Supplemental Information

CKAP4 is involved in tumor progression as a Dickkopf1 receptor

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Supplemental Methods

Antibodies

Antibodies against HSP90 (catalog 610418) and Clathrin (catalog 610500) were obtained from BD Biosciences (San Jose, CA). Antibodies against Ki-67 (catalog 9449), LRP6 (catalog 2560), pAKT (S473, catalog 9271), AKT (catalog 9272), pErk1/2 (catalog 4370), Erk1/2 (catalog 4695), pSAPK/JNK (T183/Y185, catalog 9255), SAPK/JNK (T183/Y185, catalog 9252), pSrc family (Tyr416, catalog 6943), Src (catalog 2123), p110α (catalog 4249), p85α (catalog 4257), and control IgG (Mouse (G3A1), catalog 5415 and Rabbit (DA1E), catalog 3900) were obtained from Cell Signaling Technology (Beverly, MA). Antibodies against HA (Y-11) (catalog sc-805), PKCζ (C-20) (catalog sc-216), Dkk1 (H-120) (catalog sc-25516), and GFP (B-2) (catalog sc-9996) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against FLAG (catalog NBP1-067122) and Podoplanin (catalog NBP600-1015) were obtained from Novus Biologicals (Littleton, CO). Antibodies against CKAP4 (catalog ALX-804-604), Dkk1 (catalog MAB10962 and catalog AF1096), TfR (catalog 13-6800), Prosurfactant Protein C (catalog AB3786), pAKT (S473, catalog 600-401-268), tPA (catalog 10147-1-AP), and Surfactant Protein A (catalog GTX106594) were obtained from Enzo Life Science (Farmingdale, NY), R&D Systems (Minneapolis, MN), Life Technologies (Carlsbad, CA), Merck Millipore (Billerica, MA), ROCKLAND (Limerick, PA), proteintech (Chicago IL), and GeneTex (Irvine, CA), respectively. Antibody against p85α (catalog ab22653 and catalog ABS234) was obtained from Abcam (Cambridge, United Kingdom) and Merck Millipore (Billerica, MA), respectively.

Enzyme-linked immunosorbent assay (ELISA) for measuring Dkk1 concentration

The concentration of Dkk1-FLAG secreted from MDCK cells into conditioned medium was measured by a sandwich ELISA system, which is the basically same as a previously described assay

(1). Fifty μl of 0.013 nM anti-Dkk1 goat polyclonal antibody was added to a 96-well microplate as a capture antibody and incubated for overnight at 4 °C. After washing away any unbound antibody using wash buffer (0.05% Tween 20 in PBS), reagent diluent (1% BSA in PBS) was added to the wells and incubated for 1 hour at room temperature for blocking. After washing, 50 μl of diluted conditioned medium were added to the wells and incubated for 2 hours at room temperature. After washing away any unbound substances, 50 μl of 0.013 nM anti-Dkk1 goat polyclonal antibody, which is biotinylated using Biotin Labeling Kit-NH2 (DOJINDO, Kumamoto, Japan), was added to wells and incubated for 1 hour at room temperature. After washing, 50 μl of HRP-streptavidin (DY998, R&D Systems, Inc., Minneapolis, MN) diluted in reagent diluent was added to the wells and incubated for 20 minutes at room temperature. Then, a substrate solution (DY999, R&D Systems, Inc., Minneapolis, MN) was added to the wells and allowed to react for 10 minutes. The reaction was stopped by adding 50 μl of STOP solution (Cell Signaling Technology, Beverly, MA). Color intensity was determined by a photometer at a wavelength of 450 nm. As a reference, a standard curve was prepared for every assay by making serial dilutions of recombinant Dkk1 protein ranging from 0.04 to 0.64 nM

In-Gel Digestion

In-gel digestion was performed as described (2). Gel pieces including specific bands were excised from the Silver-stained gels and destained by washing with a 1:1 solution of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate and equilibrated in 200 mM ammonium bicarbonate for 20 minutes to pH 8.0. Gel pieces were then soaked in acetonitrile for 5 minutes, acetonitrile was removed and the gel pieces were dried for 20 minutes in a vacuum. Prior to enzymatic digestion, gel pieces were reduced with 10 mM dithiothreitol in 50 mM ammonium bicarbonate at 37 °C for 30 minutes, then alkylated with 55 mM iodoacetamide in 50 mM ammonium bicarbonate for 30 minutes and dehydrated by addition of acetonitrile. The reduced and alkylated gel pieces were rehydrated in

50 mM Tris-HCl pH 9.0 and 0.5 µg/ml trypsin. Once this solution was fully absorbed by the gel pieces, enzyme-free Tris-HCl buffer was added until the gel pieces were covered. The samples were digested for 16 hours at 37 °C, extracted with 50% acetonitrile and 5% formic acid for 20 minutes and then the acetonitrile was evaporated using a Speed-Vac centrifuge. The tryptic digests were desalted with C18-StageTips, concentrated using the Speed-Vac centrifuge and reconstituted in 0.1% formic acid.

LC-MS/MS Analysis

LC-MS/MS analysis was performed as described (2) using an UltiMate 3000 Nano LC System (Thermo Fisher Scientific, Waltham, MA) coupled to Q-Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific) with a nano-electrospray ionization source. Digested sample was injected by an autosampler and enriched on a C18 reverse phase trap column (100 µm I.D. x 5 mm length, Thermo Fisher Scientific) at a flow rate of 4 µl/minute. The sample was subsequently separated by a 15-cm fused silica emitter packed in-house with reversed phase resin (RepruSil-Pur C18-AQ, 3 µm, Dr. Maisch GmbH) at a flow rate of 500 nl/minute with a linear gradient from 2% to 35% mobile phase B. Mobile phase B consisted of 90% acetonitrile with 0.1% formic acid, whereas mobile phase A consisted of 2% acetonitrile with 0.1% formic acid. The peptides were ionized using nano-electrospray ionization in positive ion mode.

The raw data files were analyzed by Mascot Distiller v2.2 (Matrix Science, Boston, MA) to create peak lists on the basis of the recorded fragmentation spectra. Peptides and proteins were identified by Mascot v2.3 (Matrix Science) against the UniProt database with a precursor mass tolerance of 10 ppm, a fragment ion mass tolerance of 0.01 Da and strict trypsin specificity allowing for up to two missed cleavage. Carbamidomethylation of cysteine residues was set as fixed modification while oxidation of methionine residues was allowed as variable modifications.

Receptor Binding Assay

To measure the binding activity of Dkk1 to CKAP4, various concentrations (0-16 nM) of Dkk1 (R & D Systems, Minneapolis, MN) was incubated with 0.5 nM GST-CKAP4-ECD or 0.5 nM GST in 500 µl of NP40 buffer by rotating for 2 hours at 4 °C. Then, GST-CKAP4-ECD and GST were collected by centrifugation followed by washing with NP40 buffer three times. The precipitates were probed with anti-Dkk1 antibody and then analyzed by ImageJ software (National Institutes of Health, Bethesda, MD).

Polarized Secretion of Dkk1 and Localization of CKAP4

MDCK/Dkk1-FLAG cells (2 x 10^5 cells) were seeded on Transwell polycarbonate filters (Corning Costar Quality Biological, Gaithersburg, MD) (3). To detect Dkk1 secreted into the culture medium of polarized MDCK cells, media from the apical and basolateral sides were incubated with Blue Sepharose for 2 hours at 4 °C, followed by centrifugation. The precipitates were probed using anti-Dkk1 antibody.

To detect cell-surface-localization of CKAP4, the apical or basolateral surface membranes of polarized MDCK cells on Transwell polycarbonate filters were selectively incubated with 0.5 mg/ml sulfo-NHS-LC-biotin (Pierce Biotechnology, Rockford, IL) for 30 minutes at 4 °C (3-5). Biotinylated cells were lysed in 500 µl of NP40 buffer with protease inhibitors (10 µg/ml leupeptin, aprotinin, and 1 mM phenylmethanesulfonyl fluoride). After 10-minutes centrifugation, 50 µl of lysates were dissolved in Laemmli's sample buffer and used as total cell lysate. Total cell lysate included membrane proteins, cytosolic proteins, and nuclear proteins. The rest of total cell lysate containing biotinylated proteins were precipitated using NeutrAvidin Agarose beads (Pierce Biotechnology) and beads were washed twice with 1 ml of NP40 buffer and once with 1 ml of 10 mM Tris-HCl pH 7.5 and the bound complexes were dissolved in 20 µl of Laemmli's sample buffer. The precipitates were probed using anti-CKAP4 antibody.

Cell Proliferation Assay

MDCK, S2-CP8, A549, and HeLaS3 cells were seeded at densities of 1×10^5 /ml in a 35-mm dish two dimensionally. MDCK and HeLaS3 cells were cultured in DMEM containing 10% FBS and S2-CP8 and A549 cells were cultured in DMEM containing 1% FBS. Cells were enumerated on the indicated days.

Three-dimensional (3D) Culture Assay

3D Matrigel culture studies were performed as previously described (6, 7) with modification. To analyze cyst formation of MDCK cells and growth of S2-CP8, A549, SUIT-2, HepG2, and HeLaS3 spheres in Matrigel (BD Biosciences, San Jose, CA), 40 μl of Matrigel was mounted on a round coverslip and incubated for 30 minutes at 37 °C to solidify the gel. MDCK cells (3×10^4 cells) suspended in DMEM containing 10% FBS and 2% Matrigel and S2-CP8, A549, SUIT-2, HepG2, and HeLaS3 cells (2×10^4 cells) suspended in DMEM containing 0.1% bovine serum albumin and 2% Matrigel were added on the solidified Matrigel and cultured for 5 days.

CTOS Preparation and Culture

CTOSs were prepared as described (8, 9). Briefly, surgical specimens and pleural effusion samples from lung cancer patients were obtained from Osaka Medical Center for Cancer and Cardiovascular Diseases, with the patients' informed consent. Tumor specimens were minced, washed, and digested with Liberase DH solution (Roche). The partially digested tissue was filtered through cell strainer (Becton Dickinson), and the tumor tissue retained in the strainer was collected to StemPro hESC (Invitrogen, Carlsbad, CA). After these tissues were cultured in suspension in StemPro hESC and transplanted subcutaneously into the flanks of NOD/SCID mice (CLEA Japan, Inc). The CTOSs were prepared from mouse xenografts.

For assessment of the inhibitory effect of anti-CKAP4 antibody on CTOS growth, CTOSs were

embedded in Matrigel Growth Factor Reduced (GFR) (BD Biosciences, Bedford, MA), cultured in StemPro hESC, and treated with anti-GST or CKAP4 antibody at the indicated doses. The growth ratio of CTOSs at day 7 relative to day 0 was analyzed by ImageJ software.

