Identification of tumorigenic cells in Kras^{G12D}-induced lung adenocarcinoma

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Supplementary Table S1.

The frequency of tumorigenic cells with at least one secondary tumor was determined by histological analysis. Total number of individual mice was presented in this table.

Supplemental Figure Legends

Supplementary Fig. S1. Genotyping for $Kras^{G12D}$ expression and immunopathological analyses for tumor formation in various organs from mice prior to and $Kras^{G12D}$ activation.

A, the primer sets for detection of the Lox-Stop-Lox cassette of genomic DNA in LSL-$Kras^{G12D}$ mice were shown. The genomic DNA from various tissues in conditional mutant mice prior to and after $Kras^{G12D}$ activation for 12 weeks was examined using PCR. B, various hematoxylin and eosin-stained tissue sections from conditional mutant mice prior to and after $Kras^{G12D}$ activation ($p < 0.05$, two-tailed *t*-test).

Supplementary Fig. S2. Characterizations of lung cell fractions with lineage-specific markers.

A, percentages of $EpCAM^{+}MHCII^{-}ICAM1^{-}$, $EpCAM^{+}MHCII^{-}ICAM1^{+}$, and $EpCAM^{+}MHCII^{+}ICAM1^{+}$ cell fractions among $CD45^{-}$ cells from conditional mutant mice prior to and after 4 and 12 weeks of $Kras^{G12D}$ activation (mean ± s.d, $p < 0.01$, $n = 3$, two-tailed *t*-test).B, lung tissues from mice after $Kras^{G12D}$ activation for 12 weeks and stained with antibodies directed against β-tubulin IV in bronchiolar and alveolar regions. C, RT-PCR analyses of gene expressions of lineage-specific markers (*P450*, *CC10*, and *SPC*), transcription factors (*Foxj1*, *FoxA2*, and *TTF-1*), surface markers (*EpCAM* and

MHCII), and GAPDH (as the internal control). Samples included $EpCAM^{+}MHCII^{-}$ and $EpCAM^{+}MHCII^{+}$ fractions of lung cells from conditional mutant mice prior to and after $Kras^{G12D}$ activation for 12 weeks, respectively.

Supplementary Fig. S3. Serial engraftment of $Kras^{G12D}$-induced primary tumor cells.

A, lungs from non-engrafted and serial engrafted mice after i.v. injection of 5 x 10^6 $CD45^{-}$ primary and secondary tumor cells into NOD-SCID mice for 12 weeks. B, FACS analysis of $CD45^{-}$ secondary tumor cells from NOD-SCID mice after i.v. injection of 5 x 10^6 $CD45^{-}$ primary tumor cells. Representative dot plots of IgG isotype controls and expression profiles of EpCAM and MHCII $CD45^{-}$ cells are shown in the top row. ICAM1 expressions by $EpCAM^{+}MHCII^{-}$ (P1) and $EpCAM^{+}MHCII^{+}$ (P2) fractions are shown in the bottom row. C, RT-PCR analyses of gene expressions of lineage-specific markers (*P450*, *CC10*, and *SPC*), transcription factors (*Foxj1*, *FoxA2*, and *TTF-1*), and GAPDH (as the internal control). Samples included $EpCAM^{+}MHCII^{-}$ and $EpCAM^{+}MHCII^{+}$ fractions of lung cells from conditional mutant mice, respectively, prior to and after $Kras^{G12D}$ activation for 12 weeks.

Supplementary Fig. S4. immunofluorescence analyses of various cell fractions from conditional mutant mice.

Immunofluorescence staining with antibodies against panCK (red) and proSPC (green), and counterstained with DAPI (blue) for the $EpCAM^{+}MHCII^{-}$ and $EpCAM^{+}MHCII^{+}$ cell fractions of lung cells from mice prior to and after $Kras^{G12D}$ activation for 12 weeks.

