Supplementary Data

Stomatin-mediated inhibition of the Akt signaling axis suppresses tumor growth

Nor Idayu A. Rahman, Akira Sato, Khurelbaatar Tsevelnorov, Akio Shimizu,
Masahiro Komeno, Mohammad Khusni Bin Ahmat Amin, Md Rasel Molla,
Joanne Ern Chi Soh, Le Kim Chi Nguyen, Akinori Wada, Akihiro Kawauchi, Hisakazu Ogita

This file includes:

Supplementary Materials and Methods

Cell culture

LNCaP cells, PC3M cells, and 22Rv1 cells were cultured in RPMI 1640 medium (Nacalai Tesque, Kyoto, Japan). VCaP cells and HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (Nacalai Tesque). HepG2 cells were cultured in Eagle Minimum Essential Medium (Wako Pure Chemical Corporation, Osaka, Japan). All media were supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, Saint Louis, MO, USA), 20 mmol/L L-glutamine (Nacalai Tesque) and 100 u/mL penicillin-streptomycin (Nacalai Tesque). Human prostate stromal cells were cultured in Stromal cell basal medium supplemented with growth factors (Lonza, Basel, Switzerland).

Mammalian expression plasmids and introduction of plasmids into cells

The full-length cDNA of human *STOMATIN* was obtained by reverse transcription PCR using RNA extracted from HeLa cells. Primers for human *STOMATIN* were as follows: 5'-ACTCGAGCGGCCGCCATGGCCGAGAAGCGGCACACAC-3' and 5'-TGGTGGAATTCTGCCTAGATGGCTGTGTTTTGC-3'. *STOMATIN* cDNA was cloned into pEGFP-N1 and pTetOne vectors (Clontech, Mountain View, CA, USA). The cDNAs of full-length human PDPK1, and N-terminus (PDPK1-N: aa 1-241) and C-terminus (PDPK1-C: aa 224-556) domain mutants of PDPK1 were obtained by reverse transcription PCR using RNA extracted from HepG2 cells. Primers for full-length human PDPK1 were as follows: 5'-AAAGAATTCATGGCCAGGACCACCAGCCAGCTG-3' and 5'-TTTGTCGACCTGCACAGCGGCGTCCGGGTGGCTC-3'. Primers for human PDPK1-N were as follows: 5'-AAAGAATTCATGGCCAGGACCACCAGCCAGCTG-3' and 5'-TTTGTCGACTGAGTTGGCCCTGGCTTGTTTGCTC-3'. Primers for human PDPK1-C were as follows: 5'-TTTGAATTCACCATGTTTGGAACAGCAAAAGTCTTATCC-3' and 5'-TTTGTCGACCTGCACAGCGGCGTCCGGGTGGCTC-3'. cDNAs of full-length human *PDPK1* and its deletion mutants were cloned into the pFLAG-CMV5a vector (Sigma-

Aldrich). The plasmid expressing AKT-DD (HA PKB T308D S473D pcDNA3) was purchased from Addgene (Watertown, MA, USA). The pSilencer 2.1-U6 hygro vector expressing shRNA for GFP, which was used as a control shRNA (shControl), was gifted from Dr. M. Ema at Research Center for Animal Life Science, Shiga University of Medical Science. For construction of pSilencer 2.1-U6 hygro vector expressing shRNA for stomatin (shStomatin), DNA coding shRNA for GFP in the vector was replaced with the hairpin siRNA template annealed with following synthetic oligos: 5'-GATCCGGAGATCCTCACAAAGGATTTCAAGAGAATCCTTTGTGAGGATCTCCTTTTTTGGAAA-3', 5'-AGCTTTTCCAAAAAAGGAGATCCTCACAAAGGATTCTCTTGAAATCCTTTGTGAGGATCTCCG-3'. All sequences inserted into the vectors were confirmed by sequencing on an ABI Prism 3100xl Genetic Analyzer (Applied Biosystems, Waltham, MA, USA). For introduction of vectors into the cells, Neon Transfection System (Invitrogen, Carlsbad, CA, USA) or Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific, Waltham, MA, USA) was applied. To generate stable LNCaP cell lines with doxycycline (Dox)-inducible expression of GFP or stomatin-GFP and stable 22Rv1 cell lines expressing shControl or shStomatin, transfected cells were selected with 100 μg/mL hygromycin (Nacalai Tesque) in RPMI medium supplemented with 10% FBS. To generate stable PC3M cell lines with Dox-inducible expression of GFP or stomatin-GFP, transfected cells were selected with 1 μg/mL puromycin (Invitrogen) in RPMI medium supplemented with 10% FBS. Colonies of antibiotic-resistant cells were isolated and expanded for the further experiments.

