

**Table S1:** Metastatic lesions in nude mice orthotopically injected with DU145 SPRY2 KD and Nsi cells (in prostates) after treatment with NAC.

Orthotopic injections and treatments	Lymph node Mets (P=0.036)	Liver Mets (P=0.049)	Diaphragm Mets (P=0.054)	Animals with Mets (P=0.025)
DUNsi Vehicle Ctrl (n=7)	3	0	2	3
DUCL61 (SPRY2 KD) Vehicle Ctrl (n=7)	0	0	0	0
DUNsi NAC (n=7)	2	0	1	3
DUCL61 (SPRY2 KD) NAC (n=7)	2	4	4	6

**Table S2.** Correlation of BPH (n=35) histoscores from our TMA were analysed by Spearman's Rank Order Correlation using SigmaPlot 11.0 software.

Spearman's Rank Order Correlation	PTEN (nuclear)	P21 (nuclear)	p-AKT
SPRY2	-0.358 P=0.05	-0.432 P=0.01	-0.322 P=0.058
PTEN (nuclear)		0.351 P=0.05	0.353 P=0.05

## **METHODS**

### **Cell lines and MEFs**

Human prostate cancer cell lines, DU145, PC3, LNCaP and CWR-22 were authenticated by LCG standards. RWPE-1, normal human prostate epithelial cell line was obtained from ATCC and grown in K-SFM (Gibco) containing bovine pituitary extract (50 µg/ml) and epidermal growth factor (EGF, 5 ng/ml). For MEFs the stable recombinations for PTEN were achieved by infecting MEFs with pBABE puro and pBABE puro-CRE (kind gift from Prof. Kevin Ryan, The Beatson Institute for Cancer Research) expressing adenoviruses and selecting the stable clones with 3µg/ml puromycin (Sigma-Aldrich). The appropriate genotypes selected for this study were checked by genotyping (Transnetyx Inc.) and western blotting. Cell proliferation assays were carried out using WST-1 reagent (Roche).

### **Animal work**

For xenograft model,  $10^7$  DU145 cells were injected in 100ul of serum free RPMI subcutaneously in CD-1 nude mice. To test the effects of FTY720 on PP2A activity, DU145 subcutaneous tumour bearing mice were injected FTY720 (10 mg/kg/d i.p. injection) for 5 days. The subcutaneous tumours were collected in PP2A activity assay buffer and homogenised using Precellys tissue homogeniser.

For orthotopic model, dorso-lateral prostates of male CD-1 nude mice were injected with  $3 \times 10^6$  cells/25 µl serum free RPMI as previously described (39). The mice were gavaged daily for 5 weeks with NAC (10 mM) as indicated. WT and SPRY2<sup>+/-</sup> mice were treated with 10 mM NAC (pH 7.5) in drinking water ad libitum for 75 days and the NAC water was changed twice weekly. Mice were sacrificed at time-points of 3 months for proliferation studies, with a further cohort aged to 6 and 12 months for tumorigenic studies. The excised prostates were placed in formalin for overnight fixation before paraffin embedding.

## Plasmids and siRNA

A 19 mer sprouty2 target sequence (5'- AACACCAATGAGTACACAGAG -3') from the 5' end of the sprouty2 cDNA previously identified employing RNAi on-line tools (Qiagen) and validated in successful siRNA KD experiments was inserted as a hairpin sequence into pTER+ plasmid (ShSPRY2). Alternatively, a 19mer non-silencing control sequence from Qiagen was also inserted into the pTER+ as a non-silencing shRNA control (shNsi). The shRNA vector pTER+ was made by inserting a modified H1 promoter cassette between the BglII and HindII sites of pcDNA4/TO (Invitrogen) as previously described (van de Wetering et al., 2003). Cells stably expressing shSPRY2 and shNsi were selected using zeocin (300 µg/ml, Invitrogen). SPRY2 expression construct used for stable and transient expression was a kind gift from Graeme Guy (Institute of Molecular and Cell Biology, Singapore). Cells stably expressing SPRY2 were selected using geneticin (500 µg/ml, Gibco). The PTEN siRNA (6251) and p53 siRNA (6562) were obtained from Cell Signaling. The PTEN expression construct (EGFP-PTEN) was generated by cloning *PTEN* cDNA (OriGene) into *EcoRI/SalI*-cut EGFP2 (Clontech) vector. The PTEN expression constructs pSG5L HA NLS PTEN (864), pSG5L HA NES PTEN (863), pSG5L HA NES PTENG129R (867) and pSG5L HA NLS PTENG129R (868) were obtained from Addgene (William Sellers, Dana Farber Cancer Institute, MA). Phosphorylation mutant (S380F and T382/383A) PTEN plasmids were generated from EGFP-PTEN plasmid using site directed mutagenesis kit (Alligent) and primers- 5'- CCTGATCATTATAGATATTTTGACACCACTGACTCTGATCC-3' (for S380F), 5'- CCTGATCATTATAGATATTCTGACGCCACTGACTCTGATCC -3' (for T382A) and 5'-CCTGATCATTATAGATATTCTGACGCCGCTGACTCTGATCC -3' (for T382/383A)

using PTENT382A plasmid as the target). Retroviral-mediated gene transfer was performed using the Phoenix packaging cells (Dr. Gary Nolan, Stanford University). Briefly Phoenix cells were transfected with retroviral plasmid and a plasmid encoding vesicular stomatitis virus glycoprotein (VSV-G), using the calcium-phosphate method. Virus containing media was collected and supplemented with 8 µg/ml polybrene (Sigma). Two rounds of infection were performed on target cells. The siRNA and plasmids were transfected using nucleofector kits from Lonza (1001, 1003 and 1005).

### **Flow cytometry analysis**

A total of  $10^5$  cells were acquired using a flow cytometer (FACScan, Becton Dickinson) and analysed using Cell Quest Pro software. The double thymidine block assay was carried out by incubating the cells in 100 mM thymidine (Sigma) containing medium twice for 12 h each as described before (Harper, 2004). ROS levels were determined using FACS by incubating the cells in PBS containing 10 mM  $H_2$ -DCFDA (Molecular Probes) for 30 minutes at 37°C as described elsewhere (Bensaad et al., 2009). For the cell cycle phase determination, cells were trypsinised, washed and fixed overnight in 70% ethanol at -20°C, rehydrated in PBS and stained for 1 h at 37°C with 10 µg/ml propidium iodide (PI). A total of 10,000 cells were acquired using a flow cytometer (FACScan, Becton Dickinson) and Cell Quest Pro software. For BrdU labelling, cells were pulse-labelled with 10 µM BrdU, ethanol-fixed and treated with 1 ml 2N HCl and 0.5% Triton X100 for 30 minutes at room temperature. After centrifugation, the pellets were resuspended in 0.1 M  $Na_2B_4O_7$  (pH 8.7) followed by washes with PBS containing 0.5% Tween 20 and 1% bovine

serum albumin (BSA). The pellets were resuspended in anti-BrdU antibody coupled with Alexa Fluor® 647 (BD 560209), and incubated for 30 minutes at room temperature. Cells were then washed twice with PBS and labelled for propidium iodide (PI), as described above, before flow cytometry analysis.

### **PP2A activity assay**

PP2A activity was measured using PP2A immunoprecipitation phosphatase assay kit by Millipore (#17-313) according to the manufacturer's protocol. Briefly, cells were scraped in phosphatase extraction buffer (20mM imidazole-HCL, 2mM EDTA, 2mM EGTA, pH 7.0, protease inhibitor cocktail, 1mM benzamidine and 1mM PMSF) and sonicated for 10 sec. Supernatant of 2000xg (5 min) was used for assay. Tissues were lysed directly in phosphatase buffer using Precellys tissue homogeniser and supernatants were used for assay. For the assay, PP2A-C subunit was immunoprecipitated and incubated with phosphopeptide provided. In cells transfected with either PP2A-C siRNA or PP2A-C expressing plamids, PP2A-A antibody (Millipore- #07-250) was used to immunoprecipitate PP2A complex. After appropriate incubations, amount of phosphates released was measured colorimetrically using malachite green phosphate detection system. For GSK3 $\beta$  associated PP2A activity assay, cell we lysed and sonicated as mentioned above and the lysates was used to immunoprecipitate GSK3 $\beta$ . The immunoprecipitates were incubated with phosphopeptide and the PP2A activity was measured.

PP2A activity was also measured using specific substrates- phosphorylated recombinant GSK3 $\beta$  and ERK2. Phosphorylated substrates were generated by carrying out in vitro kinase reaction using active AKT (Millipore #14-276) for GSK3 $\beta$

(Invitrogen #PV3365) and active MEK1 (Millipore #14-429) for ERK2 (Merk-Millipore #454854) as per manufacture's protocol using cold ATP. Phosphorylated end products – p-GSK3 $\beta$  and p-ERK2 were checked using specific phospho-antibodies (Cell Signalling #9323 and # 4370). To assay PP2A activity, PP2A enzyme complex was immunoprecipitated using either PP2A-C antibody (Millipore #17-313) or PP2A-A antibody (Millipore- #07-250). For cells transfected with either PP2A-A siRNA or plasmid, PP2A-C antibody was used. Similarly, for cells transfected with either PP2A-C siRNA or plasmid, PP2A-A antibody was used. The immunoprecipitated complex was incubated with p-GSK3 $\beta$  or p-ERK2 for 10min at 30<sup>0</sup>C shaker water bath. The samples were briefly centrifuged and supernatant was mixed with gel loading dye and process by western blotting using specific p-GSK3 $\beta$  and p-ERK2 antibodies. The blots were then stripped and probed with total GSK3 $\beta$  or ERK2 antibodies for the final read out of phosphatase activity. The relative phosphatase activity can be measured as amount of phospho protein verses total protein.

### **Xenograft and orthotopic tumour model**

For xenograft model, 10<sup>7</sup> DU145 cells were injected in 100ul of serum free medium subcutaneously in CD-1 nude mice. During 2<sup>nd</sup> week after injections mice were randomized and treated with vehicle or FTY720 till the end of 5<sup>th</sup> week. The tumour volume was measured weekly using venire calliper. For orthotopic tumour model, NAC and vehicle treatments were initiated 3 weeks after orthotopic injections. After a period of 8 weeks mice were scarified after BrdU injection. Subcutaneous and prostates tumours were excised, weighted and fixed in formalin for 24h.

## **Immunoblotting and Immunoprecipitation**

Whole cell lysates (WCL) were prepared by lysing cells in lysis buffer (50 mM Tris pH 7.6, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 1 mM sodium ortho-vanadate, 5 mM sodium fluoride and protease inhibitor cocktail, Calbiochem). Cell fractionation was carried out using kits for nuclear, cytoplasmic and membrane extraction (Pierce) as per manufacturer's guidelines. WCL from prostates were obtained by homogenising prostates in lysis buffer using Precellys tissue homogeniser. Lysates were resolved by SDS/PAGE on 4-12% gradient polyacrylamide gels (Invitrogen) at 200 V for 1 h and transferred electrophoretically onto PVDF membranes (Milipore) at 200mA for 1h. Blots were blocked for 30 min with 5% skimmed milk, rinsed and probed with the respective antibodies (in 5% BSA, 0.1% Tween-20 containing TBS) overnight at 4<sup>0</sup>C. After incubation with HRP conjugated secondary antibody, bands were detected using ECL (GE) detection reagent. Prostate and MEFs were from at least two sets of mice of all genotypes and all the samples were analyzed three times or more for confirmation. For immunoprecipitation, sepharose A beads conjugated to SPRY2, PTEN or PP2A-A antibody were incubated with total cell lysate for 16h at 4<sup>0</sup>C. The unbound material was washed off with lysis buffer and the beads were boiled in sample buffer for 10 minutes. Proteins were analysed by immunoblotting. IHC and immunoblotting was performed with the following antibodies: SPRY2 (Sigma S1444, Abcam ab50317), PTEN (Cell Signaling 9559), p-PTENser/threo (Cell Signaling 9554), p-AKTser473 (Cell Signaling 9271), p-PDK1 (Cell Signaling 3061), Caveolin-1 (Cell Signaling 3238),  $\beta$ -tubulin (Cell Signaling 2128), Lamin B (Santacruz sc-6216), p53 (Cell Signaling 2524, 2527, Vectorlabs CM5), p21 (Santacruz 2946, Cell Signaling 2947, Abcam ab2961), GAPDH (Sigma G9295), Actin (Cell Signaling 4968), GSK3 $\beta$  (Cell



Signaling 9315), p-GSK (Cell Signaling 9327), PP2A-A (Cell Signaling 2290) and PP2A-C (Cell Signaling 2038). Prostate and MEFs were from at least two sets of mice of all genotypes and all the WCL and fractionation samples are representative of at least 3 independent biological replicates. All the western blots were quantified using Image J and the values represent relative immunoreactivity of each protein normalized to respective loading control.

### **Human Tissue Microarray (TMA)**

3 x 0.6mm<sup>2</sup> cores of prostate cancer tissue, as identified by pathologists were removed from representative areas of the FFPE blocks. All samples had been taken from prostate cancer patients at the time of trans-urethral resection of the prostate (TURP). These samples consisted of tumours with Gleason 3 (n=123), 4 (n=49) to 5 (n=37), as determined independently by 2 consultant pathologists (MS and RJB).

### **Immunohistochemistry (IHC) and Immunofluorescence**

All the IHC was carried using DAKO envision HRP-tagged secondary antibodies and DAB chromogen kit. For TMA, the protein expression levels were scored by two independent observers (IA and LBS), blinded to clinical parameters, using a weighted histoscore method (H-score) at magnification ×40. Each cellular location (membranes, cytoplasm, and nuclei) was scored separately. The weighted histoscore method assesses the staining intensity and the percentage of cells stained with that intensity for the full slide. It is calculated by (1 × % cells staining weakly positive) + (2 × % cells staining moderately positive) + (3 × % cells staining strongly positive). This provides a semi-quantitative classification of staining

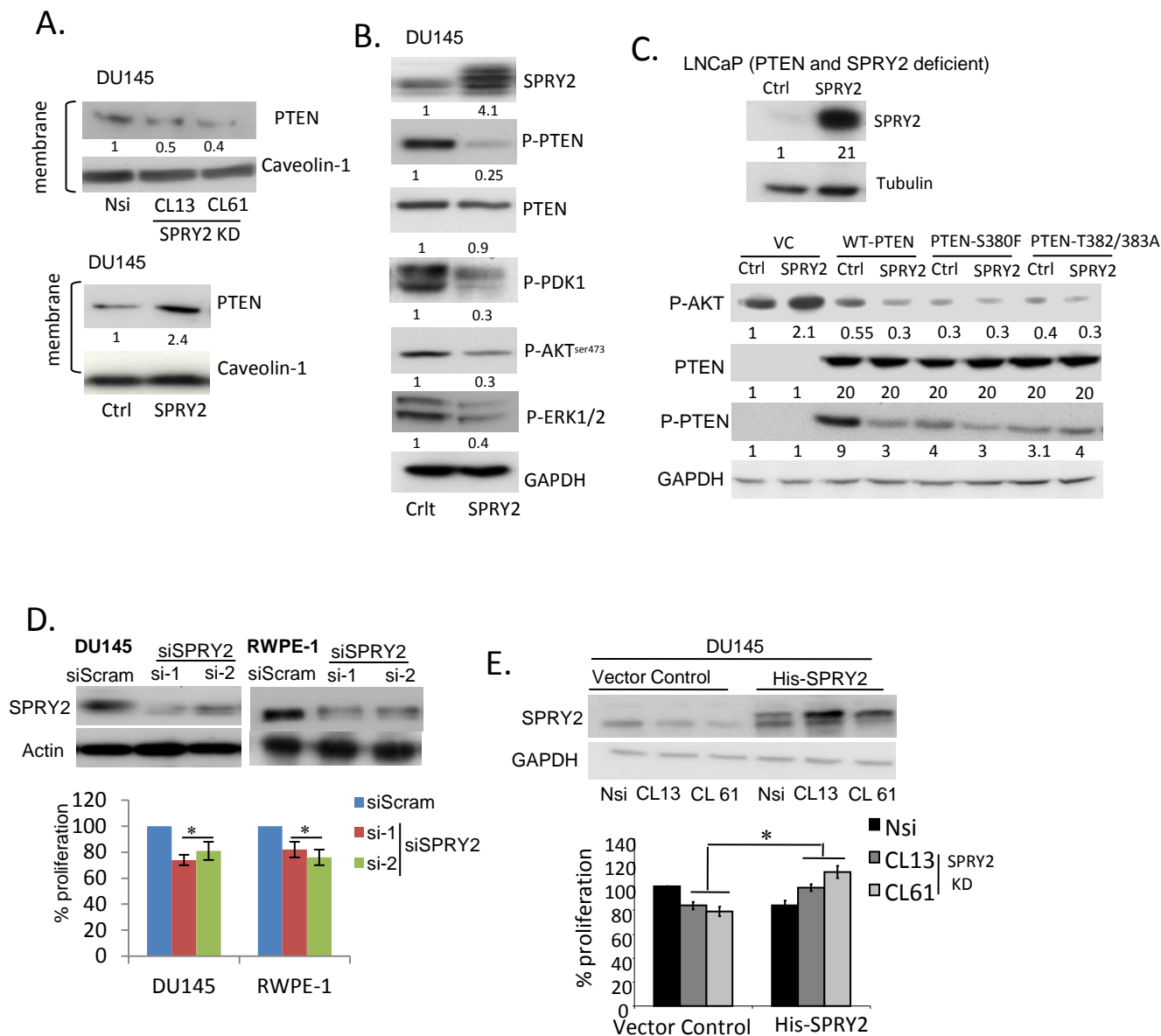
intensity, with the maximum score being 300 (if 100% of cells stained strongly positive) and minimum score being 0 (if 100% of cells are negative). For IHC on murine tissues, each genotype or treatment we stained at least 3 samples from different mice and took representative images for this manuscript. Antibodies against Ki-67 (VectorLabs VP-RM04, 1:100), BrdU (BD 347580, 1:500), PTEN (Cell Signalling 9559, 1:100), SPRY2 (Sigma S1444, 1:50), p-AKT (Cell Signalling 9277, 1:50) and p-ERK (Cell signalling 5683, 1:50) were used for IHC. For antigen retrieval, either pH 6 citrate or pH 7 Tris-EDTA buffers was used. For quantification of Ki67, BrdU, nuclear PTEN, p21 and p53 staining at least 5 different sections were counted for positively stained nuclei at 200X magnification on a light microscope.