AKT Assay

When AKT activity was measured in Figure 3E and 8C, MDCK cells were treated 1 nM nocodazole for 24 hours to arrest cells in the M phase (10) and further incubated with 166 nM anti-CKAP4 or anti-GST antibody for 4 hours. Then nocodazole was removed and the cells were stimulated with 10 nM Dkk1 or 1 nM insulin for 30 minutes in the presence of 166 nM anti-CKAP4 or anti-GST antibody.

Cell migration assay

To measure the cell activity, Transwell assays were done using a modified Boyden chamber (tissue culture treated, 6.5 mm in diameter, 10-μm thick, 8-μm pores; Transwell, Costar, Cambridge, MA) as described previously (11, 12). The lower surface of the filter was coated with 10 μg/ml type I collagen for 2 hours. Then, S2-CP8 and A549 (2.5×10^4 cells in 100 μl) cells suspended in serum-free DMEM containing 0.1% bovine serum albumin (BSA) were applied to the upper chamber. The same medium was added to the lower chamber. After the cells were incubated at 37°C for 4 to 8 hours, cells that migrated to the lower side of the upper chamber were fixed with PBS containing 4% (w/v) paraformaldehyde (PFA) and counted.

Knockdown of Protein Expression by SiRNA

Cells were transfected with a mixture of siRNAs (20 nM each) against genes of interest using RNAiMAX (Invitrogen, Carlsbad, CA). The transfected cells were then used for experiments 36-48 hours post-transfection. The following target sequences were used. Human Dkk1 #1,

5’GCTCTCATGGACTAGAAAT-3’; human Dkk1 #2, 5’-CCTGGAGTGTAAGAGCTTT-3’; human CKAP4 #1, 5’-GCTGCAGAAACTCCAGAAT-3’; human CKAP4 #2, 5’-GCAGATTAACCTCAGAAAT-3’; human t-PA #1, 5’-GGAATTCCATGATCCTGAT-3’; human t-PA #2, 5’-GCATCAAAGGAGGGCTCTT-3’; dog CKAP4, 5’-GCAGAAGGTGCAGTCTCTT-3’; dog LRP5, 5’-GCAGATTAACCTCAGAAAT-3’; dog LRP6, 5’-GCTATTGCCTTAGATCCTT-3’; dog Kremen1, 5’-CCAGATGCCTGGAAACCTT-3’; dog p110α, 5’-GCTGCTCGACTTTGCCTTT-3’; dog tPA, 5’-GGAGAGAGCTAGTGTGCAT-3’; and control siRNA (scramble) , 5’-CAGTCGCGTTTGCGACTGG-3’.

Plasmid Construction

Dkk1 was tagged with FLAG epitope and glycosylphosphatidylinositol (GPI) anchor signal sequence at the carboxy terminal tandemly (Dkk1-FLAG-GPI). Full length CKAP4 cDNA was amplified from A549 cDNA library and all mutants were constructed by using standard techniques. Lentiviral vectors were constructed by inserting cDNAs into CSII-CMV-MCS-IRES2-Bsd provided by Dr. H. Miyoshi (RIKEN BioResource Center, Ibaraki, Japan), pLVSIN-CMV Pur, or pLVSIN-EF1α Neo Vector (Takara Bio Inc., Shiga, Japan).

Lentivirus Production and Generation of Stable Transformants

To construct a lentivirus vector harboring shRNA, an oligo DNA fragment containing the H1 promoter and shRNA was cloned into CS-RfA-EVBsd using Gateway technology (Invitrogen, Carlsbad, CA). The following target sequences were used. Human Dkk1, 5’-GGGTTTCTTGGAATGACGA-3’; human CKAP4, 5’-GCAGATTAACCTCAGAAAT-3’; human p85α, 5’-CGGTACAGCAAAGAATACATA-3’; control shRNA, 5’-GTGCGTTGCTAGTACCAAC-3’. To generate lentiviruses, the lentiviral vectors were transfected with the packaging vectors, pCAG-HIV-gp and pCMV-VSV-G-RSV-Rev, into X293T

cells using FuGENE HD transfection reagent (Roche Applied Science, Basel, Switzerland) as described (13). For rescue experiments shRNA against the 3'-untranslated region of Dkk1 or CKAP4 was used in Figure 7. To generate MDCK, S2-CP8, and A549 cells stably expressing Dkk1-FLAG, Dkk1-FLAG-GPI, CKAP4-HA, Dkk1 shRNA, or CKAP4 shRNA, parental cells (5 x 10^4 cells/well in a 12-well plate) were transduced with lentivirus containing conditioned medium and 10 μg/ml polybrene. The cells were then centrifuged at 1,200 x g for 1 hour and incubated for another 24 hours.

Immunocytochemistry

For cell staining and image analysis, cells were seeded on coverslips, transwell polycarbonate filter, or Matrigel and fixed with 4% paraformaldehyde for 20 minutes at room temperature. The cells were then washed three times with PBS, blocked for 20 minutes with 1% BSA in PBS including 0.05% Tween-20 (PBST) and further incubated with primary antibodies overnight. After the cells were washed three times with PBS, they were incubated with fluorescent secondary antibodies for 1 hour at room temperature. Coverslips were washed extensively with PBS and then mounted in 50% glycerol in PBS. Processing and measurements were carried out using an LSM510 system (Carl Zeiss Microscopy Co., Ltd, Jena, Germany)

Quantitative PCR

Quantitative PCR was performed as described previously (14). Forward and reverse primers were as follows; human/dog GAPDH, 5'-TCCTGCACCACCAACTGCTT-3' and 5'-TGGCAGTGATGGCATGGAC-3'; human CKAP4, 5'-GGAGCTGAAGAGGAGTGTGG-3' and 5'-TTGACCGAGTATGCAACCAA-3'; human t-PA, 5'-CCCAGATCGAGACTCAAAGC-3' and 5'-TGGGGTTCTGTGCTGTGTAA-3'; human/dog Dkk1, 5'-AAGCGCCGAAAACGCTGCAT-3' and 5'-GATCTTGGACCAGAAGTGTC-3';dog AXIN2, 5'-CTGGCTCCAGAAGATCACAAAG-3'

and 5’-CATCCTCCCAGATCTCCTCAAA-3’; dog LRP5, 5’-TGGGGGACTTCATCTACTGG-3’ and 5’-CAGTAGCTCTAGGCCGATGG-3’; dog LRP6, 5’-GGTACTGAAAGGCGAGCAAG-3’ and 5’-AAAGCTTGCCCAATCTGCTA-3’; dog Kremen1, 5’-AAGCCAATCTCAGCGTCAGT-3’ and 5’-TCCAGATTTCCCCTGATGTC-3’.

Immunoprecipitation and Western blotting

For immunoprecipitation, cells were lysed in 1 ml of NP40 buffer (20 mM Tris-HCl pH 8.0, 10% glycerol, 137 mM NaCl, and 1% NP40) with protease inhibitors (10 μg/ml leupeptin, aprotinin, and 1 mM phenylmethanesulfonyl fluoride). After 10-minutes centrifugation, lysates were incubated with antibody for 30 minutes at 4°C and further incubated with 40 μl of 50% slurry of protein G Sepharose beads for 1 hour at 4 °C. Beads were washed three times with 1 ml of NP40 buffer and were dissolved in Laemmli’s sample buffer.

For Western blotting, samples from cell lysates and precipitates were resolved on SDS-PAGE. Samples were transfer to a nitrocellulose membrane and the membranes were blocked in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) containing 5% milk, incubated for 30 minutes at room temperature. After brief wash, the membranes were probed with antibodies against proteins of interest diluted in TBST or Can Get Signal (Toyobo, Tokyo, Japan) overnight. Then, they were washed three times with TBST for 15 minutes. After incubation with the secondary antibody for 1 hour at room temperature, membranes were washed three times with TBST for 15 minutes. Detection was performed using ECL or ECL select (GE healthcare, Buckinghamshire, England). All blots were exposed using ImageQuant LAS4000 mini system (GE healthcare, Buckinghamshire, England).

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Supplemental Table 1. Quantification of Dkk1 mRNA level

Cell lines	Relative expression of Dkk1 mRNA (fold-increase)
MDCK	1
MDCK/Dkk1-FLAG	37829.5±18121.1
A549	4596.3±1850.3
S2-CP8	1010.8±214.3
HeLaS3	147.9±35.5
X293T	45.0±1.4

The *Dkk1* mRNA levels of MDCK, MDCK/Dkk1-FLAG, A549, S2-CP8, HeLaS3, and X293T cells were measured by quantitative RT-PCR. Relative mRNA levels were normalized by *GAPDH* and are shown as fold-changes compared with levels of MDCK cells. Results are shown as means ± s.d. of three independent experiments.

Supplemental Table 2. The list of Dkk1 binding proteins

Band number	Protein name
1	FN1, DSP
2	FN1
3	MYO5A
4	FHOD3
5	DHX9
6	ACTN1, NPEPPS
7	ACTN1
8	HSPA8, HSPA2
9	PLS3, LMNA
10	CKAP4, LMNA, FUS, EIF3L
11	PKM
12	TUBB4B, TFG
13	FGG
14	ACTB, CKB, CKM
15	RPSA, SERPINB13, PKG1
16	ANXA2, YWHAZ, HSPB1, SFN
17	GAPDH, ANXA1, CAPZA1

The list of Dkk1 binding proteins was shown.

Supplemental Table 3. Immunohistochemical staining of pAKT (S473) in pancreatic and lung cancers

Cancer types	Dkk1 and CKAP4 staining	pAKT (S473) staining	
		Positive	Negative
Pancreatic ductal adenocarcinoma	Dkk1 (+) and CKAP4 (+)	18 (85.7%)	3
	Dkk1 (-) and/or CKAP4 (-)	5 (55.6%)	4
Lung adenocarcinoma	Dkk1 (+) and CKAP4 (+)	9 (75%)	3
	Dkk1 (-) and/or CKAP4 (-)	4 (50%)	4
Lung squamous cell carcinoma	Dkk1 (+) and CKAP4 (+)	9 (60%)	6
	Dkk1 (-) and/or CKAP4 (-)	1 (20%)	4

Randomly selected 30 cases of pancreatic ductal adenocarcinoma, 20 cases of lung adenocarcinoma, and 20 cases of lung squamous cell carcinoma specimens were stained with anti-Dkk1, anti-CKAP4, or anti-pAKT (S473) antibody and hematoxylin. Dkk1 (+) and CKAP4 (+), both Dkk1 and CKAP4 positive case; Dkk1 (-) and/or CKAP4 (-), either Dkk1 or CKAP4 negative case or both negative case. The number of pAKT (S473) positive or negative cases was counted.

