Therapeutic targeting of SPINK1 positive prostate cancer

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SUPPLEMENTAL INFORMATION

Supplemental information includes detailed experimental procedures and eight supplement figures.

Detailed Experimental Procedures

Cell lines and *SPINK1* Knockdown

Lentiviruses from these constructs were generated by the University of Michigan Vector Core. Viral particle infections were carried out in the presence of polybrene (8μg/ml) in 50-60% confluent 22RV1 cells. After 48 hours, infected cells were grown in 22RV1 culture media containing puromycin (2μg/ml). Three weeks later, stable cells were plated into 96 wells plate for the clonal selection. *SPINK1* knockdown was confirmed in pooled and single clones by

qPCR and single clones showing highest knockdown were further expanded. For siRNA mediated knockdowns, the most effective siRNA duplexes against *SPINK1* (J-019724-07), *EGFR* (J-003114-13), *PRSS1* (LU-006008-00-0005) or siCONTROL Non-Targeting siRNA pool (D-001210-01) were obtained from Dharmacon (Dharmacon, Thermo Scientific). All transfections were carried out in the presence of Oligofectamine (Invitrogen), according to manufacturer's instructions. After 24 hr, we carried out a second identical transfection, and cells were harvested 24 hr later for RNA isolation, invasion assays, or proliferation assays. All transient or stable knockdowns in 22RV1 cells were confirmed by qPCR.

Production of recombinant SPINK1 protein

A synthetic construct with SPINK1-2xV5 cloned into pcDNA3.1 using KpnI and XhoI restriction sites was purchased from GENEART. The full length SPINK1 cDNA including signal peptide was amplified and subcloned downstream of the Met-Arg-Gly-Ser-His_6 ($MRGSH_6$) tag coding sequence into the BamHI/PstI restriction enzyme sites of the pQE-9 expression vector (Qiagen). The plasmid was sequenced to verify integrity. Production of the recombinant protein from pQE-9 plasmids was carried out using M15 cells treated with isopropyl-1-thio-D-galactopyranoside (400mM). Bacterial cell lysates were centrifuged and the supernatants were purified by affinity chromatography using a Co^{2+}-agarose resin (Clontech, Saint-Germain-en-Laye, France). Multiple tags protein including 6XHis protein (GenScript Corp.) was used as a control. Bacterially expressed recombinant SPINK1 protein was separated on 5–30% sodium dodecyl sulfate-polyacrylamide gels under reducing conditions. In order to get concentrated SPINK1 fraction from CM of 22RV1 cells or control CM form RWPE cells, proteins were separated by ultrafiltration according to their molecular weight (MW), using membranes at a

cutoff of 3-10 kDa (SPINK1 molecular weight 6.2 kDa). Protein was transferred onto Polyvinylidene Difluoride membrane (GE Healthcare) and membrane was incubated for one hour in blocking buffer [Tris-buffered saline, 0.1% Tween (TBS-T), 5% nonfat dry milk] and probed with the SPINK1 mAb (Mobitec Inc.). After washing the blots with TBS-T, the blots were incubated with horseradish peroxidase-conjugated secondary mouse antibody and the signals visualized by enhanced chemiluminescence system as described by the manufacturer (GE Healthcare).

Quantitative PCR (QPCR)

The reverse transcriptase reaction was carried out for 60 minutes at 50ºC and the cDNA was purified using microcon YM-30 (Millipore Corp.) according to manufacturer's instruction and used as template in quantitative PCRs. All oligonucleotide primers used in this study were synthesized by Integrated DNA Technologies. Briefly, reactions were performed with SYBR Green Master Mix (Applied Biosystems) and 25ng of both the forward and reverse primers for *SPINK1* (5'-TGTCTGTGGGACTGATGGAA-3' and 5'-AGGCCCAGATTTTTGAATGA-3'); *PRSS1* (5'-GCCTGGACGCTCCTGTGCTG-3' and 5'-CTGGGCACAGCCATCACCCC-3') and *EGFR* (5'-GGGCCAGGTCTTGAAGGCTGT-3' and 5'-ATCCCCAGGGCCACCACCAG-3') using the manufacturer recommended thermocycling conditions. For each experiment, threshold levels were set during the exponential phase of the qPCR reaction using the StepOne software. The amount of each target gene relative to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; forward 5'-TGCACCACCAACTGCTTAGC-3' and reverse primers 5'-GGCATGGACTGTGGTCATGAG-3') for each sample was determined using the comparative threshold cycle (Ct) method. Threshold levels for each experiment were set during

the exponential phase of the QPCR reaction using Sequence Detection Software version 1.2.2 (Applied Biosystems).

Cell Motility Assay

22RV1 or stable shNS vector and shSPINK1 cells were plated on a lawn of microscopic fluorescent beads on collagen coated 96-well plates (Cellomics, Thermo Scientific). Motile cells push the beads and create phagokinetic tracks behind each cell. The cleared track area is proportional to the magnitude of cell motility. Plates were analyzed and images were captured using standard light microscopy.

Soft Agar Colony Assay

A 50μL base layer of agar (0.6% agar in DMEM with 10% FBS) was allowed to solidify in a 96-well flat-bottom plate prior to the addition of 75μL stable sh*NS* vector and sh*SPINK1* cell suspension containing 4,000 cells in 0.4% agar in DMEM with 10% FBS. The cell containing layer was then solidified at 4^{o}C for 15 minutes prior to the addition of 100μL of MEM with 5% FBS. Colonies were allowed to grow for 21 days before imaging under a light microscope.

