NOTCH2 in LADC oncospheres. Related to Figure 4. (A) Co-immunoprecipitation of ELF3 with PKCι in LADC oncospheres. Immunoblot analysis for PKCι and ELF3 of total cell lysates (Input, Inp), and immunorecipitations using IgG control (IgG) or PKCι (PKCι) antibody, from A549 and H358 oncospheres. (B) QPCR analysis of NOTCH1 and NOTCH2 expression in A549, H358 and H23 NT and ELF3 KD oncospheres. Data are expressed as fold NT control +/-SEM. n=4.

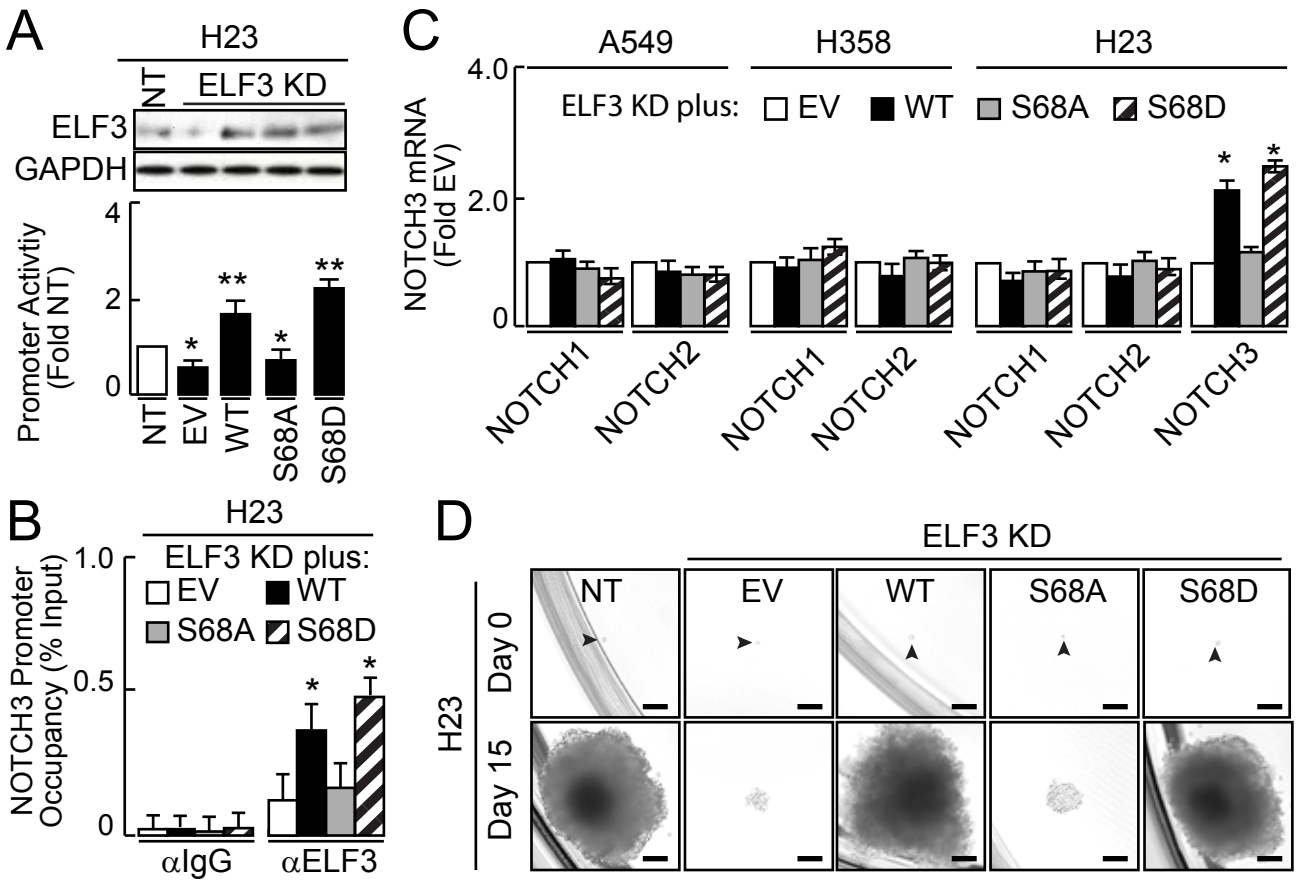
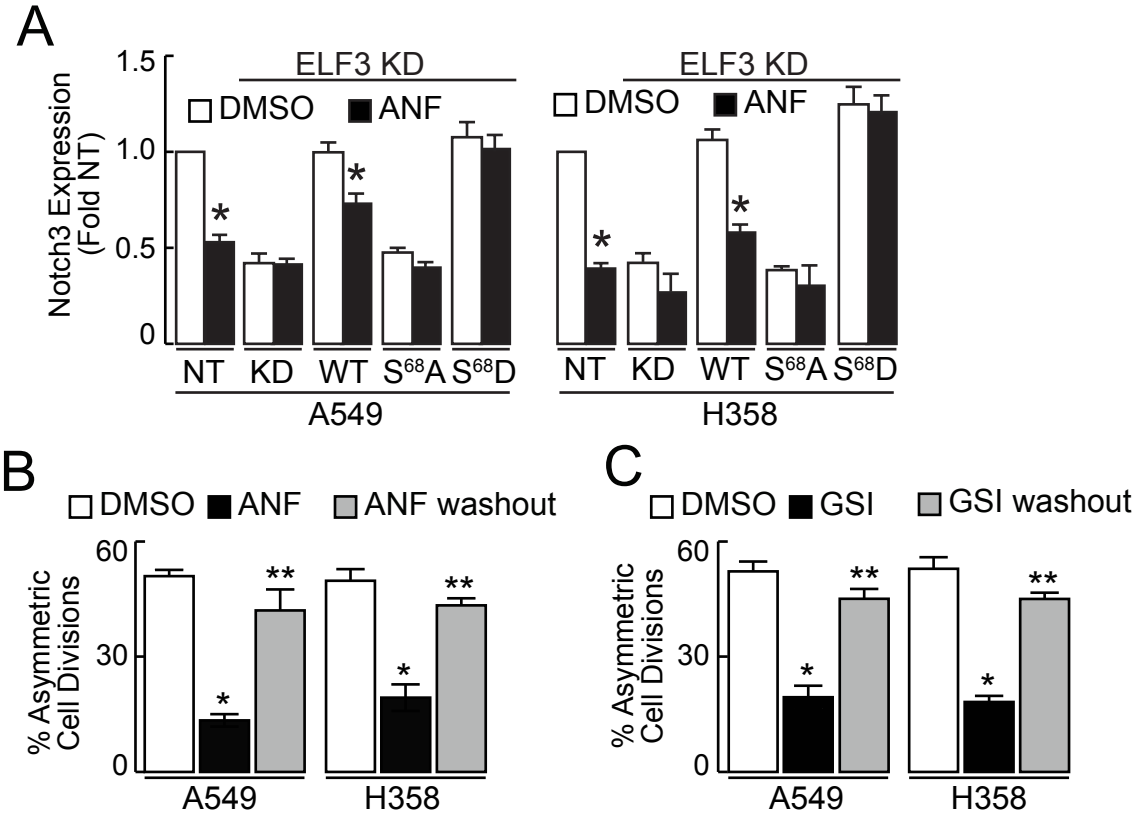
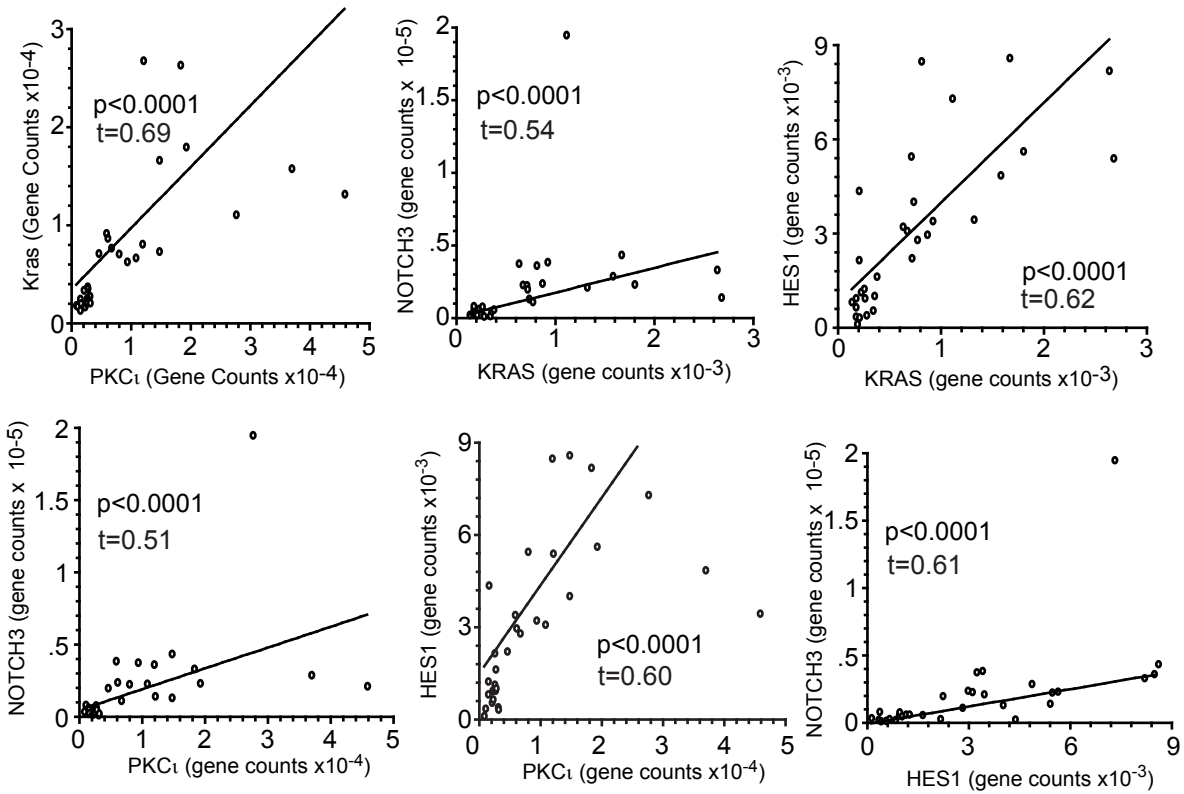