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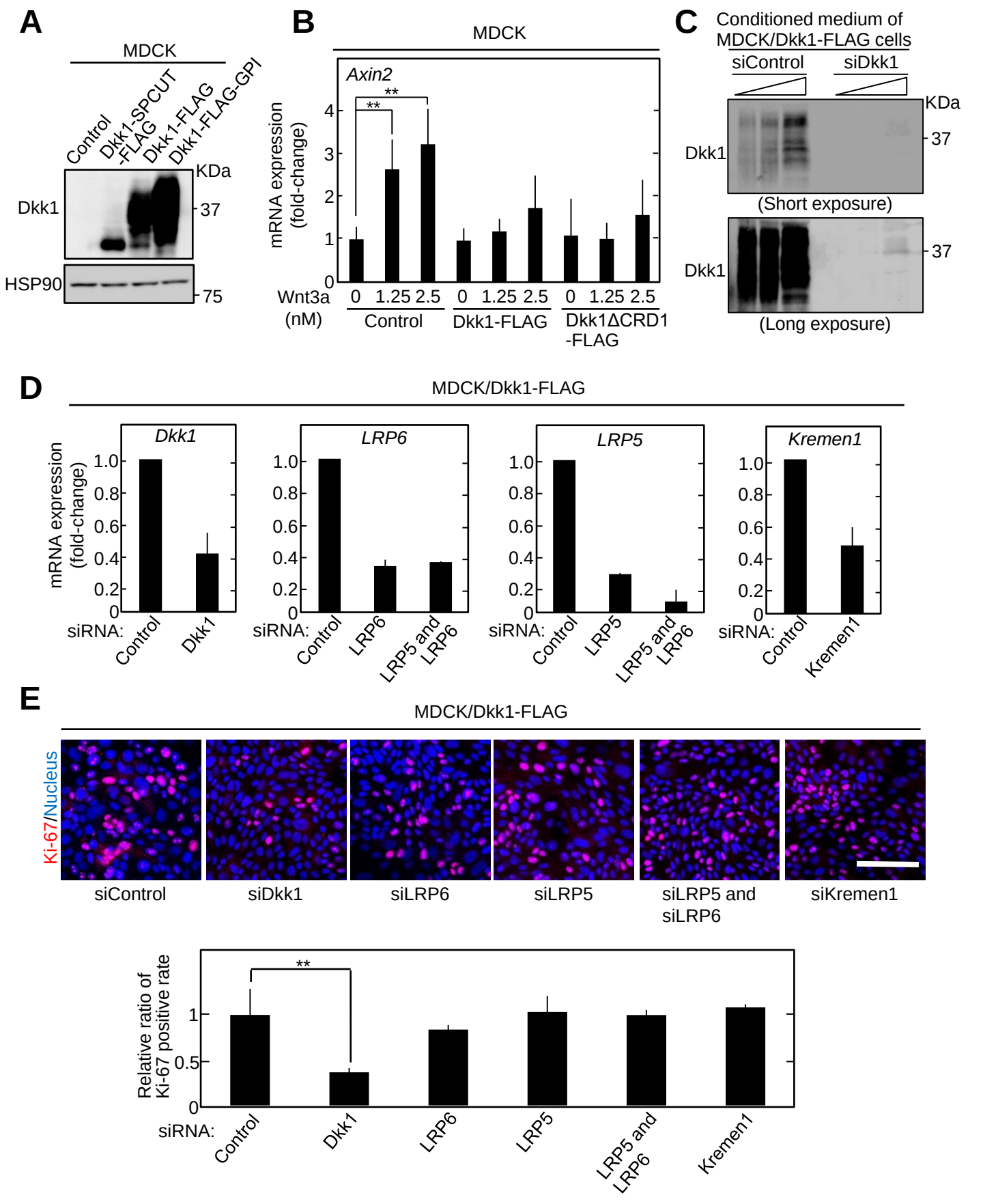


Supplemental Figure 1. Dkk1 promotes MDCK cell proliferation.

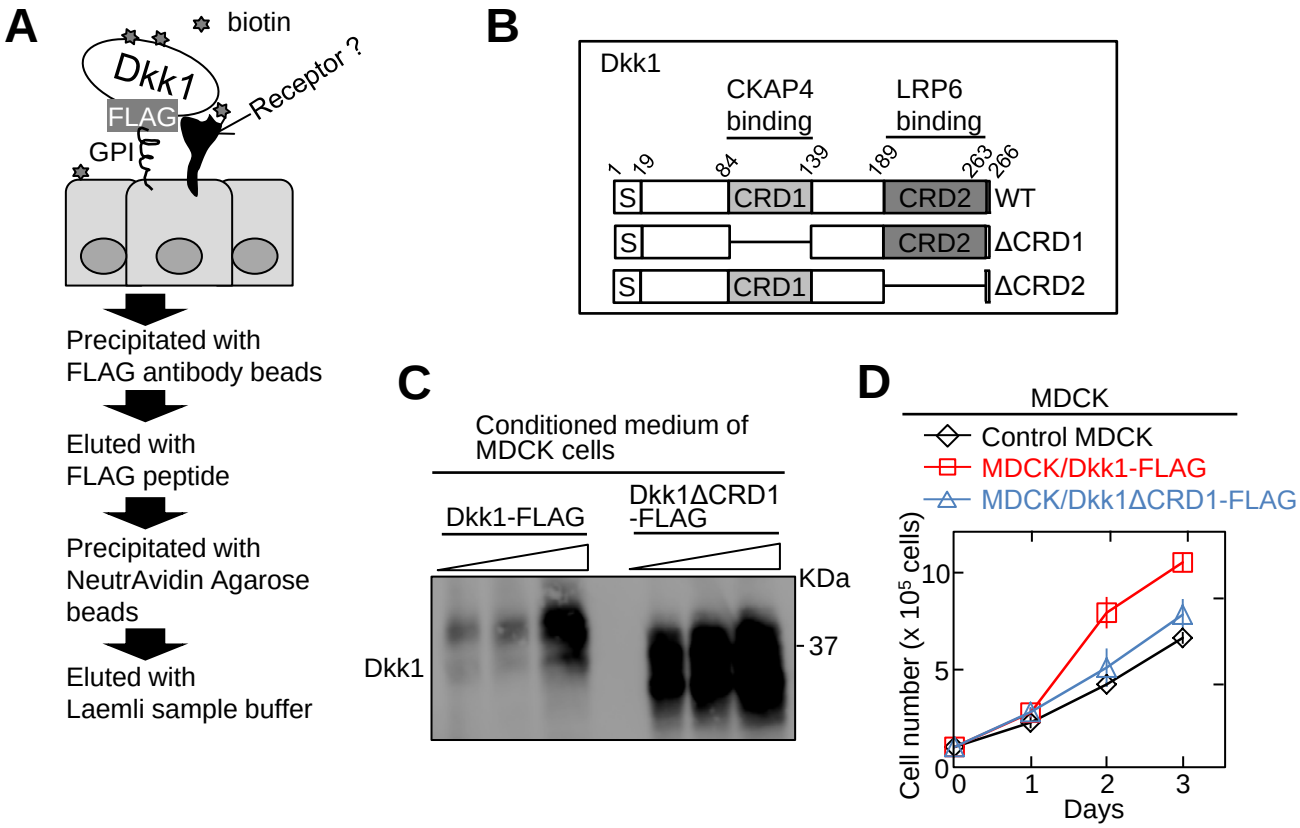
(A) Lysates of control MDCK, MDCK/Dkk1-FLAG, MDCK/Dkk1-SPCUT-FLAG and MDCK/Dkk1-FLAG-GPI cells were probed with anti-Dkk1 and anti-HSP90 antibodies. HSP90 was used as a loading control.

(B) After control MDCK, MDCK/Dkk1-FLAG, and MDCK/Dkk1ΔCRD1-FLAG cells treated with 1.25 or 2.5 nM Wnt3a for 24 hours, *Axin2* mRNA levels were measured. Results are expressed as fold-changes compared with the *Axin2* mRNA level in control cells without Wnt3a treatment. Results are shown as means ± s.d. of three independent experiments. **, $P < 0.01$ (compared with the buffer treatment group (0 nM Wnt3a), 2-tailed Student's *t* test).

(C) Different volumes (2, 5, and 10 μl) of conditioned medium from MDCK/Dkk1-FLAG cells transfected with control (scramble) or Dkk1 siRNA were probed with anti-Dkk1 antibody. Upper and lower panels show the same membrane with short and long exposure times, respectively.

(D) After MDCK/Dkk1-FLAG cells were transfected with control (scramble), Dkk1, LRP6, LRP5, LRP5 and LRP6, or Kremen1 siRNA, *Dkk1*, *LRP5*, *LRP6*, and *Kremen1* mRNA levels were measured by quantitative RT-PCR. Relative mRNA levels were normalized by *GAPDH* and are shown as fold-changes compared with mRNA levels in control siRNA transfected cells.

(E) MDCK/Dkk1-FLAG cells transfected with control (scramble), Dkk1, LRP6, LRP5, LRP5 and LRP6, or Kremen1 siRNA were cultured for 5 days and the cells were stained with anti-Ki-67 antibody (red) and DRAQ5 DNA Dye (blue). Ki-67-positive cells are expressed as the percentage of total cells per field. Results are shown as means ± s.d. of three independent experiments. **, $P < 0.01$ (compared with the Control group, 2-tailed Student's *t* test). Scale bar, 100 μm.



Supplemental Figure 2. The binding of Dkk1 to CKAP4 is required for MDCK cellular proliferation.