Quantification of panCK$^+$ and panCK$^+$proSPC$^+$ cells on cytospun slides for the EpCAM$^+$MHCII$^-$ and EpCAM$^+$MHCII$^+$ cell fractions (mean ± s.d, $p < 0.01$, $n = 3$, two-tailed *t*-test).

Supplementary Fig. S5. inherent and mutated Kras expressions of various lung cell fractions from conditional mutant mice.

A, two sets of primers specific for inherent and mutated Kras, separately, were designed as shown. B, RT-PCR analyses of inherent and mutated Kras expressions in the EpCAM$^+$MHCII$^-$ plus EpCAM$^+$MHCII$^+$ cells from lungs of conditional mutant mice prior to and after Kras^{G12D} activation for 12 weeks. The *GAPDH* was used as internal controls. C, The PCR products of inherent and mutated Kras, separately, were further examined by DNA sequencing.

Supplemental Materials and methods

Mice

LSL-Kras^{G12D} knock-in mice were obtained from NCI Mouse Models of Human Cancers Consortium. *CCSP-rtTA* and *TetO-Cre* transgenic mice were obtained from Jackson Laboratory. *CCSP-rtTA/TetO-Cre/LSL-Kras^{G12D}* conditional mutant mice were treated with doxycycline in drinking water (1 mg/ml; Sigma) beginning when the mice were about 6~8 weeks old. All mice were housed in a pathogen-free environment, and experiments were performed with the approval of the Institute of Cellular and Organismic Biology, Academia Sinica and Animal Use Committee.

Genotyping for conditional mutant mice

Transgenic and knock-in mice were identified using PCR primers specific for each transgene. The *CCSP-rtTA* transgenic mice, forward *CCSP* promoter region: (5'-ACT GCC CAT TGC CCA AAC AC-3') and the reverse *rtTA* coding sequence: (5'-AAA ATC TTG CCA GCT TTC CCC-3'); *TetO-Cre* transgenic mice, forward *TetO* region: (5'-GCC ATC CAC GCT GTT TTG-3') and reverse *Cre* coding sequence: (5'-AGA GAC GGA AAT CCA TCG CTC G-3'); *LSL-Kras^{G12D} knock-in* mice, forward *STOP* element region (5`-AGC TAG CCA CAA TGG CTT GAG TAA GTC TGC A-3`), forward wild type *Kras* (5`- GTC GAC AAG CTC ATG CGG GTG-3`) and reverse *Kras* (5`- CCT TTA CAA GCG CAC GCA GAC TGT AGA-3`). Amplification of PCR products for *CCSP-rtTA* and *TetO-Cre* transgenic mice were performed as follows: denaturation at 95°C for 5 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s, followed by a 5-min extension at 72°C, and for *LSL-Kras^{G12D}* knock-in and wild type Kras^{G12} allele were performed as follows: denaturation at 95°C for 5 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 67°C for 45 s, and extension at 72°C for 45 s, followed by a 5-min extension at 72°C.

Fluorescence-activated cell sorting (FACS) analysis of total lung cells

Single-cell suspension prepared from lung tissues of the conditional mutant mice was performed as described (1). Briefly, the conditional mutant mice were killed, and their lungs were removed and cut into small pieces. After washing in Hank's buffer containing penicillin (100 units/ml) and streptomycin (100 μg/ml), the tissues were treated with 0.1% protease type-XIV (Sigma) in Joklik's MEM (Sigma) at 4°C overnight.

Afterwards, lung tissues were transferred to 10% FCS/Joklik's MEM, pipetted more than 40 times to release cells from lungs tissues and then filtered through a 100-μm nylon cell strainer. The remaining debris of lung tissues in nylon cell strainer were further homogenized by syringe, and washed twice in PBS supplemented with 5% fetal or newborn calf serum, thus to maximize the suspension of the lung cells to be released.

