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Supplemental Information

Monocarboxylate Transporter 4 Is a Therapeutic Target in Non-small Cell Lung Cancer with Aerobic Glycolysis Preference

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Supplemental data
Figure S1

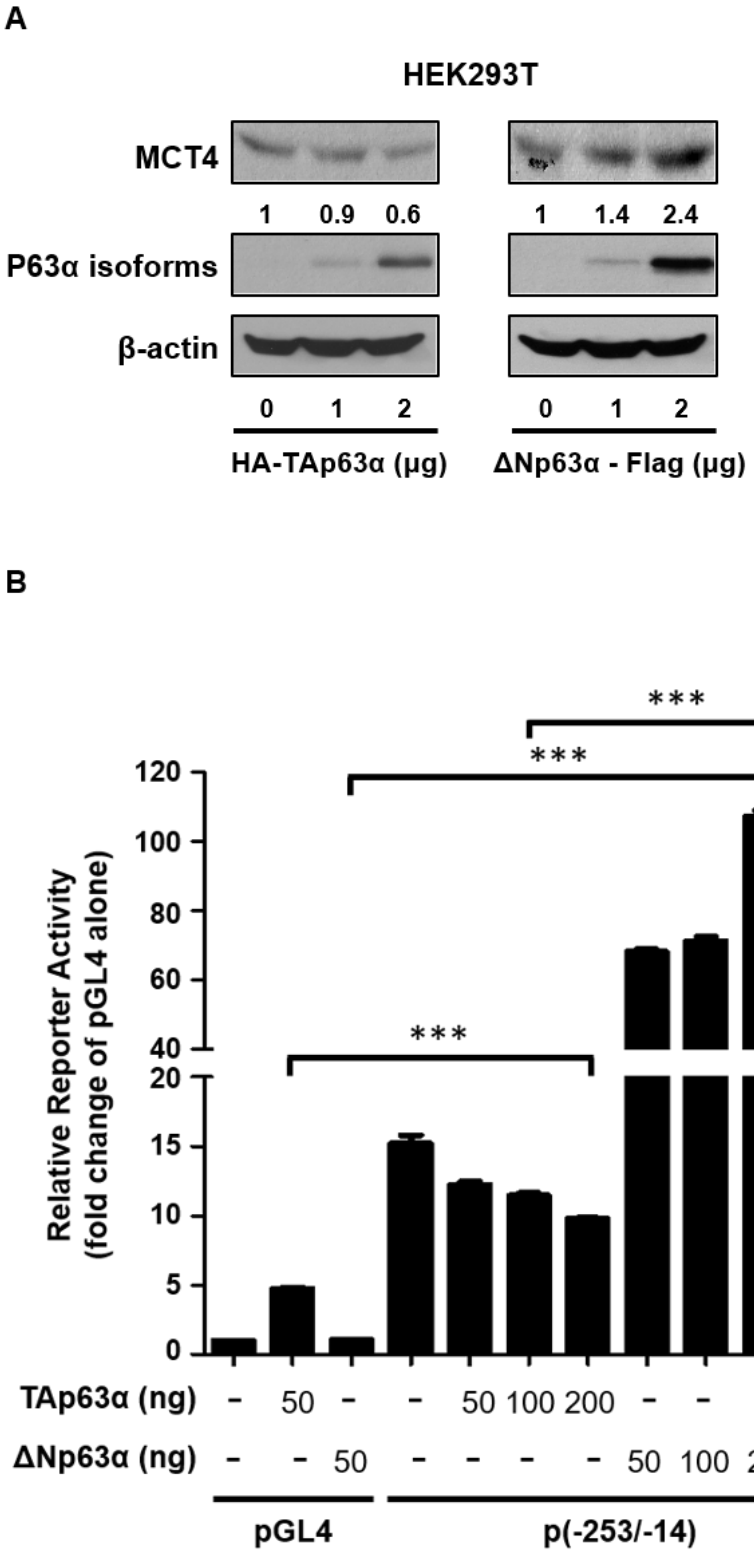

Figure S1 - TAp63α will suppress the transcription of *SLC16A3* gene while ΔNp63α would promote the transcription of *SLC16A3* gene in HEK293T cells

(A) The protein level of MCT4 was detected by immunoblotting in the HEK293T cells with TAp63α or ΔNp63α overexpression. (B) HEK293T cells were co-transfected with vector, TAp63α or ΔNp63α plasmids, indicated luciferase reporter constructs, and pRL-SV40 as a transfection control. Luciferase activity was measured 48 hours post-transfection. The promoter activity of the specific region between -253 to -14 on the *SLC16A3* promoter was expressed as a ratio of firefly/Renilla luciferase activities in HEK293T cells with TAp63α or ΔNp63α overexpression.