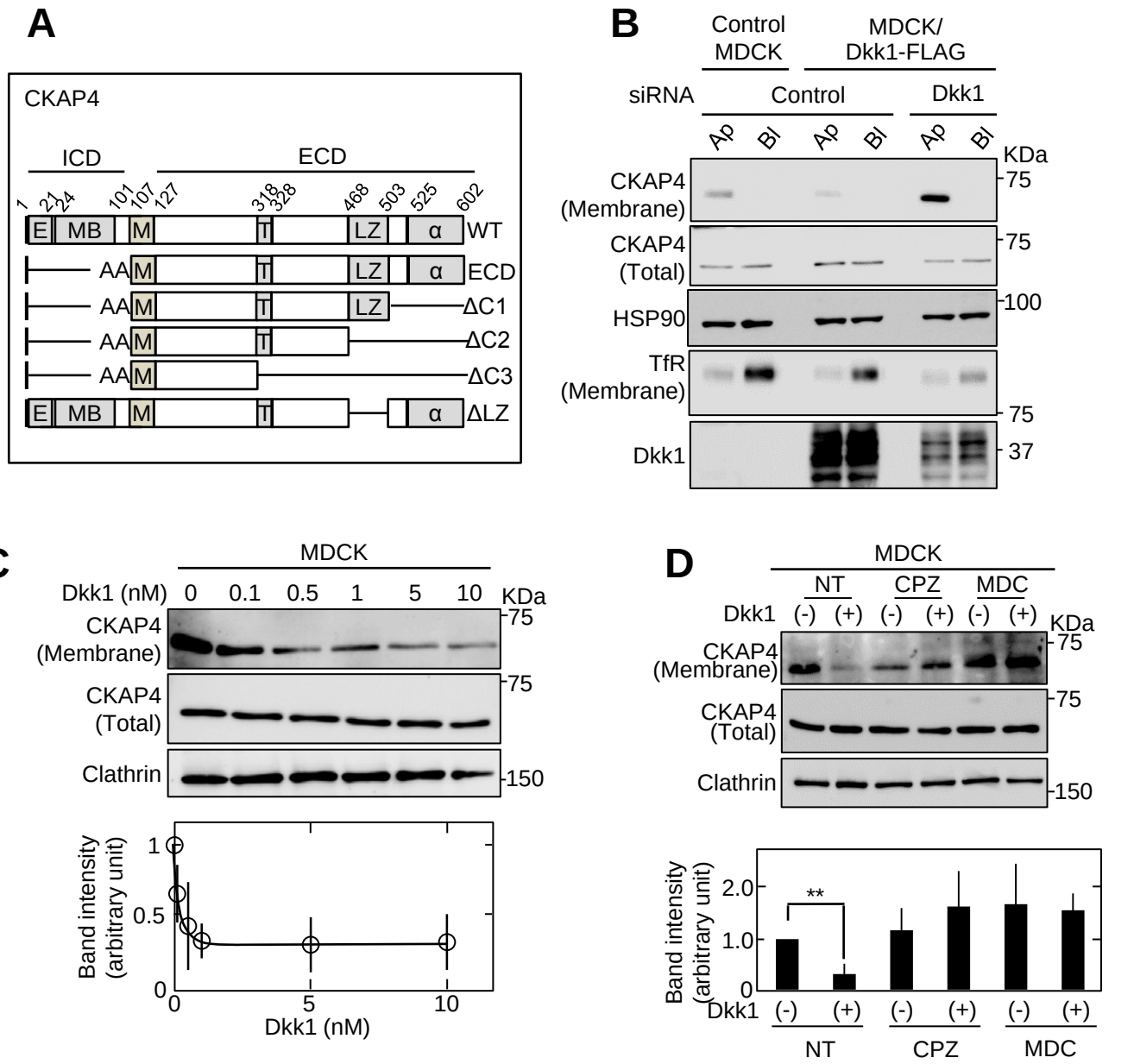
(A) Schematic diagram illustrating the experimental procedure for the screening of Dkk1 binding proteins at the apical surface. The FLAG epitope and glycosylphosphatidylinositol (GPI) anchor signal sequence were tagged to Dkk1 at its carboxyl terminal in tandem (Dkk1-FLAG-GPI) and Dkk1-binding proteins were purified from Dkk1-GPI-FLAG expressing MDCK cells.

(B) Schematic illustration of human Dkk1 deletion mutants used in this study. WT, wild type; S, signal peptide; CRD, cysteine-rich domain.

(C) Different volumes (1, 2, and 5 μl) of conditioned medium from MDCK/Dkk1-FLAG and MDCK/Dkk1ΔCRD1-FLAG cells were probed with anti-Dkk1 antibody.

(D) Cell numbers of control MDCK, MDCK/Dkk1-FLAG, or MDCK/Dkk1ΔCRD1-FLAG cells were counted. Results are shown as means ± s.d. of three independent experiments.

Kimura et al., Supplemental Figure 3



Supplemental Figure 3. CKAP4 acts as a Dkk1 receptor.

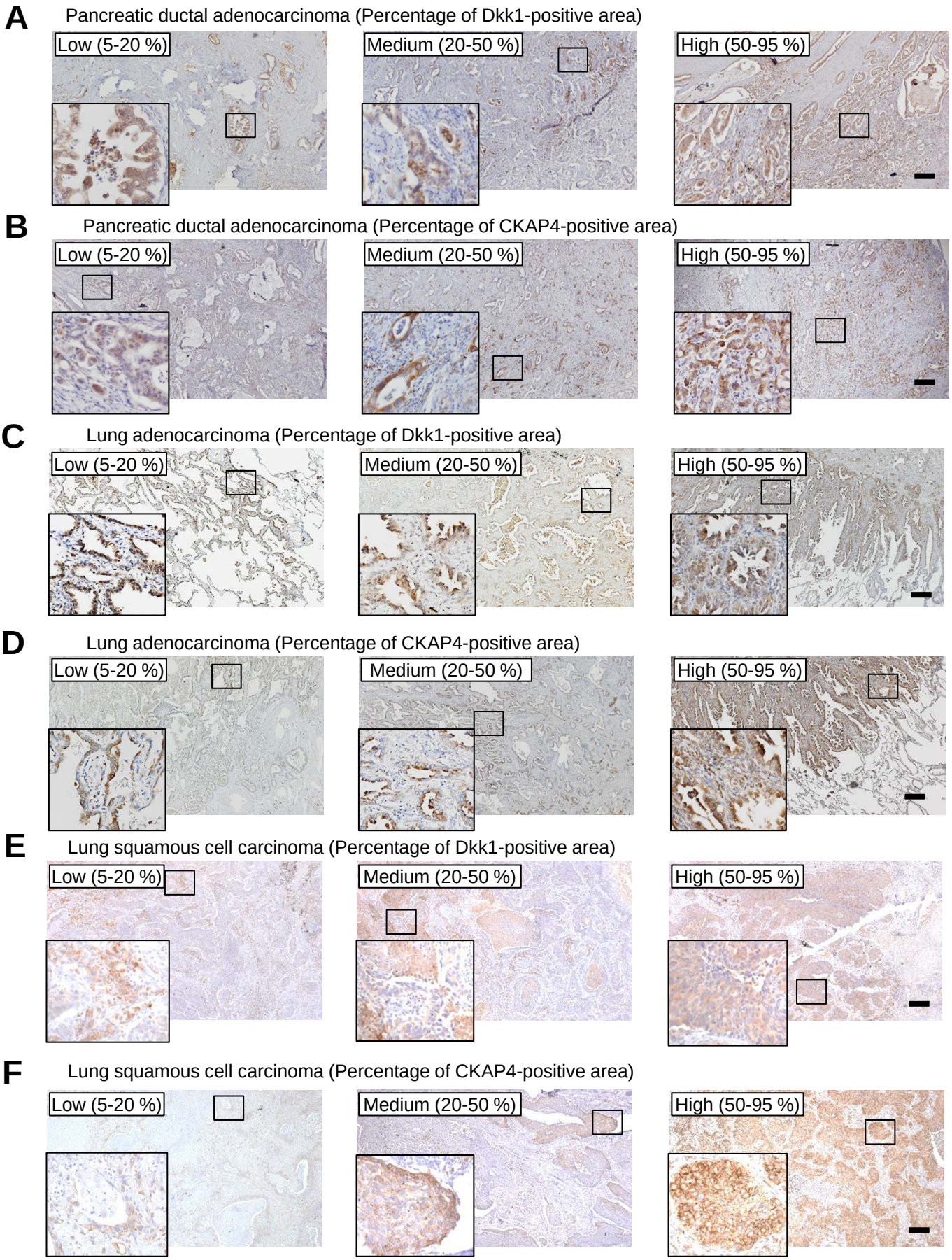
(A) Schematic illustration of human CKAP4 deletion mutants used in this study. WT, wild type; ICD, intracellular domain; ECD, extracellular domain; E, ER anchoring domain; MB, microtubule binding domain; M, transmembrane domain; T, tyrosine sulfation region; LZ, leucine zipper domain; α, α helix domain.

(B) After control MDCK or MDCK/Dkk1-FLAG were transfected with control (scramble) or Dkk1 siRNA, cell surface proteins were biotinylated and precipitated with NeutrAvidin Agarose beads. The precipitates were probed with the indicated antibodies. TfR was used as a basolateral membrane protein marker. HSP90 was used as a loading control. SiRNA against the coding region of Dkk1 was used.

(C) Top panel, MDCK cells were stimulated with the indicated concentration of Dkk1 for 1 hour and cell surface proteins were biotinylated and precipitated with NeutrAvidin Agarose beads. The precipitates were probed with anti-CKAP4 and anti-clathrin antibodies. Bottom panel, CKAP4 (Membrane) and CKAP4 (Total) band intensities were analyzed by Image J software and the membrane level normalized to total level was expressed as arbitrary units compared with buffer treatment (0 nM Dkk1). Clathrin was used as a loading control. Results are shown as means ± s.d. of three independent experiments.

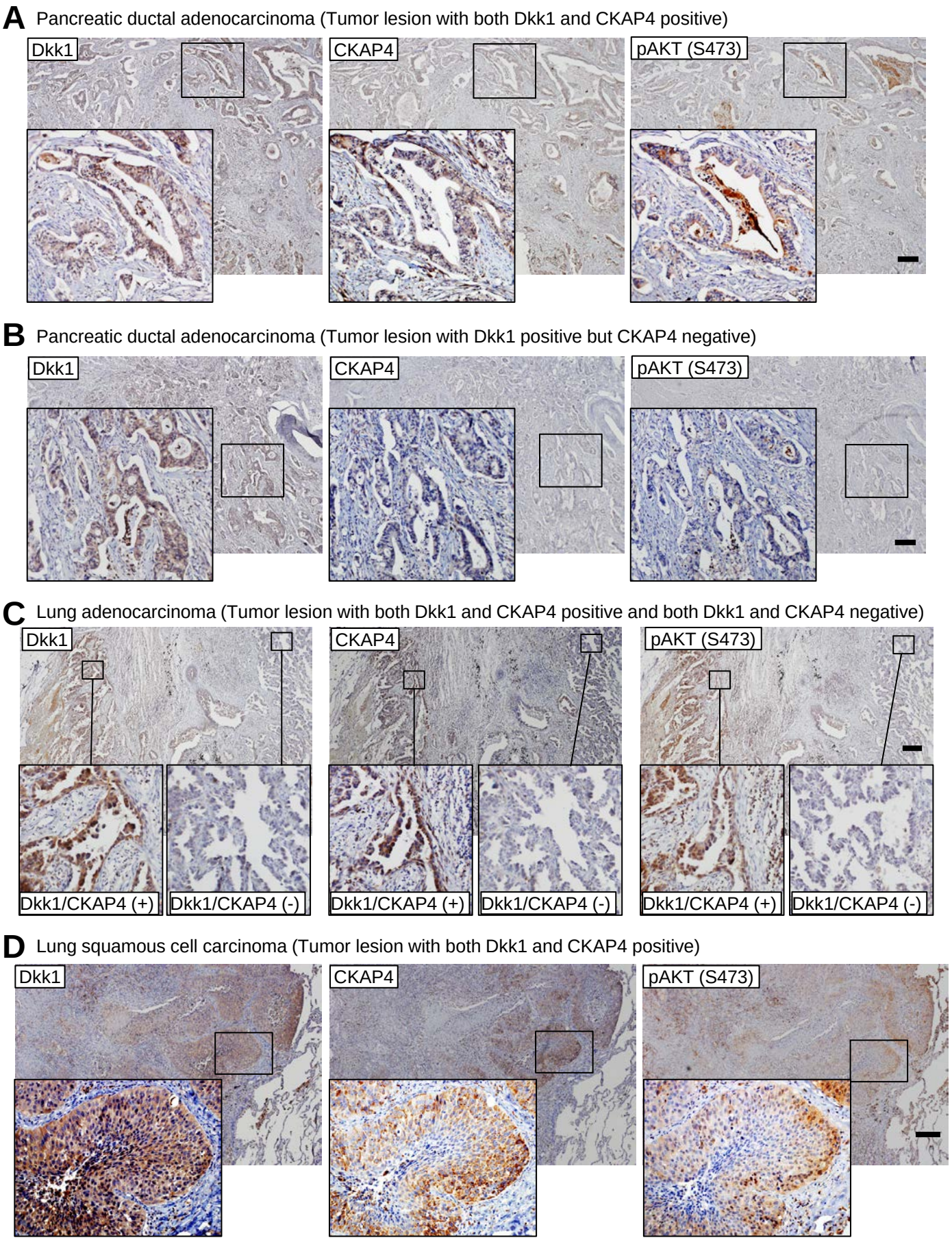
(D) Top panel, MDCK cells pretreated with 10 μg/ml chlorpromazine (CPZ) or 50 μM monodansylcadaverine (MDC) for 30 minutes at 37 ° C were stimulated with 10 nM Dkk1 for 30 minutes. Cell surface proteins were biotinylated and precipitated with NeutrAvidin Agarose beads. The precipitates were probed with the indicated antibodies. Bottom panel, CKAP4 (Membrane) and CKAP4 (Total) band intensities were analyzed by ImageJ software and the membrane level normalized to total level was expressed as arbitrary units compared with that of control MDCK cells without Dkk1 treatment. Clathrin was used as a loading control. Results are shown as means ± s.d. of three independent experiments. **, $P < 0.01$ (2-tailed Student's *t* test).