Antibodies for Fluorescence-activated cell sorting (FACS) analysis

To define surface markers, mouse lung cell suspensions were reacted with various antibodies and reagents including biotin-conjugated anti-CD45 (eBioscience, USA), FITC-conjugated antibody to Sca1 (eBioscience), FITC-conjugated antibody to ICAM1 (Pharmingen, USA), phycoerythrin (PE)-conjugated antibody to EpCAM (eBioscience), and allophycocyanin (APC)-conjugated antibody to MHCII (eBioscience); and FITC-conjugated rat IgG2a (eBioscience), FITC-conjugated Hamster immunoglobulin G1 (IgG1) (Pharmingen), PE-conjugated rat IgG2a (eBioscience), and APC-conjugated rat IgG2b (eBioscience) as relevant isotype controls. After washing, samples were further reacted with APC-Cy7-conjugated streptavidin and propidium iodide (Pharmingen) to eliminate $CD45^+$ and dead cells. Cell sorting was performed using a BD FACS Aria or FACSVantage. (Becton Dickinson, USA). Analysis was done using a BD Canto II bench top analyzer (Becton Dickinson), and data were analyzed using BD FACSDiva software (Becton Dickinson). To detect the phosphoproteins, the fractionated cells were fixed with 2 % formaldehyde for 10 minutes at room temperature, and then fixed in 100 % methanol for 16 hours at -20°C. After incubation of rabbit IgG isotype (Santa Cruz, USA) and primary antibodies, fractionated cells were washed and incubated with Alexa488-

conjugated anti-rabbit antibody (Molecular Probe, USA) for 1 h at room temperature.

Antibodies for immunofluorescence and immunohistochemical staining

The primary antibodies for immunohistochemical and immunofluorescence staining were: β-tubulin IV (Sigma, USA), CC10 (Santa Cruz), proSPC (Millipore, USA), pan-cytokeratin (Millipore), Ki67 (DAKO, USA); phosphor-p44/p42 MAPK (Thr202/Tyr204), total ERK1/2, phosphor-Stat3, (Tyr705), and phosphor-Akt (Ser473) (Cell Signaling, USA). The secondary antibodies for immunohistochemical staining: donkey anti-goat, goat anti-rabbit, goat anti-mouse, and goat anti-rat antibodies (Vector, USA); for immunofluorescence staining: Alexa488-conjugated donkey anti-goat IgG, goat anti-rabbit IgG, goat anti-mouse IgG (Invitrogen, USA), and Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, USA).

Immunohistochemical staining

Hematoxylin and eosin (H&E) staining and immunohistochemical staining were performed on formalin-fixed paraffin sections. After overnight incubation of primary antibodies, tissue slides were washed and incubated with biotinylated-labeled secondary antibodies for 1 h at room temperature, followed by horseradish peroxidase (HRP)-conjugated streptavidin (Vector). All sections were counterstained with Mayer's hematoxylin.

Immunofluorescence staining

Fractionated cells were cytospun onto glass slides and fixed in 4%

paraformaldehyde/phosphate-buffered saline (PBS) for 15 min at room temperature, permeabilized with 0.5% Triton X-100 in PBS for 5 min, and then blocked with 5% bovine serum albumin (BSA)/PBS for 30 min. Slides were incubated at 4 °C with primary antibodies. After overnight incubation, cells were washed and incubated for 1 h at room temperature with secondary antibodies. Cells were then counterstained with DAPI (Pharmingen). The percentages of β-tubulin IV$^+$, proSPC$^+$, CC10$^+$, and pan-cytokeratin$^+$ cells were calculated by counting the proportion of positive cells from at least 400 DAPI-labeled cells.

Colony-formation assay and measurement of cytokines

The colony-formation assay was as described (2). Briefly, fractionated cells resuspended in 90 μL of Matrigel (Pharmingen) prediluted 1:1 (vol/vol) with media were added to a 24-well transwell filter insert (Polyester transwell insert; Corning) in a 24-well tissue culture plate containing 450 μL of media. The medium was changed every other day. The number of colonies per insert was counted with an inverted microscope. All images are representative of cultures grown for 10~12 days. Conditioned medium of PD98059 - or DMSO (sigma)-treated cell cultures were collected after 2 days of incubation. Cytokines were quantified using the Beadlyte mouse 22-Plex Cytokine Detection system (Millipore).