For immunofluorescence, the cells grown on glass bottom microplates (Mat Tek) were fixed in 4% PFA for 15 m followed by 10 m incubation in 100% chilled methanol in -20°C. The primary antibody incubation was carried out at 4°C over night after blocking for 1 h in PBS with 1% FBS and 0.5% BSA. For IF of tissues, FFPE samples were deparaffinised and hydrated followed by the primary antibody incubation at 4°C over night after blocking for 1 h in NTB buffer (5% normal goat serum, 1% BSA, 0.1% Triton X-100). Fluorophores, Alexa-488 and 555 tagged secondary antibodies were purchases from Invitrogen. DAPI (Vetashield) was used for nuclear counter staining.

REFERENCES:

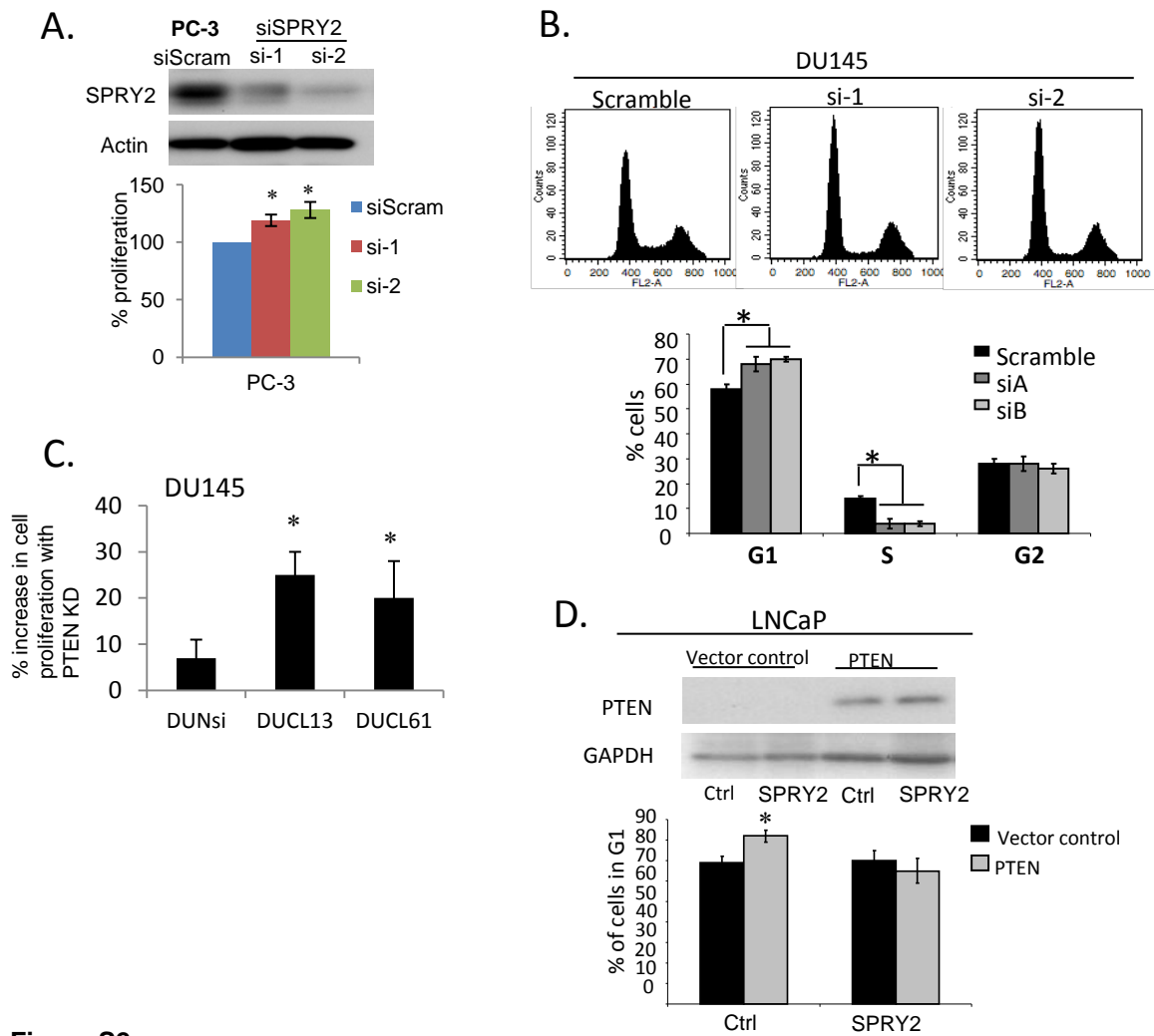
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**Figure S1.**

(A) Membrane fractions of SPRY2 KD and SPRY2 over-expression DU145 cells were analysed by Western blotting (WB).  
 (B) Whole cell lysates (WCL) of stable SPRY2 over-expressing DU145 clones were analysed by WB.  
 (C) Total cell lysates of vector control (VC) and SPRY2 expressing LNCaP cells transfected with plasmids expressing WT-PTEN and phosphorylation mutant PTEN (PTEN-S380F and PTEN-T382/383A) were analysed by western blotting 12 hours post transfection.  
 (D) DU145 and RWPE-1 cells were transfected with scrambled (control, siScram) siRNA or two different siRNAs against SPRY2 (si-1 and si-2). WCL were analysed by western blotting and cell proliferation was measured by WST-1 assay (\* $P < 0.01$ ;  $n = 3$ , analysed by Mann-Whitney test). As compared to respective controls, SPRY2 KD in PTEN proficient RWPE-1 and DU145 cells showed decreased cell proliferation.  
 (E) His tagged WT SPRY2 transfected Nsi control and SPRY2 KD DU145 cells were analysed by western blotting and WST-1 assay (\* $P < 0.01$ ;  $n = 3$ , analysed by Mann-Whitney test).  
 All the western blots were quantified using Image J and the values represent relative immunoreactivity of each protein normalized to respective loading control.



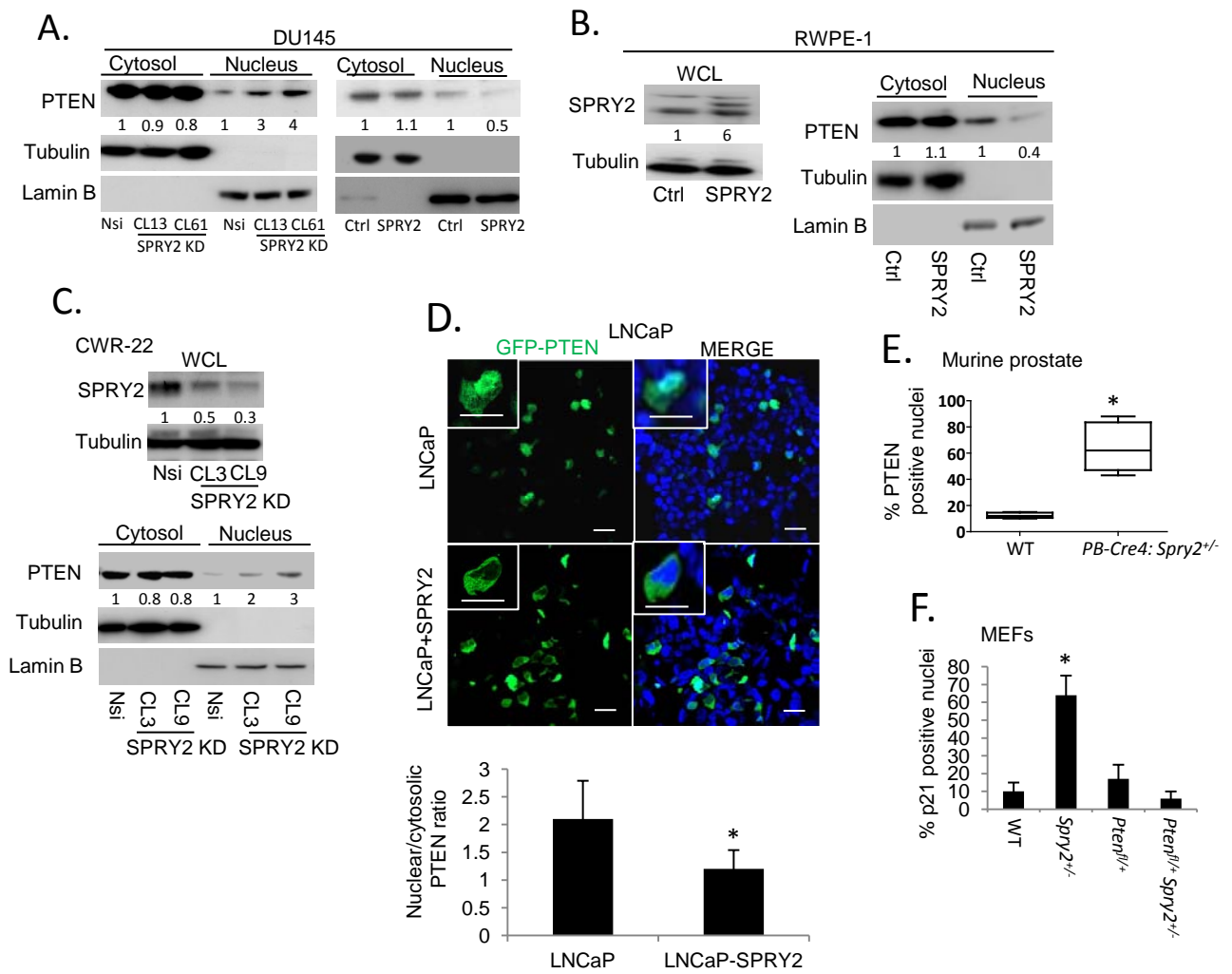
**Figure S2.**

(A) PTEN deficient PC-3 cells were transfected with scrambled (control, siScram) siRNA or two different siRNAs against SPRY2 (si-1 and si-2). WCL were analysed by western blotting and cell proliferation was measured by WST-1 assay (\* $P < 0.01$ ;  $n = 3$ , analysed by Mann-Whitney test).

(B) DU145 cells transfected with scrambled (negative control) or two different siRNA sequences directed against SPRY2 (si-1 and si-2) were analysed after 48 h for cell cycle profile. Data are presented as the mean  $\pm$  SD (\* $P < 0.01$ ;  $n = 3$ , analysed by Mann-Whitney test).

(C) SPRY2 KD clones of DU145 cells were transfected with PTEN siRNA were analysed by WST-1 assay (\* $P < 0.001$ ;  $n = 3$ , analysed by Mann-Whitney test).

(D) SPRY2-LNCaP cells transfected with WT-PTEN were analysed by western blotting and cells in G1 were quantified.



**Figure S3.**

(A) Cytosolic and nuclear fractions of DU145 SPRY2 KD and over-expressing cells with respective vector controls were analysed for western blotting.

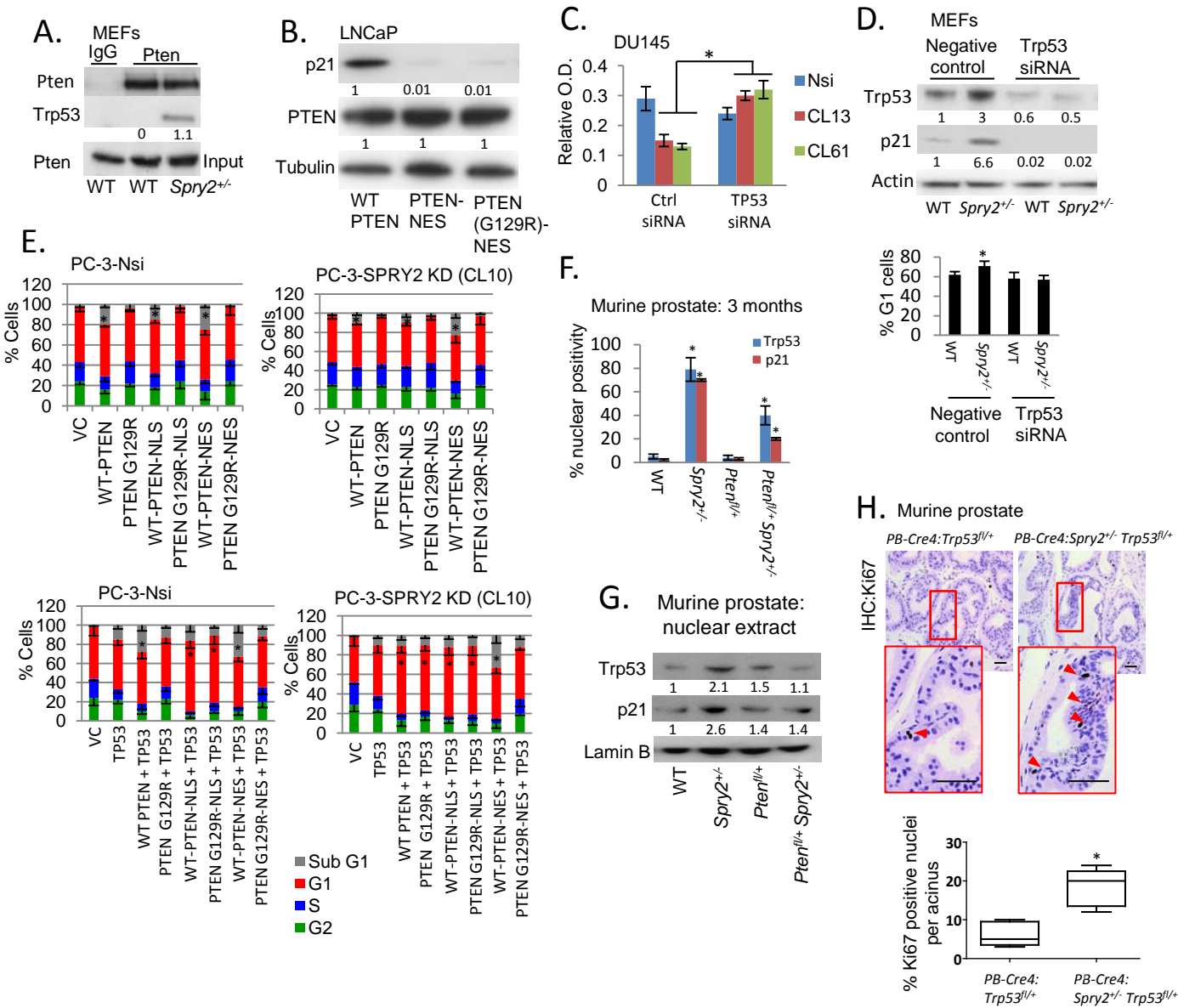
(B-C) Western blot analysis of whole cell lysates, cytosolic and nuclear fractions from (B) PTEN proficient RWPE-1 cells with stable over-expression of SPRY2 and (C) PTEN proficient CWR-22 cells with stable shRNA KD of SPRY2.

(D) Representative confocal images of LNCaP and SPRY2-LNCaP cells following transfection with GFP-PTEN. Nuclear and cytoplasmic fluorescence was analysed using ImageJ. Scale bars: 50µm. (\*P<0.01; number of cells analysed per treatment = 20, analysed by Mann-Whitney test).

(E) Quantitation of Pten positive nuclei as mentioned in Figure 3C. (\*P<0.01; n= 5, analysed by Mann-Whitney test). Box and whisker plots show median (lines within boxes), interquartile range (bounds of boxes), and upper and lower range (whiskers).

(F) Quantitation of p21 positive nuclei as mentioned in Figure 3D (\*P<0.01; n= 3, analysed by Mann-Whitney test).

All the western blots were quantified using Image J and the values represent relative immunoreactivity of each protein normalized to respective loading control.



**Figure S4.**

(A) WB analysis of Pten immunoprecipitated (IP) from MEFs.

(B) Western blot analysis for LNCaP cells transfected with indicated plasmids.

(C) SPRY2 stable KD clones and Nsi control DU145 were transfected with control and siRNA targeting TP53 for 48h and analysed by WST-1 assay for cell proliferation. Data are presented as the mean  $\pm$  SD (\* $P < 0.001$ ;  $n = 3$ , Mann-Whitney test).

(D) MEFs transfected with Trp53 siRNA were analysed by WB quantified for cells in G1. (\* $P < 0.01$ ;  $n = 3$ , Mann-Whitney test).

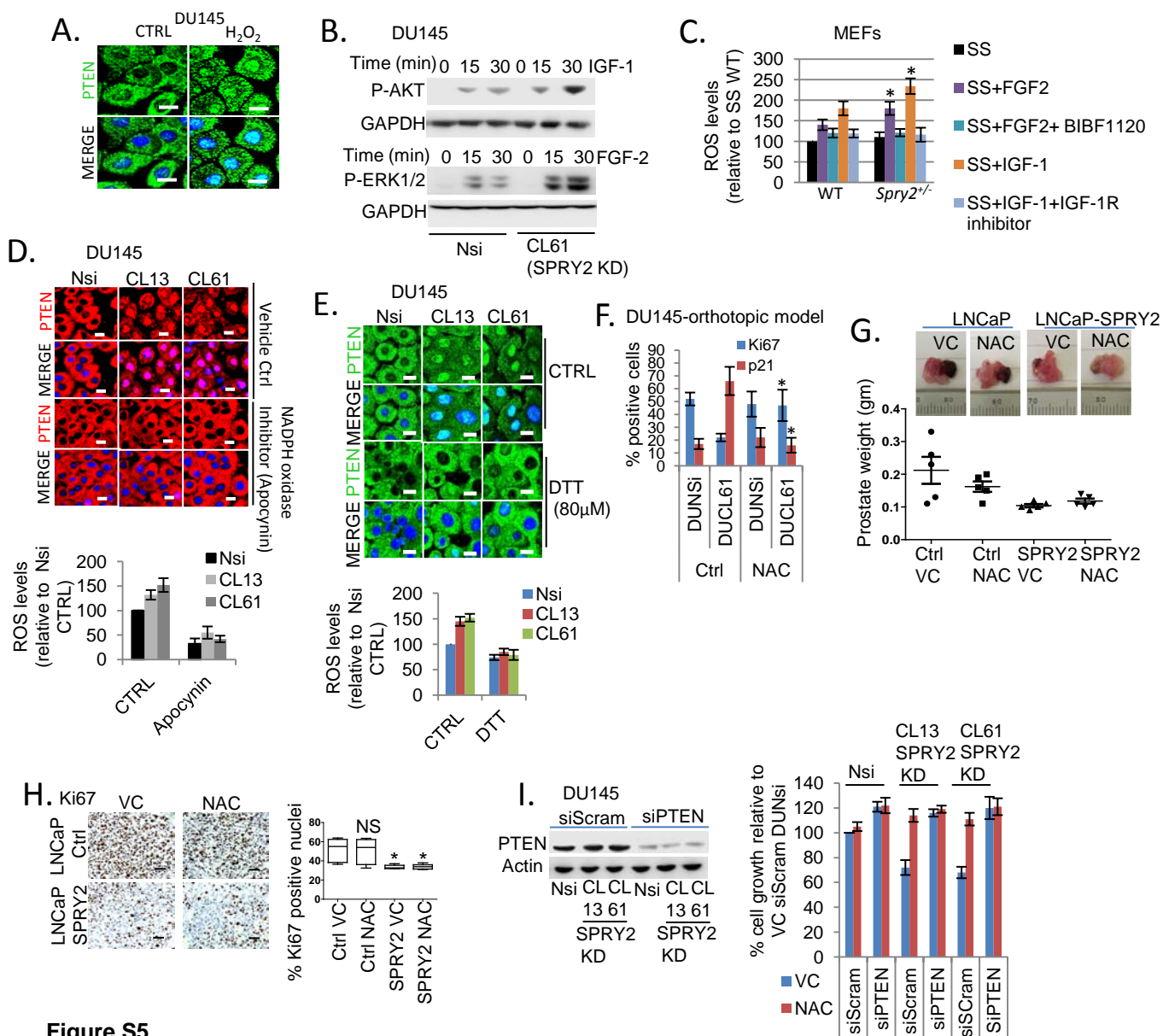
(E) Top panel: SPRY2 KD and Nsi vector control PC-3 cells transfected with indicated PTEN plasmids were analysed for cell cycle profile after 24h (\* $P < 0.05$  relative to vector control,  $n = 3$ ). Bottom panel: As above with additional transfection with WT-TP53. (\* $P < 0.05$  relative to WT-TP53,  $n = 3$ , Mann-Whitney test).