Immunohistochemistry and immunofluorescence staining

FFPE sections were obtained from formalin-fixed xenografted tumors and, antigen retrieval was performed by microwaving sections in citrate buffer (pH6.0) for 10min, followed by cooling and rinse in water. Sections were further blocked in hydrogen peroxidase for 5 min followed by incubation in anti-Ki-67 antibody at 1:400 dilutions (AbCam, Cat# ab15580) for 30min at room temperature. Sections were further incubated in EnVision+ for 30min at room

temperature; immunoreactive nuclei were visualized with the Vectastain Elite ABC Kit using diaminobenzidine (DAB) as the substrate (Vector Laboratories, Inc.). Finally, sections were counterstained with Harris Hematoxlyin (Fisher), dehydrated, and mounted with Permount (Fisher). Immunoreactive positive Ki-67 nuclei were scored blindly for both groups in 400X magnification.

For immunofluorescence staining, sh*NS*-luciferase and sh*SPINK1*-luciferase cells were grown in chamber slides at sub-confluent density. Cells were fixed using chilled methanol after washing with 1X PBS. The chamber slides containing cells were then blocked in PBS-T containing 5% normal donkey serum for 1 hour at room temperature. Slides were incubated overnight at 4°C with a mouse anti-SPINK1 antibody (H00006690-M01; Abnova) 1: 1000 dilution, then washed, and followed by secondary antibodies (anti-mouse Alexa 555 1:1000 dilutions) for 1 hour. Slides were mounted using Vectashield mounting medium containing DAPI (Vector Laboratories) after washing with PBS-T and PBS. Fluorescence images were captured using a Zeiss Microscope (Carl Zeiss) equipped with a high resolution CCD camera controlled by ISIS image processing software (Metasystems).

Immunoprecipitation and Western blot analysis

Briefly, transiently EGFR expressing HEK293 cells were washed twice PBS supplemented with protease inhibitor. Cells were lysed in Triton X-100 lysis buffer (20mM MOPS, pH 7.0, 2mM EGTA, 5mM EDTA, 30mM sodium fluoride, 60mM β-glycerophosphate, 20mM sodium pyrophosphate, 1mM sodium orthovanadate, 1% Triton X-100, protease inhibitor cocktail (Roche). Cell lysates (0.5-1.0mg) were then pre-cleaned with protein A/G agarose beads (Santa Cruz) by incubation for 1 hour with shaking at room temperature followed by

centrifugation at 2000xg for 1 minute. Recombinant SPINK1-GST (Proteintech Group Inc.), GST (AbCam) or GST-VEGF Receptor 2 protein (Cell Signaling) (80µg/ml) were added to the pre-cleaned protein lysates and incubated at 4°C overnight. Similarly, 22Rv1 cells lysate in Kinet lysis buffer was mixed with SPINK1-GST recombinant protein (80µg/ml) and incubated at 4°C overnight. After adding 2µg of each antibody (mouse IgG, Millipore; SPINK1 mAb, Mobitec Inc.; EGFR mAb, Cell Signaling) lysates were further incubated with shaking at 4°C for 4 hours prior to addition of 20µL protein A/G agarose beads (Santa Cruz). The mixture was then incubated with shaking at 4°C for another 4 hours prior to washing the lysate-bead precipitate (centrifugation at 2000xg for 1 minute) 3 times in Triton X-100 lysis buffer or Kinet lysis buffer. Beads were finally precipitated by centrifugation, resuspended in 25µL of 2X loading buffer and boiled at 80°C for 10 minutes. Samples were then analyzed by SDS-PAGE Western blot analysis as described below.

Western Blot Analysis

Cell lysates were prepared in RIPA lysis buffer (Thermo Scientific), supplemented with complete proteinase inhibitor and phosphatase inhibitor mixture (Roche). Fifteen micrograms of each protein extract was boiled in sample buffer, separated by SDS-PAGE, and transferred onto Polyvinylidene Difluoride membrane (GE Healthcare). rSPINK1 stimulated 22RV1 phospho-EGFR blot was performed as described before (*1*). The membrane was incubated for one hour in blocking buffer (Tris-buffered saline, 0.1% Tween (TBS-T), 5% nonfat dry milk) and incubated overnight at 4ºC with anti-phospho-MEK or -ERK or -EGFR or -AKT antibodies or total -MEK or -ERK or -AKT and -EGFR antibodies (Cell Signaling); trypsin1 polyclonal antibody (Abcam) and PSA monoclonal antibody (Dako). Following three washes with TBS-T, the blot

was incubated with horseradish peroxidase-conjugated secondary antibody and the signals visualized by enhanced chemiluminescence system as described by the manufacturer (GE Healthcare).

EGFR cross linking immunoblotting

22RV1 cells were stimulated with rSPINK1 (100 ng/ml) or EGF (10 ng/ml) in 6-well plates for 0, 5, 10, 30, 60 and 90 min at 37^{o}C. At the end of each time point cells were washed twice with 1X PBS. Cells were then treated with cross linking reagent BS3 [Bis-(sulfosuccinimidyl) suberate] to a final concentration of 5mM and incubated on ice for 2 h. The quench solution (1M Tris, pH 7.5, 1:100 dilutions) was then added to a final concentration of 10mM and incubated for 15 min on ice. The cells were then lysed with RIPA buffer supplemented with protease inhibitor and phosphatase inhibitors (Roche). EGFR dimerization was analyzed by Non-reducing immunoblot.