A
H23
NT
ELF3 KD
ELF3
GAPDH
Promoter Activity (Fold NT)
4
2
0
NT
EV
WT
S68A
S68D
B
H23
ELF3 KD plus:
EV
WT
S68A
S68D
NOTCH3 Promoter Occupancy (% Input)
1.0
0.5
0
αIgG
αELF3
C
A549
H358
H23
ELF3 KD plus:
EV
WT
S68A
S68D
NOTCH3 mRNA (Fold EV)
4.0
2.0
0
NOTCH1
NOTCH2
NOTCH1
NOTCH2
NOTCH1
NOTCH2
NOTCH3
D
ELF3 KD
NT
EV
WT
S68A
S68D
H23
Day 0
Day 15

Figure S5: Effect of PKCι-mediated ELF3 phosphorylation on NOTCH3 expression and oncosphere growth. Related to Figure 5. (A) ELF3 protein expression (top) and NOTCH3 promoter reporter activity (bottom) assayed in NT and ELF3 KD oncospheres stably transduced empty vector (EV) or vector encoding WT-ELF3 (WT), S68A-ELF3 (S68A), or S68D-ELF3 (S68D) mutants. Activity is plotted as fold NT+EV +/-SEM, n=5, *$p<0.05$ compared to NT+EV, **$p<0.05$ compared to ELF3-KD+EV. (B) ChIP analysis assessing ELF3 occupancy of the NOTCH3 promoter. Data are presented as % input +/-SEM, n = 3; *$p<0.05$ compared to EV. Data are representative of two independent experiments. (C) QPCR of NOTCH1, NOTCH2 and NOTCH3 (H23 only) expressed as fold EV +/-SEM, n=3. *$p<0.05$ compared to EV. (D) Representative micrographs of H23 oncosphere growth. Scale bars 50 μm.


A
ELF3 KD
DMSO
ANF
Notch3 Expression (Fold NT)
1.5
1.0
0.5
0
NT
KD
WT
S68A
S68D
A549
H358
B
DMSO
ANF
ANF washout
% Asymmetric Cell Divisions
60
30
0
A549
H358
C
DMSO
GSI
GSI washout

D



E

Gene Association		P Value	Log Odds Ratio
KRAS	NOTCH1	0.536	-0.021
KRAS	NOTCH2	0.331	-0.171
KRAS	NOTCH4	0.066	-0.566
PRKCI	NOTCH1	0.545	0.029
PRKCI	NOTCH2	0.294	0.386
PRKCI	NOTCH4	0.187	0.432

F

Gene Association		P Value	Log Odds Ratio
KRAS	PRKCI	0.196	-0.4
KRAS	NOTCH3	0.252	0.294
KRAS	HES1	0.196	-0.526
PRKCI	NOTCH3	0.545	0.029
PRKCI	HES1	0.294	0.386
NOTCH3	HES1	0.187	0.432

Figure S6. Effect of ANF and GSI on asymmetric cell division. Related to Figure 7. (A) Effect of ANF (1 μM) on NOTCH3 expression in NT and ELF3 KD oncospheres expressing WT ELF3, S68A or S68D ELF3. Results are expressed as fold DMSO-treated NT cells +/-SEM. n=3, *p<0.05 compared to DMSO. Effect of ANF (B) and GSI (C) on asymmetric cell divisions in A549 and H358 oncospheres. Data presented as % asymmetric cell divisions +/-SEM. n=3, *p<0.05 compared to DMSO; **p<0.05 compared to indicated drug prior to washout. (D) 2x2 scatter plots demonstrating associations between expression of *KRAS*, *PRKCI*, *NOTCH3* and *HES1* in a primary LADC gene expression dataset (Kalari et al., 2012). Linear regression line is indicated as well as the Kendall τ and p-value for each association. (E) Gene expression analysis of TCGA dataset for correlations between oncogenic *KRAS* or *PRKCI* with *NOTCH1*, *NOTCH2* or *NOTCH4* in LADC tumors. (F) Gene expression analysis of a LSCC TCGA dataset for correlation between *KRAS* or *PRKCI* with *NOTCH3* or *HES1* in primary LSSC tumors. See Supplemental Experimental Procedures for details.