(F) Quantitation of nuclear Trp53 and p21 in prostates of WT, *Spry2*<sup>-/-</sup>, *Pten*<sup>fl/+</sup> and *Pten*<sup>fl/+</sup>*Spry2*<sup>-/-</sup> mice (related to figure 4E; \* $P < 0.01$ ;  $n = 3$ , analysed by Dunnett's Multiple Comparison Test).

(G) WB analysis of nuclear extracts from murine prostates of indicated genotypes.

(H) Representative Ki67 IHC images and quantification in prostates of indicated mice. Scale bars: 50 $\mu$ m. \* $P < 0.01$ ,  $n = 3$ , analysed by Mann-Whitney Test). Box and whisker plots show median (lines within boxes), interquartile range (bounds of boxes), and upper and lower range (whiskers).

All the western blots were quantified using Image J and the values represent relative immunoreactivity of each protein normalized to respective loading control.



**Figure S5.**

(A) Representative IF images stained for PTEN in DU145 treated with 1mM H<sub>2</sub>O<sub>2</sub> for 1 h. Scale bars: 50μm.

(B) Western blot analysis of WCL from SPRY2 KD DU145 cells serum starved (24h) and treated with FGF2 (10ng/ml) or IGF-1 (100ng/ml) for 0, 15 or 30 minutes.

(C) Levels of intracellular ROS in (WT and *Spry2*<sup>-/-</sup>) MEFs were assayed using DCFDA dye. Following 24 h serum starvation, MEFs were treated with growth factors [FGF2 (10ng/ml), IGF-1 (100ng/ml)] alone or with their respective inhibitors [FGFR inhibitor- BIBF1120 (0.4 μM) and IGF-1R inhibitor, PPP (2 nM)] in serum free medium for 30 m. (\*P<0.05, n=3, analysed by Mann-Whitney test). SS- serum starved.

(D-E) Representative IF images stained for PTEN and intracellular ROS levels assayed using DCFDA dye in DU145 treated with (D) NADPH oxidase inhibitor, Apocynin, at 20μM for 16h and (E) ROS quencher, dithiothreitol (DTT) at 80 μM for 16h.

(F) Quantification of Ki67 and p21 in Nsi and SPRY2 KD DU145 orthotopic tumours (related to figure 5E). Data are presented as the mean ± SD (\*P<0.05; n=3, analysed by Mann-Whitney test). Scale bars: 50μm.

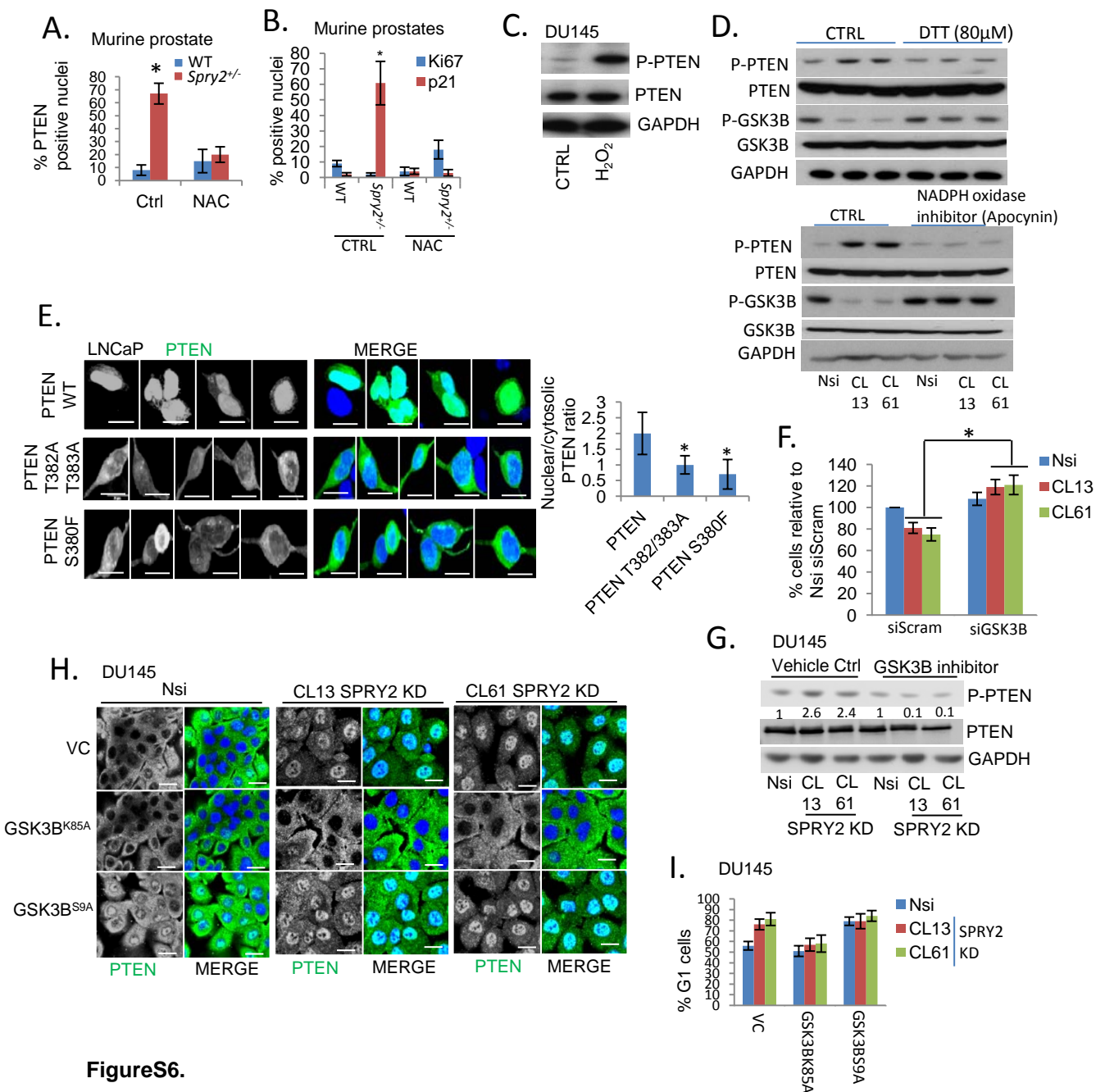
(G) Representative images and prostate weights of nude mice orthotopically injected with LNCaP Ctrl or LNCaP-SPRY2 (stable SPRY2 expressing cells) and treated with vehicle control (VC) or NAC (\*P<0.01). The whisker plots show median (lines) and upper and lower range (whiskers).

(H) Representative images of Ki67 IHC and quantification in orthotopic tumours from nude mice orthotopically injected with LNCaP Ctrl or LNCaP-SPRY2 (stable SPRY2 expressing cells) and treated with vehicle control (VC) or NAC (\*P<0.01, analysed by Dunnett's Multiple Comparison Test). Box and whisker plots show median (lines within boxes), interquartile range (bounds of boxes), and upper and lower range (whiskers). Scale bars: 100μm.

(I) Nsi and SPRY2 KD DU145 cells were treated with sisram (control siRNA) or PTEN siRNA and WCL were analysed by WB. Nsi and SPRY2 KD DU145 clones with transient PTEN KD (or control siScram) were treated with vehicle control (VC) or 5 μM NAC for 48h and analysed for cell growth.

All the western blots were quantified using Image J and the values represent relative immunoreactivity of each protein normalized to respective loading control.





**FigureS6.**

(A-B) Quantification of (A) Pten positive and (B) Ki67 and p21 positive nuclei in prostates of indicated mice treated with 10mM NAC in drinking water ad libitum for 75 days. Data are presented as the mean  $\pm$  SD (\* $P < 0.01$ ;  $n = 3$ , analysed by Mann-Whitney test) (related to figure 6A-B).

(C) WCL from DU145 cells treated with 1mM H<sub>2</sub>O<sub>2</sub> for 1 h were analysed by WB.

(D) WCL from DU145 cells treated with ROS quencher/ anti-oxidant, dithiothreitol (DTT) at 80  $\mu$ M for 16h and NADPH oxidase inhibitor, Apocynin at 20 $\mu$ M for 16h were analysed by WB.

(E) Representative confocal images of SPRY2 null LNCaP cells 12 h post transfection with WT and phosphorylation mutants (S380F and T382/383A) GFP tagged PTEN. Nuclear and cytoplasmic fluorescence was analysed using ImageJ. Data are presented as the mean  $\pm$  SD (\* $P < 0.05$ ; number of cells analysed = 15, analysed by Mann-Whitney test). Scale bars: 50 $\mu$ m.

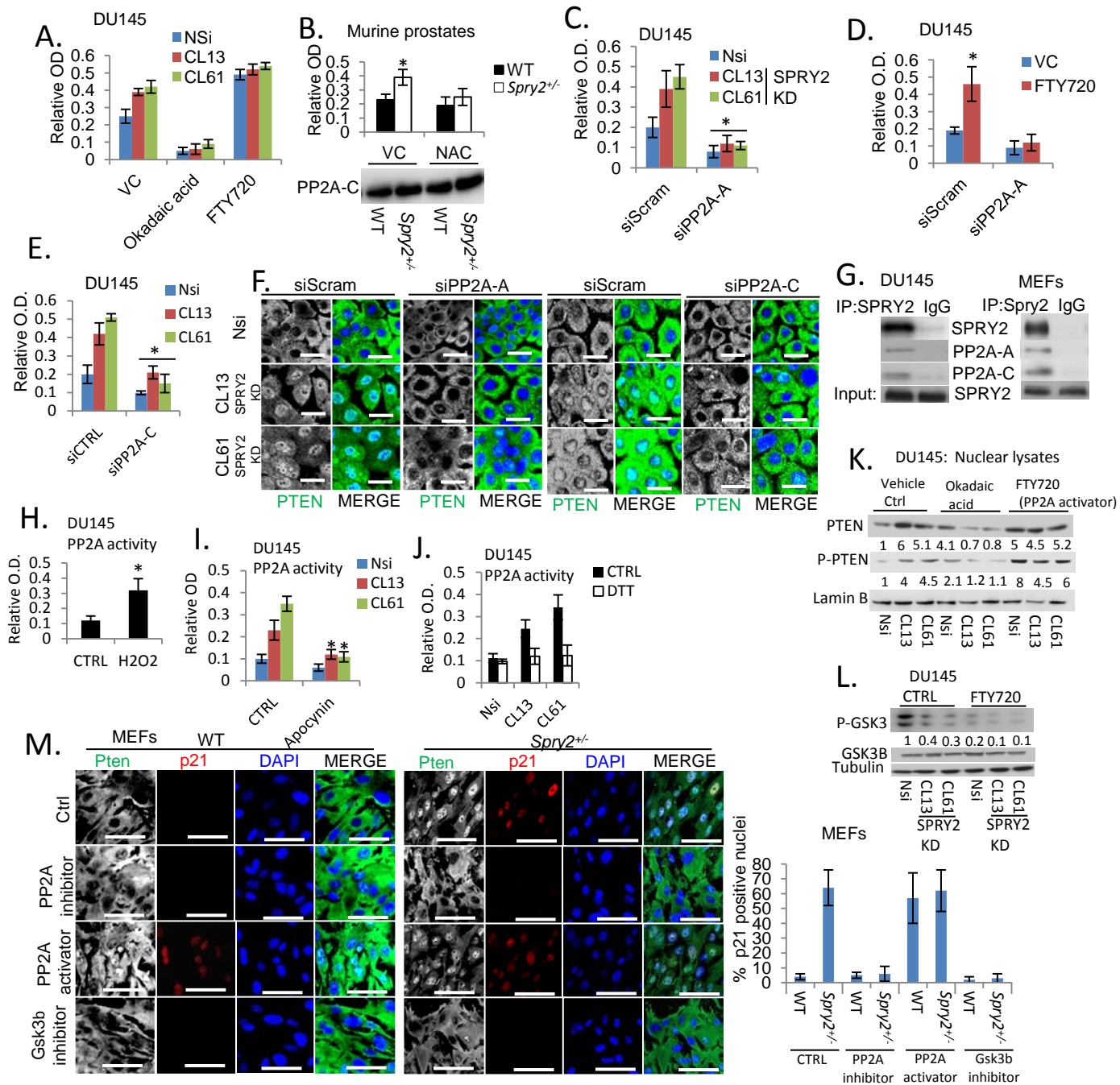
(F) Quantification of cell proliferation assayed by WST-1 assay in DU145 SPRY2 KD cells treated with GSK3B siRNA.

(G) WB analysis of DU145 SPRY2 KD clones treated with GSK3B inhibitor- SB216763 (5 $\mu$ M for 12 h).

(H) Representative IF images stained for PTEN in DU145 SPRY2 KD clones with stable expression of kinase dead (GSK3BK85A) and constitutively active (GSK3BS9A) GSK3B. Scale bars: 50 $\mu$ m.

(I) Quantification of DU145 SPRY2 KD with stable expression of kinase dead (GSK3BK85A) and constitutively active (GSKBS9A) GSK3B cells in G1.

All the western blots were quantified using Image J and the values represent relative immunoreactivity of each protein normalized to respective loading control.



**FigureS7.**

(A) PP2A activity was measured in indicated DU145 cells treated with okadaic acid (1 $\mu$ M) and FTY720 (10 $\mu$ M) for 2h by immunoprecipitating PP2A-C subunit.

(B) PP2A activity was measured in murine prostates as indicated (\* $P$ <0.05, analysed by Mann-Whitney test) and PP2A-C IP was analysed by WB.

(C) PP2A activity was assayed in indicated DU145 cells treated with PP2A-A siRNA by immunoprecipitating PP2A-C (\* $P$ <0.05, analysed by Mann-Whitney test).

(D-E) PP2A activity was assayed in DU145 cells treated with (D) PP2A-siRNA and FTY720 (5 $\mu$ M) for 12h and (E) treated with PP2A-C siRNA by immunoprecipitating PP2A-A (\* $P$ <0.01, analysed by Mann-Whitney test).

(F) Representative IF images stained for PTEN in DU145 SPRY2 KD clones treated with PP2A-A and PP2A-C siRNA. Scale bars: 50 $\mu$ m.

(G) Western blot analysis for SPRY2 immunoprecipitation in DU145 and MEF cells.

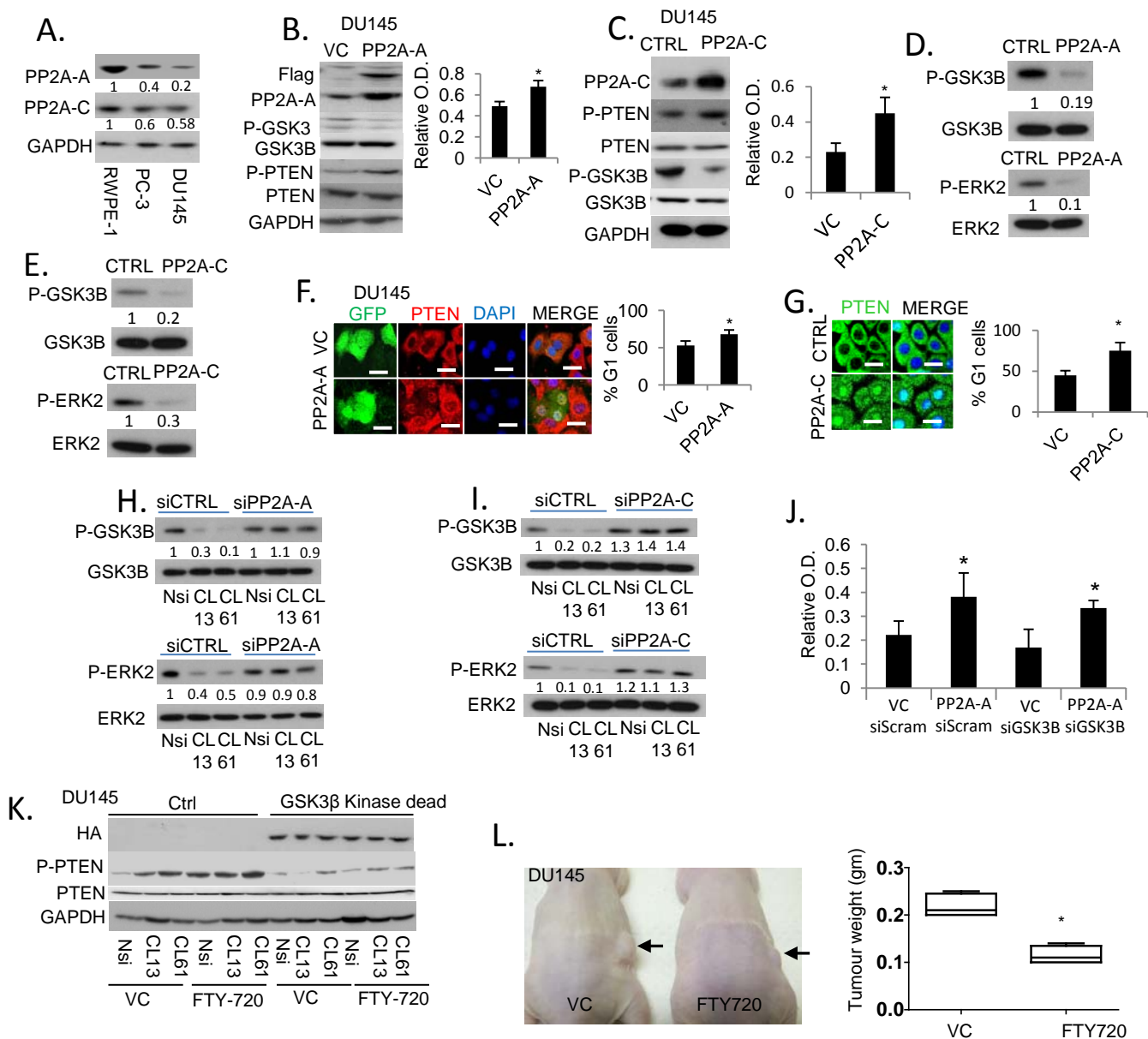
(H-J) PP2A activity was measured in DU145 cells treated with (H)1mM H<sub>2</sub>O<sub>2</sub> for 1h; (I) 20 $\mu$ M of NADPH oxidase inhibitor, Apocynin for 16h and (J) 80 $\mu$ M DTT for 16h.