RT-PCR and Real-time quantitative PCR

Total RNA from sorted cell populations was extracted using an RNeasy spin column (Qiagen, USA) kit. One microgram of total RNA used for complementary

(c)DNA was made using cDNA synthesis with a Sensiscript Reverse Transcriptase kit (Qiagen), which was performed as per the manufacturer's suggestions. The primers for the reverse-transcription (RT)-PCR were *Kras* (sum of inherent and mutated Kras): 5'-AGC ACG ACC CTA CGA TAG AGG ACT CCT -3' (forward), inherent *Kras*: 5'-GAG TAT AAA CTT GTG GTG GTT GGA GCT GG-3' (forward), mutated *Kras*: 5'-GAG TAT AAA CTT GTG GTG GTT GGA GCT GA-3' (forward), and 5'-CTA ATG TAT AGA AGG CAT CGT CAA CAC CC-3' (reverse); *Sprouty-2*: 5'-TGT GAG GAC TGT GGC AAG TGC-3' (forward) and 5'-TTT AAG GCA ACC CTT GCT-3' (reverse); *gp130*: 5'-TGT CAG CAC CAA GGA TTT-3' (forward) and 5'-GTA GCT GAC CAT ACA TGA AGT G-3' (reverse); *EpCAM*: 5'-TTG CTC CAA ACT GGC GTC TA-3' (forward) and 5'-ACG TGA TCT CCG TGT CCT TGT-3' (reverse); *MHCII* (*H2-Eb*): 5`-GAG AAC CTG CGC TTC GAC AGC-3' (forward) and 5'-CAC CTG GCA GGT GTA AAC CTC-3' (reverse); *P450* (*2F2*): 5'-TCA TCG ACT GCT TCC TCA CAA A-3' (forward) and 5'-CAT CAG CAG GGT ATC CAT ATT GAA-3' (reverse); *CC10*: 5'-CCA GCT GAA GAG ACT GGT GGA T-3' (forward) and 5'-TTA CAC AGA GGA CTT GTT AGG ATT TTC T-3' (reverse); *SPC*: 5'-TTT CCT AGG CCT TGC TGT-3' (forward) and 5'-TTT GTG ATA GGA TCC CCC-3' (reverse); *FoxJ1*:5'-CAC CCG GCA AGC CCA CAT CGT C -3' (forward) and 5'-CCT TGC CGG GCT CAT CCT TCT CC-3' (reverse); *FoxA2*: 5'-TGG TCA CTG GGG ACA AGG GAA-3' (forward) and 5'-GCA ACA ACA GCA ATA GAG AAC-3' (reverse); *TTF-1*: 5'-CAT CTC CCG CTT CAT GGG-3' (forward) and 5'-CCG CTG TCC TGC TGC AGT-3' (reverse); and *GAPDH*: 5'-ACC ACA GTC CAT GCC ATC TGT A-3' (forward) and 5'-TCC ACC ACC CTG TTG CTG TA -3' (reverse).

A real-time quantitative PCR system was used to quantify the relative mRNA levels in the samples as described previously (3). The primers were designed for Fast SYBR Green system (Applied Biosystems). Briefly, the PCR was carried out in a thermal cycler (ABI PRISM 7900 Sequence Detection System; Applied Biosystems) in the following sequence: reaction at 50°C for 2 min, at 95°C for 10 min, and subsequently the PCR was repeated for 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. The authenticity of PCR products was verified by 1.5% agarose gel electrophoresis. The relative concentration of each mRNA was calculated using the $\Delta\Delta C_T$ method according to the manufacturer's user manual. In other word, the threshold cycle (C_T) values of the target gene mRNA and the internal controls in the samples were first measured. The ΔC_T value of the study sample was calculated by the following formula: ΔC_T = C_T of target gene−C_T of GAPDH (designated as 'sample ΔC_T'). Similarly for the ΔC_T value of the calibrator, the C_T values of the target gene and GAPDH were obtained and the ΔC_T was calculated (designated as the 'calibrator ΔC_T'). Finally, the $\Delta\Delta C_T$ was calculated using the following formula: $\Delta\Delta C_T$ = ΔC_T (sample)−ΔC_T (calibrator); the relative value of each mRNA was calculated by the formula: $2^{-\Delta\Delta CT}$. The primers for the real-time quantitative PCR system were *CD133*: 5'-TCA TCG CTG TGG TCG TCA TTG -3' (forward) and 5'-GTC CGC TGG TGT AGT GTT GTA G-3' (reverse); *GAPDH*: 5'-AAC TTT GGC ATT GTG GAA GG-3' (forward) and 5'-GGA TGC AGG GAT GAT GTT CT-3' (reverse).