Cell proliferation assay

LNCaP, HEK293T, PC3M, HepG2 or 22Rv1 cells transfected as indicated were plated on cell culture dishes. LNCaP or PC3M cells with the Dox-inducible expression of stomatin-GFP or GFP were treated with 1 μg/mL Dox (Tokyo Chemical Industry, Tokyo, Japan) from the day of cell seeding or 6 d after seeding. When the apoptosis inhibitor Z-VAD (R&D Systems, Minneapolis, MN, USA) was used in assays, cells were treated with both 5 μmol/L Z-VAD

and 1 μg/mL Dox. Cells were counted by using the cell counting chamber (Erma, Tokyo, Japan) at the indicated time points.

Immunofluorescence staining

For cell staining, cells grown on glass coverslips coated with fibronectin were fixed with 4% (w/v) paraformaldehyde (PFA) and permeabilized with 0.2% (w/v) Triton X-100 for 5 min. For tissue staining, cryosections were fixed in 4% PFA, washed with PBS for 15 min, and permeabilized with 0.3% Triton X-100 for 5 min. Non-specific staining was reduced by blocking with 3%–5% bovine serum albumin for 30 min at room temperature. Samples were incubated overnight at 4°C with the primary antibody at appropriate dilutions as described in Supplementary Table S1. The secondary fluorescent antibodies Alexa Fluor 488 and Alexa Fluor 555 (1:200-1000 dilution) (Thermo Fisher Scientific) were applied for 1 h at room temperature in the dark. After washing samples with PBS, nuclei were stained with DAPI (1:200 dilution) (Dojindo, Kumamoto, Japan) for 5 min. The cells were viewed using a confocal microscope (FV1000-D, Olympus, Tokyo, Japan, or TCS SP8 X, Leica, Wetzlar, Germany). To visualize the cell membrane, Wheat Germ Agglutinin Red Molecular probe (Invitrogen) was added into the culture media at a ratio of 1:250, and cells were incubated in the CO_2 incubator at 37°C for 8 min. The cells were washed with Hank's Balanced Salt Solution (HBSS; Thermo Fisher Scientific), directly fixed with 4% paraformaldehyde in PBS (Nacalai Tesque) for 15 min, and visualized on a fluorescence microscope.

TUNEL assay

LNCaP cells transfected with stomatin-GFP or GFP plasmids were stained using the Click-iT Plus TUNEL assay with Alexa Fluor 647 dye (Invitrogen) according to the manufacturer's protocol. DAPI (1:500 dilution) was applied for 15 min at room temperature. TUNEL-positive cells among GFP-positive cells were counted by confocal fluorescence microscopy.

Histological analysis of tumors

Cryosections of mouse xenograft tumors were fixed in 4% PFA, washed with PBS for 15 min, and stained with hematoxylin and eosin. The images were viewed and taken using a light microscope (FXA, Nikon, Tokyo, Japan).

siRNA transfection

Stomatin and FOXO3a siRNAs and negative control RNA (Scramble) were produced using the CUGA7 *in vitro* transcription kit (Nippon Gene, Tokyo, Japan). HSP90 siRNA was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). siRNA sequences were as follows: stomatin siRNA #1 5'-GGAGAUCCUCACAAAGGAU-3', stomatin siRNA #2 5'-GCAGACACUGACCACCAUU-3', FOXO3a siRNA 5'-GGACAATAGCAACAAGTAT-3', and Scramble 5'-CAGUCGCGUUUGCGACUGG-3'. siRNA transfection was performed using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen).

Immunoprecipitation

Cells were lysed in RIPA lysis buffer (50 mmol/L Tris-HCl [pH 7.5], 150 mmol/L NaCl, 0.5% deoxycholate sodium, 0.1% SDS, 1% Nonidet P-40, 1 mmol/L phenylmethylsulfonylfluoride (PMSF), 1 μg/mL leupeptin, 1 μg/mL aprotinin, 5 mmol/L sodium fluoride, and 1 mmol/L sodium orthovanadate) and centrifuged at 20,000 x *g* for 10 min. The supernatants were pre-incubated with protein G Sepharose beads (GE Healthcare, Fairfield, CT, USA) and after centrifugation, supernatants were incubated with primary antibody (1:100 dilution) overnight at 4°C. Next, 15–20 μL of protein G Sepharose beads were added and samples were incubated for 2 h at 4°C. Samples were centrifuged at 3,000 × *g* for 1 min at 4°C, and beads were washed three times with NP-40 lysis buffer (20 mmol/L Tris-HCl [pH 8.0], 137 mmol/L NaCl, 10% glycerol, 1% Nonidet P-40, 1 mmol/L PMSF, 1 μg/mL leupeptin, 1 μg/mL aprotinin, 5 mmol/L sodium fluoride, and 1 mmol/L sodium orthovanadate), eluted in loading buffer, and used in western blotting.

Immunoblotting

Cells were lysed in RIPA lysis buffer, and the lysates were centrifuged at 20,000 x *g* for 10 min. The supernatants were separated by SDS-PAGE, followed by blotting on polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was incubated with primary antibody overnight in 5% skim milk at 4°C. Horseradish peroxidase (HRP)-labeled secondary antibody (GE Healthcare) was applied for 2 h in 5% skim milk. To visualize the protein bands, the membrane was treated with HRP substrate (Luminata Forte, Millipore Corp., Billerica, MA, USA) for 5 min and observed on a luminescent image analyzer LAS-4000 (Fujifilm Life Science, Tokyo, Japan). Band densities were analyzed using ImageJ software.