Kimura et al., Supplemental Figure 4



Supplemental Figure 4. Dkk1 and CKAP4 are expressed in pancreatic and lung cancer.
Pancreatic ductal adenocarcinoma (**A** and **B**), lung adenocarcinoma (**C** and **D**), and lung squamous cell carcinoma (**E** and **F**) specimens were stained with anti-Dkk1 (**A**, **C**, and **E**) or anti-CKAP4 (**B**, **D**, and **F**) antibody and hematoxylin. Black boxes show enlarged images. Areas staining positive for Dkk1 or CKAP4 were classified as follows. Low, 5-20%; Medium, 20-50%; High, 50-95%. Scale bars, 50 μm.

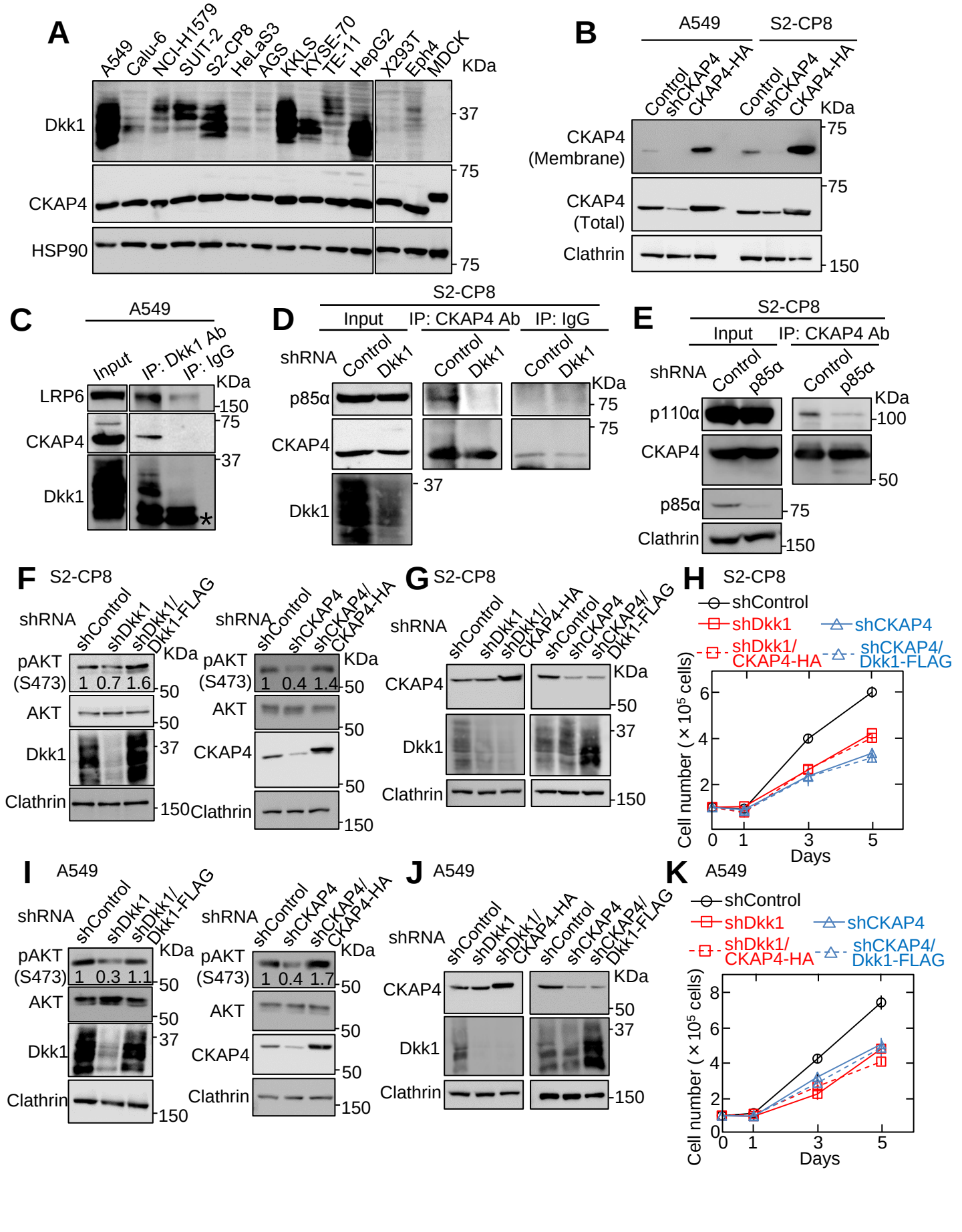
Kimura et al., Supplemental Figure 5



Supplemental Figure 5. Coexpression of Dkk1 and CKAP4 promotes pAKT positivity in pancreatic and lung cancers.

Pancreatic ductal adenocarcinoma (**A and B**), lung adenocarcinoma (**C**), and lung squamous cell carcinoma (**D**) specimens were stained with anti-Dkk1, anti-CKAP4, or anti-pAKT (S473) antibody and hematoxylin. Black boxes show enlarged images. Scale bars, 200 μm.

Kimura et al., Supplemental Figure 6



Supplemental Figure 6. Dkk1-CKAP4 signaling is required for cancer cell proliferation.

(A) Lysates of lung adenocarcinoma cells (A549, Calu-6, and NCI-H1975), pancreatic cancer cells (SUIT-2 and S2-CP8), cervical cancer cells (HeLaS3), gastric cancer cells (AGS and KKLS), esophageal squamous cell carcinoma cells (KYSE-70 and TE-11), hepatoblastoma cells (HepG2), embryonic kidney cells (X293T), mouse mammary epithelial cells (Eph4), and dog kidney tubule cells (MDCK) were probed with the indicated antibodies. HSP90 was used as a loading control.

(B) A549 and S2-CP8 cells stably expressing CKAP4 shRNA or CKAP4-HA and cell surface proteins were biotinylated and precipitated with NeutrAvidin Agarose beads. The precipitates were probed with anti-CKAP4 and anti-clathrin antibodies. Clathrin was used as a loading control.

(C) Lysates of A549 cells were immunoprecipitated with anti-Dkk1 antibody or non-immune IgG and probed with the indicated antibodies. *, IgG.

(D) Lysates of S2-CP8 cells stably expressing control or Dkk1 shRNA were immunoprecipitated with anti-CKAP4 antibody or non-immune IgG and probed with the indicated antibodies.

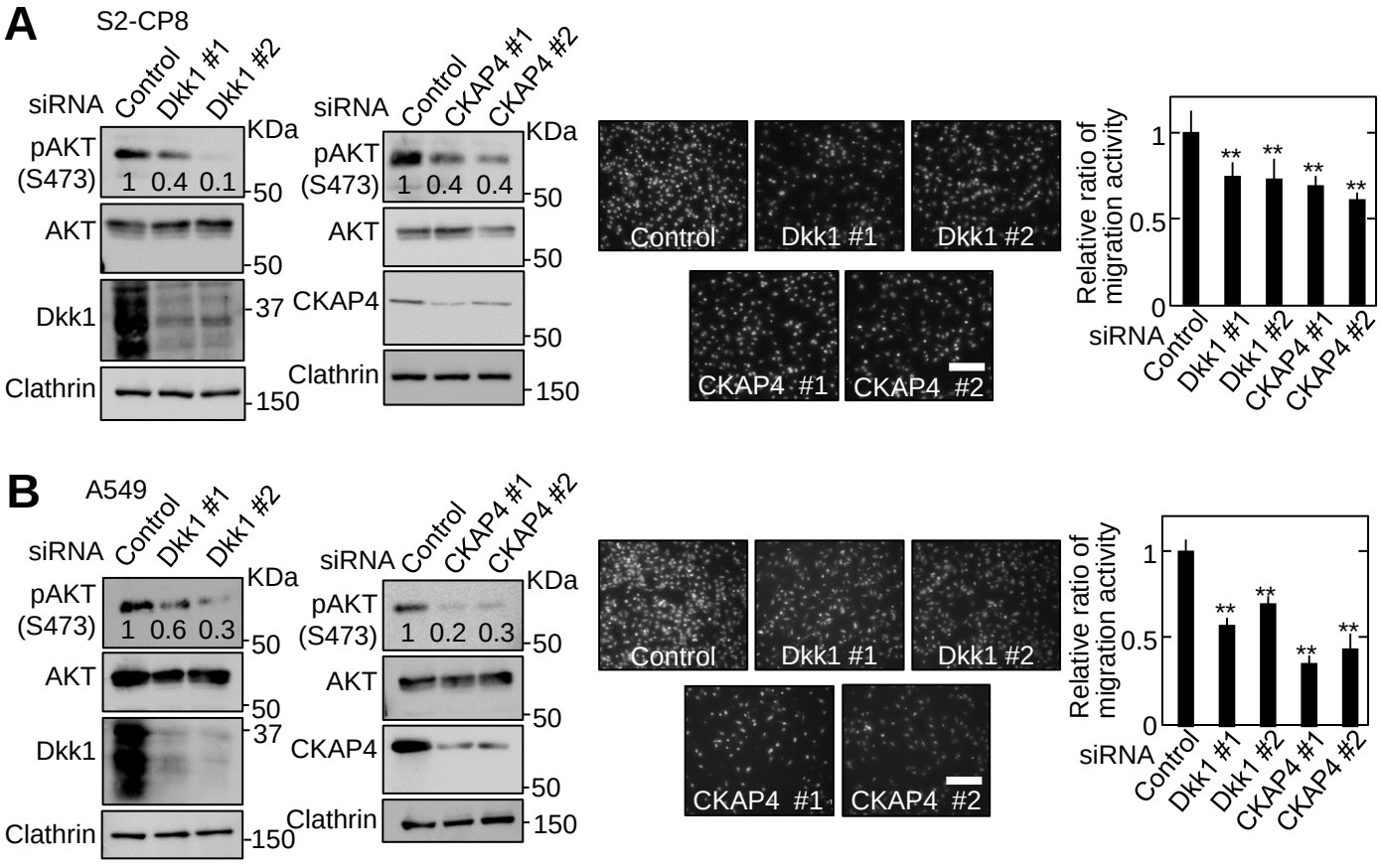
(E) Lysates of S2-CP8 cells stably expressing control or p85α shRNA were immunoprecipitated with anti-CKAP4 antibody and probed with the indicated antibodies. Clathrin was used as a loading control.