Supplemental Experimental Procedures

Culture of lung cancer cell lines and TIC enrichment

Human lung adenocarcinoma (LADC) and squamous carcinoma (LSCC) cell lines (A549, H358, H23, H661, H1437, H1703, H1299, H520 and ChagoK1) were purchased from and grown as described by American Type Culture Collection (ATCC) (Manassas, VA). Cell growth media, F-12K Mixture Kaighn's modification, RPMI Medium 1640 and DMEM was purchased from Gibco Life Technologies (Life Technologies, Grand Island, NY). Supplementary components FBS, Sodium pyruvate, Sodium Bicarbonate, HEPES, L-Glutamine and Pen strep were purchased from Gibco Life Technologies. Prior to use, cell culture media was supplemented following the ATCC instructions specific for each cell type provided online (www.atcc.org). TICs from LADC (A549, H358, H23, H661 and H1437) and LSCC (H1703, H1299, H520, and ChagoK1) were enriched as described previously (Justilien et al., 2014). Briefly, cells were grown in serum-free DMEM-F12 (1:1) medium (Life Technologies). Growth media was modified by adding 50 μg/ml insulin and 0.4% Albumin Bovine Fraction V from (Sigma-Aldrich St. Louis, MO), N-2 Plus Media Supplement and B-27 Supplement (Life Technologies), 20 μg/ml EGF and 10 μg/ml basic FGF purchased from (PeproTech, Rocky Hill, NJ). Cells were plated in ultra-low attachment flasks (Corning, Corning, NY) having a neutral, hydrophilic hydrogel coating to prevent "stem-like" cells from attachment-mediated differentiation by maintaining cells in suspension. To expand TICs, media was removed by centrifuging at 800 RPM followed by 1X-PBS wash. TICs were treated with TryLE (Life Technologies) to dissociate large colonies. Freshly supplemented media was added and TICs were propagated in low adherent flasks. TICs were re-differentiated by removing them from low adherence culture as described above. TICs were centrifuged at 800 RPM for 5 min, washed using 1X-PBS and oncospheres dissociated in 0.25% Trypsin-EDTA (1X) (Life Technologies). Dissociated TICs were grown in normal tissue culture plates containing adherent growth media specific for each cell line.

Clonal expansion and MTT assays

For clonal expansion analysis parental cells, TICs and re-differentiated TICs were harvested and mechanically dissociated using a cell strainer. Dissociated cells were plated into 96-well ultra-low attachment tissue culture plates (Corning, Corning, NY) after dilution to achieve delivery of a single cell/100 μl of modified cancer stem cell media in each well. Wells containing a single cell were marked at Day 0 and followed for clonal expansion by photomicroscopy over a 15 day period. Oncosphere diameters were determined using Image-Pro Plus version 7 software for quantitation and measurements (Media Cybernetics, Bethesda, MD). In some experiments, γ-Secretase Inhibitor XX (GSI; Cat: 565789-1MG, Calbiochem, EDM Millipore Corp., Billerica, MA) or Auranofin (ANF; SantaCruz, Cat: sc-202476) were added to cultures as described in the figure legends. Drugs and media were refreshed every third day. Standard MTT assays were performed to measure cellular viability according to supplier's instructions (Promega, Madison, WI) using a Spectra Max M5 from Molecular Devices (Sunnyvale, CA) for signal detection.

Anchorage-independent growth assays

Anchorage-independent growth was assessed by the ability of cells suspended in agarose to form colonies (SeaPlaque GTG Agarose from Lonza, Rockland, ME). Complete 2X media was mixed with 1.5% agarose at 1:1 ratio to achieve a final agar concentration of 0.75% in growth media plated into 35 mm tissue culture dishes to create bottom layer of soft agar. Single cell suspensions containing 5,000 cells per plate were mixed in soft agar and dispensed over the solidified bottom layer of soft agar. For ANF and Notch inhibition studies, the final concentration of 1μM (ANF) and 5μM (GSI) was used in both layers of soft agar. Plates were incubated at 37^{o}C in 5% CO_2 and colony growth assessed after 4-5 weeks after staining with Giemsa (EMD Millipore, Darmstadt, Germany). Briefly, plates were fixed in methanol for 20 min followed by two 1X-PBS wash. Giemsa stain was diluted (1:20) in 1X-PBS and fixed colonies were stained at room temperature for 1-2 hr. Stained plates were washed with 1X-PBS and plates were imaged using BioSpectrum (UVP, Upland, CA). Colony size and number were determined using Image-Pro Plus version 7 software.

Immunoblot Analysis

Cells were pelleted and washed twice with ice-cold 1X-PBS. For protein expression analysis, total cell lysates were resolved using gradient SDS-PAGE (Novex, Life Technologies, Carlsbad, CA) and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore). Membranes were incubated with the appropriate primary antibodies followed by incubation with HRP-conjugated secondary antibodies purchased from Santa Cruz Biotechology (Santa Cruz, CA). Proteins bands were visualized by chemiluminescence detection (Perkin-Elmer Life Sciences) using KODAK BioMax MR Film (Carestream Health, Rochester, NY). Antibodies were as follows:

Rabbit polyclonal antibodies: βTubulin (1: 3000) GAPDH (1: 2000) and phospho-T410-PKCι (1:1000) from Cell Signaling Technology (Danvers, MA), ELF3 (1:3000) and NOTCH3 (1:3000) from Abcam (Cambridge, MA). Mouse monoclonal antibody: PKCι (1: 2000) from BD Pharmingen (San Jose, CA). Normal rabbit and mouse IgG antibodies were purchased from SCBT.