The expressions of miRNAs and internal control *snoRNA-234* were determined by TaqMan miRNA Assays Kit (Applied Biosystems) according to the manufacturer's protocol. Briefly, miR-specific reverse transcription was performed for each miRNA

from 10 ng of total RNA in 15uL reaction volume by using TaqMan microRNA Reverse Transcription Kit, and subsequent qPCR amplification was carried out using miR-specific primers on the 7300 Sequence Detection System (Applied Biosystems). Expressions of miRNAs and internal control snoRNA-234 were determined by TaqMan miRNA Assays Kit (Applied Biosystems) according to the manufacturer's protocol. Briefly, miR-specific reverse transcription was performed for each miRNA from 10 ng of total RNA in 15uL reaction volume by using TaqMan microRNA Reverse Transcription Kit, and subsequent qPCR amplification was carried out using miR-specific primers on the 7300 Sequence Detection System (Applied Biosystems). For miRNA quantification assays, The threshold cycle (Ct) data were determined using default threshold setting and total RNA input was normalized to the Ct values of common internal control, snoRNA- 234.

Western blot analysis and measurement of phosphoproteins

For the preparation of whole-cell lysates, cells were lysed in modified RIPA buffer (Millipore) and 1X protease inhibitor cocktail (Roche, USA). Protein concentrations were measured using Bio-Rad protein assay dye regent (Bio-Rad, USA). Proteins (10 to 20 μg) were separated on 12% SDS-PAGE and transferred onto PVDF membrane (Millipore). The membranes were probed with antibodies recognizing Kras (Santa Cruz), phosphor-MEK1/2 (Ser217/221), total MEK1/2, phosphor-p44/p42 MAPK (Thr202/Tyr204), total ERK1/2, phosphor-Stat3 (Tyr705), Stat3 and phosphor-Akt (Ser473), and Akt (Cell Signaling), Sprouty-2 (Abcam) and β-actin (Sigma) and then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies. Bands were visualized using the enhanced chemiluminescence (ECL) reagents

(PerkinElmer). Band intensities were quantified using AlphaErase FC V6.0 software (AlphaInnotech). In addition, quantification of the relative expression levels in each cell fractions of mice are performed after normalization with the controls. Phosphoproteins were quantified using the MILLIPLEX MAP 8-Plex Multi-Pathway Signaling Kit (Millipore). The 8-plex Multi-Pathway Signaling kit for phosphoprotein was used to detect changes in phosphorylated p44/p42 MAPK (ERK1/2) (Thr185/Tyr187), Stat3 (Ser727), JNK (Thr183/Tyr185), p70 S6 kinase (Thr412), IkB-α (Ser32), Stat5A/B (Tyr694/699), CREB (Ser133), and p38 (Thr180/Tyr182) in cell lysates using the Luminex® system.

Cell culture

M210B4 were purchased from ATCC (USA) and the medium for this cell line is 10% FBS/RPMI-1640.