(K) Western blot analysis of nuclear lysates of DU145 cells treated with okadaic acid (1  $\mu$ M for 2 h) and FTY720 (10  $\mu$ M for 12 h).

(L) Western blot analysis of total lysates of SPRY2 KD DU145 cells treated with FTY720 (10  $\mu$ M for 12 h).

(M) Representative immunofluorescence images and quantification of WT and *Spry2*<sup>+/-</sup> MEFs stained for Pten (green) and p21 (red) treated with okadaic acid (0.5  $\mu$ M for 1 h), FTY720 (10 $\mu$ M for 6h) and SB216763 (5 $\mu$ M for 12h). Data are presented as the mean  $\pm$  SD (\* $P$ <0.05; n=3, analysed by Mann-Whitney test). Scale bars: 50 $\mu$ m.

All the western blots were quantified using Image J and the values represent relative immunoreactivity of each protein normalized to respective loading control.



**Figure S8.**

(A) WCL from RWPE-1 normal non-tumourigenic prostate cells, PC-3 and DU145 prostate cancer cells were analysed by WB. (B-C) PP2A activity and WB analyses in DU145 cells transfected with (B) PP2A-A sub-unit and (C) PP2A-C sub-unit. (\* $P < 0.01$ , analysed by Mann-Whitney test).

(D-E) PP2A activity was measured by in vitro phosphatase assay and analysed by WB using two different PP2A substrates, phosphorylated recombinant GSK3B and p-ERK2, in DU145 cells transfected with (D) PP2A-A where PP2A-C sub-unit was immunoprecipitated or (E) PP2A-C sub-unit where PP2A-A sub-unit was immunoprecipitated for PP2A phosphatase assay.

(F) IF images of PTEN (red) and quantification of cells in G1 of DU145 cells transfected with PP2A-A sub-unit. Both vector control and PP2A-A expressing plasmids expressed GFP from an independent promoter. Scale bars: 50 $\mu$ m. (\* $P < 0.01$ , analysed by Mann-Whitney test).

(G) IF images of PTEN (green) and quantification of cells in G1 of DU145 cells transfected with PP2A-C sub-unit. Scale bars: 50 $\mu$ m. (\* $P < 0.01$ , analysed by Mann-Whitney test).

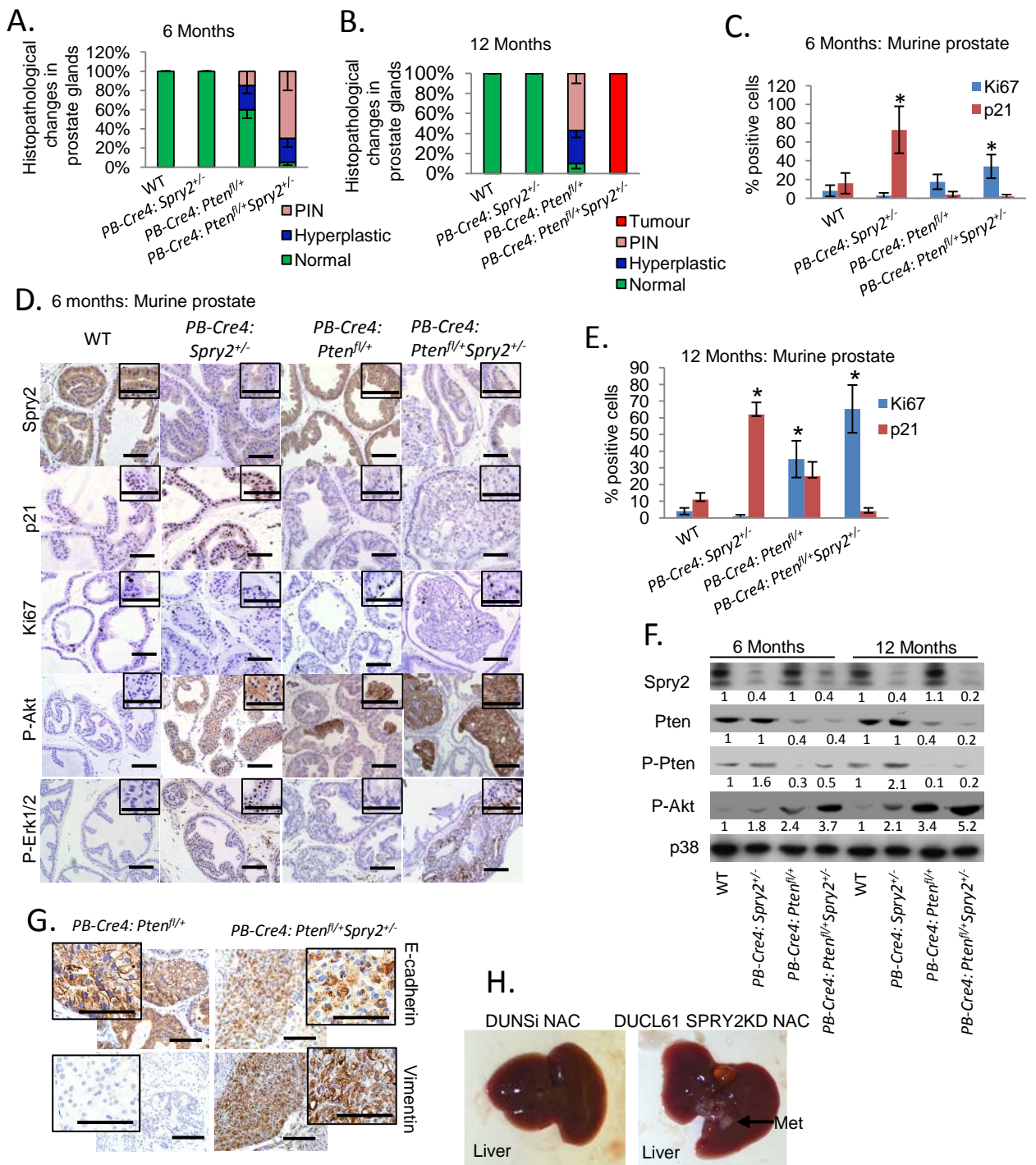
(H-I) PP2A activity was measured by in vitro phosphatase assay and analysed by WB using two different PP2A substrates, phosphorylated recombinant GSK3B and p-ERK2, in DU145 cells transfected with control siRNA and (H) PP2A-A siRNA where PP2A-C sub-unit was immunoprecipitated or (I) PP2A-C siRNA where PP2A-A sub-unit was immunoprecipitated for PP2A phosphatase assay.

(J) PP2A activity assay in DU145 cells transfected with PP2A-A and treated with GSK3B siRNA. (\* $P < 0.01$ , analysed by Mann-Whitney test).

(K) DU145 SPRY2 KD cells with stable expression of Kinase dead GSK3BB (K85A) were treated with 5  $\mu$ M FTY720 for 12 h and analysed by WB.

(L) Subcutaneous DU145 tumour weights and representative images of nude mice treated with PP2A activator FTY720 (10 mg/kg/d i.p. injection) for 5 days (\* $P < 0.01$ , analysed by Mann-Whitney test). Box and whisker plots show median (lines within boxes), interquartile range (bounds of boxes), and upper and lower range (whiskers).





## FigureS9.

(A-B) Quantification of histo-pathological changes in the prostates from mice as indicated (n=6) at 6 and 12 months (A and B respectively, related to Figure 9A).

(C) Quantification of Ki67 and p21 in prostates of 6 month old mice as indicated (n=3, \*P<0.01, analysed by Mann-Whitney test).

(D) Representative IHC images of prostate tissue from 6 month old mice as indicated (n=3). Scale bars: 50µm.

(E) Quantification of Ki67 and p21 in prostates of 12 month old mice as indicated (n=3, \*P<0.05, analysed by Mann-Whitney test).

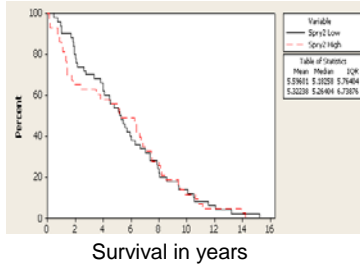
(F) Western blot analysis of total prostate lysates from 6 and 12 month old mice as indicated (n=3).

(G) Representative IHC images for E-cadherin and Vimentin staining in murine prostates of *PB-Cre4: Pten<sup>fl/+</sup>* and tumours from *PB-Cre4: Pten<sup>fl/+</sup>Spry2<sup>+/-</sup>*. PIN lesions in *PB-Cre4: Pten<sup>fl/+</sup>* mice show membrane E-cadherin where as tumours from *PB-Cre4: Pten<sup>fl/+</sup>Spry2<sup>+/-</sup>* show delocalised E-cadherin with associated increase in Vimentin suggesting epithelial to mesenchymal transition (EMT). Scale bars: 50µm.

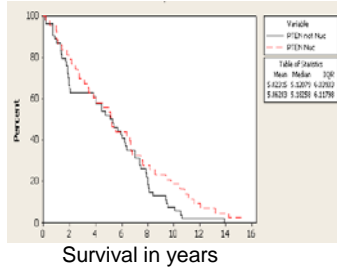
(H) Representative images of livers from NAC treated nude mice orthotopically injected with either DU145 Nsi or SPRY2 KD (CL61) cells. Arrow indicates liver metastasis.

All the western blots were quantified using Image J and the values represent relative immunoreactivity of each protein normalized to respective loading control.

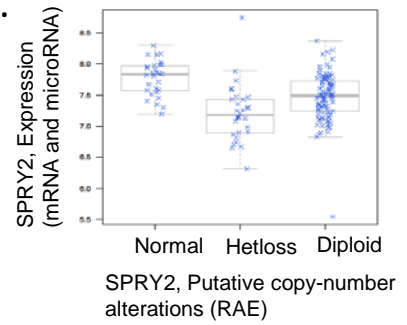
**A.** Survival of low vs high Sprouty2 expression



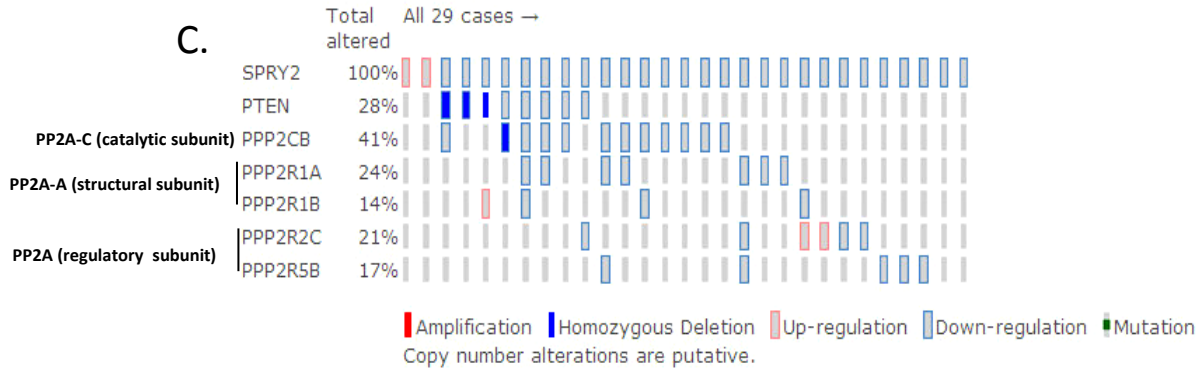
Survival of nuclear PTEN vs non-nuclear PTEN



**B.**



**C.**



**FigureS10.**

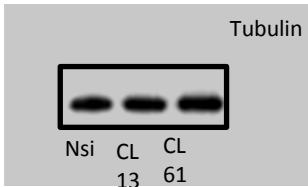
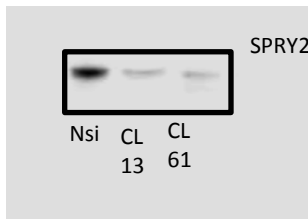
(A) Kaplan Meier survival plot for PC patients stratified according to the levels of nuclear PTEN or SPRY2 expression.

(B) SPRY2 expression analysis in clinical PC dataset obtained from cBio Cancer Genomic Portal. Box and whisker plots show median (lines within boxes), interquartile range (bounds of boxes), upper and lower range (whiskers), and each cross represented a case.

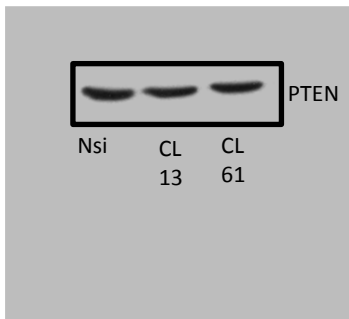
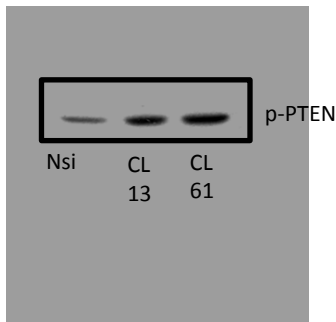
(C) Heat map generated from primary tumours with SPRY2 alterations (29/131 cases) using prostate cancer dataset from cBio genomic portal.

**Uncut gels**

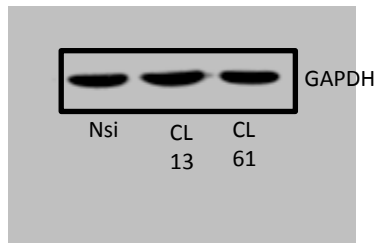
Figure 1A.

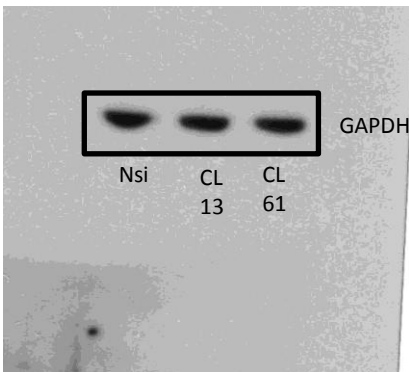
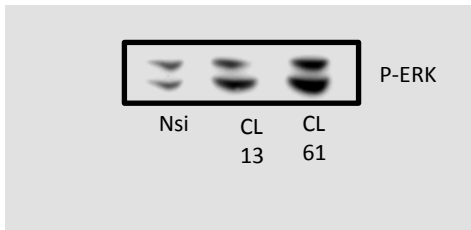
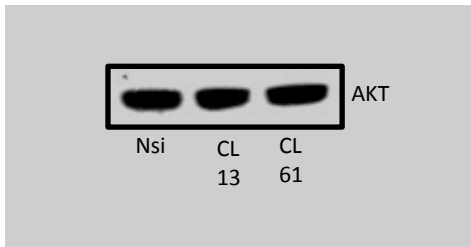
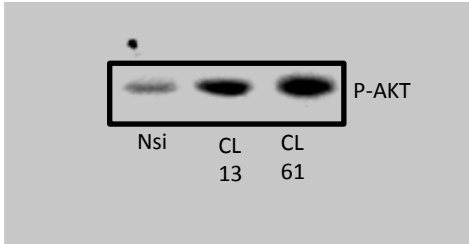
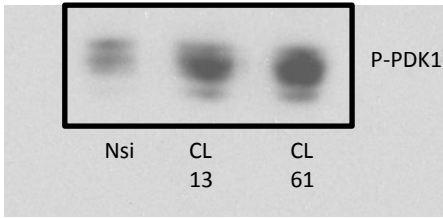


Blot was cut into two. The bottom half was probed for SPRY2 and the upper half was probed for Tubulin (loading control).



Blot was cut into two. The top half was first probed for P-PTEN (anti-rabbit) and then stripped and re-probed for PTEN (anti-mouse). The lower half was probed for GAPDH as loading control.



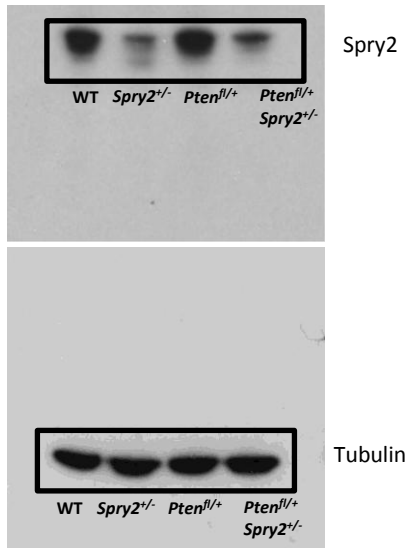


Blot was cut into two. The top half was probed for p-AKT, total AKT and p-PDK1 and the lower half was probed for p-ERK, ERK and GAPDH.