(F and I) Lysates of S2-CP8 (F) cells and A549 cells (I) stably expressing control shRNA, Dkk1 shRNA, or Dkk1 shRNA and Dkk1-FLAG (left panels) or control shRNA, CKAP4 shRNA, or CKAP4 shRNA and CKAP4-HA (right panels) were probed with the indicated antibodies.

(G and J) Lysates of S2-CP8 cells (G) and A549 cells (J) stably expressing control shRNA, Dkk1 shRNA, or Dkk1 shRNA and CKAP4-HA (left panels) or control shRNA, CKAP4 shRNA, or CKAP4 shRNA and Dkk1-FLAG (right panels) were probed with the indicated antibodies.

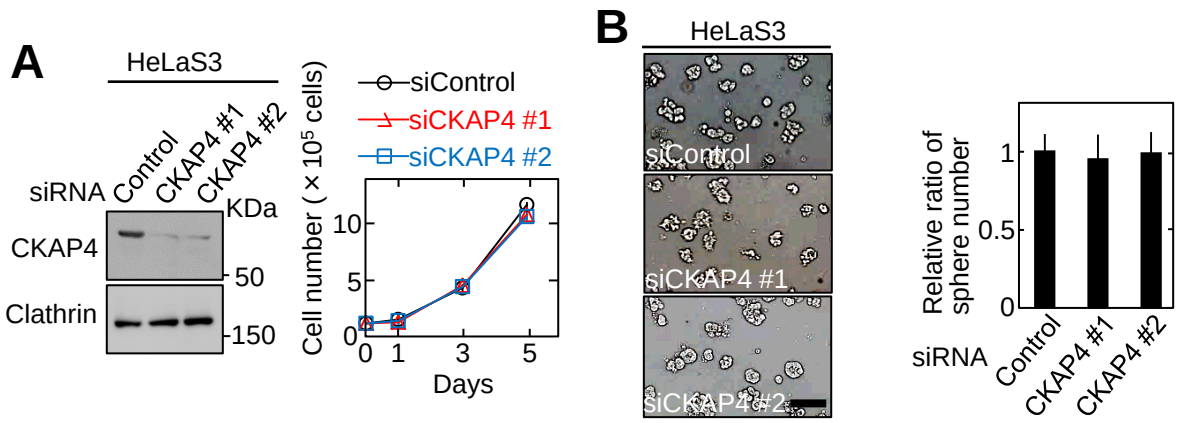
(H) S2-CP8 cells used in Supplemental Figure 6G were subjected to the 2D cell proliferation assay. Results are shown as means ± s.d. of three independent experiments.

(K) A549 cells used in Supplemental Figure 6J were subjected to the 2D cell proliferation assay. Results are shown as means ± s.d. of three independent experiments.



Supplemental Figure 7. Dkk1-CKAP4 signaling is required for cancer cell migration.

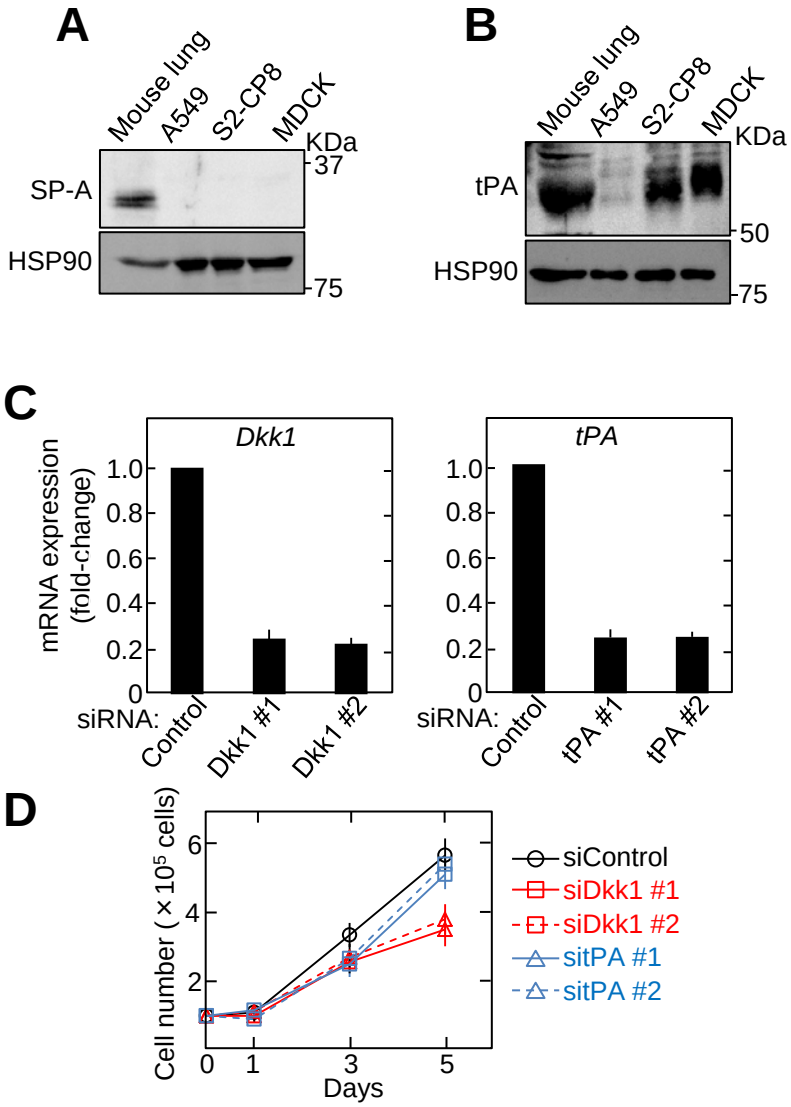
(A and **B)** Left panels, S2-CP8 cells (**A**) and A549 cells (**B**) were transfected with control (scramble), Dkk1, or CKAP4 siRNA and lysates were probed with the indicated antibodies. Clathrin was used as a loading control. Right panel, the cells were subjected to migration assays for 4 hours and stained with DRAQ5 DNA dye. Migration activity is expressed as the ratio compared with that of control siRNA transfected cells. Scale bars, 200 μm. Results are shown as means ± s.d. of three independent experiments. **, $P < 0.01$ (compared with the Control group, 2-tailed Student's *t* test).



Supplemental Figure 8. CKAP4 is not essential for cellular proliferation in Dkk1 minimally expressed cells.

(A) Left panels, HeLaS3 cells were transfected with control (scramble) or CKAP4 siRNA and lysates were probed with anti-CKAP4 and anti-clathrin antibodies. Right panel, the cells were subjected to the 2D cell proliferation assay.

(B) Left panels, HeLaS3 cells (2 x 10^4 cells) transfected with control (scramble) or CKAP4 siRNA were cultured for 5 days in 3D Matrigel. Right panel, the number of sphere per field (n = 5) was counted and results are shown compared with the number of control sphere. Scale bar, 500 μm.



Supplemental Figure 9. tPA is not involved in A549 and S2-CP8 cell proliferation.

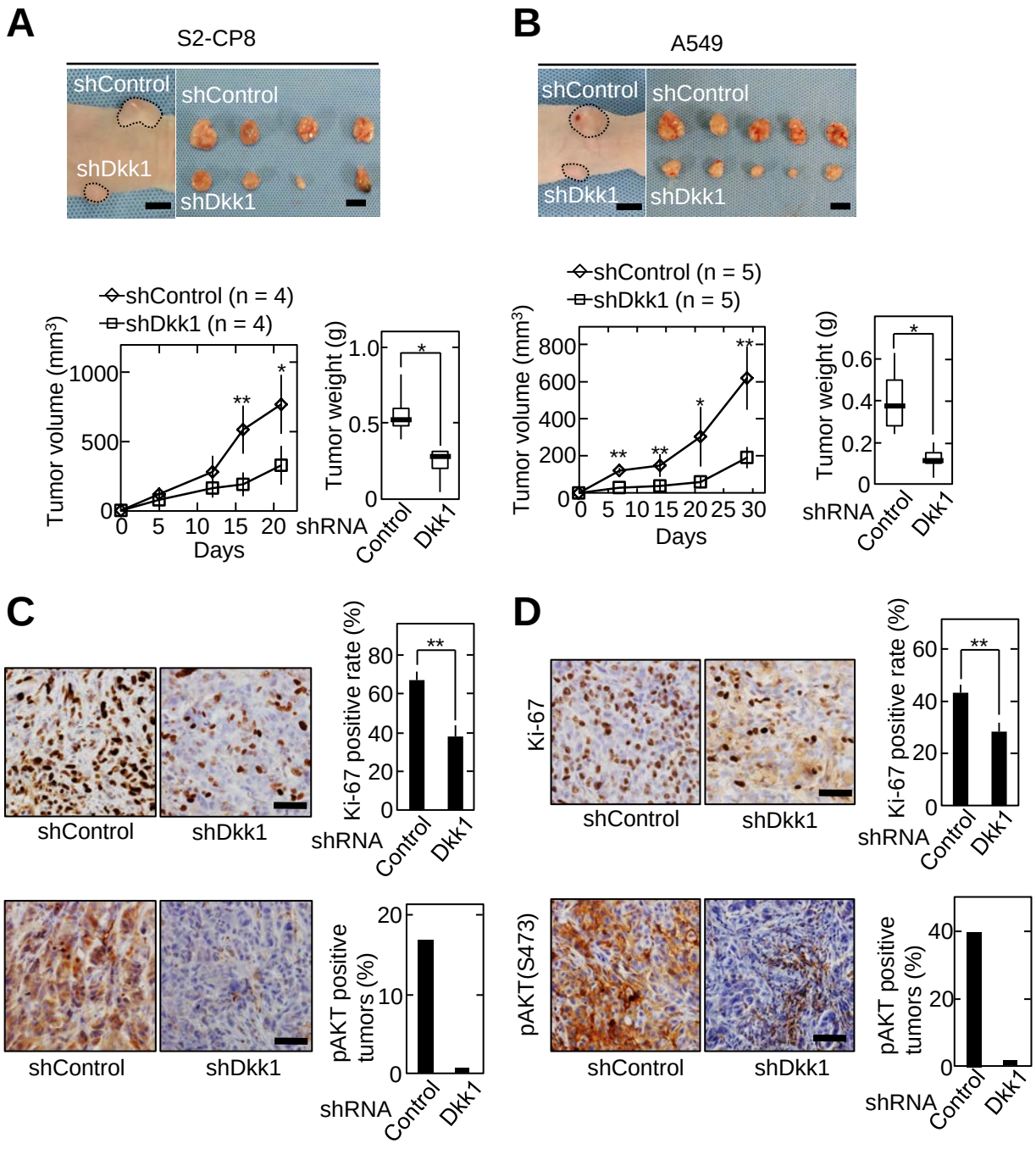
(A) Lysates of mouse lung, A549 cells, S2-CP8, and MDCK cells were probed with anti-SP-A and anti-HSP90 antibodies. HSP90 was used as a loading control.