Purification of $EpCAM^{+}MHCII^{-}$ plus $EpCAM^{+}MHCII^{+}$ cells

The $EpCAM^{+}MHCII^{-}$ plus $EpCAM^{+}MHCII^{+}$ cells were purified using phycoerythrin (PE)-conjugated antibody to EpCAM (eBioscience) and microbeads conjugated antibody to PE (Miltenyi Biotec, USA). Afterwards, aliquots of samples were run through MACS column (Miltenyi Biotec, USA) with magnet according to the manufacturer's instructions. After further washings, the bead-attached cells were eluted and analyzed for EpCAM/MHCII expressions and lysed in modified RIPA buffer (Millipore) and 1X protease inhibitor cocktail (Roche).

Reference

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3. Lin RJ, Lin YC, Chen J, Kuo HH, Chen YY, Diccianni MB, et al. microRNA signature and expression of Dicer and Drosha can predict prognosis and delineate risk groups in neuroblastoma. Cancer Res. 2010;70:7841-50.

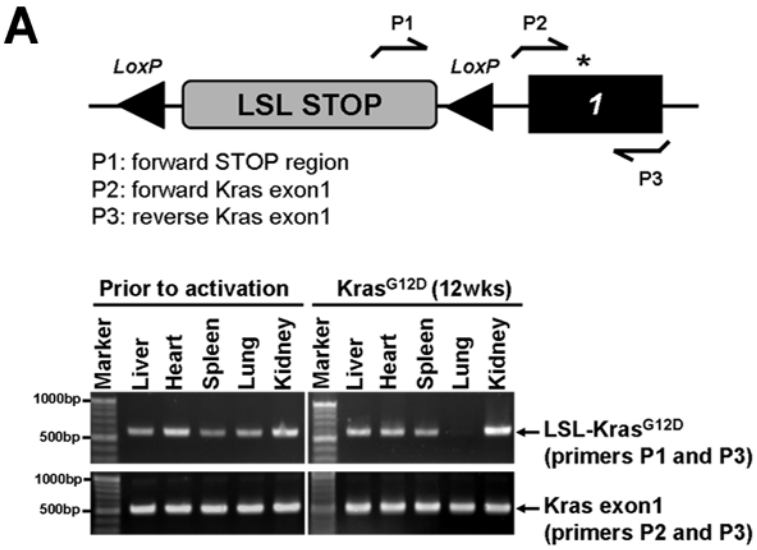
Supplementary Table S1

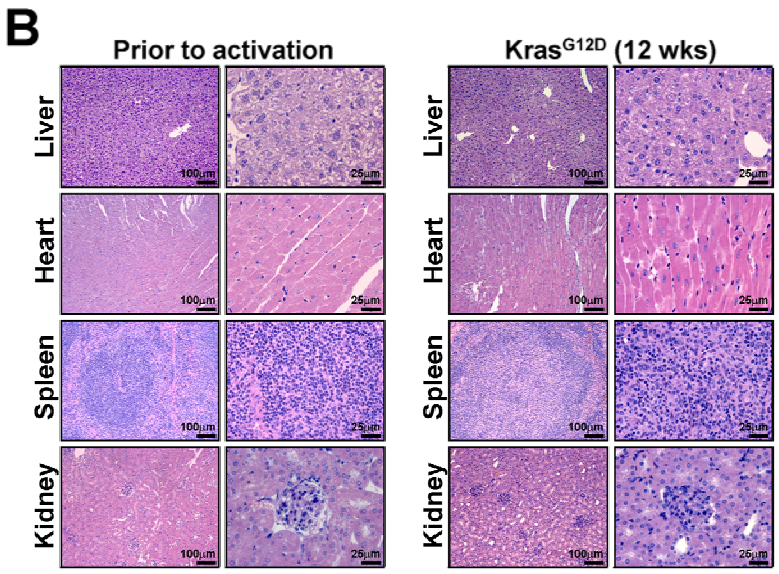
TableS1. Engraftment results of cell fractions

	Number of cells injected		
Cell fraction	$5X10^5$	$1X10^5$	$5X10^4$
$EpCAM^+MHCII^-$	6/6 (100%)[a]	6/6 (100%)[b]	5/5 (100%)[b]
$EpCAM^+MHCII^+$	6/6 (100%)[a]	4/6 (66%)[b]	1/5 (20%)[b]