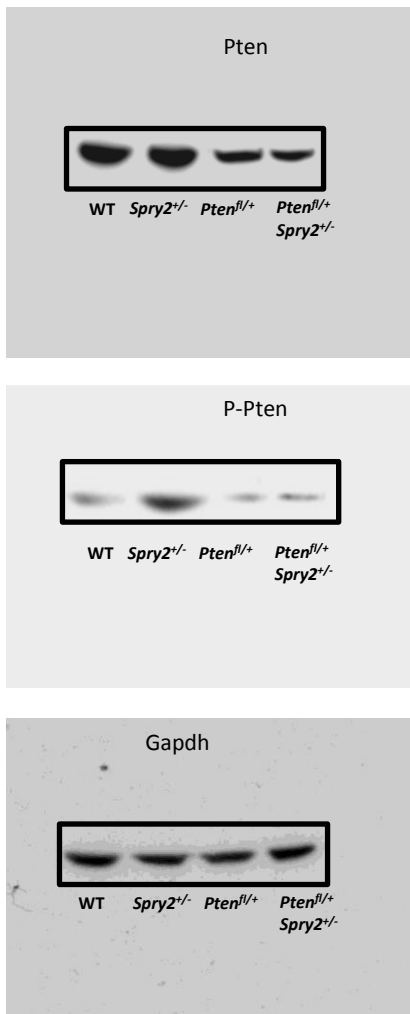
The top half was first probed for p-AKT (anti-mouse) and stripped and re-probed for P-PDK1 (anti-rabbit). The blot was further stripped and re-probed for AKT (anti-goat). The lower half was first probed for p-ERK (anti-rabbit), stripped and re-probed for ERK (anti-mouse) and probed for GAPDH (HRP tagged antibody) as the loading control.



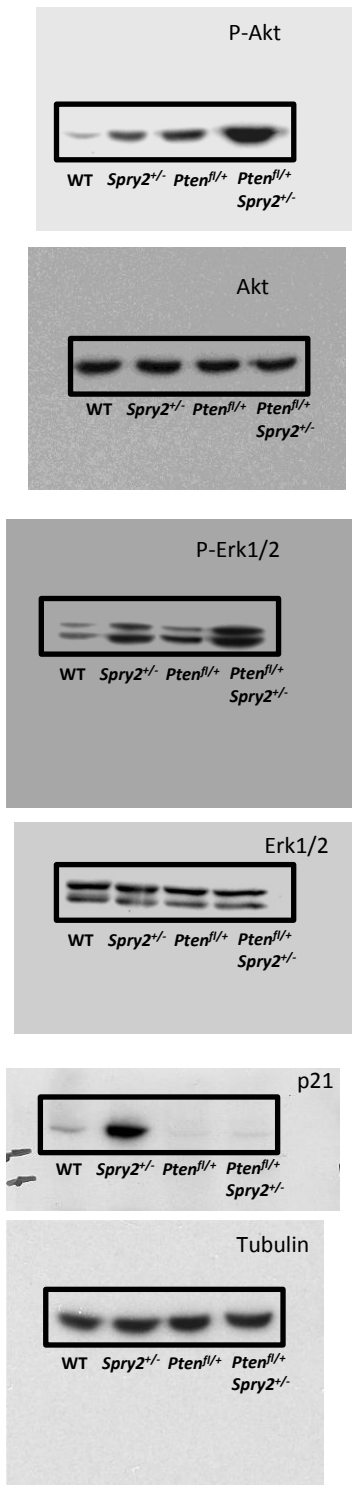
Figure 1B.



Blot was cut into two. The bottom half was probed for Spry2 and the upper half was probed for Tubulin (loading control).



Blot was cut into two. The top half was first probed for P-Pten (anti-rabbit) and then stripped and re-probed for Pten (anti-mouse). The lower half was probed for Gapdh as loading control.

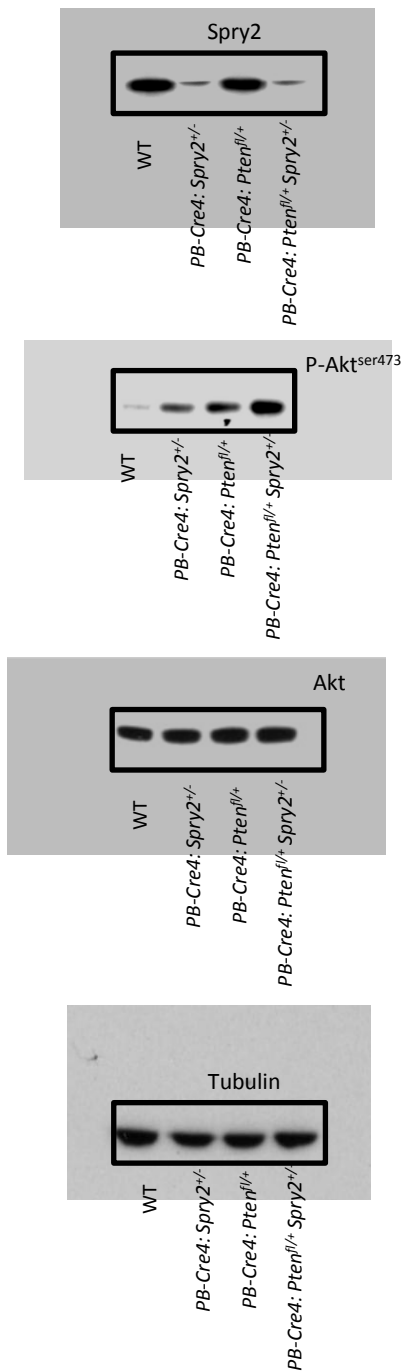


The blot was cut into three. The top part of the blot was probed for p-Akt (anti-rabbit) and then stripped and probed for Akt (anti-goat). This was further stripped and re-probed for Tubulin (anti-mouse) as loading control.

The middle part was first probed for p-Erk1/2 (anti-rabbit) and then stripped and probed for total Erk1/2 (anti-mouse).

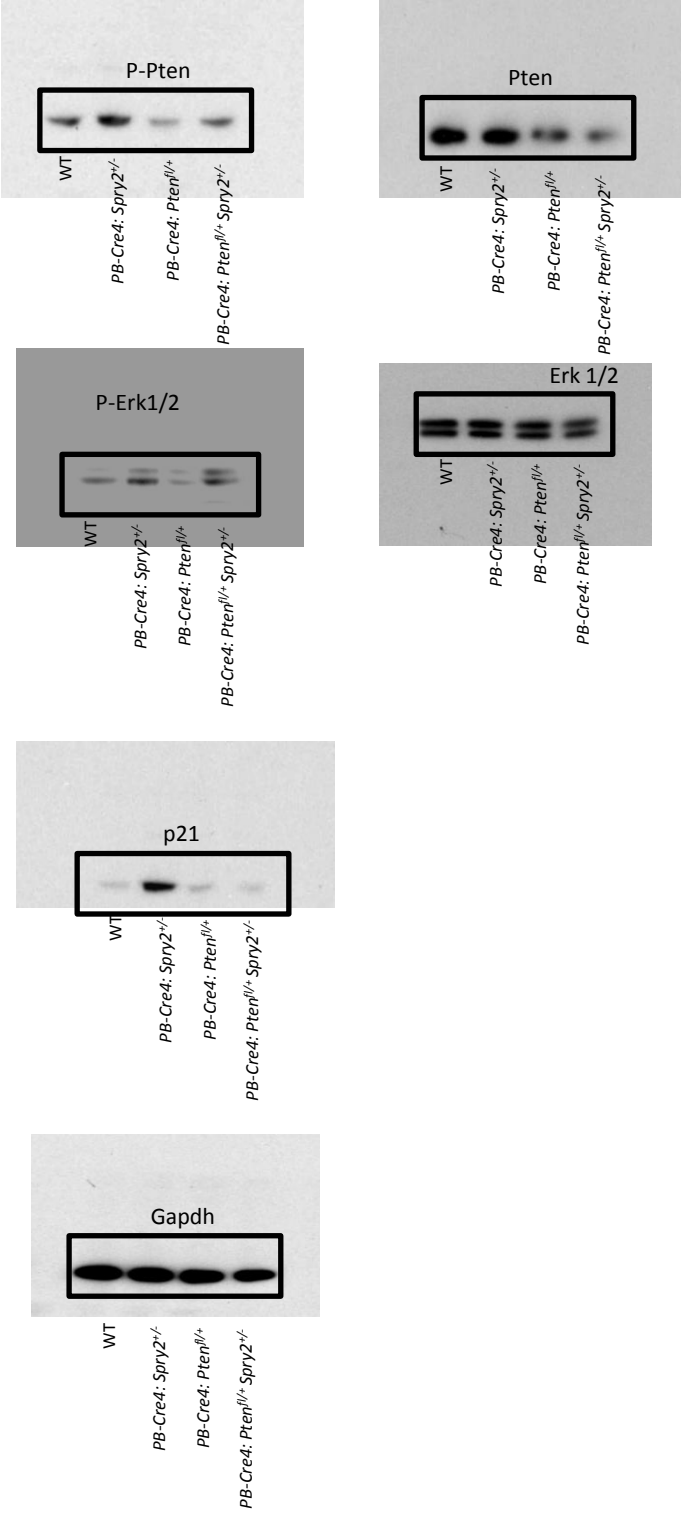
The lower part was probed for p21 (anti-mouse).

Figure 1C.



Blot was cut into two. The bottom half was probed for Spry2 . The upper half was first probed for p-Akt (anti-mouse) and then stripped and re-probed for Tubulin (anti-mouse) as the loading control. The blot was further stripped and re-probed for Akt (anti-goat).

Figure 1C.



Blot was cut into three. The top part of the blot was probed for p-Pten (anti-rabbit) and then stripped and re-probed for Pten (anti-mouse). The middle part was first probed for p-Erk (anti-rabbit), stripped and re-probed for Erk1/2 (anti-mouse) and stripped again and probed for Gapdh (HRP tagged anti-body) as the loading control. The bottom part of the blot was probed for p21.

Figure 2A.

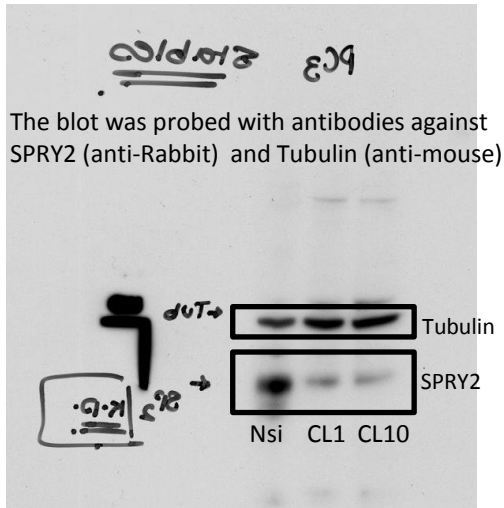


Figure 2B.

The blot was probed with antibodies against SPRY2 (anti-Rabbit) and Tubulin (anti-mouse)

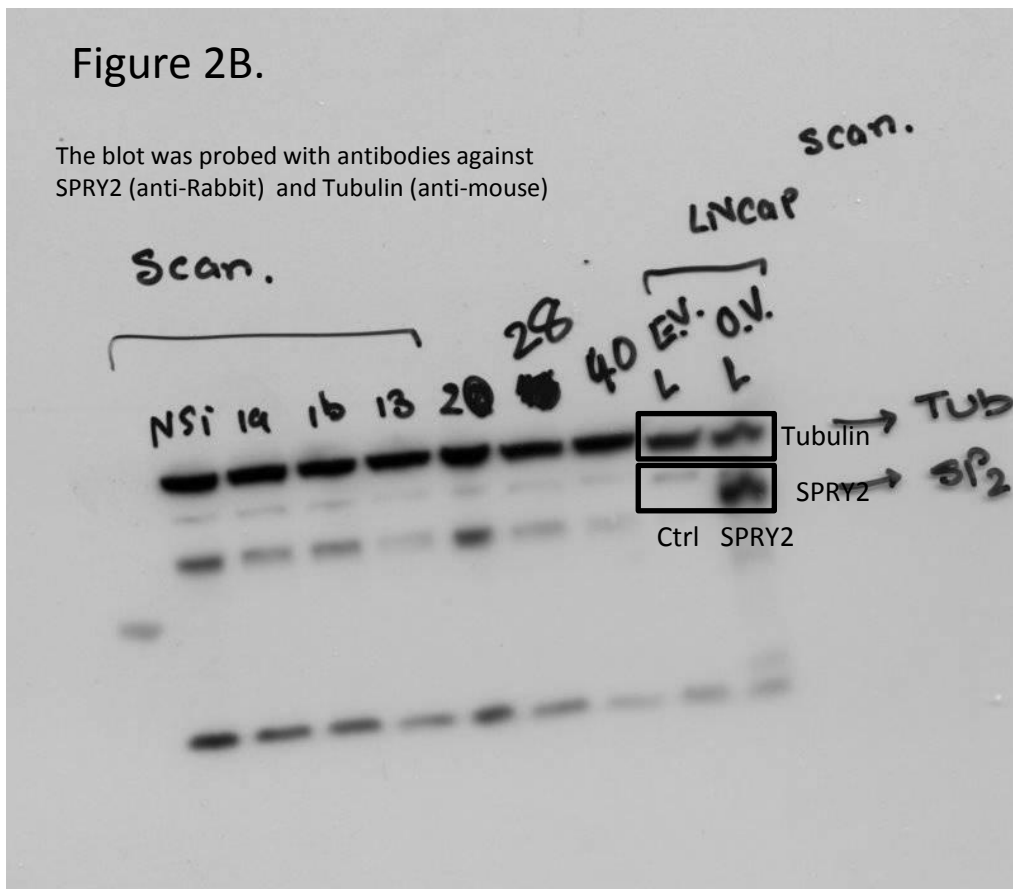
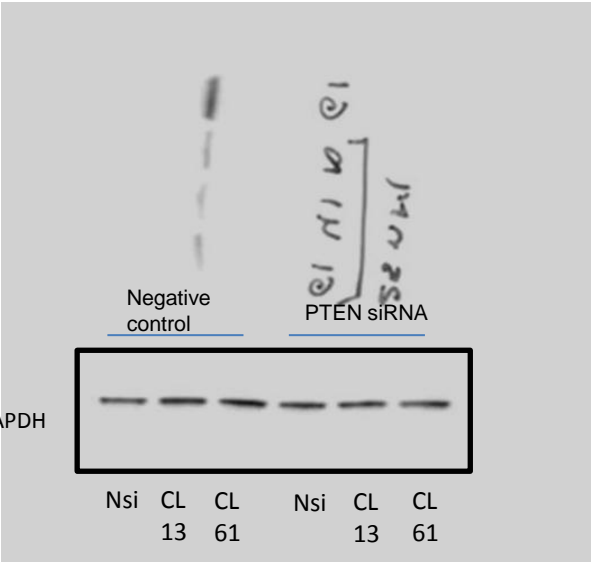
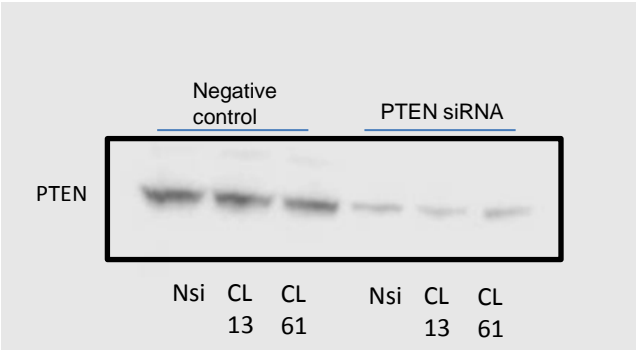
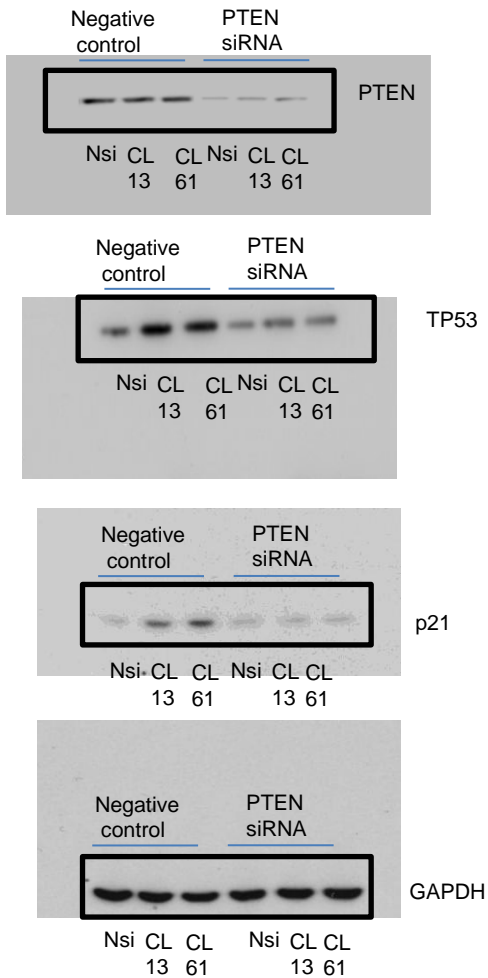


Figure 2F.



Blot was cut into two. The top half was probed for PTEN and lower half was probed for GAPDH as loading control.

Figure 4A.



Blot was cut into three. The top part was probed for PTEN (anti-rabbit) and then stripped and re-probed for TP53 (anti-mouse). The middle part was probed for GAPDH as loading control. The lower part was probed for p21.

Figure 4B.