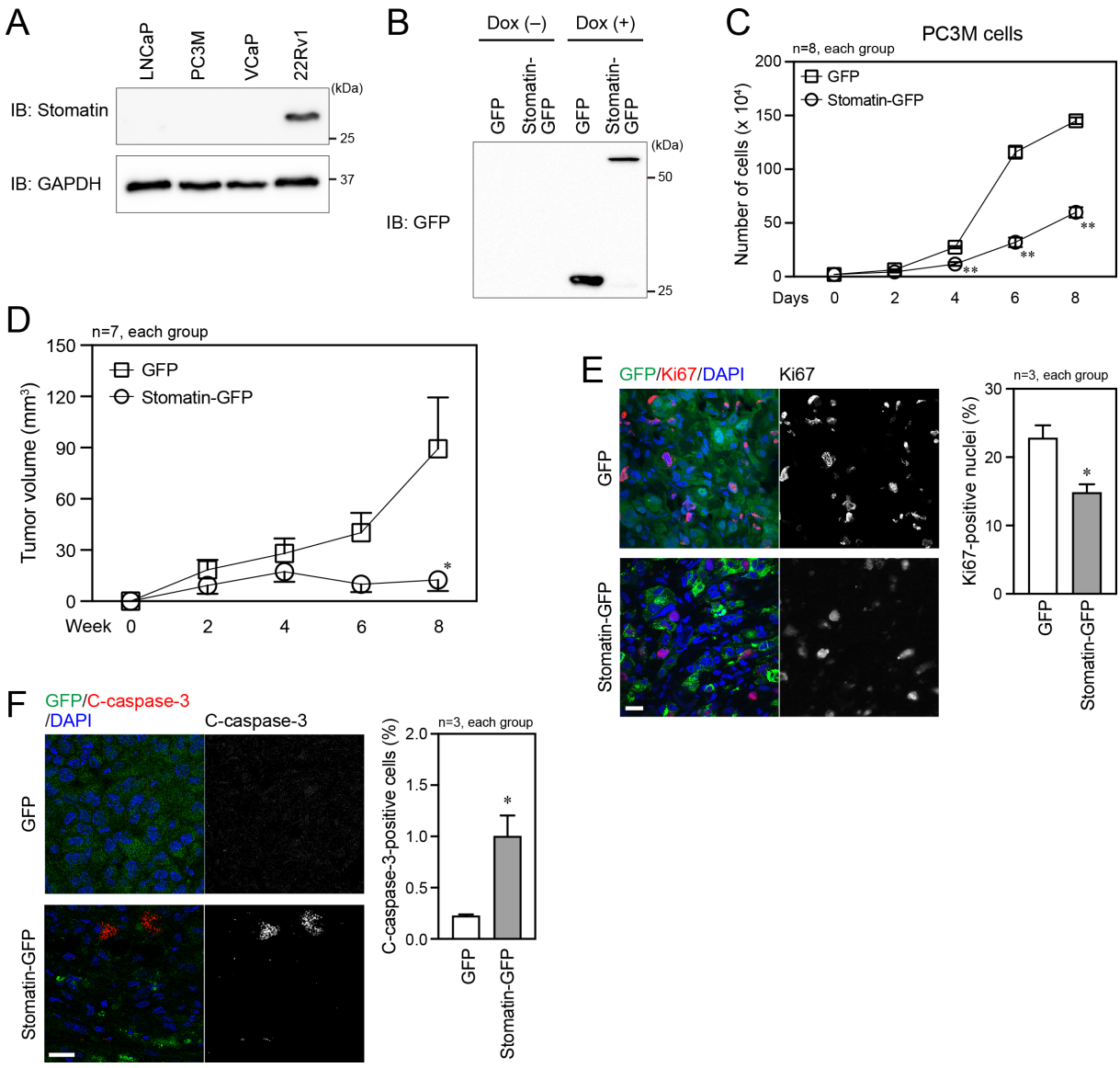
Bacterial expression plasmids

The full-length cDNAs of human *STOMATIN* and human *PDPK1* were obtained by reverse transcription PCR using RNA extracted from HeLa cells. Primers for human *STOMATIN* were as follows: 5'-CTGGGATCCATGGCCGAGAAGCGGCACAC-3' and 5'-TCCGCTCGAGCTAGCCTAGATGGCTGTGTTTTGC-3'. Primers for human *PDPK1* were as follows: 5'-GGGGAGCTCCATGGCCAGGACCACCAGCCAGCTG-3' and 5'-GGGGTCGACTCACTGCACAGCGGCGTCCGGGTGGCTC-3'. *STOMATIN* and *PDPK1* cDNAs were cloned into pGEX-6P-1 vector (MERCK, Kenilworth, NJ, USA) and pQE-31 vector (Creative Biogene, Shirley, NY, USA), respectively, to generate recombinant proteins. All sequences inserted into the vectors were confirmed by sequencing.