22RV1 Xenograft Models

Mice were anesthetized using a cocktail of xylazine (80-120 mg/kg IP) and ketamine (10mg/kg IP) for chemical restraint before implantation. Eight mice were included in each group. Mice implanted with 22RV1-luciferase or PC-3 luciferase cells were randomly divided into different groups, and treated twice a week with SPINK1 mAb (Mobitec Inc.) or control mouse IgG antibody (Innovative Research) at the dose of 10mg/kg body weight. Growth in tumor volume was recorded weekly using digital calipers, and tumor volumes were calculated using the formula (π/6) (L × W2), where L = length of tumor and W = width. Antitumor activity was determined from the analyses of tumor growth inhibition, defined as the decrease in the mean

tumor volume for SPINK1 mAb treated mice versus mouse IgG, mAb treated mice. *In vivo* bioluminescent imaging was performed weekly up to week 4 using the IVIS-200 imaging system (Xenogen Corp.). Mice were injected 150mg/kg luciferin intra-peritoneal 12 min before imaging. All images were collected and analyzed with Living Image software (Xenogen Corp.). All procedures involving mice were approved by the University Committee on Use and Care of Animals (UCUCA) at the University of Michigan and conform to their relevant regulatory standards.

Serum Toxicity Marker Analyses

At the end of the xenograft study, mice were anaesthetized and blood was collected by cardiac puncture. Blood was transferred into a 1.5 ml eppendorf tube and kept on ice for 45 min, followed by centrifugation at 8000 rpm for 10 min at 4^{o}C. Clear supernatant containing serum was collected and transferred into a sterile 1.5 ml eppendorf tube. All serum markers were measured using dry-slide technology on IDEXX Vettest 8008 biochemical analyser (IDEXX, France). About 50 μL of the serum sample was loaded on the VetTest pipette tip followed by securely fitting it on the pipettor and manufacturer's instructions were followed for further analyses.

1. T. Marchbank, G. Weaver, M. Nilsen-Hamilton,R.J. Playford, Pancreatic secretory trypsin inhibitor is a major motogenic and protective factor in human breast milk. *Am J Physiol Gastrointest Liver Physiol* **296,** G697-703 (2009).