Immunofluorescence microscopy

For immunofluorescence analysis, cells were plated on 0.5% gelatin-coated coverslips and fixed in cold 4% paraformaldehyde purchased from Electron Microscopy Sciences (Hatfield, PA) for 10 min on ice. Cells were washed with PBS twice and permeabilized in 0.15% Triton X-100 in PBS. For detection of cell surface CD133, the permeabilization step was omitted. Fixed cells were pre-blocked for 30 min using 1X- Bovine Serum Albumin (BSA from Sigma-Aldrich) in PBS and then incubated with primary antibodies diluted in P-BSA at 37°C for 1 hr. ELF3 and ELF3 mutants were localized by staining with ELF3 antibody from Abcam (1:100) and for CD133 using CD133/1 (W6B3C1) (IF, 1: 10) from Miltenyi Biotech (San Diego, CA). After incubation, cells were washed 4 times for 5 min in PBS containing 0.5% BSA (PBS/BSA) on ice. Then cells were incubated with secondary Alexa Flour 488 goat anti-rabbit and Alexa Flour 568 goat anti-mouse antibodies from Invitrogen at 37C for 1 hr, followed by four washes of 1X-PBS/BSA on ice. Coverslips were mounted on slides using Prolong Gold anti-fade from Molecular Probes containing 4, 6-diamidino-2-phenylindole (DAPI). Images were captured at 100X using an oil immersion lens. Cells were counted at 40X magnification.

RNA extraction and QPCR mRNA expression analysis

To determine mRNA expression cells or tissue were processed using RNAqueous phenol-free total RNA isolation protocols (Ambion, Life Technologies, Grand Island, NY). Extracted RNA was subjected to DNase treatment to eliminate DNA contamination according to provider's instructions using TURBO DNA-free™ (Ambion, Life Technologies). DNase-treated RNA was quantified and processed for synthesis of cDNA using reagents (DNTPs, RT-Buffer and RT-Enzyme) from Applied Biosystems (AB, Life Technologies). cDNA synthesis reactions were performed using thermocycler (Biometra, Goettingen, Germany). Gene expression was assessed using TaqMan Fast Universal PCR Master Mix or Fast SYBR Green Master Mix from Applied Biosystems (AB, Life Technologies). QPCR amplification analysis was performed using Applied Biosystems ViiA7 thermal cycler (Foster City, CA). QPCR reagents were purchased from Applied Biosystems (Foster City, CA) or custom designed from Invitrogen. Relative mRNA expression values were determined by using GAPDH, 28S and β-Actin as internal control. See Table for QPCR Primers used.

Mouse orthotopic and ectopic injections

Firefly luciferase-labelled A549 and H358 transfectants (PKCι, ELF3 and NOTCH3 KD) oncospheres or parental cells were suspended in 10% Growth Factor Reduced Matrigel Matrix (BD Biosciences, San Jose, California) in PBS and injected orthotopically into the left lungs (final volume 50μl) of immune-deficient mice using a 28-gauge needle. To assess tumor initiating potential different cell numbers (10-1,000,000 cells as indicated) oncospheres or parental cells were orthotopically injected into the lung. Tumor growth was monitored by bioluminescence imaging using IVIS (Caliper Life Sciences-Xenogen, Hopkinton, MA), after intraperitoneal injection of luciferin (150 mg/kg) in live mice. Bioluminescent signals were quantified using Living Image software (Caliper Life Sciences-Xenogen). After five weeks, mouse lungs were harvested for gene expression analysis and formalin-fixed for tissue histology, hematoxylin+eosin (H & E) staining was performed as previously described (Regala et al., 2009; Regala et al., 2011). Extreme limiting dilution analysis was performed as described (Hu and Smyth, 2009) using the online analysis tool (http://bioinf.wehi.edu.au/software/elda/). For drug studies, A549 cells (100,000 cells) were suspended in 10% Growth Factor Reduced Matrigel Matrix (BD Biosciences) in serum free media and subcutaneously injected into the flanking of immune-deficient mice using a 28-gauge needle in a final volume of 50 μl. After fifteen days, tumor-bearing mice were randomly divided into four groups receiving sodium chloride (0.9%) as a vehicle control, ANF (9 mg/kg), GSI (200 μg/kg; (Mizugaki et al., 2012)) or combined ANF (9 mg/Kg) and GSI (200 μg/kg) by IP injection daily, 6 days a week for eight weeks. Tumor growth was monitored by caliper measurement and tumor volume was estimated using the formula (π/6 x L x W x H) as described previously (Tomayko and Reynolds, 1989). After eight weeks, mice were harvested and tumors were isolated surgically and final tumor weights were determined. Serial transplantations of TIC-derived tumors were performed as described previously (Justilien et al., 2014). All animal experiments were performed under an approved IACUC protocol (#A10811).