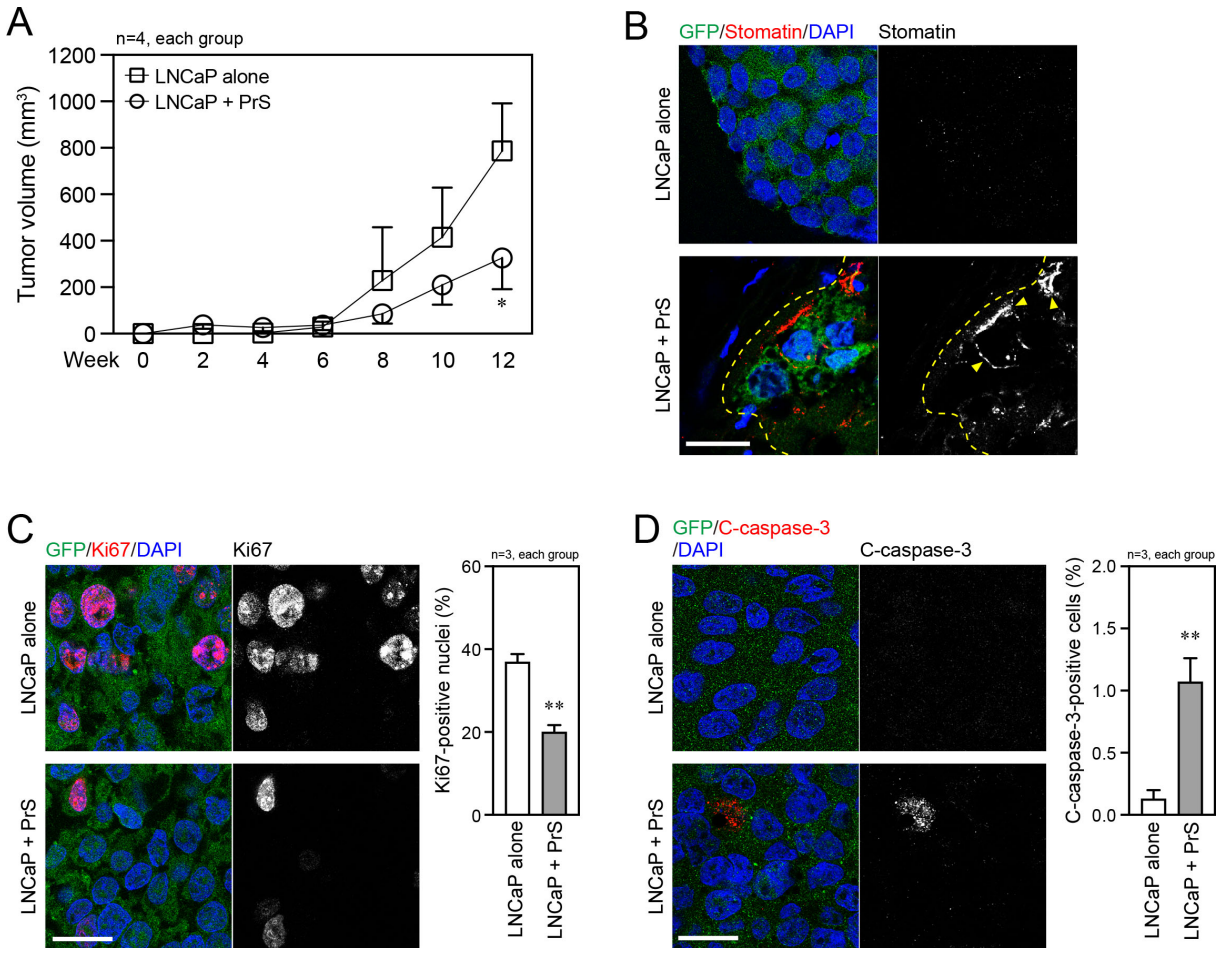
Glutathione-*S*-transferase (GST)-pull down assay

Bacterial cells of the DH5α *E. coli* strain transformed with the pGEX-6P or pGEX-6P-stomatin plasmids were lysed in Tris buffer (50 mmol/L Tris-HCl [pH 7.4], 150 mmol/L NaCl, 1% Triton-X 100, 1 mmol/L PMSF, 1 μg/mL leupeptin, 1 μg/mL aprotinin), sonicated by ultrasonic homogenizer (MICROTEC, Chiba, Japan), and centrifuged at 20,000 x *g* for 10 min. The supernatants were incubated with Glutathione Sepharose 4B beads (GE Healthcare)