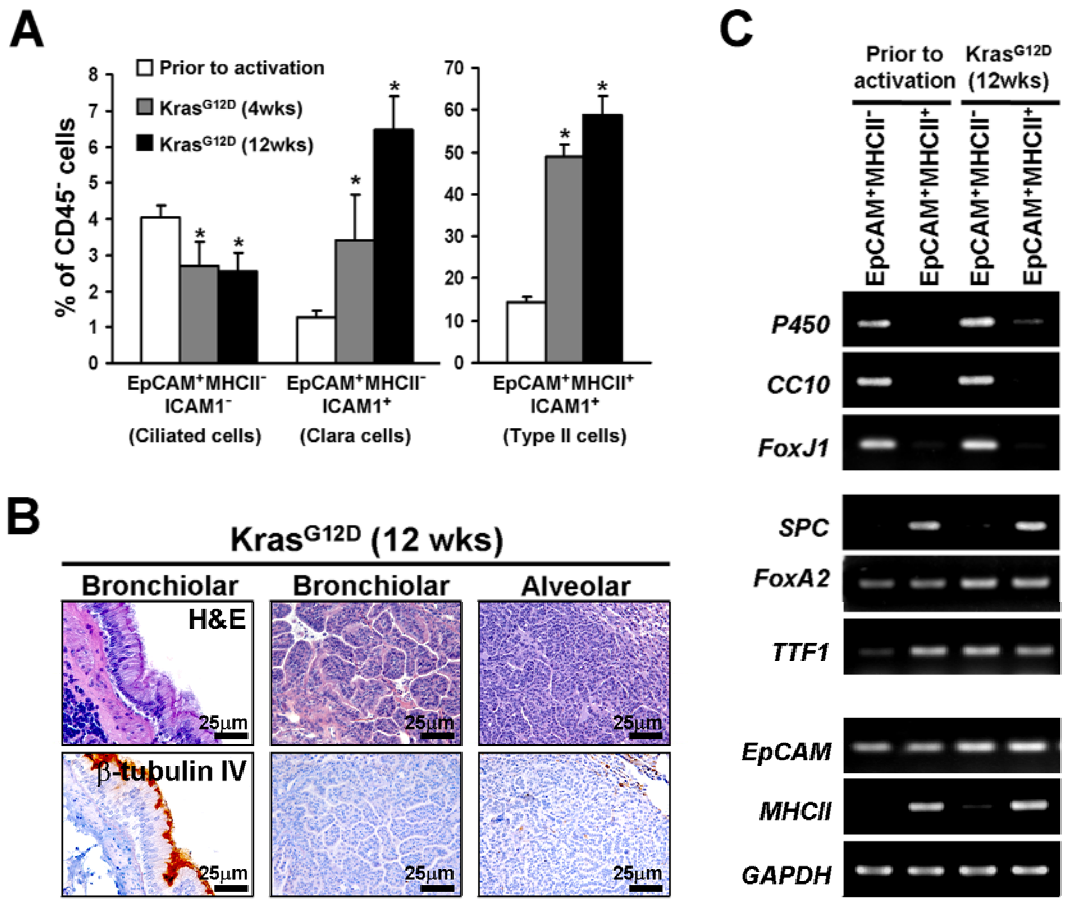
[a]Tumor foci more than 250nm in diameter.
[b]Tumor foci more than 100nm in diameter.