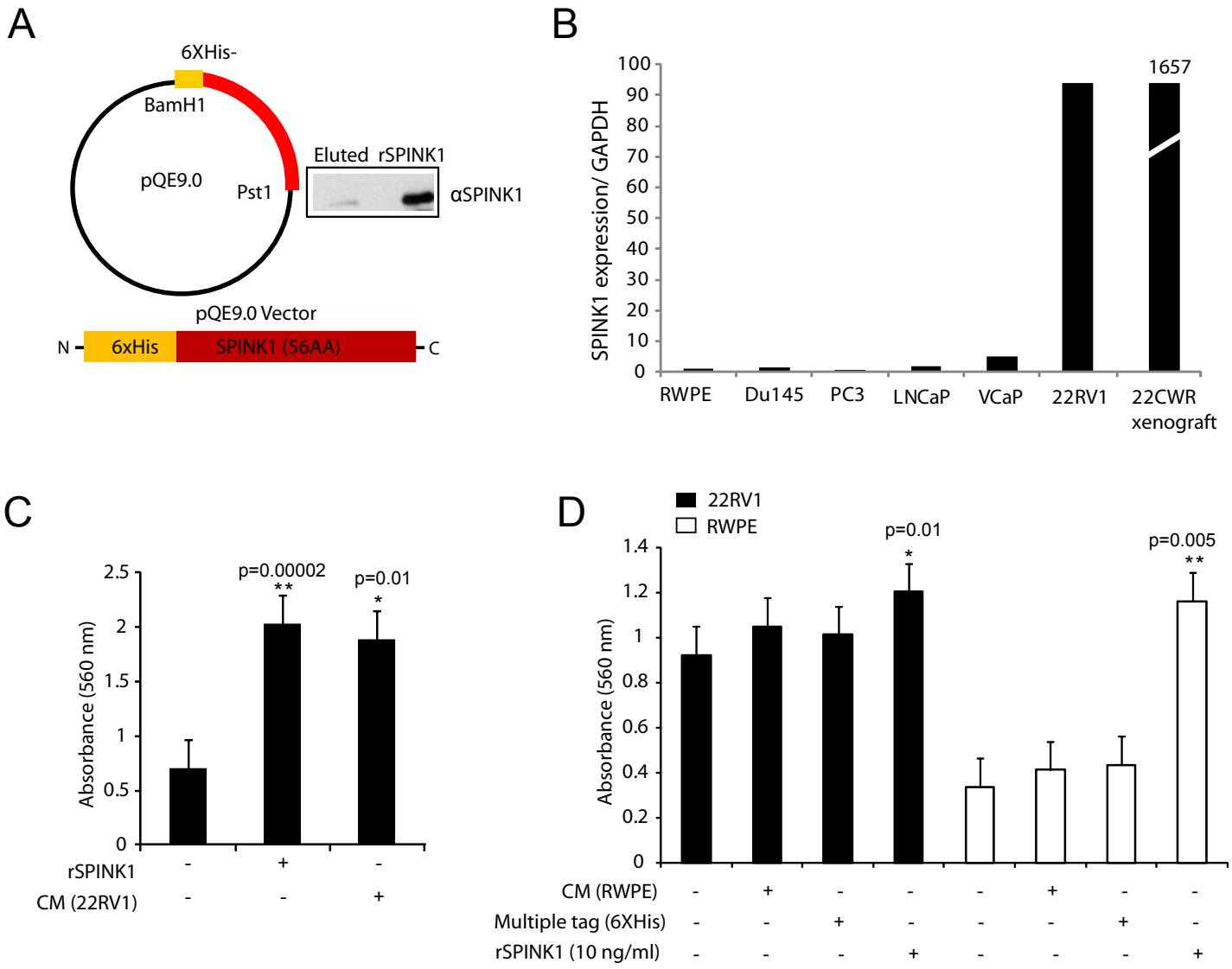

Fig. S1. rSPINK1 or CM collected from 22RV1 cells induces invasion in benign or cancer cells. A, Expression of recombinant N-6XHis-tag SPINK1 protein in bacterial expression system. Bacterial expression vectors (pQE-9) was constructed to produce N-6XHis-tag SPINK1 recombinant protein (rSPINK1) from human cDNA. rSPINK1 protein was isolated as described in methods and lysates were resolved on 15% SDS-PAGE and immunoblotted with the anti-SPINK1 antibody. **B,** Expression of SPINK1 in a prostate cell line panel by quantitative RT-PCR. **C,** Effect of conditioned medium (CM) collected from 22RV1 cells (10 kd fraction) or rSPINK1 protein on breast cancer MCF7 cells invasion in Boyden chamber Matrigel invasion assay. **D,** Effect of conditioned medium (CM) collected from RWPE cells (10 kd fraction), mutiple tag protein (including 6XHis) or rSPINK1 protein on 22RV1 or RWPE cells invasion in Boyden chamber Matrigel invasion assay. Data represented in the bar graph represents mean +/- SEM; P values from significant two-sided Student's t tests are given (* = <0.05, ** = <0.001).

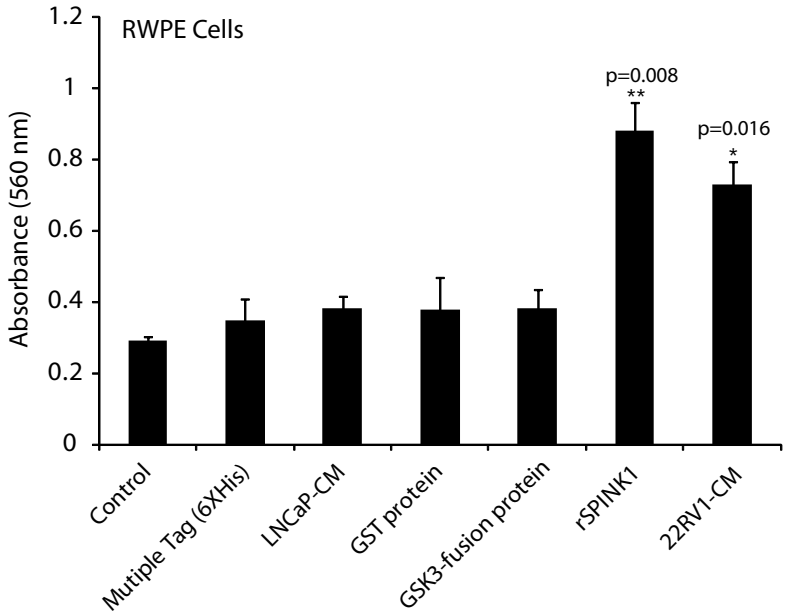

Fig. S2. CM collected from 22RV1 cells induces cell invasion, but not CM from LNCaP cells. Benign immortalized RWPE prostate epithelial cells treated with multiple tag (6XHis), CM collected from LNCaP cells or 22RV1 cells (10 kd fraction), GST protein, GSK3-fusion protein (GST tag) and rSPINK1 protein showing invasion in Boyden chamber Matrigel invasion assay. In each condition 10 ng/ml protein was used. Data represented in the bar graph represents mean +/- SEM; P values from significant two-sided Student's t tests are given (* = <0.05, ** = <0.001).