(B) Lysates of mouse lung, A549 cells, S2-CP8, and MDCK cells were probed with anti-tPA and anti-HSP90 antibodies. HSP90 was used as a loading control.

(C) S2-CP8 cells were transfected with control (scramble), Dkk1, or tPA siRNA, and *Dkk1*and *tPA* mRNA levels were measured by quantitative RT-PCR. Relative mRNA levels were normalized by *GAPDH* and are shown as fold-changes compared with levels in control siRNA transfected cells.

(D) S2-CP8 cells transfected with indicated siRNAs were subjected to the 2D cell proliferation assay. Results are shown as means ± s.d. of three independent experiments.

Kimura et al., Supplemental Figure 10

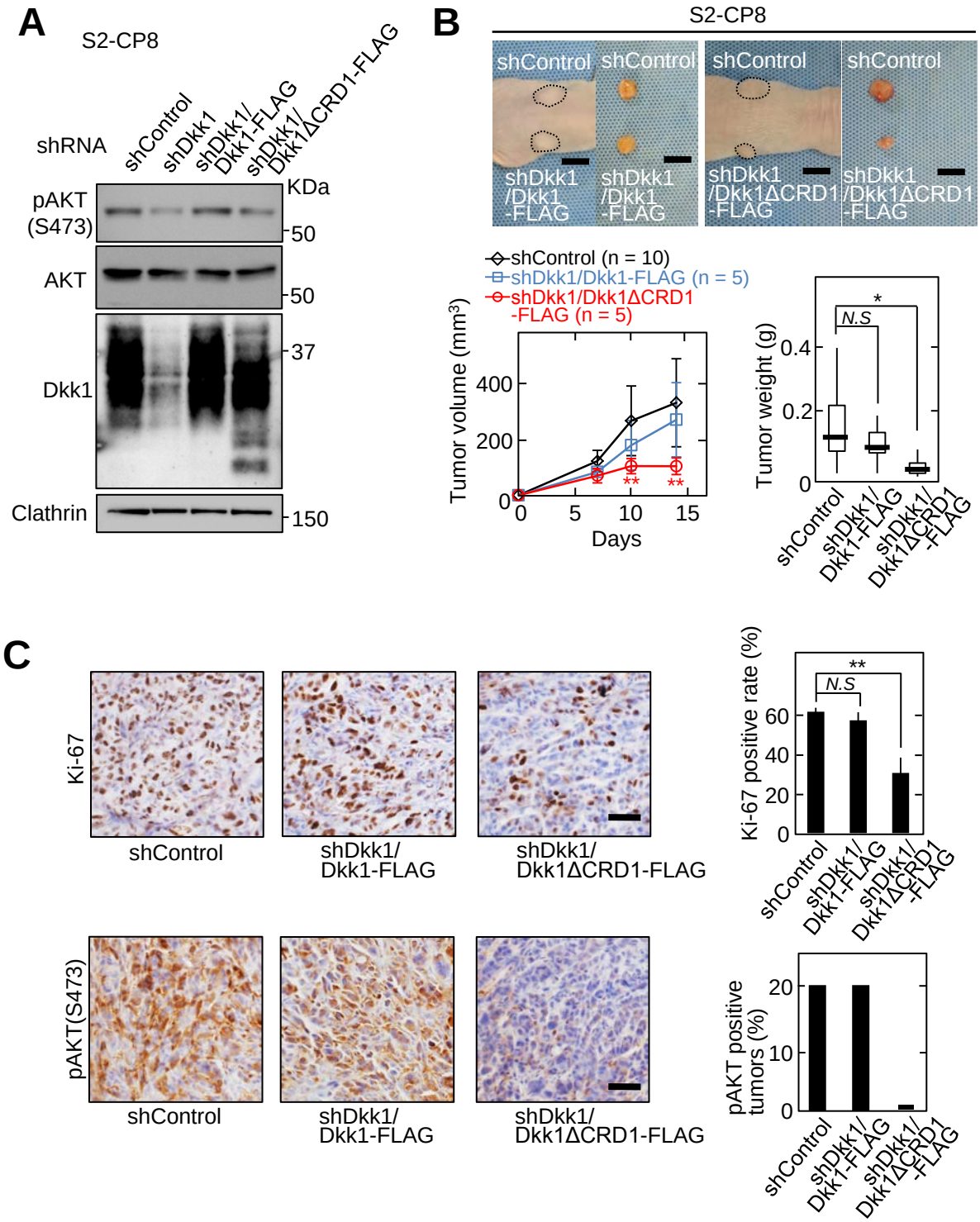


Supplemental Figure 10. Dkk1 signaling is required for cancer cell proliferation.

(A and **B)** S2-CP8 cells (**A**) and A549 cells (**B**) stably expressing control or Dkk1 shRNA were subcutaneously implanted into immunodeficient mice (n = 4) (for S2-CP8 cells) and (n = 5) (for A549 cells). Top panel, representative appearance of one mouse (left picture) and extirpated xenograft tumors (right picture) are shown. Dashed lines show the outline of xenograft tumors. The volumes (bottom left panel) and weights (bottom right panel) of the xenograft tumors were measured. Results are plotted as box and whiskers where the median is represented with a line, the box represents the 25^{th}-75^{th} percentile and error bars show the 5^{th}-95^{th} percentile.

(C and D) Sections prepared from xenograft tumors of S2-CP8 cells (**C**) and A549 cells (**D**) were stained with hematoxylin and anti-Ki-67 (top panel) or anti-pAKT (bottom panel) antibody. Ki-67-postitive cells are expressed as the percentage of positively stained cells compared with total cells per field (n = 5 fields) in the right panel. Percentages of pAKT (S473)-positive tumors in the total xenograft tumors tested are shown in the right panel. Results are shown as means ± s.d. of three independent experiments. *, $P < 0.05$; **, $P < 0.01$ (2-tailed Student's *t* test). Scale bars, 10 mm (**A and B**); 50 μm (**C and D**).

Kimura et al., Supplemental Figure 11



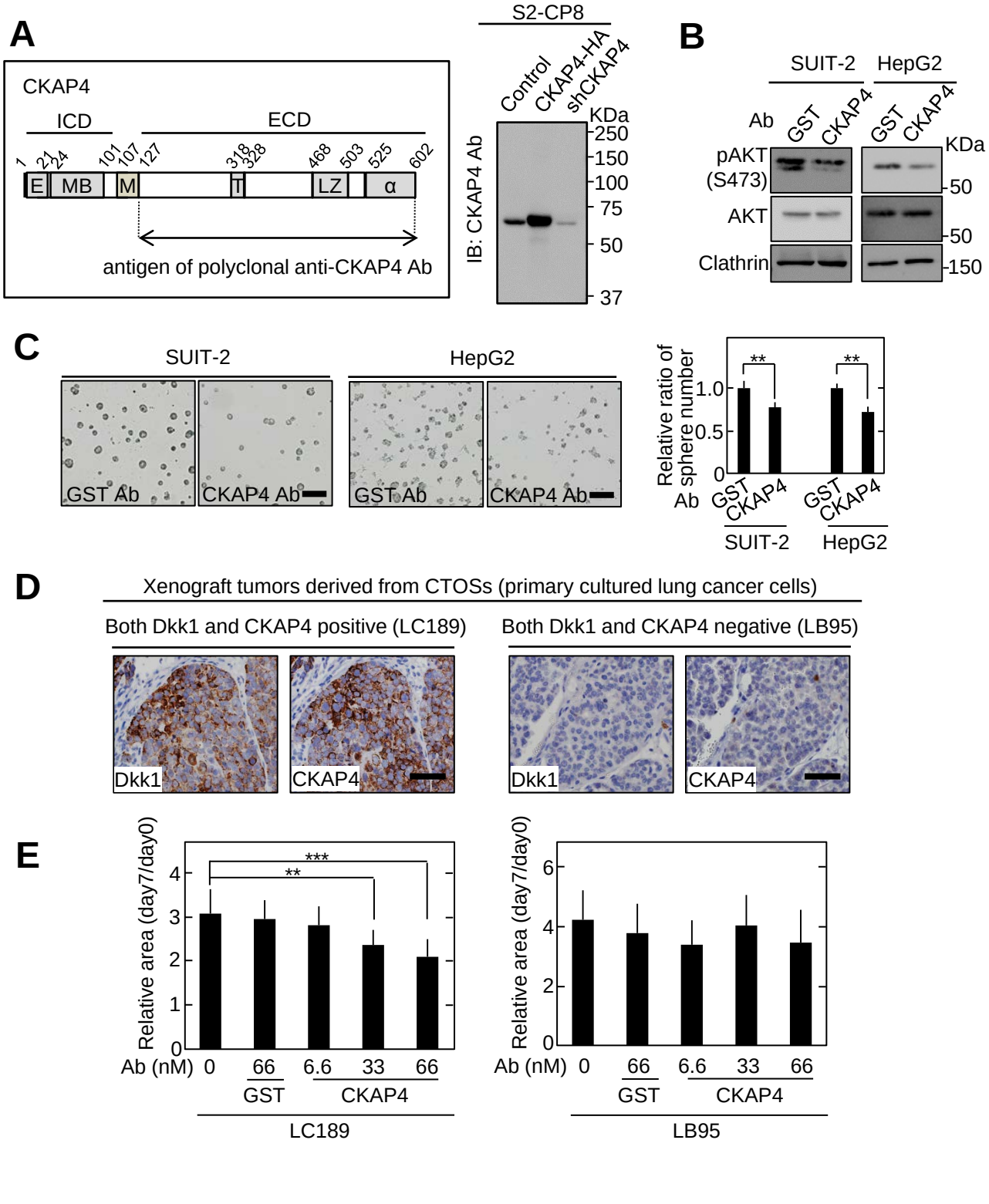
Supplemental Figure 11. The binding of Dkk1 to CKAP4 is required for xenograft tumor formation.