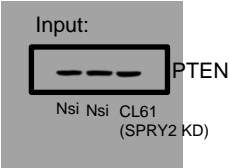
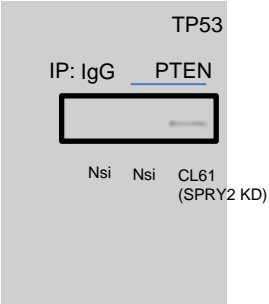
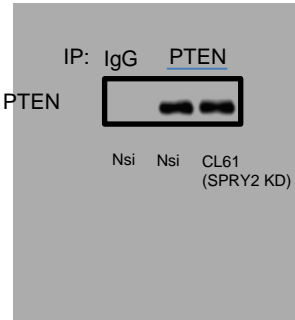
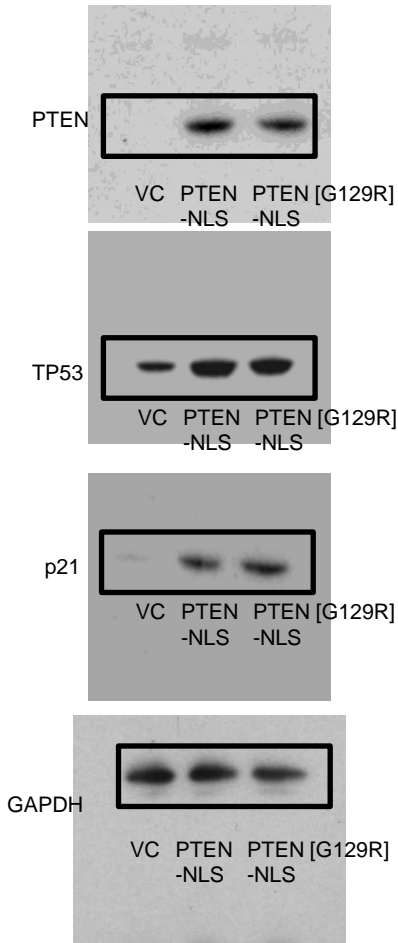


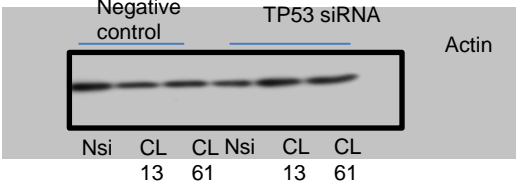
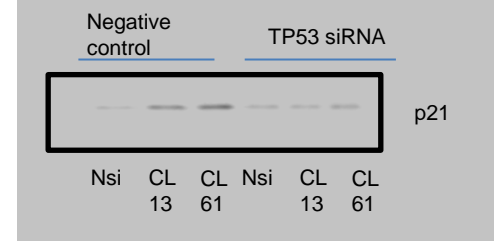
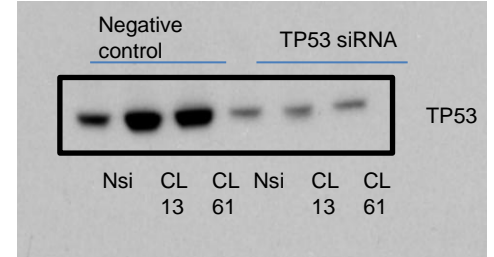


Figure 4C.



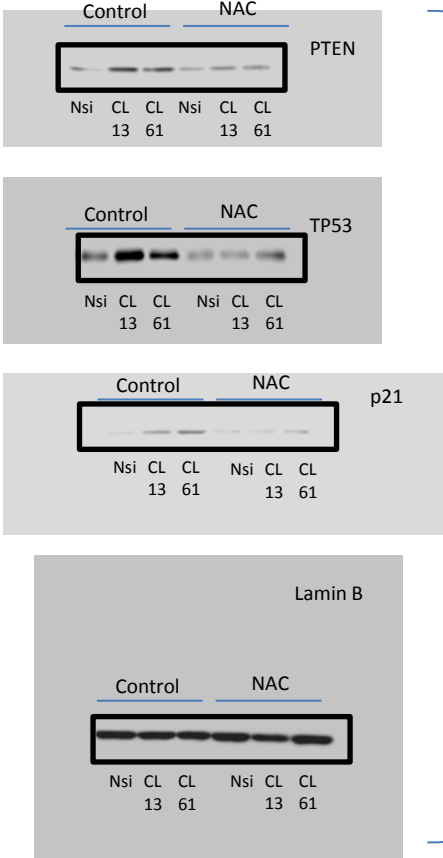
Blot was cut into three. The top part was probed for PTEN (anti-rabbit) and then stripped and re-probed for TP53 (anti-mouse). The middle part was probed for GAPDH as loading control. The lower part was probed for p21.

Figure 4D.



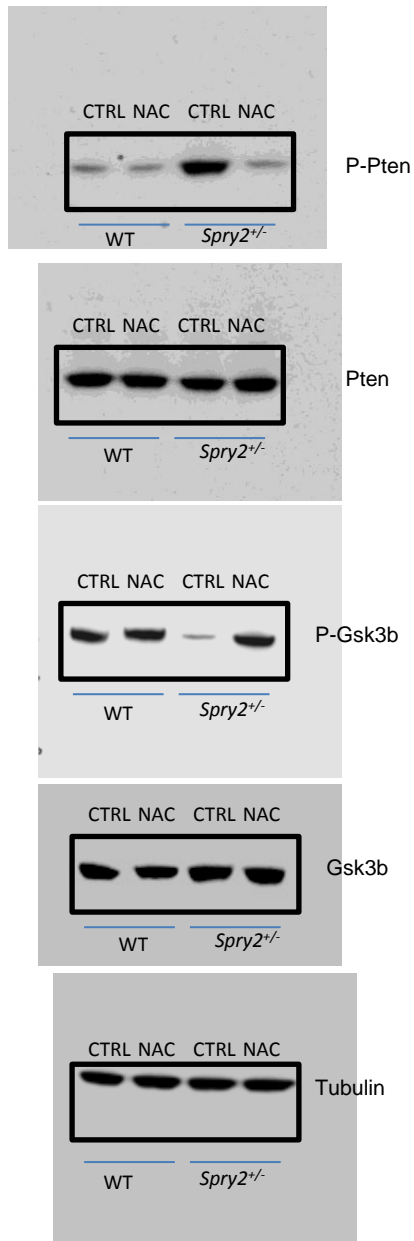
Blot was cut into two. The top half was probed for TP53 (anti-mouse) and then stripped and re-probed for Actin (anti-rabbit). The lower half was probed for p21.

Figure 5D.



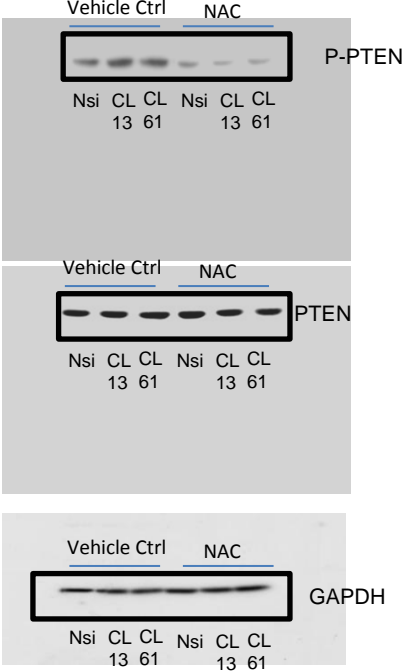
Blot was cut into two. The top half was first probed for PTEN (anti-rabbit) and then stripped and re-probed for TP53 (anti-mouse). This blot was further stripped and re-probed for Lamin B (anti-goat) as loading control. The lower half was probed for p21.

Figure 6C.

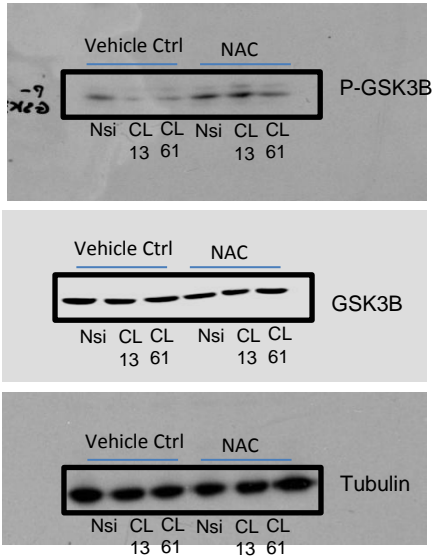


Blot was cut into two. The top half was first probed for p-Pten (anti-rabbit) and then stripped and re-probed for Pten (anti-mouse). This blot was further stripped and re-probed for Tubulin (anti-goat) as loading control. The lower half was probed for p-Gsk3b (anti-rabbit) stripped and re-probed for Gsk3b (anti-mouse).

Figure 6D.

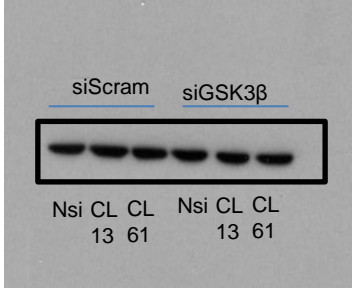
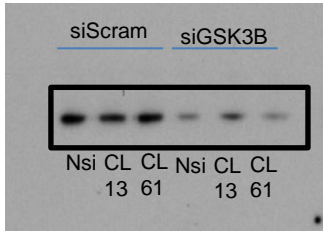


Blot was cut into two. The top half was probed for P-PTEN (anti-rabbit) and stripped and re-probed for PTEN (anti-mouse). The lower half probed for GAPDH as loading control.

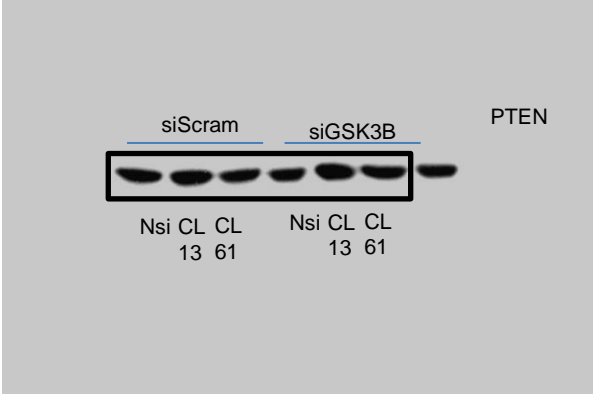
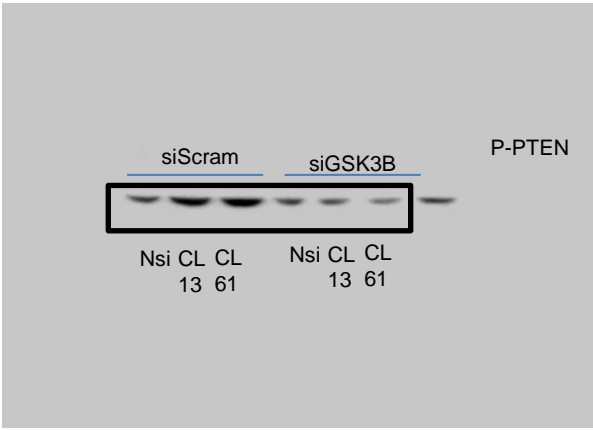


Blot was cut into two. The bottom half was probed for p-GSK3B (anti-rabbit), stripped and re-probed for GSK3B (anti-mouse). The top half was probed for Tubulin as loading control.

Figure 6E.



Blot was cut into two. The bottom half was probed for GSK3B and top half was probed for Tubulin as loading control.



Blot was cut into two. The top half was probed for p-PTEN (anti-rabbit), stripped and re-probed for PTEN (anti-mouse). The lower half was probed for GAPDH as loading control.

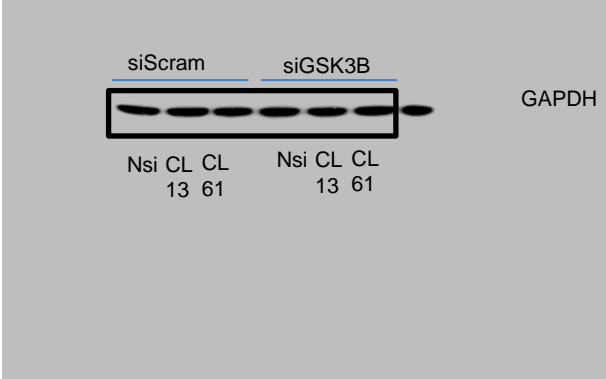


Figure 6G.

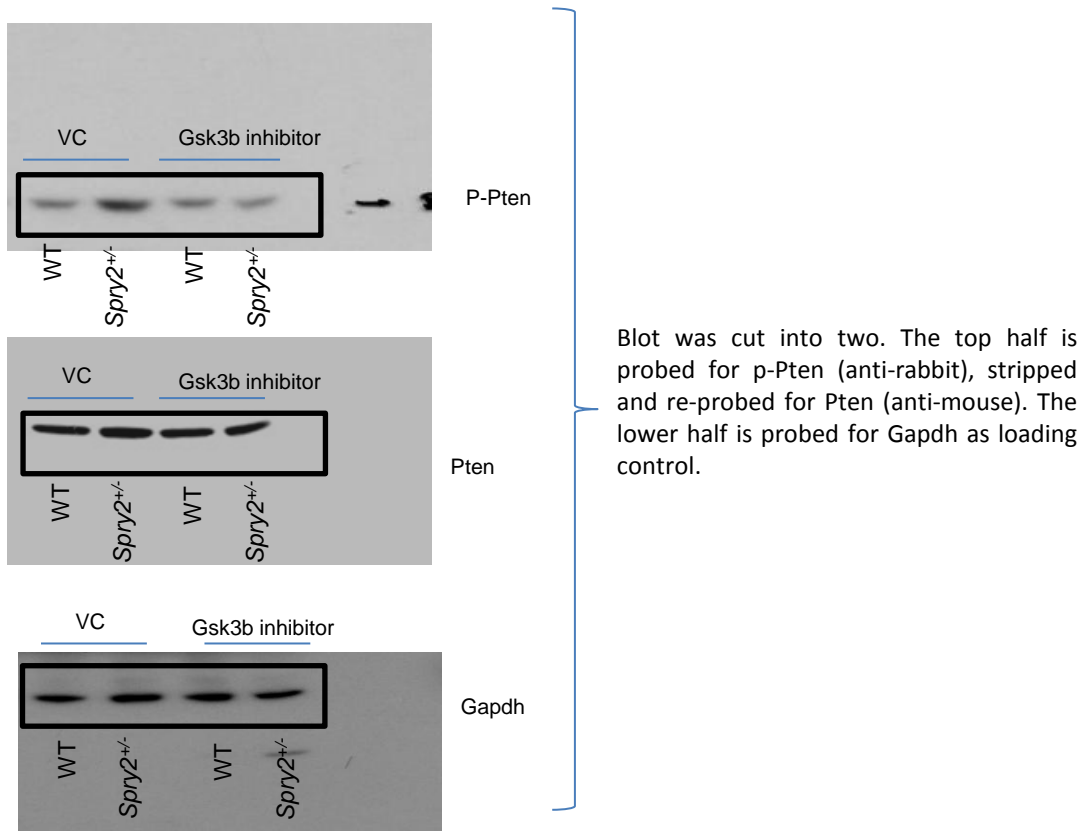
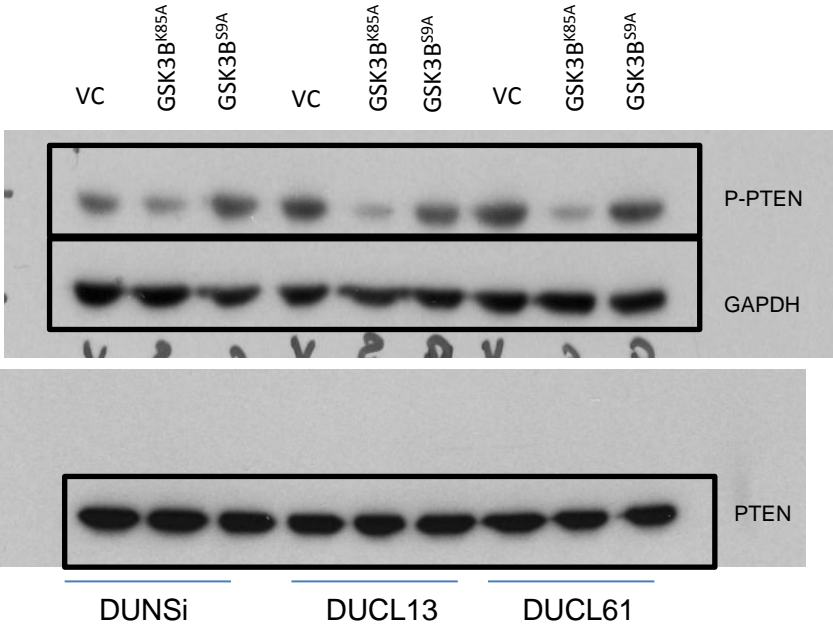
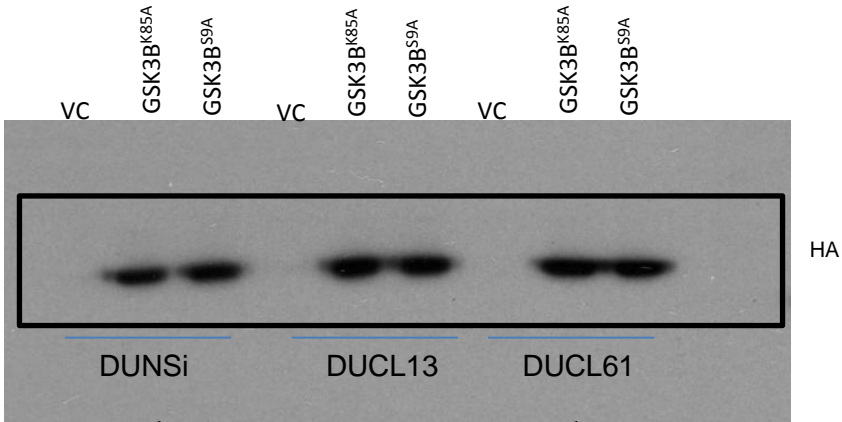


Figure 6H.



The blot was probed for p-P-TEN (anti-rabbit) and GAPDH (HRP tagged antibody) as loading control. Blot was cut and upper part was stripped and re-probed for PTEN (anti-mouse).



The blot was first probed for HA (anti-rabbit) and then was stripped and re-probed for Tubulin (anti-mouse) as loading control.