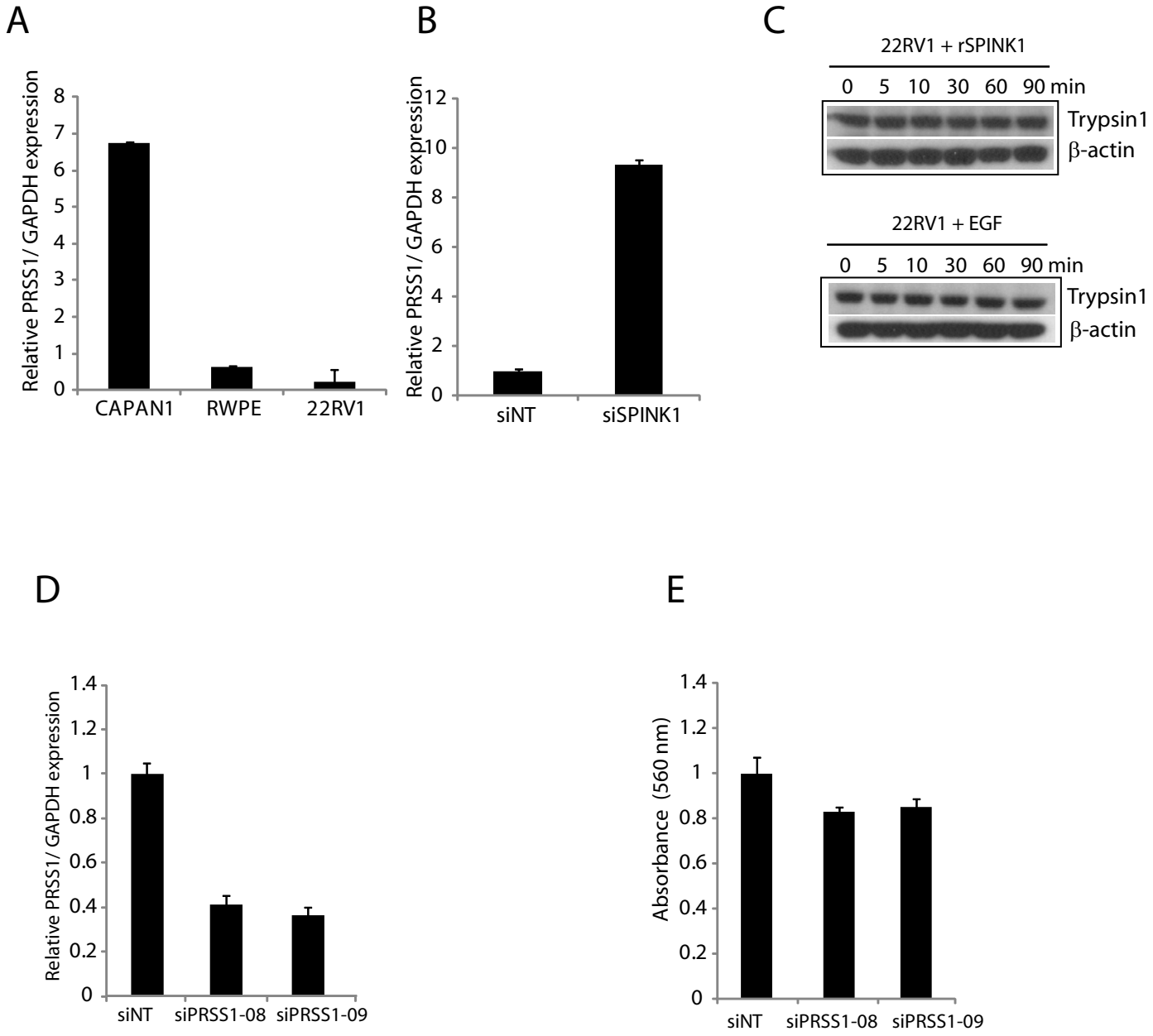

Fig. S3. *PRSS1* (trypsin1) knockdown in 22RV1 cells has no effect on SPINK1 mediated cell invasion. **A,** Expression of *PRSS1* (trypsin1) by qPCR. Pancreatic cancer cells, CAPAN1 was used as a control. **B,** Same as A except *SPINK1* was knocked down in the 22RV1 cell line using siRNA against *SPINK1*. **C,** Western blot showing trypsin levels in the 22RV1 cells stimulated with rSPINK1 or EGF at different time points as indicated.

D-E, *PRSS1* was knocked down in the 22RV1 cell line using multiple siRNA constructs. From parallel experiments, **D**, *PRSS1* expression was determined by qPCR and **E,** the effect on invasion was determined by Boyden chamber Matrigel invasion assay. Experiment was run in quadruplicates for each condition (mean +/- SEM shown).

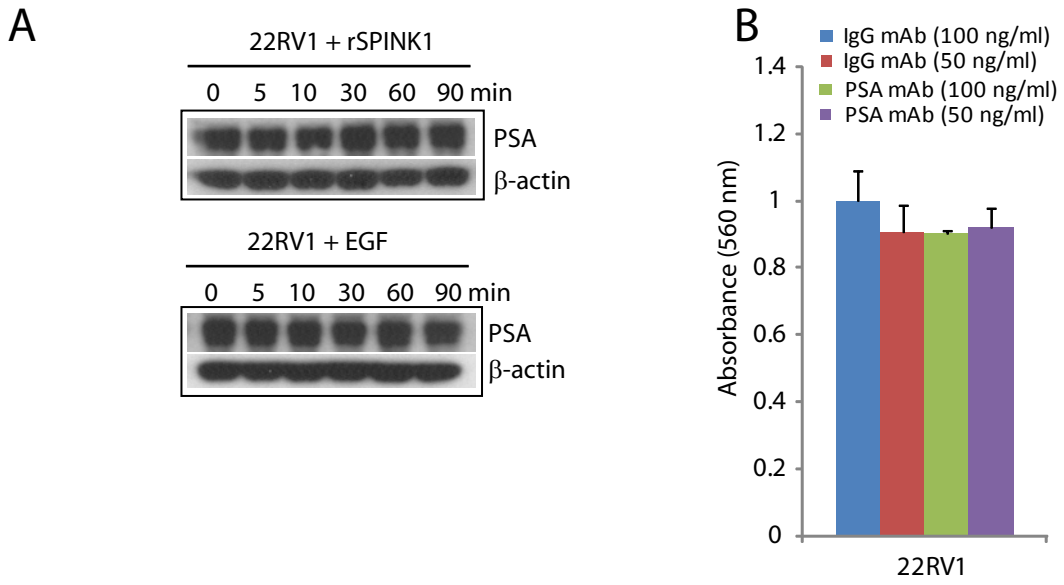

Fig. S4. Exogenous rSPINK1 has no effect on PSA in 22RV1 cells. A, Western blot showing no change in PSA level in 22RV1 cell line stimulated with rSPINK1 (100 ng/ml) or EGF (10 ng/ml). **B,** Matrigel invasion assay using 22RV1 cell line in the presence of IgG or PSA monoclonal antibody. Experiment was run in quadruplicates for each condition (mean +/- SEM shown).