Lentiviral shRNA plasmids and transfections

Cells were transduced with recombinant lentivirus and stable transfectants were selected for puromycin resistance as described previously (Frederick et al., 2008). Efficiency of PKCι, ELF3 and NOTCH3 KD was assessed by measuring the mRNA levels by QPCR and protein expression by immunoblot as described above. To generate ELF3 phosphorylation site mutants, piRES-puro-ELF3 (Addgene) was used as a template. Forward and reverse primers incorporating either a Serine to Alanine or a Serine to Aspartic acid conversion were used for site-directed mutagenesis. Resulting plasmids were sequenced to ensure no PCR-mediated errors were introduced. Primer sequences are provided in Table S1. For the detection of protein levels in EV and KD samples, total cell lysates from were prepared in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 0.1% SDS and 1% sodium deoxycholate). At the time of use, RIPA buffer was supplemented with freshly prepared protease inhibitor mixture (Roche Applied Science), phosphatase inhibitor mixture I and II (Sigma) and subjected to immunoblotting as described previously (Regala et al., 2005).

Co-immunoprecipitation assay and Western Blot Analysis

To assess PKCι and ELF3 interaction in TICs, co-immunoprecipitation assays were performed with slight modifications as previously described (Ali et al., 2012). LADC TICs were harvested in sonication buffer after 1X-PBS wash and subjected to sonification using a Branson Sonifier150 (Fisher Scientific. Pittsburgh, PA). Cell lysates were centrifuged at 4^{o}C to remove debris. Pre-clearing was performed using protein A or G beads (Santa Cruz Biotechnology) for 3 hours. Cell lysates were centrifuged at 4^{o}C to obtain clear supernatant. Lysates were incubated overnight at 4°C with 5 µg of PKCι (N 20) antibody or non-specific rabbit polyclonal IgG (Santa Cruz Biotechnology) with gentle rocking. Immuno-complexes were isolated, washed with cold wash buffer and resolved by SDS/PAGE followed by immunoblot analysis. Antibody dilutions used were: anti-PKC □(1: 2000;BD Pharmingen) and Anti-ELF3 (1:3000, ab133621; Abcam). Secondary HRP-conjugated goat anti-mouse (1:2000) and goat anti-rabbit-IgG (1:20000) were from Santa Cruz Biotechnology. Protein bands were visualized using ECL Primer WB detection reagent (Amersham, GE Healthcare, Buckinghamshire, UK).

Chromatin Immunoprecipitation (ChIP) assays

LADC TICs were harvested by centrifugation and re-suspended in 1% formaldehyde for 5 min at room temperature. The protein-DNA cross-linking reaction was quenched using 250 mM glycine for 5 min. Cells were washed with 1X-PBS and re-suspended in lysis buffer containing 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris·HCl, (pH 8.0), 25 µM MG-132, and 1X Complete protease inhibitor mixture (Roche). After 10 min of incubation on ice, cells were sonicated to acquire ~500 base pairs long DNA fragments as determined by agarose gel electrophoresis and ethidium bromide staining. After sonication, cells were centrifuged at 12,000 x g for 10 min to remove debris, the clarified supernatant was transferred to fresh tubes and one percent of the supernatant was saved as input to compare and analyze antibody pull-down. The remainder of each supernatant was incubated overnight at 4^{o}C with 6 µg of specific or non-specific IgG antibody as indicated in the figures. After addition of A/G-conjugated agarose beads (Santa Cruz), samples were incubated at 4^{o}C on a tube rocker for 2 hrs. Agarose beads were sequentially washed with the following buffers: a) low salt wash [20 mM Tris·Cl, (pH 8.1), 150 mM NaCl, 1% Triton X-100, 2 mM, EDTA, and 1× Complete protease inhibitor mixture (Roche)], b) high salt wash [20 mM Tris·Cl, (pH 8.1), 500 mM NaCl, 1% Triton X-100, 2 mM EDTA], c) LiCl wash [10 mM Tris·Cl, (pH 8.1), 250 mM LiCl, 1% deoxycholate, 1% Nonidet P-40, 1 mM EDTA], and d) T.E wash (twice) [10 mM Tris·Cl, (pH 8.1), 1 mM EDTA]. All the washes were performed at 4^{o}C for 10 min followed by centrifuge at 4000 RPM (5 min) to remove the supernatant. After washing, bound DNA was eluted in 1% SDS, 100 mM NaHCO3. Cross-linking was reversed by incubating at 65°C overnight in elution buffer after addition of 200 mM NaCl. DNA was purified using Phenol:Chloroform:Isoamyl alcohol (25:24:1, V/V from Invitrogen, Carlsbad, CA) and Chloroform: Isoamyl alcohol (24:1, V/V from Sigma, St. Louis, MO) using phase lock gel light (5 PRIME, Gaithersbury, MD). Finally, DNA was ethanol precipitated in the presence of 10 µl glycogen (5 mg/ml) and suspended in T.E buffer. For enrichment analysis, QPCR was performed using primer sets designed to amplify the promoter regions of the human NOTCH3 gene (see Supplemental Table: Primers and reagents used in the experimental procedures below) using SYBR® Green (Life Technologies, Grand Island, NY) dye detection on an Applied Biosystems ViiA7 thermal cycler. The comparative Ct method was used for quantitation, and ChIP enrichment was determined as % input. ChIP primers were designed using the Primer3 program available online (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). For the extraction of promoter sequences of genes related to this study, the UCSC Genome Bioinformatics online program was used (http://www.genome.ucsc.edu). Putative ELF3 binding sites were identified using TF search (http://www.cbrc.jp/research/db/TFSEARCH.html) and BIOBASE (www.gene-regulation.com/pub/programs.html)