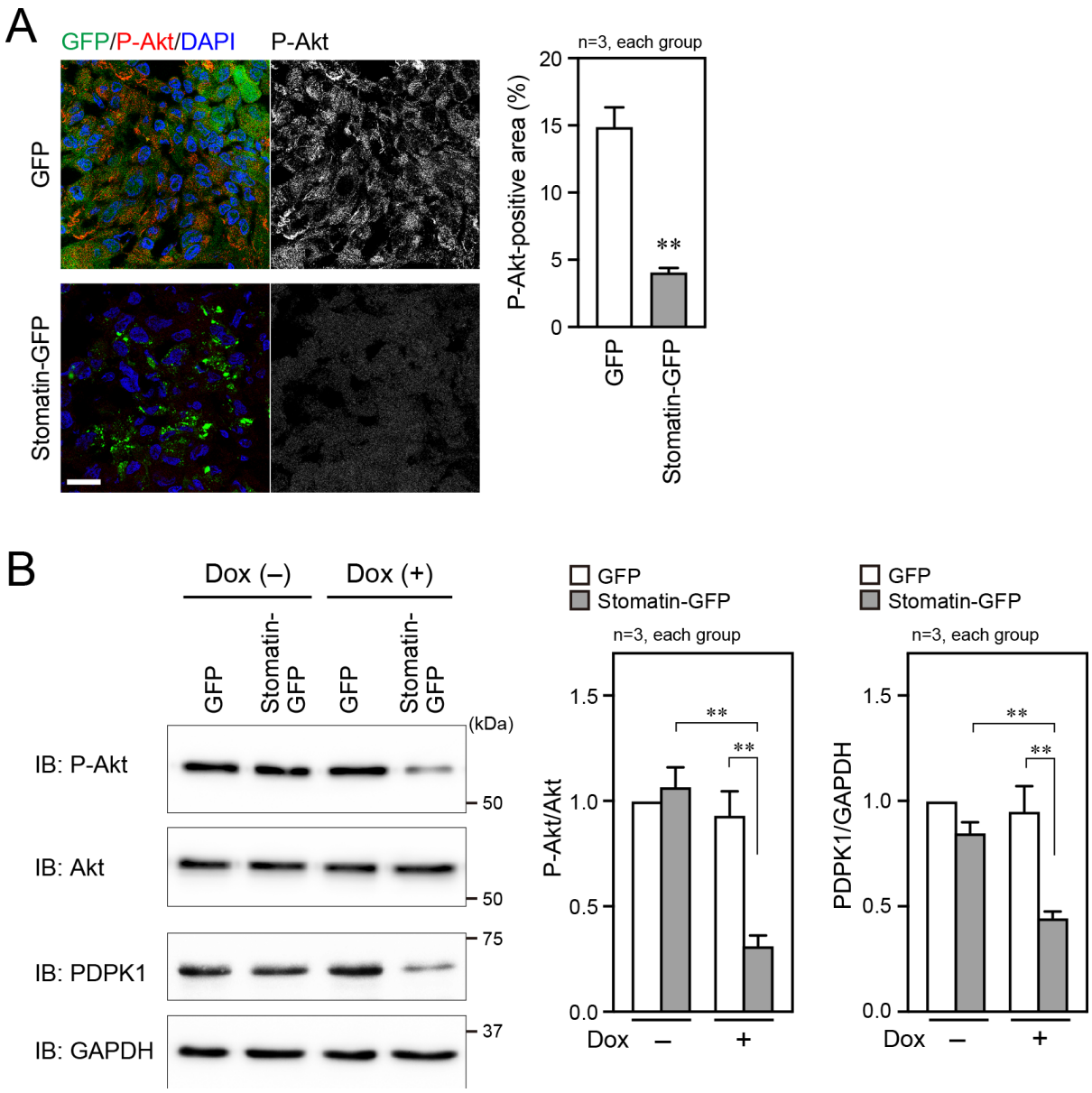
overnight at 4°C and the beads bound to GST or GST-tagged stomatin were washed three times with Tris buffer after centrifugation. 6 x His-tagged PDPK1 (His-PDPK1) was produced in the bacterial cells transformed with the pQE-31-PDPK1 plasmid and purified with HiTrap Chelating HP column (Cytiva, Gland, Switzerland). LNCaP cell supernatant or purified His-PDPK1 was incubated with the beads bound to GST or GST-tagged stomatin overnight at 4°C. Next, beads were washed three times with Tris buffer after centrifugation (3,000 × *g* for 1 min at 4°C), eluted in loading buffer, and used in Coomassie Brilliant Blue (CBB) staining and western blotting.