Figure S2

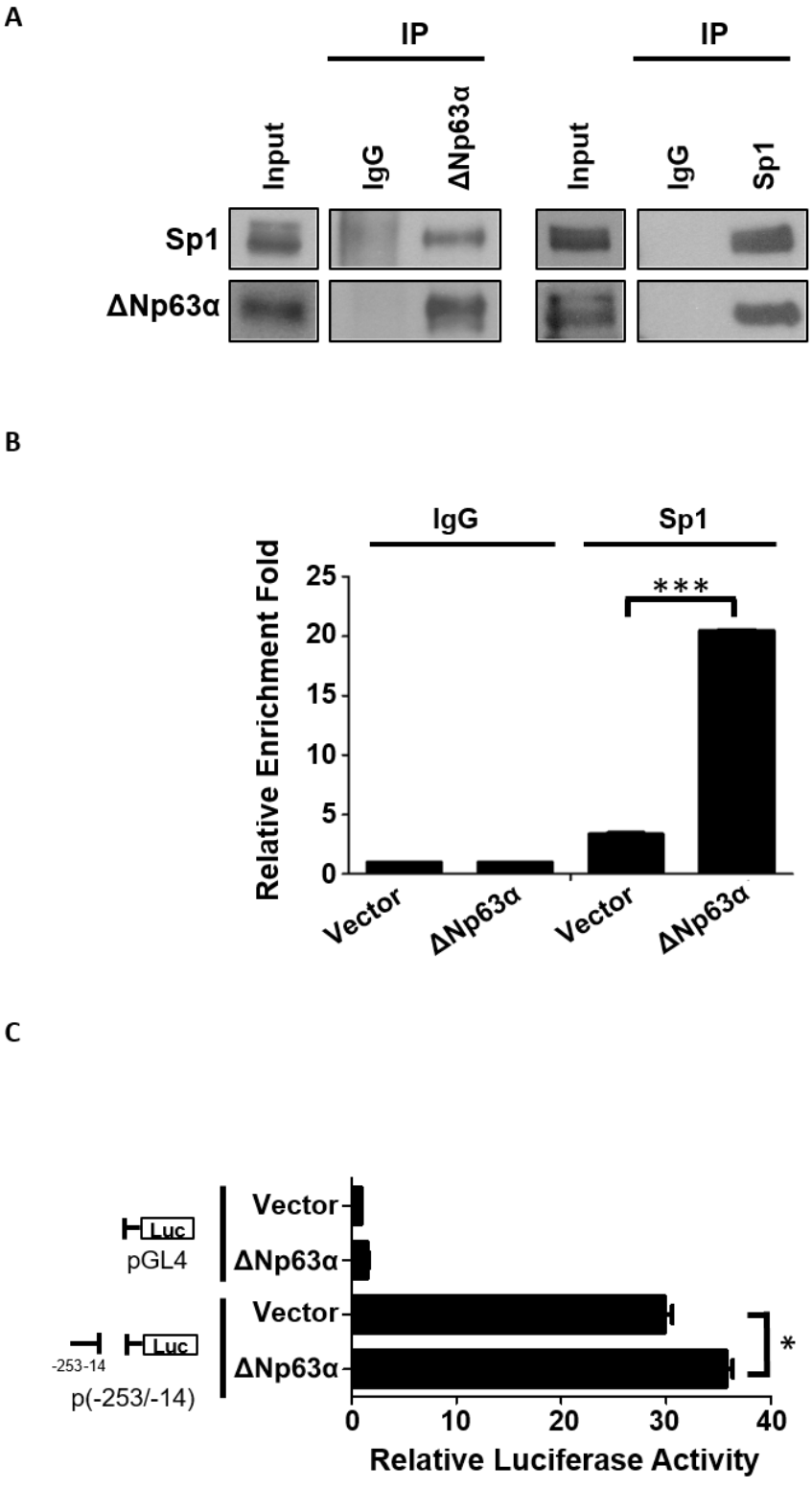
A
IP
IP
Input
IgG
ΔNp63α
Input
IgG
Sp1
Sp1
ΔNp63α
B
IgG
Sp1
Relative Enrichment Fold
25
20
15
10
5
0
***
Vector
ΔNp63α
Vector
ΔNp63α
C
Luc
pGL4
Vector
ΔNp63α
Luc
-253-14
p(-253/-14)
Vector
ΔNp63α
*
0
10
20
30
40
Relative Luciferase Activity