Supplementary Figure S1

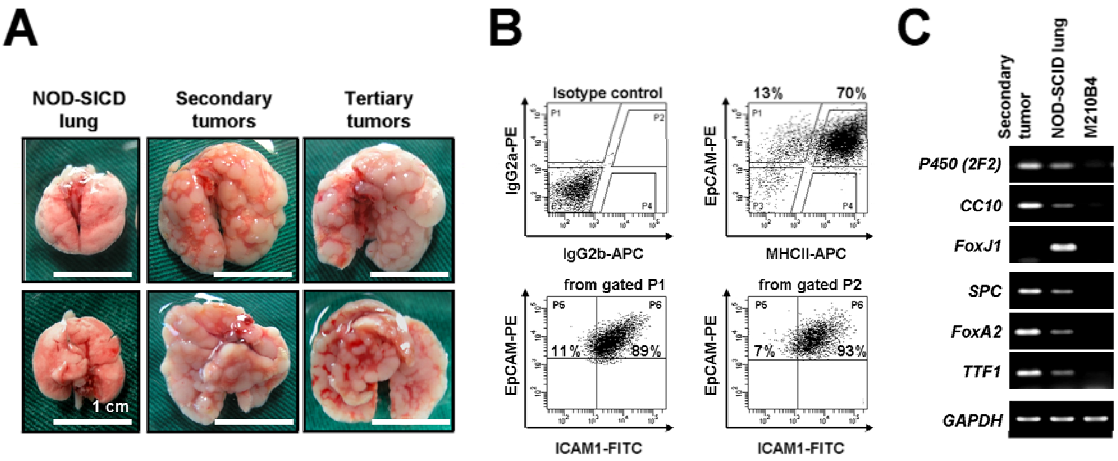




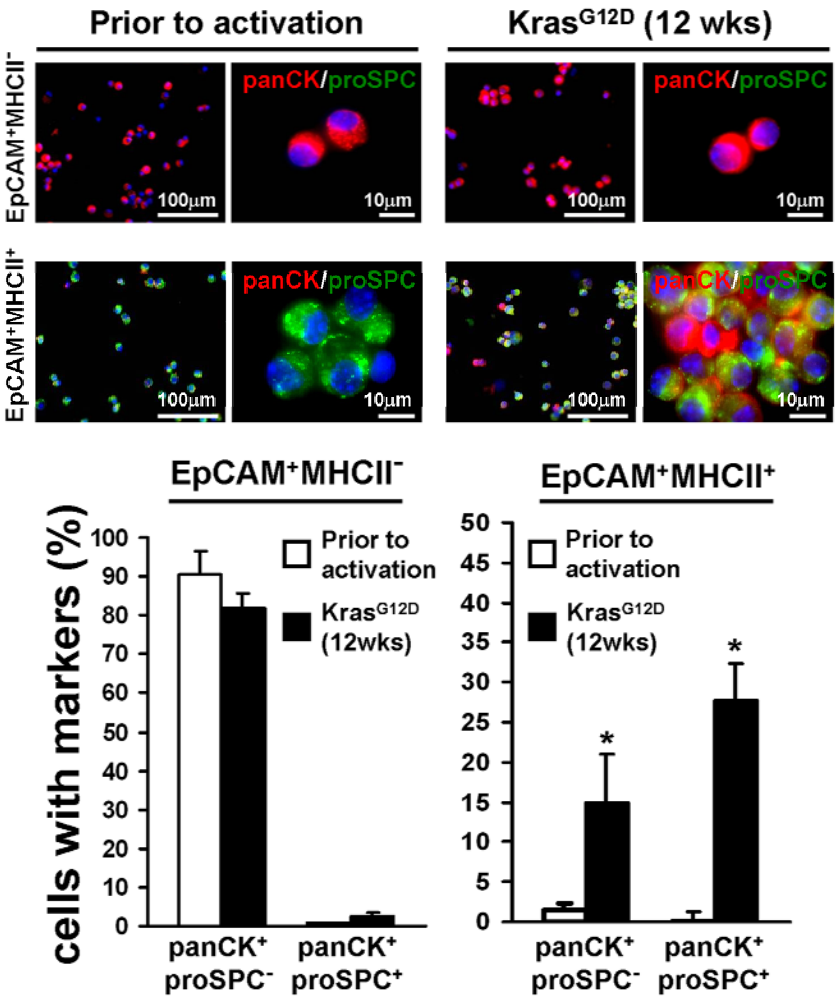
Supplementary Figure S2



Supplementary Figure S3



Supplementary Figure S4



Supplementary Figure S5