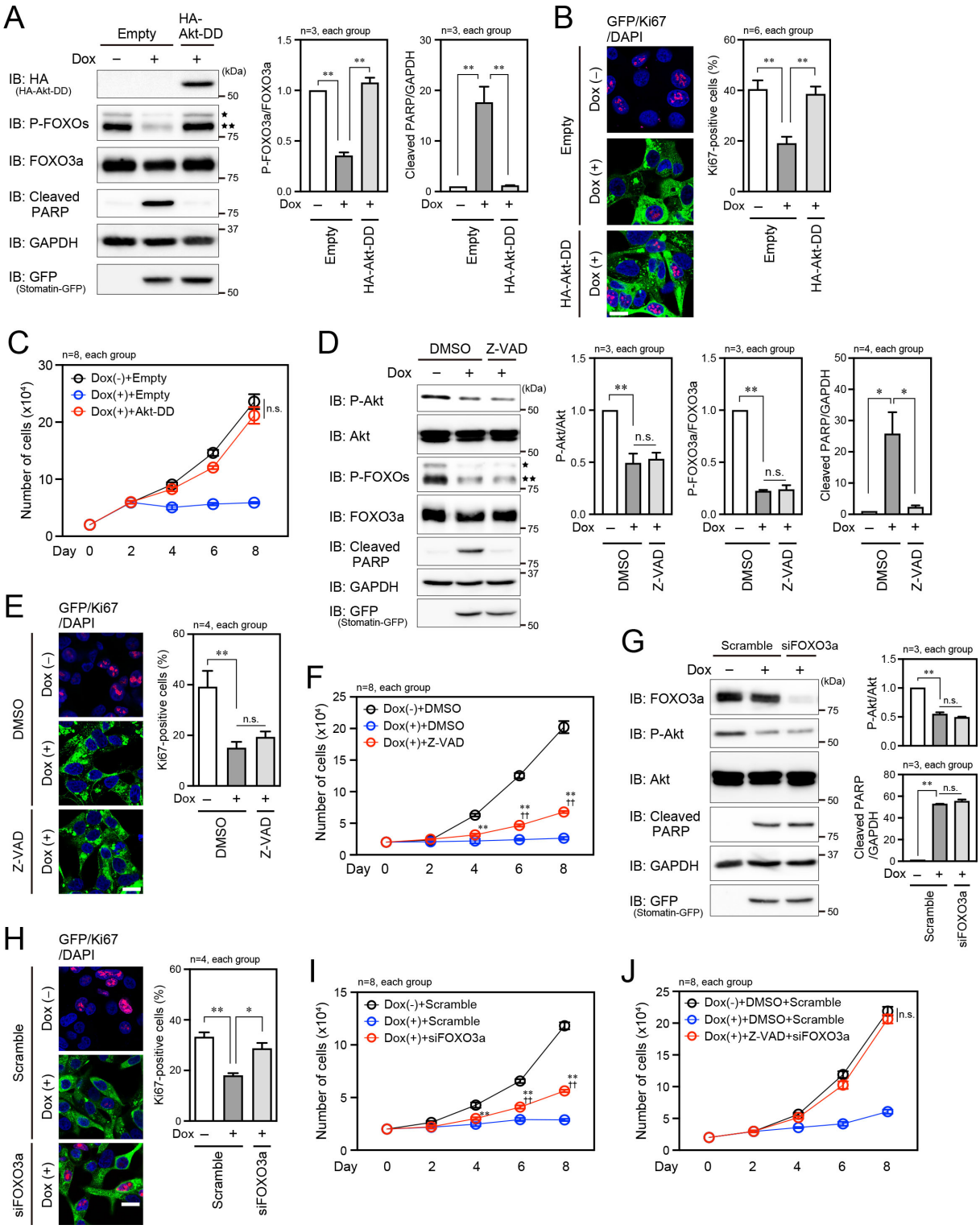
Supplementary Figure 1. Inhibition of tumor growth by Dox-inducible stomatin expression in PC3M prostate cancer cells. (**A**) Evaluation of endogenous stomatin expression in several prostate cancer cells by western blotting. (**B**) PC3M cells expressing the Dox-inducible GFP or stomatin-GFP were treated with or without 1 μg/mL Dox for 48 h. Cell lysates were analyzed by western blotting to confirm the expression of GFP or stomatin-GFP after Dox treatment. (**C**) Number of PC3M cells at the indicated time points. At day 0, 2.0×10^4 cells in each group were seeded on the cell culture dish and were treated with Dox just after seeding. (**D**) Tumor volume at the indicated time points after the subcutaneous injection of PC3M cells expressing the Dox-inducible stomatin-GFP or GFP in NOD/SCID mice. Mice were treated with Dox in the drinking water just after the injection of PC3M cells. (**E** and **F**) (Left panels) Immunohistochemical analyses of tumors formed subcutaneously in the mice with the indicated antibodies. Scale bars, 20 μm. (Right panels) Quantification of Ki67-stained nuclei (**E**), and C-caspase-3-positive cells (**F**), respectively. In (**C**–**F**), data are means ± SEM from independent experiments of which number is indicated in each figure. * P<0.05 and ** P<0.01 vs. GFP.