Figure S2 - ΔNp63α facilitates the binding of Sp1 to the promoter region of *SLC16A3* and stimulates the promoter activity of *SLC16A3*

(A) The interaction between ΔNp63α and Sp1 was detected by immunoprecipitation method in PC9 cells overexpressed ΔNp63α. PC9 cell lysates were immunoprecipitated with anti-ΔNp63α, anti-rabbit IgG (left panel), anti-Sp1, or anti-rabbit IgG (right panel) antibodies and immunoblotted with the indicated antibodies. (B) The interaction of Sp1 with the specific region between -253 to -14 on the *SLC16A3* promoter was determined by the ChIP assay in PC9 cells with or without ΔNp63α overexpression. PC9 cells were transfected with vector or ΔNp63α plasmids for 30 h, and then ChIP assays were performed using anti-Sp1 antibody. Immunoprecipitated chromatin was analyzed using specific TaqMan probe with primers specific to the *SLC16A3* promoter, between -253 to -14. (C) PC9 cells were co-transfected with vector or ΔNp63α plasmids, indicated luciferase reporter constructs, and pRL-SV40 as a transfection control. Luciferase activity was measured 48 hours post-transfection. The promoter activity of the specific region between -253 to -14 on the *SLC16A3* promoter was expressed as a ratio of firefly/Renilla luciferase activities in PC9 NSCLC cells with or without ΔNp63α overexpression.

Figure S3

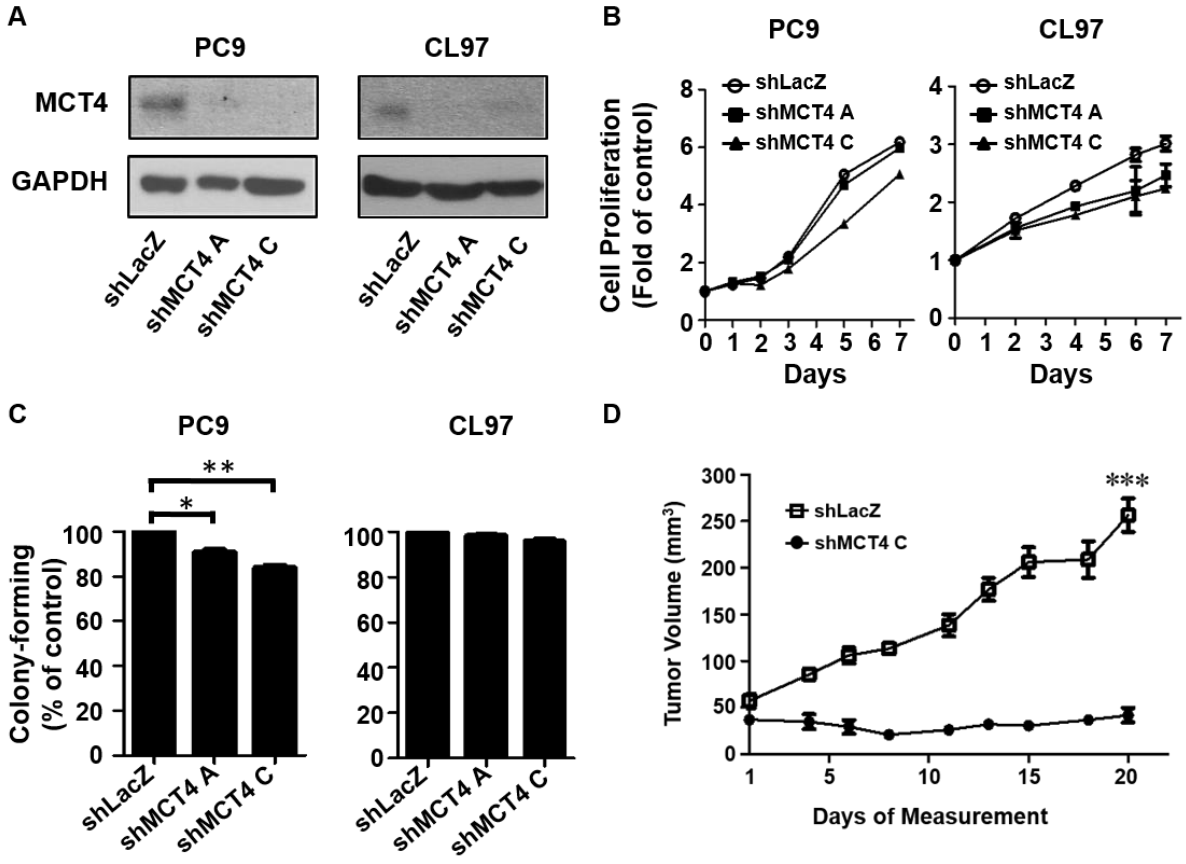
A
PC9
CL97
MCT4
GAPDH
shLacZ
shMCT4 A
shMCT4 C
B
PC9
CL97
shLacZ
shMCT4 A
shMCT4 C
Cell Proliferation (Fold of control)
Days
C
PC9
CL97
Colony-forming (% of control)
D
shLacZ
shMCT4 C
Tumor Volume (mm³)
Days of Measurement