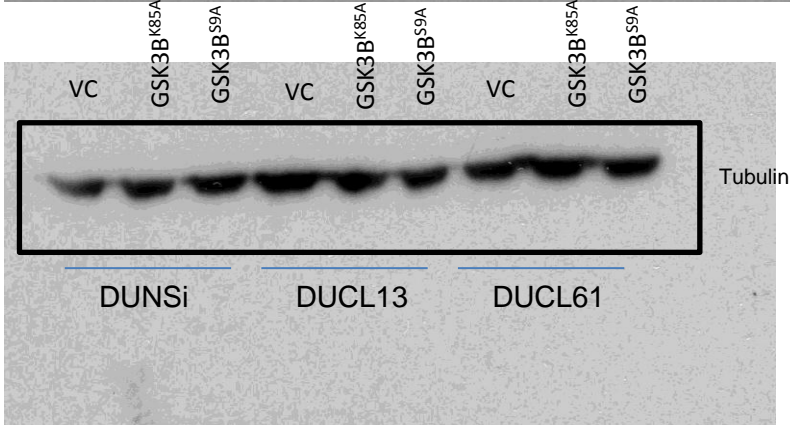
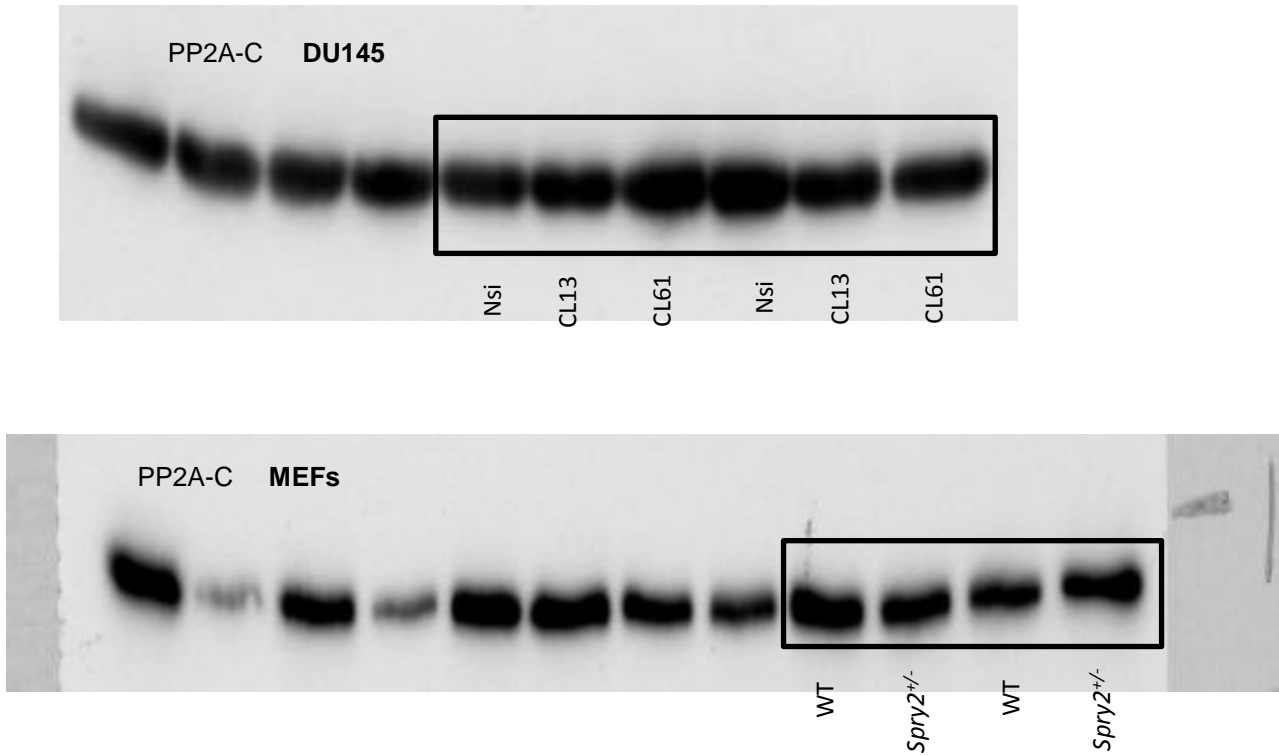




Figure 7A.

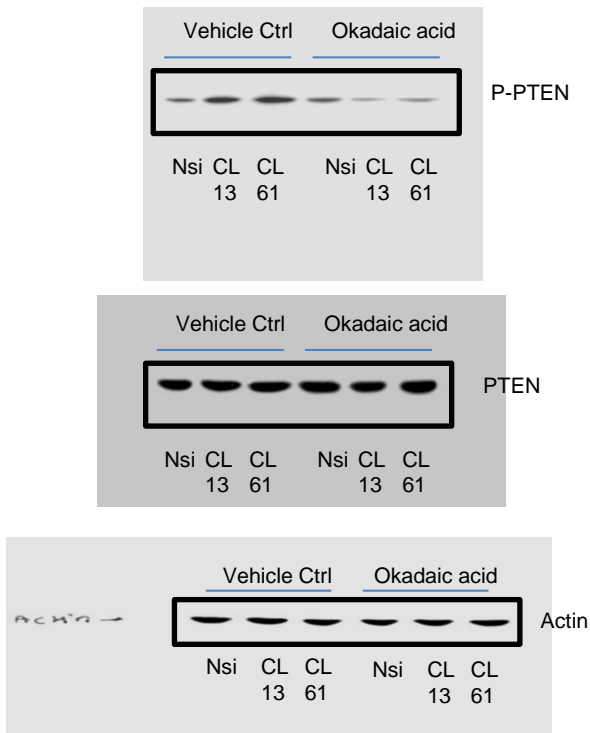


This data represents IP control for PP2A activity displayed in Figure 7A.

After the phosphatase assay, the beads with bound antibody and PP2A-C were wash three times with TBS. The washed beads were boiled with gel loading buffer and 1M DTT as reducing agent. Approximately 10 ul per assay sample was loaded on SDS-PAGE gel. The blot was cut to eliminate heavy and light anti-body chains and probed for PP2A-C (anti-rabbit). Anti- mouse PP2A-C antibody was used to immunoprecipitate PP2A-C from respective lysates for the PP2A activity assay.

As shown here, the total amount of PP2A-C immunoprecipitated is comparable in all samples indicating that the differences seen in activity are not due to different amounts of PP2A-C pulled down.

Figure 7B.



The blot was cut and upper part was probed for p-PTEN (anti-rabbit) and then stripped and re-probed for PTEN (anti-mouse). The lower part was probed for Actin (anti-mouse) as loading control.

Figure 7C.

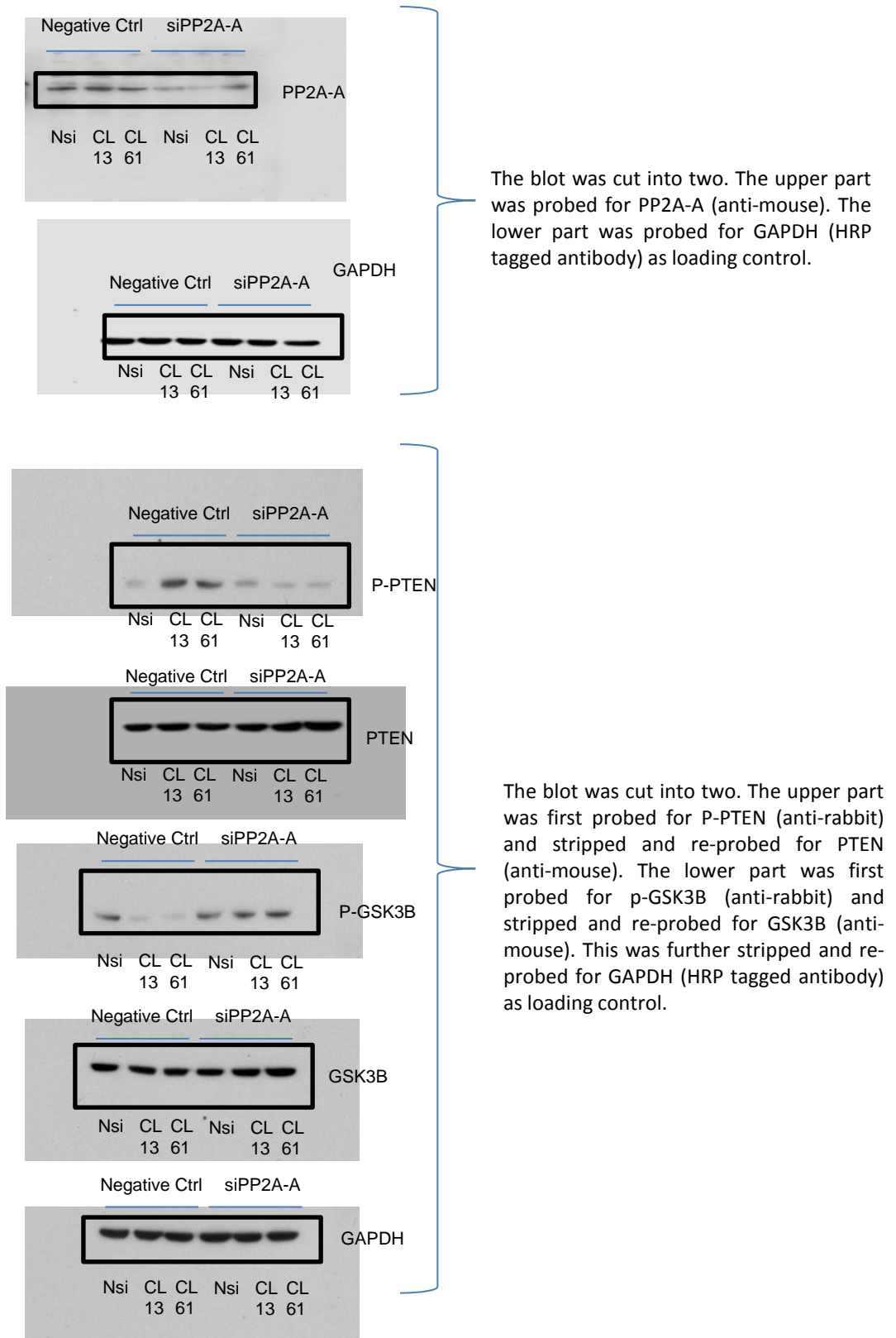
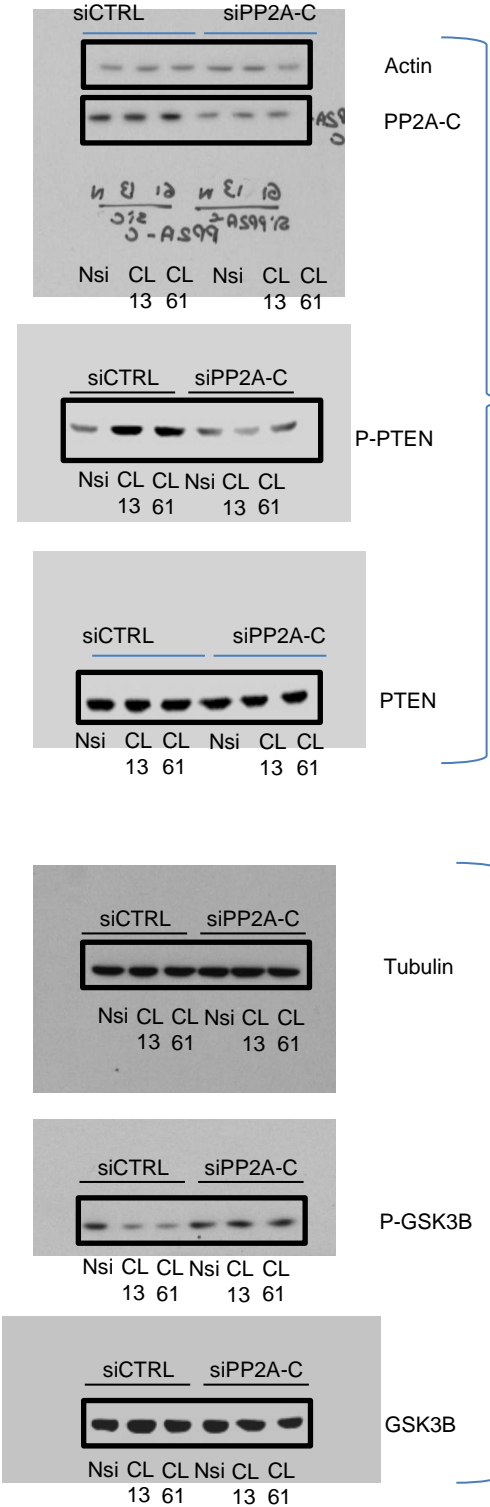


Figure 7D.



The blot was cut into two. The upper part was first probed for p-PTEN (anti-rabbit) and stripped and re-probed for PTEN (anti-mouse). The lower part was probed for PP2A-C (anti-rabbit) and Actin (anti-mouse) as the loading control.

The blot was cut into two. The upper part was probed for Tubulin (anti-mouse) as the loading control. The lower part was probed for p-GSK3B (anti-rabbit), stripped and .re-probed for GSK3B (anti-mouse)

Figure 7E.

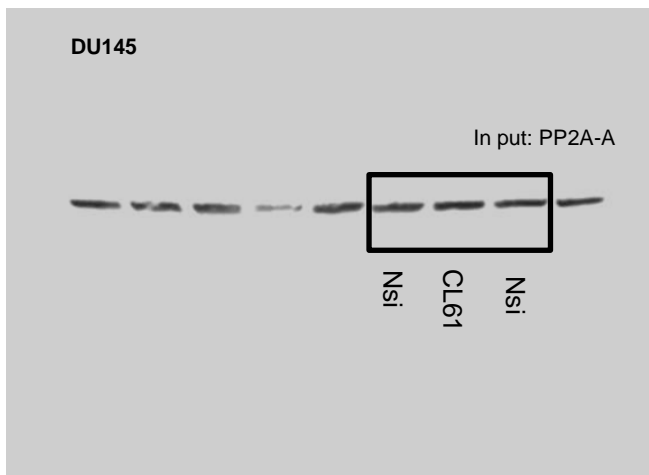
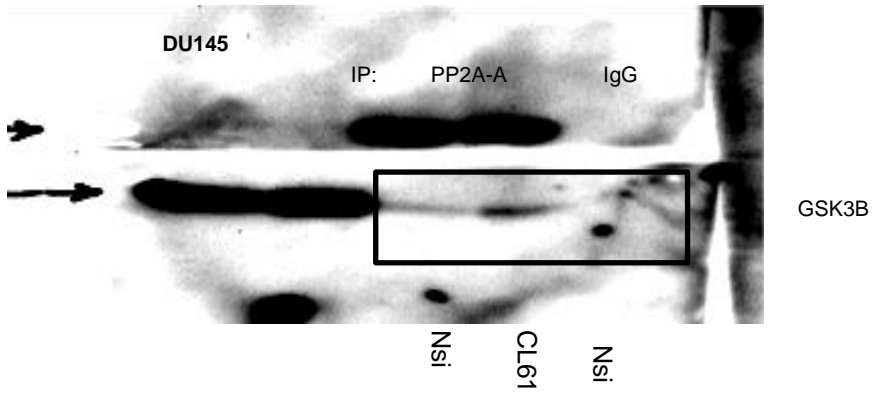
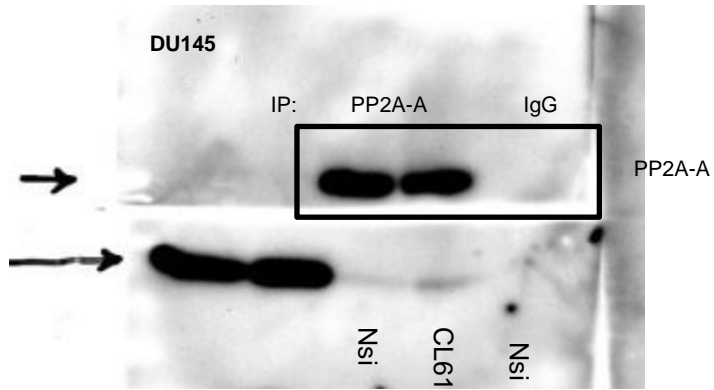


Figure 7E.

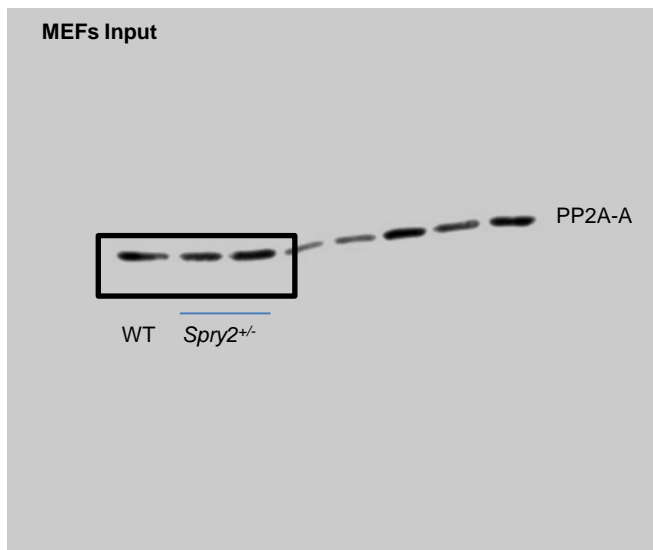
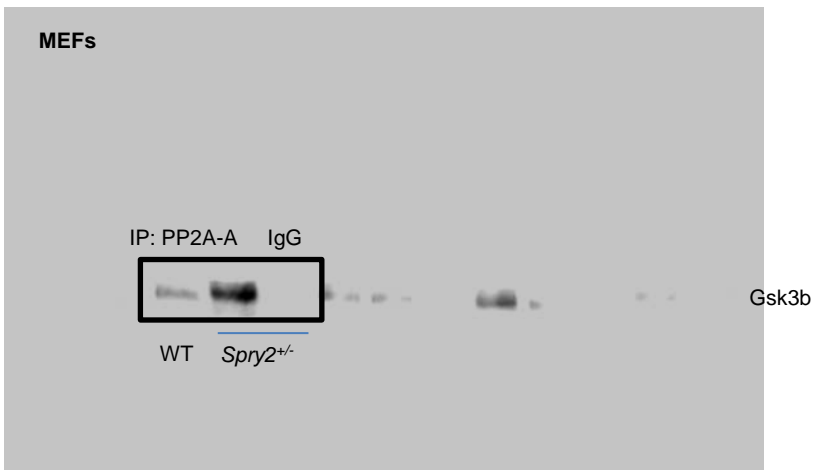
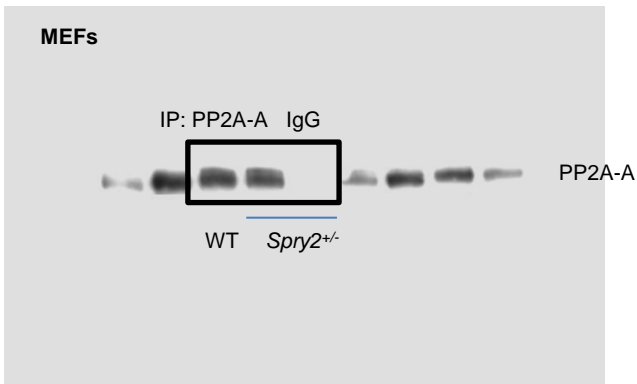


Figure 7F.