(A) Lysates of S2-CP8 cells stably expressing control shRNA, Dkk1 shRNA, Dkk1 shRNA and Dkk1-FLAG, or Dkk1 shRNA and Dkk1ΔCRD1-FLAG were probed with the indicated antibodies.

(B) S2-CP8 cells stably expressing control shRNA, Dkk1 shRNA and Dkk1-FLAG, or Dkk1 shRNA and Dkk1ΔCRD1-FLAG were subcutaneously implanted into immunodeficient mice. Top panel, representative appearance of one mouse (left picture) and extirpated xenograft tumors (right picture) are shown. Dashed lines show the outline of xenograft tumors. The volumes (bottom left panel) and weights (bottom right panel) of the xenograft tumors were measured. Results are plotted as box and whiskers where the median is represented with a line, the box represents the 25^{th}-75^{th} percentile and error bars show the 5^{th}-95^{th} percentile.

(C) Sections prepared from xenograft tumors of S2-CP8 cells stably expressing control shRNA (n = 10), Dkk1 shRNA and Dkk1-FLAG (n = 5), or Dkk1 shRNA and Dkk1ΔCRD1-FLAG (n = 5) were stained with hematoxylin and anti-Ki-67 (top panel) or anti-pAKT (bottom panel) antibody. Ki-67-postitive cells are expressed as the percentage of positively stained cells compared to total cells per field (n = 5 fields) in the right panel. Percentages of pAKT (S473)-positive tumors in the total xenograft tumors tested are shown in the right panel. Results are shown as means ± s.d. of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; *N.S., not significant* (compared with the shControl group, 2-tailed Student's *t* test). Scale bars, 10 mm (**B**); 50 μm (**C**).

Kimura et al., Supplemental Figure 12



Supplemental Figure 12. Anti-proliferative effect of anti-CKAP4 antibody on Dkk1- and CKAP4-expressing cancer cells.

(A) Left panels, schematic illustration shows antigen of anti-CKAP4 rabbit polyclonal antibody (CKAP4 pAb). Right panel, lysates of S2-CP8 cells stably expressing CKAP4-HA cDNA or CKAP4 shRNA were probed with anti-CKAP4 antibody.

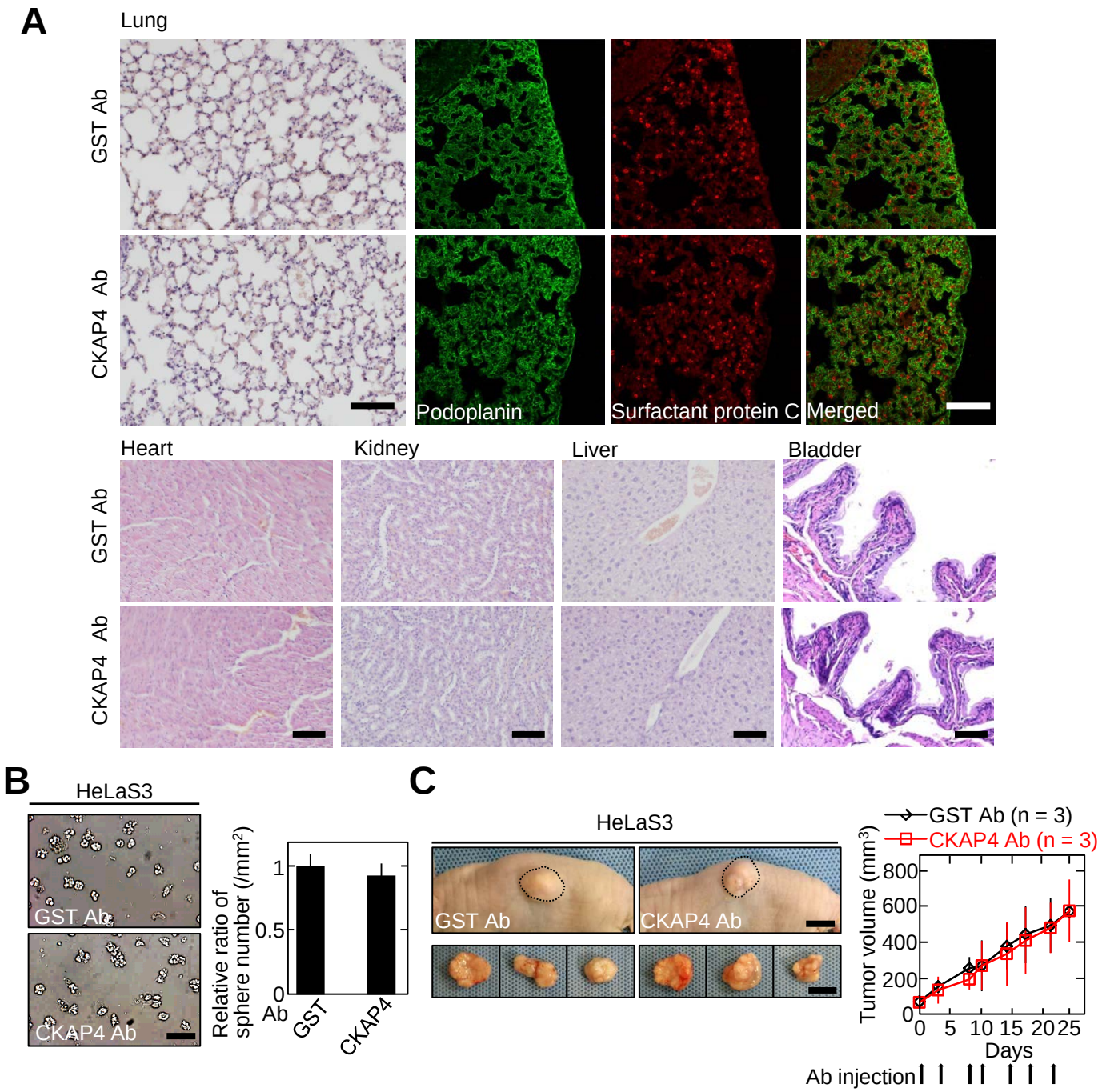
(B) Lysates of SUIT-2 cells and HepG2 cells treated with 33 nM anti-CKAP4 or anti-GST antibody for 4 hours were probed with the indicated antibodies.

(C) Left panels, phase contrast images of SUIT-2 or HepG2 cells treated with anti-GST antibody or CKAP4 antibody were cultured for 5 days in 3D Matrigel. Right panel, the number of anti-CKAP4 antibody-treated spheres per field (n = 5) was expressed as the ratio of that of anti-GST antibody-treated spheres. **, $P < 0.01$ (2-tailed Student's *t* test).

(**D**) Xenograft tumors derived from CTOSs were stained with hematoxylin and anti-Dkk1 or anti-CKAP4 antibody.

(**E**) The CTOSs were embedded in Matrigel, cultured in StemPro hESC, and treated with the indicated doses of anti-GST (n = 12) or CKAP4 (n = 12) antibody. Growth ratio of CTOSs at day 7 relative to day 0 was shown as means ± s.d. **, $P < 0.01$; ***, $P < 0.001$ (compared with the buffer treatment group (0 nM Ab), 2-tailed Student's *t* test). Scale bars, 200 μm (**C**); 50 μm (**D**).

Kimura et al., Supplemental Figure 13

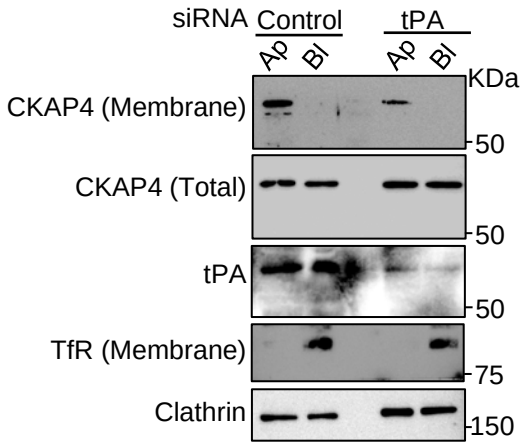


Supplemental Figure 13. Anti-CKAP4 antibody does not impair normal tissues nor affect HeLaS3 cellular proliferation.

(A) Histopathological examination of HE-stained normal tissues (lung, heart, kidney, liver, and bladder) in mice administered the anti-GST antibody or anti-CKAP4 antibody. Sections prepared from the mouse lung were stained with anti-podoplanin (green) and anti-surfactant protein C (red) antibodies. Podoplanin and surfactant protein C were used as type I and type II pneumocytes marker, respectively.

(B) Left panels, phase contrast images of HeLaS3 cells (2×10^4 cells) cultured in the presence of anti-CKAP4 or anti-GST antibody for 5 days in 3D Matrigel. Right panel, the number of sphere per field (n = 5) was counted and results are shown compared with the number of control sphere.

(C) HeLaS3 cells were subcutaneously implanted into immunodeficient mice. Anti-CKAP4 (150 μg/body) (n = 3) or anti-GST antibody (150 μg/body) (n = 3) was injected into the intraperitoneal cavity twice per week. Left panel, representative appearance of one mouse (top picture) and extirpated xenograft tumors (bottom picture) are shown. Dashed lines show the outline of xenograft tumors. Right panel, the volumes of the xenograft tumors were measured and results are shown as means ± s.d. Scale bars, 100 μm (**A**); 500 μm (**B**); 10 mm (**C**).



Supplemental Figure 14. tPA is not involved in the apical localization of CKAP4 in MDCK cells. After polarized MDCK cells were transfected with control (scramble) or tPA siRNA, cell surface proteins were biotinylated and precipitated with NeutrAvidin Agarose beads. The precipitates were probed with the indicated antibodies. TfR was used as a basolateral membrane protein marker. Clathrin was used as a loading control. SiRNA against the coding region of tPA was used.