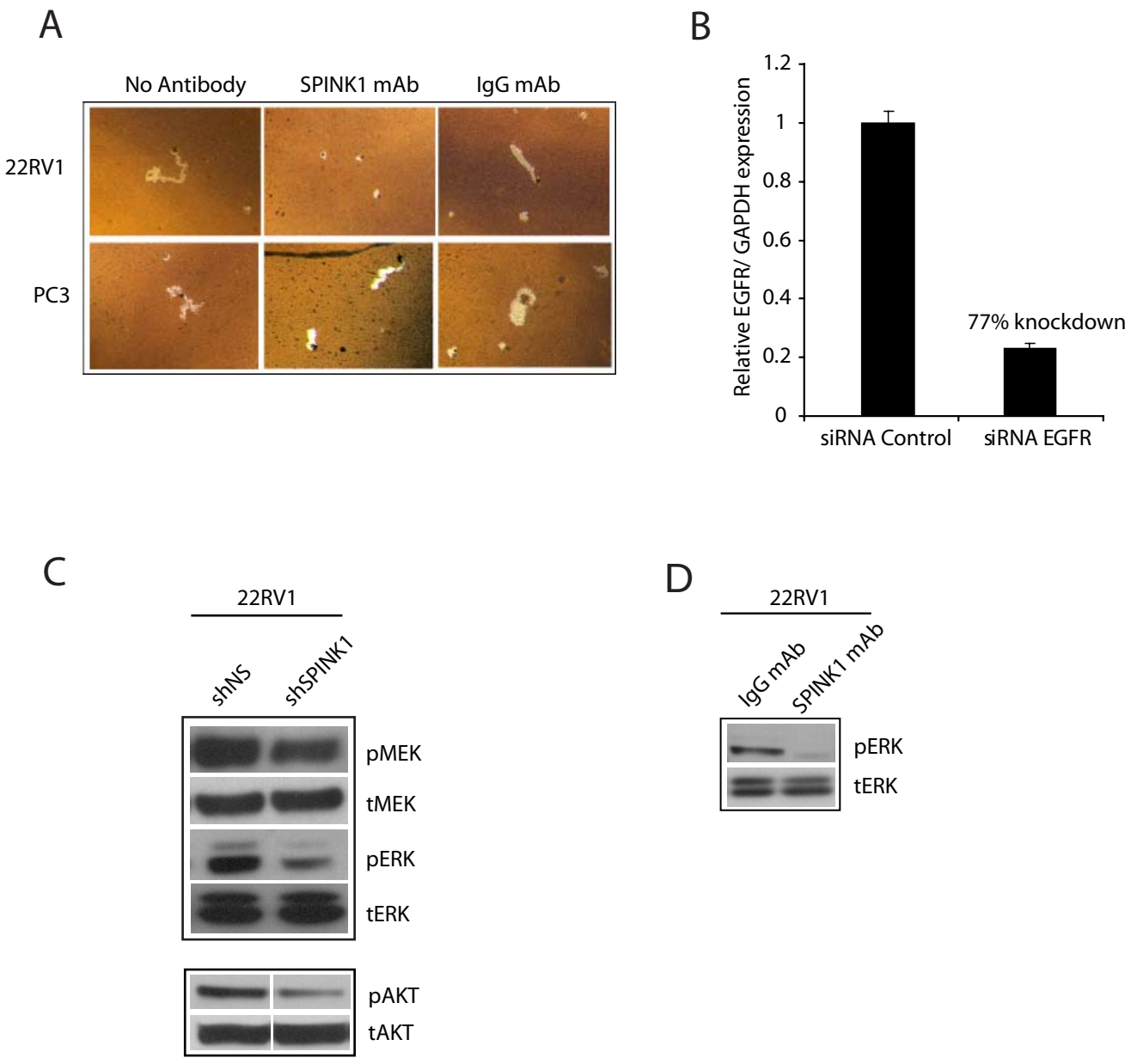

Fig. S5. SPINK1 mAb reduces SPINK1+ cell motility and SPINK1 knockdown alters MAPK pathway. **A**, Cell motility assay was carried out by plating 22RV1 cells in the presence or absence of the SPINK1 mAb or IgG mAb on a lawn of microscopic fluorescent beads on collagen coated 96-well plates. 22RV1 cells showed reduced cell motility in the presence of SPINK1 mAb as compared to IgG, whereas no effect of SPINK1 mAb was observed in the PC3 cells (400X magnification). Cleared track areas are proportional to the magnitude of cell motility. **B,** Quantitative RT-PCR showing decrease in *EGFR* expression in the 22RV1 cells. **C,** Western blot showing pMEK, pERK, pAKT, tMEK, tERK and tAKT expression levels in sh*NS* and sh*SPINK1* 22RV1 cells (single clone). **D,** Same as C, except pERK and tERK levels in the 22RV1 cells treated with IgG or SPINK1 mAb. pMEK, pERK and pAKT denotes phosphorylated-MEK, -ERK or -AKT and tMEK, tERK or tAKT denotes total-MEK, -ERK or -AKT levels.