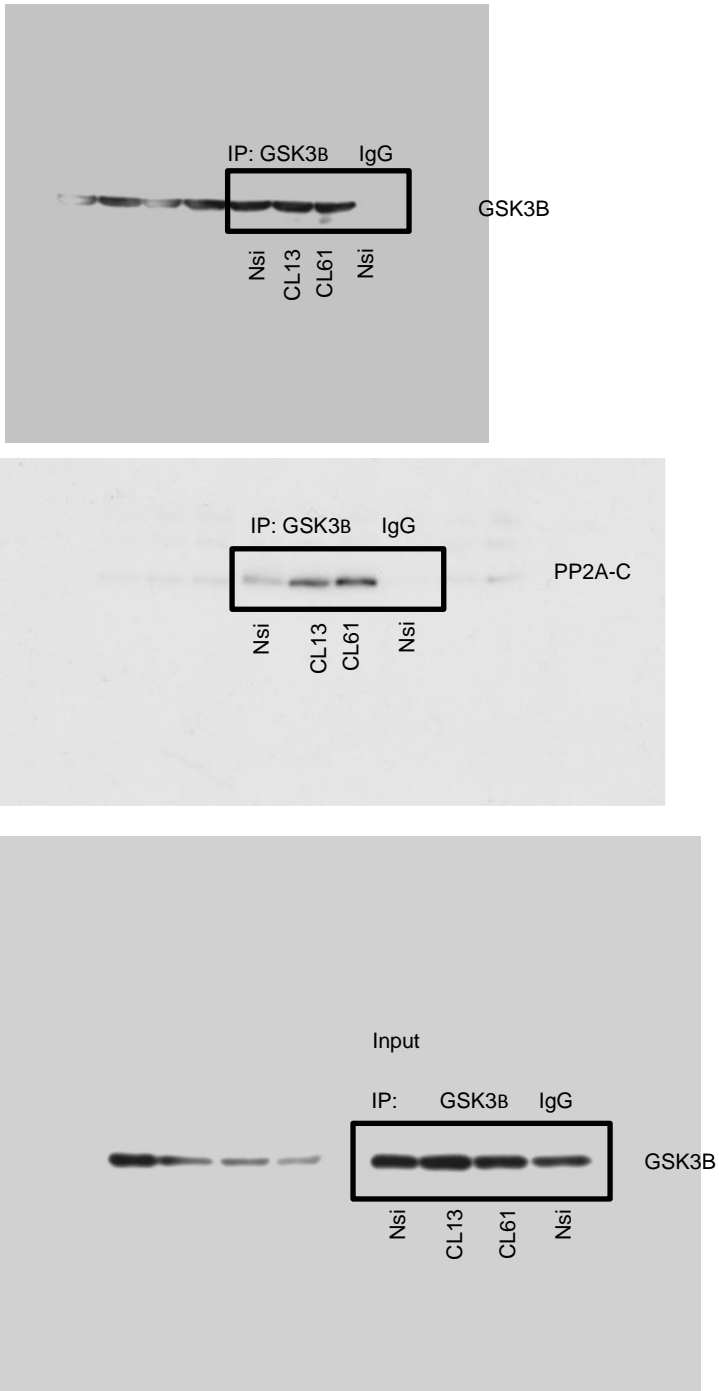
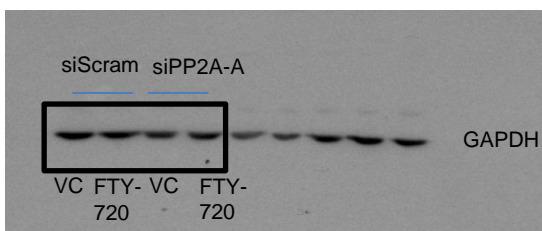
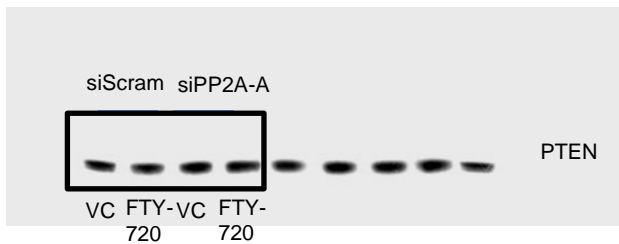
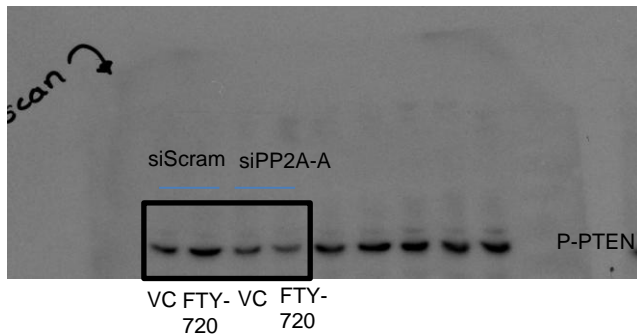


Figure 8B.



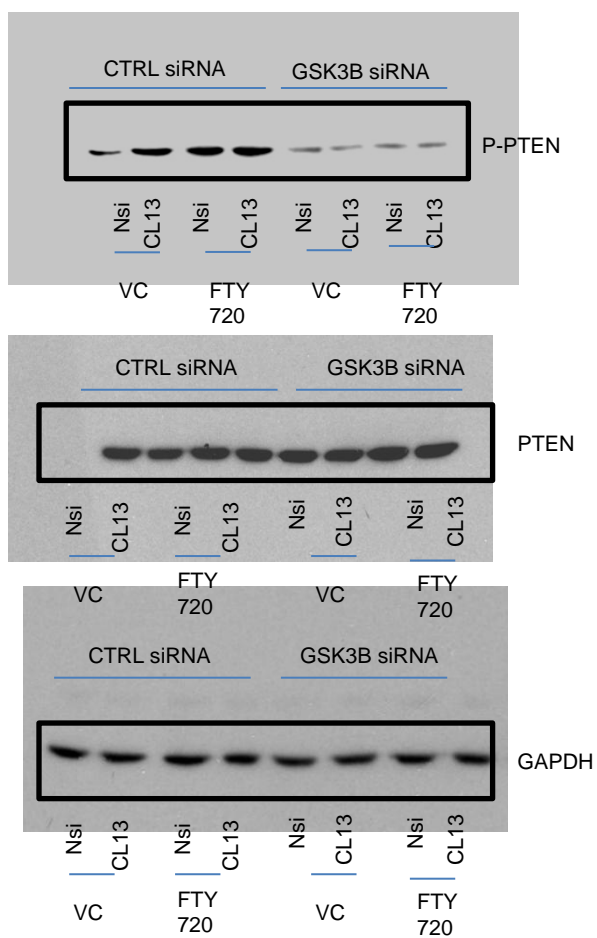
The blot was cut into two. The upper part was probed for PP2A-A and the lower part was probed for GAPDH as loading control.



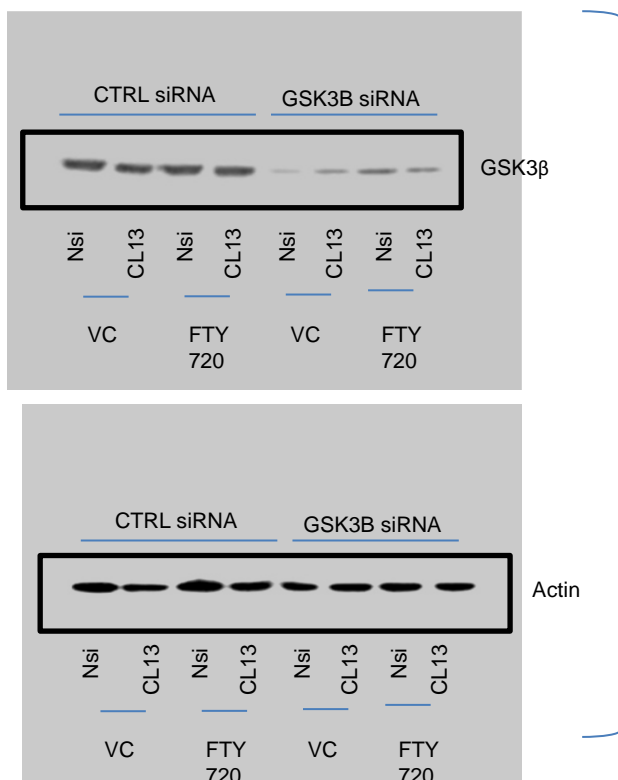
The blot was cut into two. The upper part was probed for p-PTEN (anti-rabbit) and stripped and re-probed with PTEN (anti-mouse). The lower part was probed for GAPDH as loading control.



Figure 8C.



The blot was cut into two. The upper part was probed for p-PTEN (anti-rabbit and stripped and re-probed for PTEN (anti-mouse)). The lower part was probed for GAPDH as loading control.



The blot was first probed for GSK3B (anti-rabbit) and then stripped and re-probed for Actin (anti-mouse) as loading control.

Figure 9A.

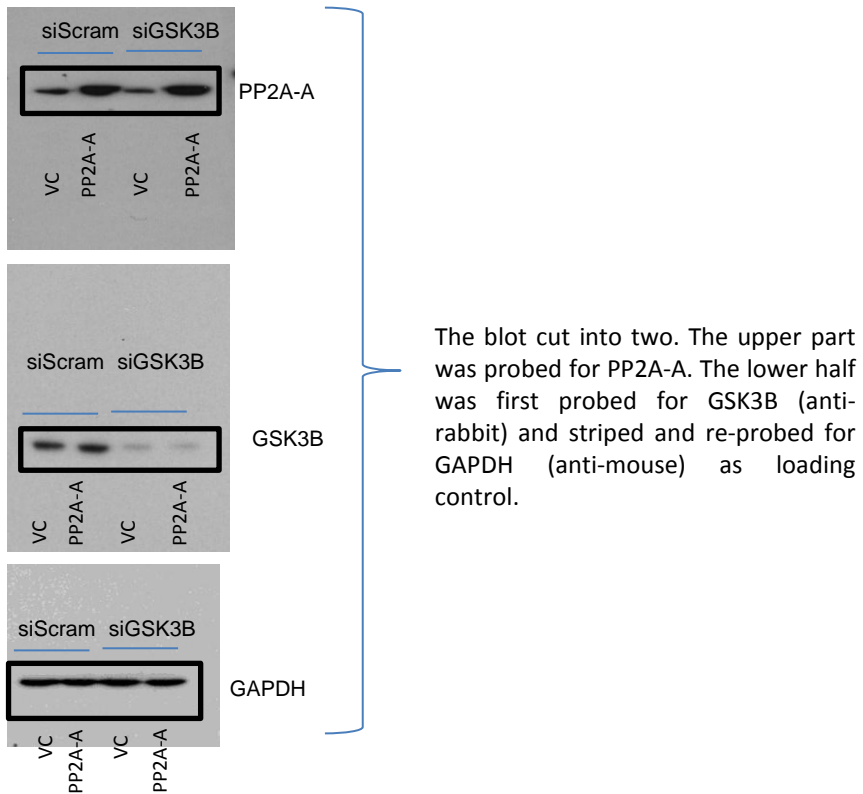
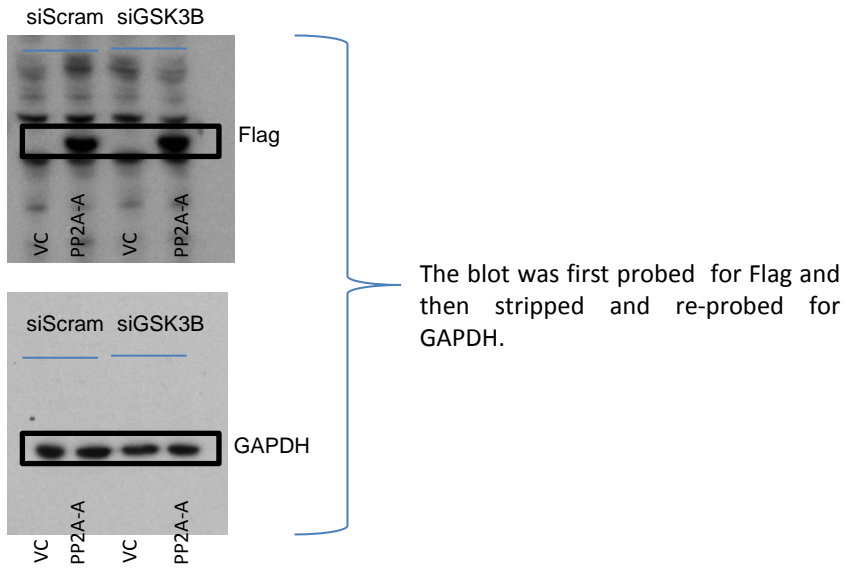
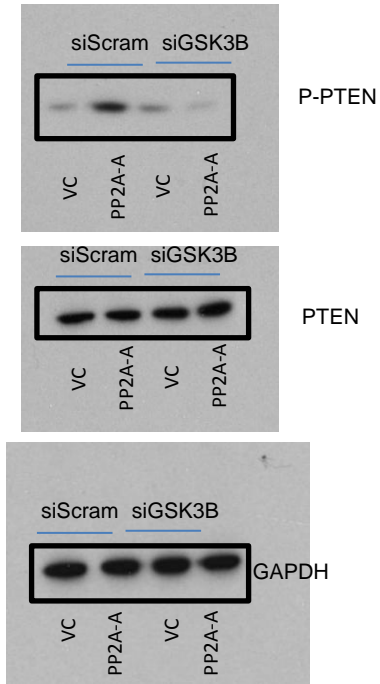
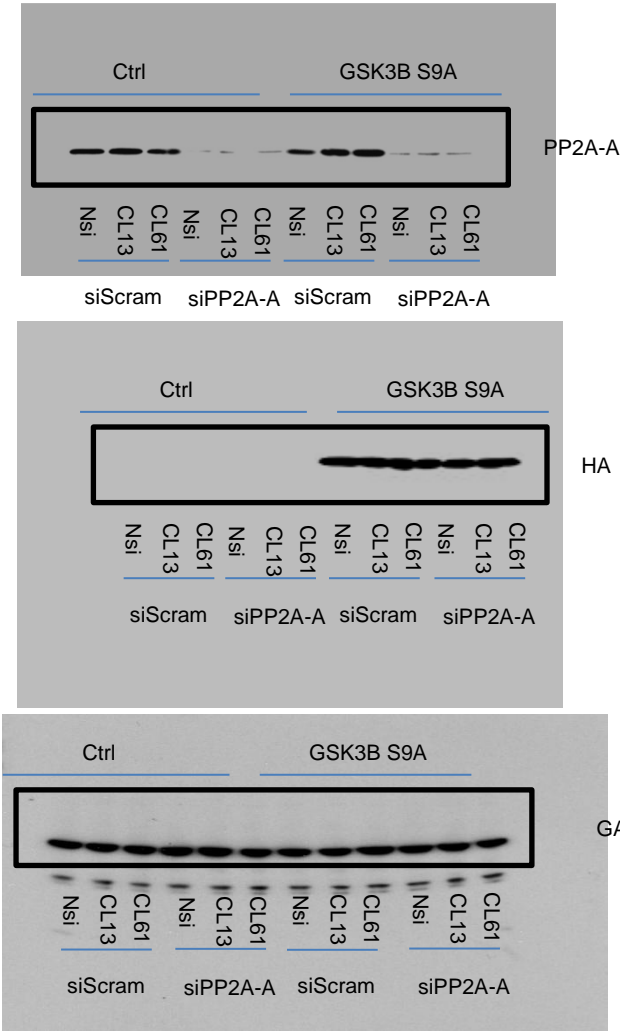


Figure 9A.



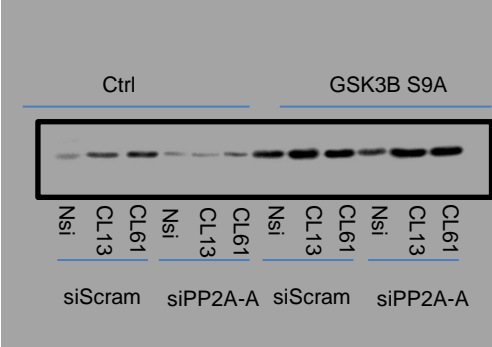
The blot cut into two. The upper part was first probed for p-PTEN (anti-rabbit) and then stripped and re-probed for PTEN (anti-mouse). The lower part was probed for GAPDH as loading control.

Figure 9C.

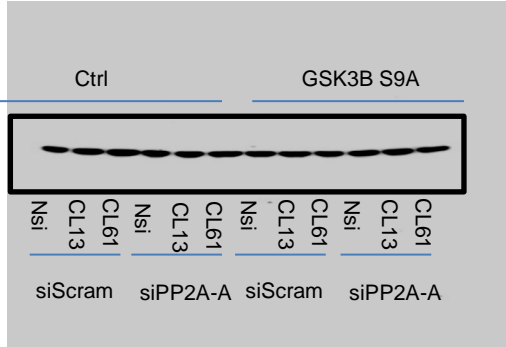


The blot cut into two. The upper part was first probed for PP2A-A (anti-rabbit) and then stripped and re-probed for HA (anti-mouse). The lower part was probed for GAPDH as loading control.

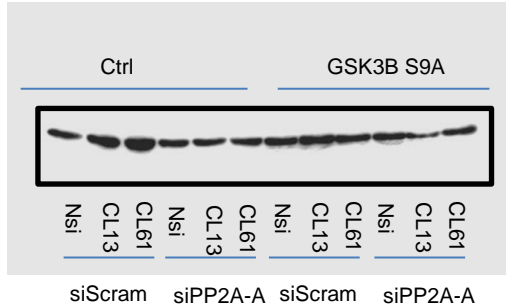
Figure 9C.



P-PTEN



PTEN



GAPDH

The blot cut into two. The upper part was probed for p-PTEN (anti-rabbit) and stripped and re-probed for PTEN (anti-mouse). The lower part was probed for GAPDH as loading control.