Figure S3 – Knockdown of MCT4 has mild effects *in vitro* but has significant anti-proliferation effect *in vivo* in the OXPHOS-preference NSCLC cell subtype

(A) The protein level of MCT4 after transduction with shRNA virus targeting *SLC16A3* gene or LacZ control was detected by immunoblotting in PC9 and CL97 cells. (B) The cell proliferation rate of PC9 and CL97 cells after transduction with shRNA virus targeting *SLC16A3* gene or LacZ control was detected by SRB assays. The cells were fixed at the indicated day and detected as described in Methods. The graph indicated the fold change between each day vs. day 0. (C) The colony formation ability of PC9 and CL97 cells was detected by colony formation assays after transduction with shRNA virus targeting *SLC16A3* gene or LacZ control. The colonies were fixed, stained, and dissolved as described in Methods. The graph indicated the total absorbance values at 490 nm in shMCT4 groups relative to that in shLacZ group, which were set to 100%. (D) The growth curve of PC9 subcutaneous tumors in nude mice with or without MCT4 knockdown by shRNA virus.

Figure S4

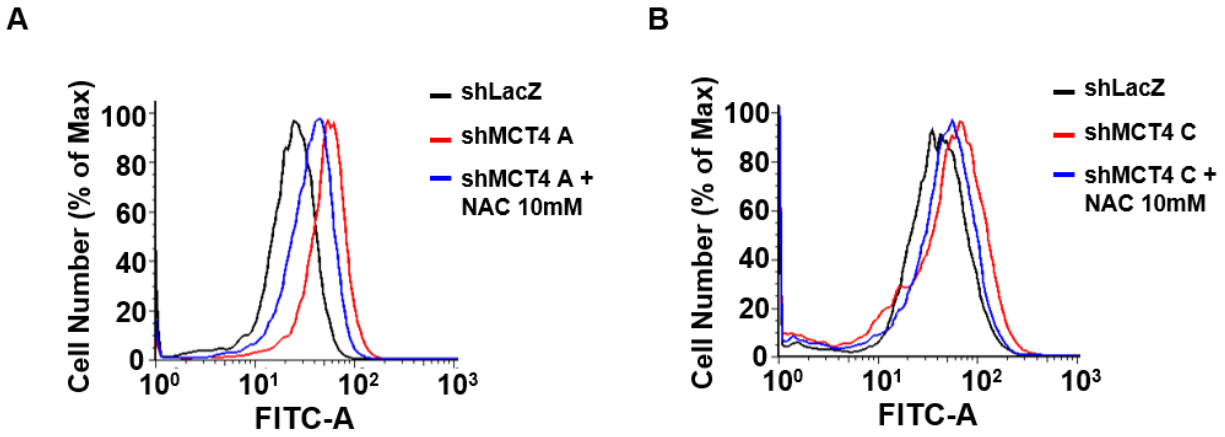
A
Cell Number (% of Max)
100
80
60
40
20
0
10^0
10^1
10^2
10^3
FITC-A
shLacZ
shMCT4 A
shMCT4 A +
NAC 10mM
B
Cell Number (% of Max)
100
80
60
40
20
0
10^0
10^1
10^2
10^3
FITC-A
shLacZ
shMCT4 C
shMCT4 C +
NAC 10mM