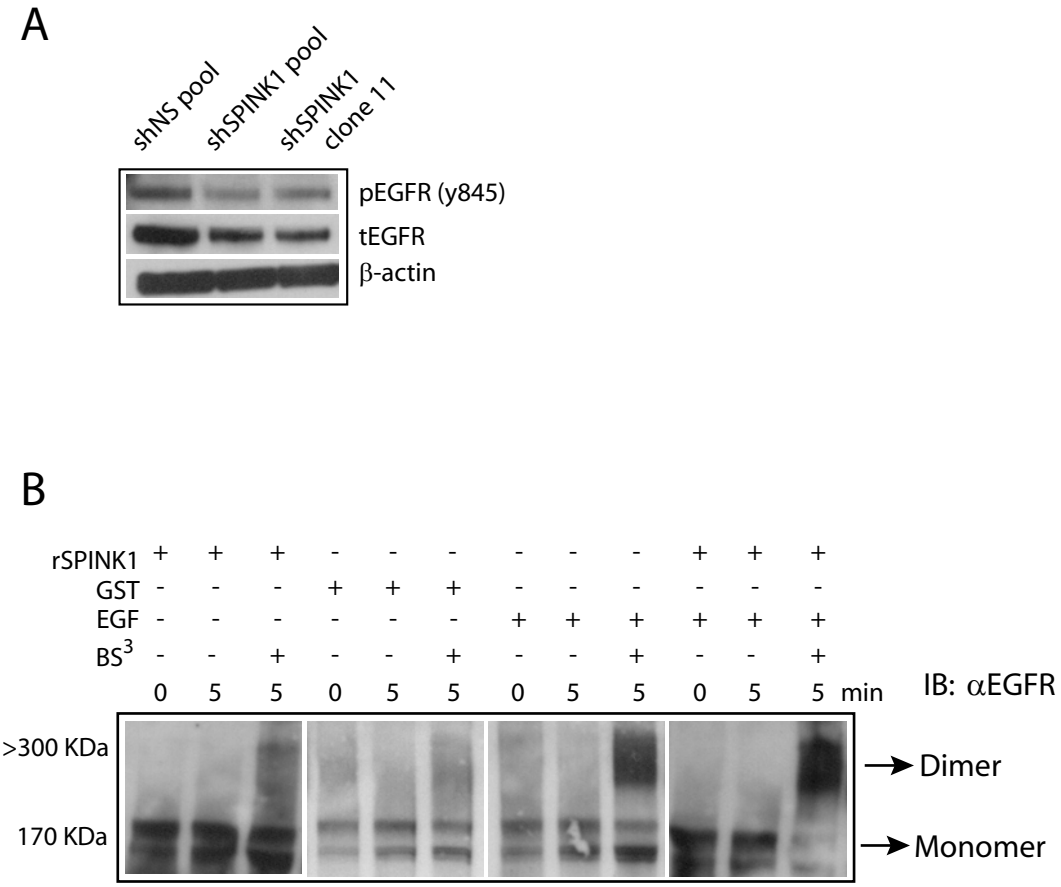

Fig. S6. Exogenous SPINK1 induces EGFR dimerization and phosphorylation. A, Western blot showing EGFR phosphorylation in the stable sh*NS,* sh*SPINK1* pool and in single sh*SPINK1* clone. **B,** Non-reducing Western blot showing EGFR dimerization after stimulating with rSPINK1 (100 ng/ml) and EGF (10 ng/ml) as indicated in the presence or absence of crosslinking reagent BS^3 (3mM in PBS). pEGFR denotes phosphorylated-EGFR and tEGFR denotes total-EGFR.