Supplementary Figure 2. Inhibition of tumor growth by co-injection of LNCaP cells together with PrS cells in the mice. (**A**) Tumor volume at the indicated time points after the subcutaneous injection of LNCaP cells expressing the Dox-inducible GFP alone or co-injection of LNCaP cells together with PrS cells (LNCaP + PrS). Mice were treated with Dox in the drinking water three days after the injection of the cells. (**B**) Confirmation of stomatin expression in LNCaP cells after co-injection of LNCaP and PrS cells by immunohistochemistry. Dotted lines: the boundary of LNCaP cells and PrS cells. (**C** and **D**) (Left panels) Immunohistochemical analyses of tumors with the indicated antibodies. Scale bars, 20 μm. (Right panels) Quantification of Ki67- and C-caspase-3-positive nuclei and cells, respectively. In (**A**, **C** and **D**), data are means ± SEM from independent experiments of which number is indicated in each figure. * P<0.05 and ** P<0.01 vs. LNCaP alone.

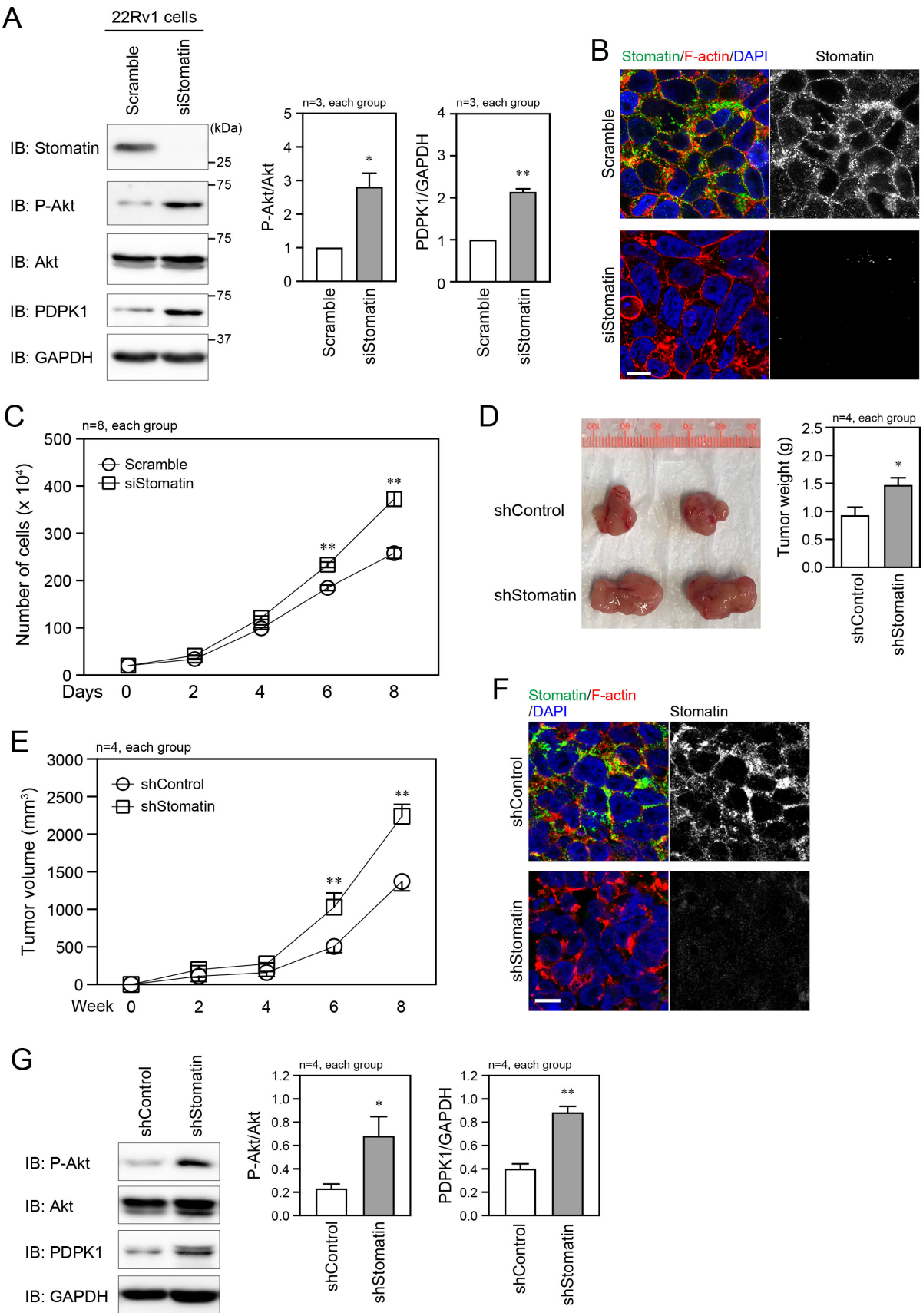


Supplementary Figure 3. Inhibition of Akt phosphorylation and PDPK1 expression by stomatin in PC3M cells. (**A**) (Left panels) Immunohistochemical staining of phosphorylated Akt (P-Akt) in tumors formed after the subcutaneous injection of PC3M cells expressing the Dox-inducible stomatin-GFP or GFP in NOD/SCID mice. Mice were treated with Dox in the drinking water just after the injection of PC3M cells. Scale bars, 20 μm. (Right panel) Quantification of P-Akt-positive area. (**B**) (Left panels) PC3M cells expressing the Dox-inducible GFP or stomatin-GFP were treated with or without 1 μg/mL Dox for 48 h. Cell lysates were analyzed by western blotting with the indicated antibodies. (Right panels) Quantification of relative band density of P-Akt and PDPK1. The relative band density in the group of PC3M cells expressing the Dox-inducible GFP without Dox treatment was set at 1.0. Data are means ± SEM from independent experiments of which number is indicated in each figure. ** P<0.01 vs. GFP (**A**) or between the groups (**B**).