Figure S4 – NAC can effectively reduce intracellular ROS induced by MCT4 knockdown in CL1-5 NSCLC cells

(A and B) The intracellular ROS level was detected in CL1-5 NSCLC cells with or without 10 mM NAC treatment at 72 hours after transduction with shRNA virus targeted *SLC16A3* gene or LacZ control by flow cytometry with DCFH-DA dye.

Figure S5

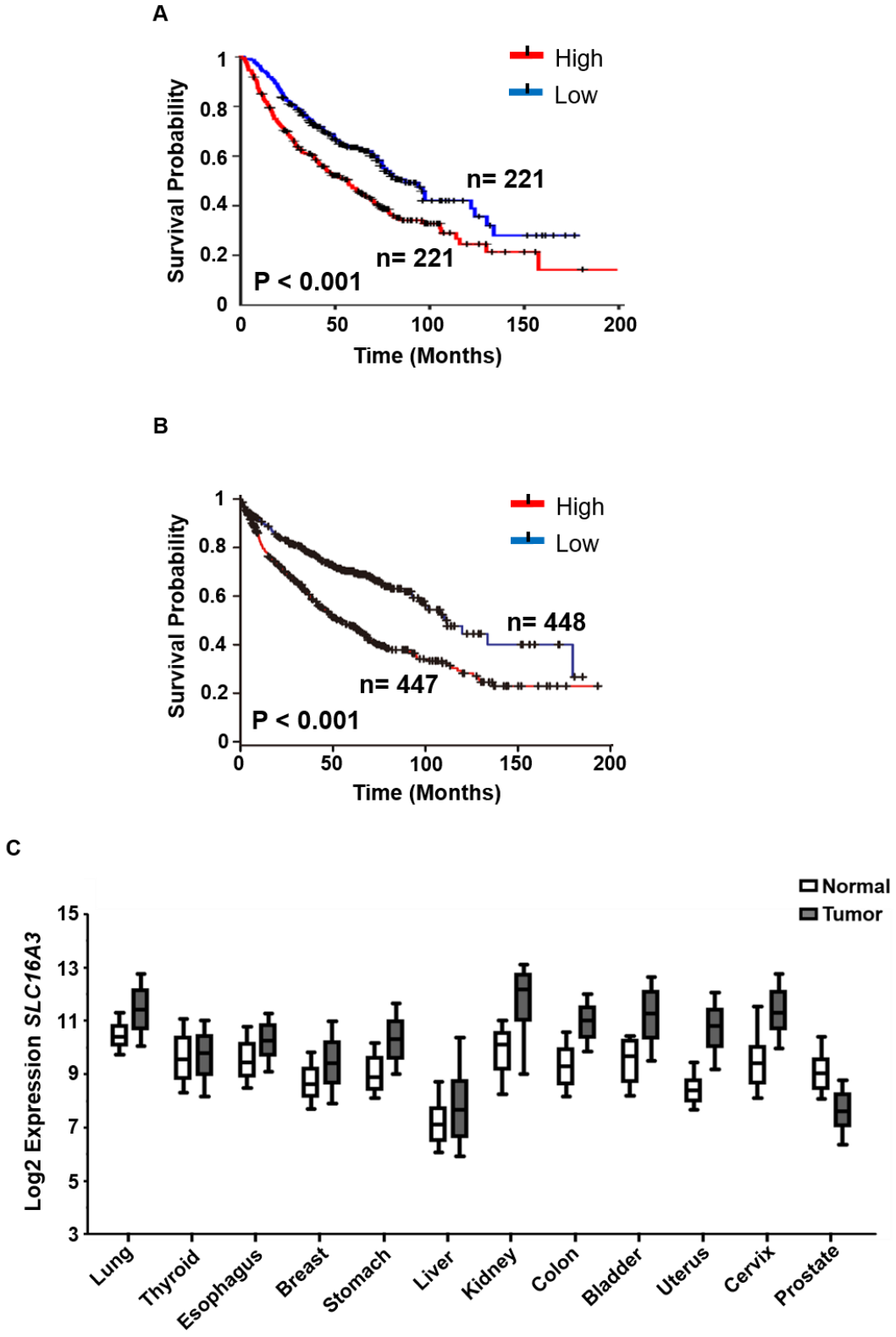
A
High
Low
Survival Probability
n= 221
n= 221
P < 0.001
Time (Months)
B
High
Low
Survival Probability
n= 448
n= 447
P < 0.001
Time (Months)
C
Normal
Tumor
Log2 Expression SLC16A3
Lung
Thyroid
Esophagus
Breast
Stomach
Liver
Kidney
Colon
Bladder
Uterus
Cervix
Prostate