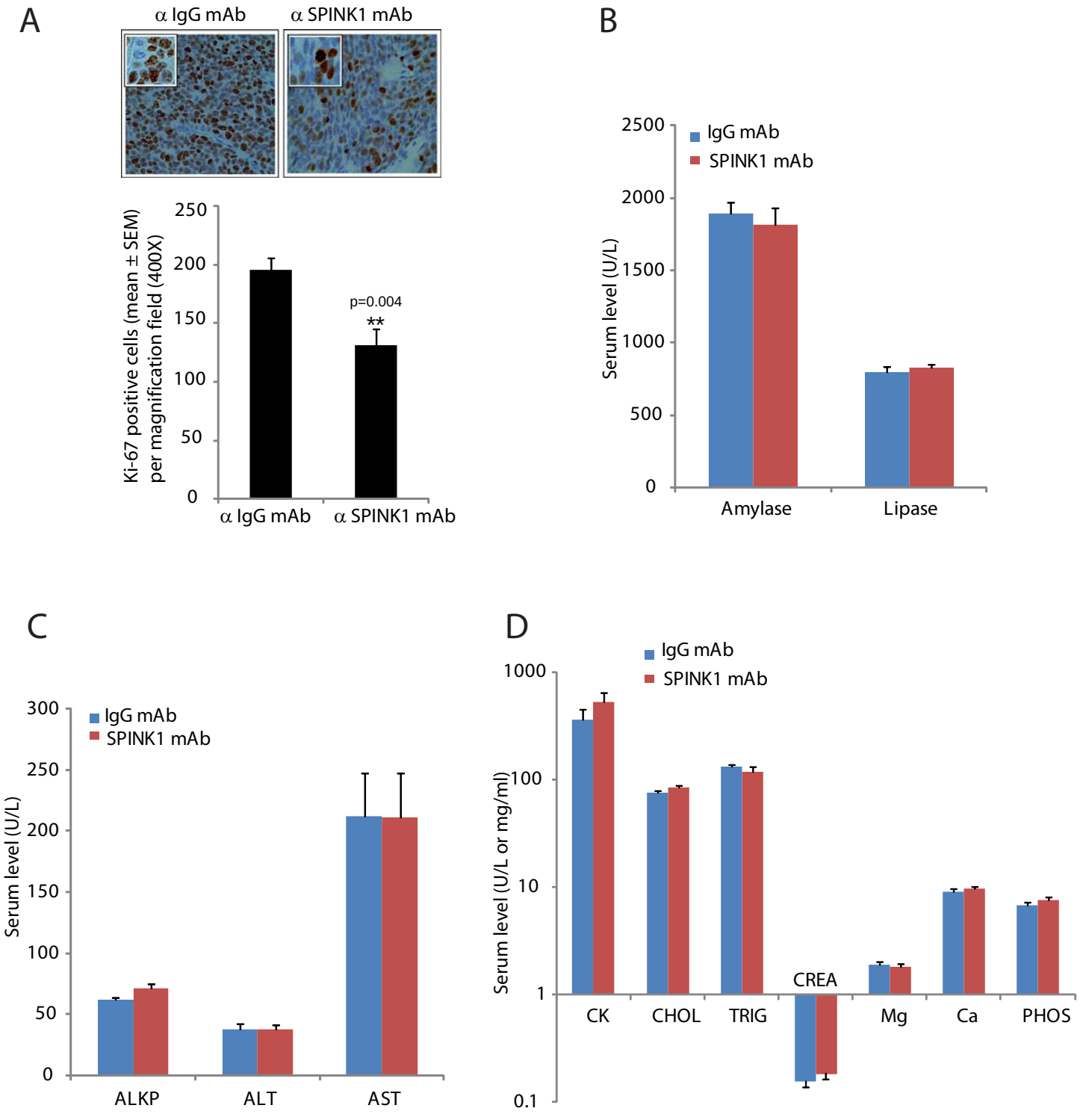

Fig. S7. SPINK1 mAb induces decrease in tumor proliferation index, but has no effect on toxicity markers. A, Ki-67 immunohistochemical (IHC) staining of tumor tissue showing Ki-67 positive nuclei in SPINK1 mAb treament group as comapred to control IgG. Representative photographs were taken at 400X magnification. **B,** Pancreatic toxicity markers showing amylase and lipase levels (U/L) in the serum samples collected from control IgG or anti-SPINK1 mAb treated mice. **C,** Same as **B,** except hepatic toxicity markers showing alkaline phosphatase (ALKP), alanine aminotranferease (ALT) and aspartate aminotransferase (AST) levels (U/L). **D,** Same as **B**, except general health profile markers showing CK:creatinine kinase (U/L); CHOL: cholesterol; TRIG: triglycerides; CREA: creatine; Ca: calcium; Mg: magnesium; PHOS: phosphorus (mg/ml).

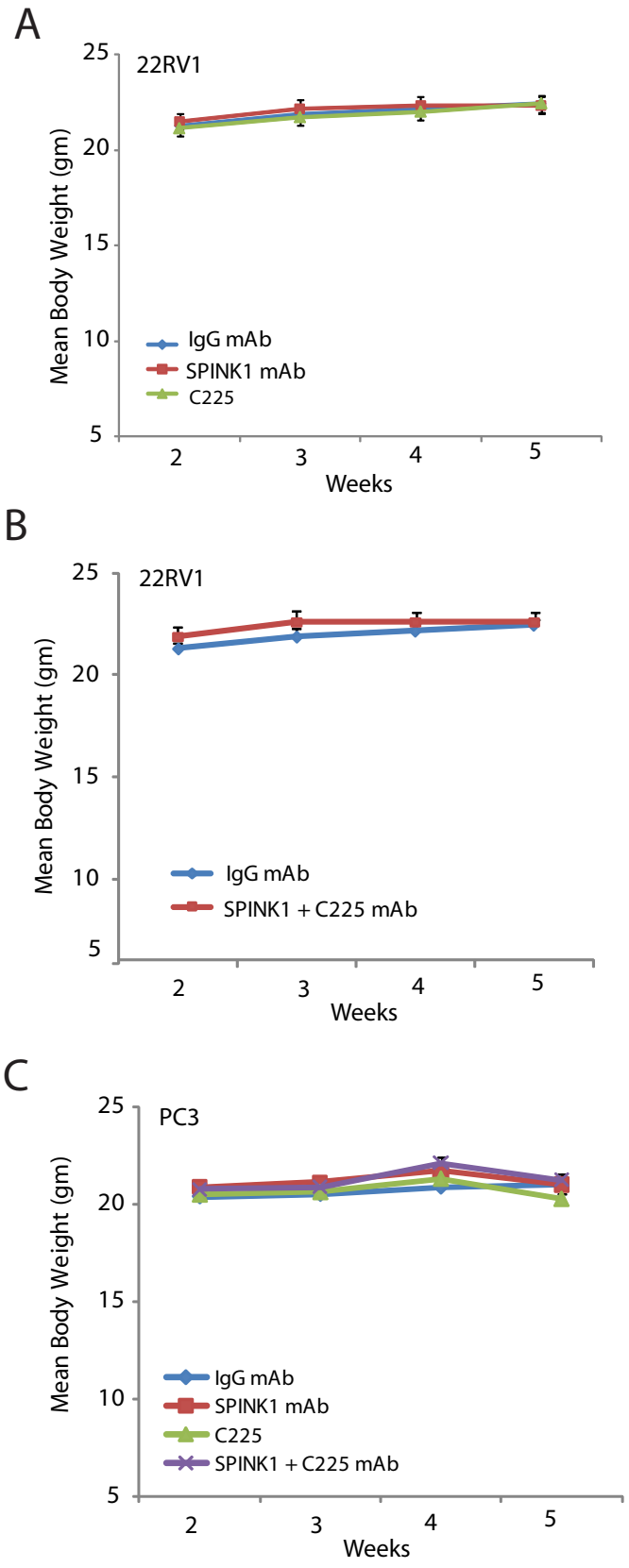

Fig. S8. Anti-IgG or -SPINK1 mAb or C225 administration has no effect on mouse body weight. A, Body weight was recorded for the mice treated with control IgG or anti-SPINK1 monoclonal antibodies. **B,** same as **A** except mice were treated with a combination of SPINK1 monoclonal antibody and C225. **C,** same as **A** except mice were xenografted with PC3 cells.