NOTCH3 promoter luciferase assays

NOTCH3 promoter sequence was obtained using online UCSC Genome Bioinformatics. NOTCH3 promoter sequence (~1kb) was PCR cloned into pGL4.14 [luc2/Hygro] (Promega, Madison, WI). A forward primer incorporating a KpnI site and a reverse primer incorporating a BglII site were used to generate a PCR product that was ligated into the KpnI/BglII sites of the multicloning site of the receiving vector. The resulting vector was sequenced to ensure no PCR-mediated errors were introduced. Primer sequences are provided in Table S1. Cells were transfected with 10 ng of Renilla and 300 ng of pGL4-NOTCH3 promoter plasmid using lipofectamine in low adherent culture conditions to analyze NOTCH3-Luc expression in the absence and presence of ELF3 and PKCι. 48 hr after transfection cells were lysed and Notch3 luciferase expression was analyzed using the Dual-Luciferase assay system (Promega) following the manufacturer's instructions and a GloMax 96 microplate luminometer with dual injectors (Veritas microplate luminometer, Promega). Promoter activity was calculated as fold change over Renilla. Notch3 luciferase activity was also assessed in the absence (EV) and presence of ELF3 mutants (WT, S68A and S68D); cells were co-transfected with Renilla, NOTCH3-Luc and the indicated ELF3 mutant and analyzed as described above.

In-vitro PKCι kinase assays and mass spectrometry analysis

PKCι in vitro kinase assays were performed to assess PKCι-mediated ELF3 phosphorylation as described previously (Justilien et al., 2011). Purified cell free recombinant full-length human GST-tagged ELF3 (Abnova, Taipei, Taiwan) was incubated in the presence or absence of recombinant, active human PKCι (Millipore, Danvers, MA) in reaction buffer (20 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$, 10 mM $MgCl_2$, and 40 μg/ml phosphatidic acid) supplemented with 200 μM ATP for 30 min at 25°C. Reactions were stopped by addition of 2× Laemmli buffer, and the proteins were resolved by SDS-PAGE. Protein bands were visualized using GelCode Blue Stain Reagent (Thermo scientific, Rockford, IL) and bands corresponding to ELF3 were excised and prepared for mass spectrometry analysis as described previously (Justilien et al., 2014). Briefly, stained bands were treated with 50 mM sodium thioisulfate and 15 mM potassium ferricyanide in water followed by multiple water washes to de-stain the samples. De-stained bands were reduced (50 mM Tris, pH 8.1 and 50 mM tris (2-carboxyethyl)) phosphine for 40 min at 55°C and further alkylated in the dark for 40 min at room temperature using 40 mM iodoacetamide. Protein bands were either Lys-C, trypsin or chymotrypsin digested in situ (0.0002% Zwittergent 3-16, 20 mM Tris, pH 8.1), for 4 hr to overnight, at 37°C, followed by peptide extraction (20 μl of trifluoroacetic acid (2%) and then 60 μl of acetonitrile). The pooled extract samples were concentrated using SpeedVac concentrator (Savant Instruments, Holbrook, NY). For protein identification, samples were treated with formic acid (0.15%) and tri-fluoroacetic acid (0.05%) by nano-flow liquid chromatography electrospray tandem mass spectrometry (nanoLC-ESI-MS/MS) using a ThermoFinnigan LTQ Orbitrap Hybrid Mass Spectrometer (ThermoElectron Bremen, Germany) coupled to an Eksigent nanoLC-two-dimensional HPLC system (Eksigent, Dublin, CA). The peptide digest was back-loaded onto a 250-nl OPTI-PAK trap (Optimize Technologies, Oregon City, OR custom-packed with Michrom Magic C8 solid phase (Michrom Bioresources, Auburn, CA). Chromatography analysis was performed using formic acid (0.2%) in both solvents (A= acetonitrile (2%) water (98%) and B= acetonitrile (80%), isopropyl alcohol (10%), water (10%)), and running a gradient (2%-50%) at 325 nl/min over 1 hr through a Michrom Magic C18 (75 μm × 200 mm) packed tip capillary column. The LTQ Orbitrap mass spectrometer experiment was set to perform a Fourier transform full scan from 375 to 1600 m/z with resolution set at 60,000 (at 400 m/z), followed by linear ion trap MS/MS scans on the top five [M + 2H]2+ or [M + 3H]3+ ions. The MS/MS raw data were converted to DTA files using extract_msn.exe from Bioworks 3.2 and aligned to theoretical fragmentation patterns of tryptic peptide sequences from the SwissProt using both SEQUESTTM (ThermoElectron, San Jose, CA) and MascotTM (Matrix Sciences London, UK) search algorithms. All the searches were conducted for the prediction of phospho- Ser, Thr and Tyr residue modifications. All protein modifications were considered confirmed when individual peptide scores were above 95% percentile for the probability and ranked number one of all the hits for the respective MS/MS spectra. Identification of phosphorylation sites on phosphorylated peptides was manually validated.