Figure S5 - High expression of MCT4 can be observed in many types of cancer tissues and serves as a poor prognostic factor for NSCLC patients

(A and B) The correlation between *SLC16A3* gene expression level and overall survival of NSCLC patients. The correlation was measured using Kaplan-Meier survival curve analysis with 202856_s_at probe in Shedden and KM-plotter datasets. (C) The RNA expression data of *SLC16A3* gene across tissues and cancer types. The data were downloaded from Genotype Tissue Expression project (GTEx) and The Cancer Genome Atlas (TCGA) and normalized by a pipeline developed by Wang and his colleagues.[1-3] The line in the middle of the box was plotted at the median and the whiskers were drawn down to the 10th percentile and up to the 90th.

Figure S6

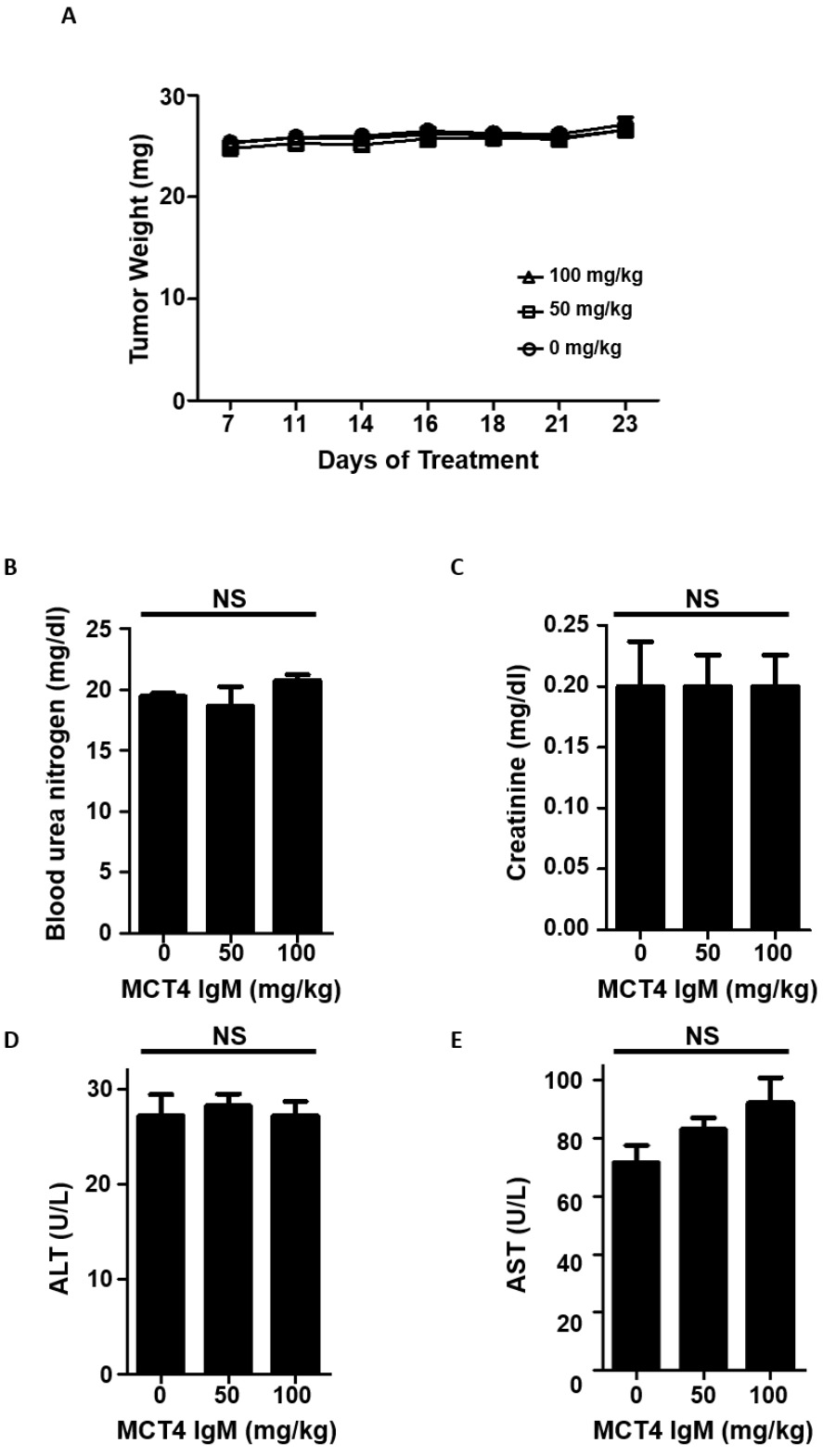
A
Tumor Weight (mg)
30
20
10
0
7
11
14
16
18
21
23
Days of Treatment
100 mg/kg
50 mg/kg
0 mg/kg
B
NS
Blood urea nitrogen (mg/dl)
25
20
15
10
5
0
0
50
100
MCT4 IgM (mg/kg)
C
NS
Creatinine (mg/dl)
0.25
0.20
0.15
0.10
0.05
0.00
0
50
100
MCT4 IgM (mg/kg)
D
NS
ALT (U/L)
30
20
10
0
0
50
100
MCT4 IgM (mg/kg)
E
NS
AST (U/L)
100
80
60
40
20
0
0
50
100
MCT4 IgM (mg/kg)