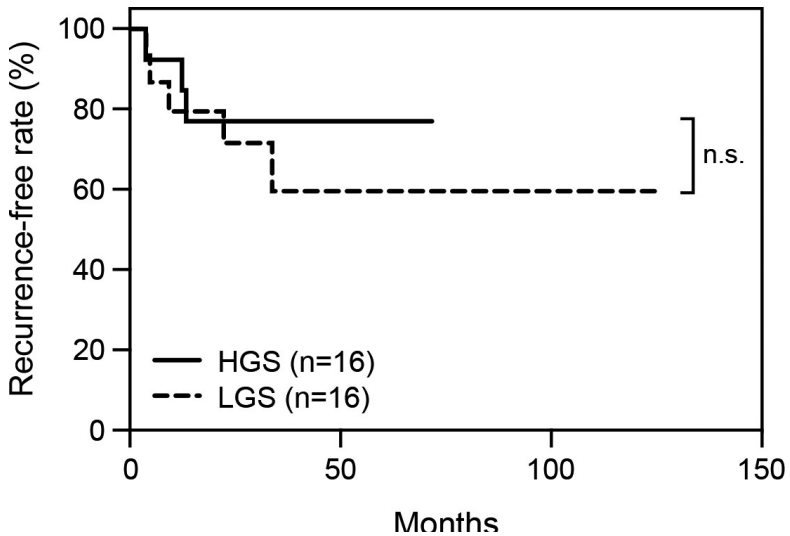
Supplementary Figure 4. Stomatin–Akt signaling axis for regulating prostate cancer cell proliferation and apoptosis. (**A**, **D** and **G**) (Left panels) LNCaP cells expressing the Dox-inducible stomatin-GFP, together with HA-Akt-DD transfection (**A**), Z-VAD treatment (**D**) or siFOXO3a transfection (**G**), were treated with or without 1 μg/mL Dox for 48 h. Cell lysates were analyzed by western blotting with the indicated antibodies. In (**A** and **D**), ★ P-FOXO1, and ★★ P-FOXO3a. (Right panels) Quantification of relative band densities in western blotting. The relative band density in the group of LNCaP cells without Dox treatment was set at 1.0. (**B**, **E** and **H**) Ki67 staining in LNCaP cells (Left panels) and quantification of the ratio of Ki67-positive cells (Right panels). (**C**, **F**, **I** and **J**) Number of indicated LNCaP cells at the indicated time points. At day 0, 2.0×10^4 cells in each group were

seeded on the cell culture dish with indicated treatment. * $P<0.05$ and ** $P<0.01$; †† $P<0.01$ vs. Dox-treated LNCaP cells with empty vector transfection (**C**), DMSO (**F**) or scramble RNA transfection (**I**).



Supplementary Figure 5. Inhibitory effect of endogenous stomatin on prostate cancer cell growth. (**A**) (Left panels) Cell lysates of 22Rv1 prostate cancer cells transfected with siStomatin or scramble RNA were analyzed by western blotting with the indicated antibodies. (Right panels)

Quantification of relative band densities of P-Akt and PDPK1. The relative band density in the scramble was set at 1.0. (**B**) 22Rv1 cells transfected with siStomatin or scramble RNA were stained with the anti-stomatin antibody (green). Plasma membrane and nuclei were visualized with Alexa Fluor 546-conjugated phalloidin (red) and DAPI (blue), respectively. Scale bar, 10 μm. (**C**) Number of 22Rv1 cells at the indicated time points after transfection of siStomatin or scramble RNA. Just after transfection, 2.0×10^5 cells in each group were seeded on the cell culture dish. (**D**) (Left panel) Tumors extracted from the mice 8 weeks after injection of the 22Rv1 cells stably expressing shControl or shStomatin. (Right panel) Summary graph of tumor weight. (**E**) Tumor volume at the indicated time points after injection of the cells. (**F**) Immunohistochemical staining of stomatin in tumors as described in (**B**). Scale bar, 10 μm. (**G**) (Left panels) Tumor lysates were analyzed by western blotting with the indicated antibodies. (Right panels) Quantification of relative band densities of P-Akt and PDPK1 as described in (**A**). In (**A**, **C**, **D**, **E** and **G**), data are means ± SEM from independent experiments of which number is indicated in each figure. * $P<0.05$ and ** $P<0.01$ vs. Scramble or shControl.



Supplementary Figure 6. Kaplan-Meier curve for the recurrence-free rate after the operation in the lower and higher GS groups.