Primers and reagents used in the experimental procedures.

A. Real-Time QPCR Probes (Taqman)

Target	ABI Assay ID or Sequence (5' to 3')
PKCι	Forward Primer: cgttcttccgaaatgttgattg
	Reverse Primer: tccccagaaatatttggtttaaagg
	Reporter: ttgctccatcatatcc (Justilien et al., 2014)
MMP10 Hs00233987_m1	
CD133	Hs01009245_m1
NOTCH1	Hs01062014_m1
NOTCH2	Hs01050719_m1
NOTCH3	Hs01128541_m1
NOTCH4	Hs00965889_m1
ELF3	Hs00963881_m1
GAPDH	Hs99999905_m1
18S-rRNA	Hs99999901_s1
JAG1	Hs00164982_m1
JAG2	Hs00171432_m1
DLL1	Hs00194509_m1
DLL2	Hs01085096_m1
DLL4	Hs00184092_m1

B. ChIP RT-QPCR Primers (SYBR)

Target	Sequence (5' to 3')
NOTCH3 Primer A:	Forward: ttgagccagataggctgagg
	Reverse: tgttcacacatgcacacaca
NOTCH3 Primer B:	Forward: catgggcttgcatttgtg
	Reverse: ccccagtacacacccaga

C. RNAi Targeting Sequences

PKCι	KD1= gcctggatacaattaaccatt (Frederick et al., 2008; Justilien and Fields, 2009)
	KD2= cctgaagaacatgccagattt
NOTCH3	KD1= ccaatgccaactgaagaggat
	KD2= gccaataaggacatgcaggat
ELF3	KD= gccatgaggtactactacaaa

D. NOTCH3 Promoter Cloning Primers

Forward: t**GGTACC**aaagctgtgtggggtatgggg

Reverse: a**AGATCT**gaaacccaggccgagcatg

Bold and underlined sequence indicating restriction sites introduced for cloning.

E. ELF3 Mutagenesis Primers

S68(A) mutant:

Forward: ggaacagccccagttctgggcgaagacgcaggttctggactgg

Reverse: ccagtccagaacctgcgtcttcgcccagaactggggctgttcc

S68(D) mutant:

Forward: ggaacagccccagttctgggacaagacgcaggttctggactgg

Reverse: ccagtccagaacctgcgtcttgtcccagaactggggctgttcc

Bold and underlined sequence indicates introduced mutation at Serine 68.

F. ELF3 Sequencing Primers

ELF3 (seq) Forward 1:	ACGTCTCCGGATTCGAATT
ELF3 (seq) Forward 2:	CCCCAGCGATGGTTTTCGTG
ELF3 (seq) Reverse 1:	CTTCCAGCCGCTTGAGTTTT
ELF3 (seq) Reverse 2:	AGCTGGGCATGGAGTTGGTC

Supplemental References

Ali, S. A., Dobson, J. R., Lian, J. B., Stein, J. L., van Wijnen, A. J., Zaidi, S. K., and Stein, G. S. (2012). A RUNX2-HDAC1 co-repressor complex regulates rRNA gene expression by modulating UBF acetylation. J Cell Sci *125*, 2732-2739.

Frederick, L. A., Matthews, J. A., Jamieson, L., Justilien, V., Thompson, E. A., Radisky, D. C., and Fields, A. P. (2008). Matrix metalloproteinase-10 is a critical effector of protein kinase Ciota-Par6alpha-mediated lung cancer. Oncogene *27*, 4841-4853.

Hu, Y., and Smyth, G. K. (2009). ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. J Immunol Methods *347*, 70-78.

Mizugaki, H., Sakakibara-Konishi, J., Ikezawa, Y., Kikuchi, J., Kikuchi, E., Oizumi, S., Dang, T. P., and Nishimura, M. (2012). gamma-Secretase inhibitor enhances antitumour effect of radiation in Notch-expressing lung cancer. Br J Cancer *106*, 1953-1959.

Regala, R. P., Justilien, V., Walsh, M. P., Weems, C., Khoor, A., Murray, N. R., and Fields, A. P. (2011). Matrix metalloproteinase-10 promotes Kras-mediated bronchio-alveolar stem cell expansion and lung cancer formation. PLoS One *6*, e26439.

Tomayko, M. M., and Reynolds, C. P. (1989). Determination of subcutaneous tumor size in athymic (nude) mice. Cancer Chemother Pharmacol *24*, 148-154.