Figure S6 - The IgM monoclonal antibody treatment is relatively safe, resulting in minimal weight loss and no obvious toxicity to the kidney or liver tissue

(A) Body weight, (B) blood urea nitrogen, (C) Creatinine, (D) ALT, (E) AST for mice from each group. NS: non-significant.

Figure S7

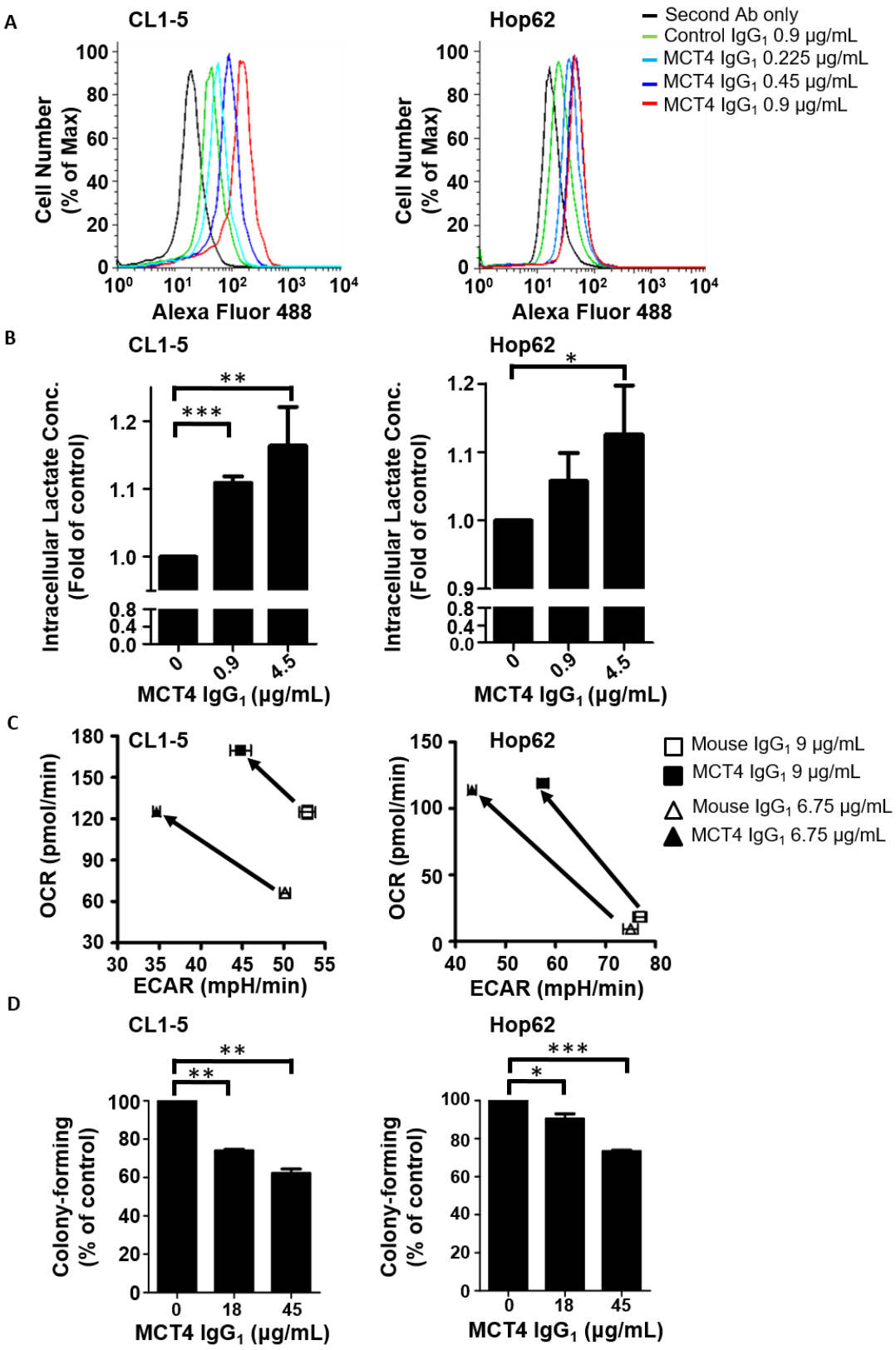
A
CL1-5
Hop62
Second Ab only
Control IgG1 0.9 μg/mL
MCT4 IgG1 0.225 μg/mL
MCT4 IgG1 0.45 μg/mL
MCT4 IgG1 0.9 μg/mL
Cell Number (% of Max)
Alexa Fluor 488
B
Intracellular Lactate Conc. (Fold of control)
MCT4 IgG1 (μg/mL)
C
OCR (pmol/min)
ECAR (mpH/min)
Mouse IgG1 9 μg/mL
MCT4 IgG1 9 μg/mL
Mouse IgG1 6.75 μg/mL
MCT4 IgG1 6.75 μg/mL
D
Colony-forming (% of control)

Figure S7 - Anti-MCT4 IgG_1 monoclonal antibody can neutralize the function of MCT4 and inhibit the proliferation of the aerobic glycolysis-preference NSCLC cell subtype

(A) The binding of our MCT4 IgG_1 antibody with cell-surface MCT4 in CL1-5 and Hop62 cells was detected using Alexa Fluor 488 conjugated anti-mouse IgG antibody by flow cytometry. (B) Intracellular lactate concentration of CL1-5 and Hop62 cells was measured by a lactate assay kit after incubating with MCT4 IgG_1 antibody or mouse IgG_1 isotype control for 24 hours. (C) The basal ECAR and OCR levels of CL1-5 and Hop62 cells was measured using a Seahore XF analyzer after incubating with MCT4 IgG_1 antibody or mouse IgG_1 isotype control for 48 hours. (D) The anti-proliferative effect of MCT4 antibody in CL1-5 and Hop62 cells. The colonies were fixed, stained, and dissolved as described in Methods after incubating with MCT4 IgG_1 antibody or mouse IgG_1 isotype control for 7 days. The graph indicated the total absorbance values at 490 nm in MCT4 IgG_1 antibody group relative to that in mouse IgG_1 isotype control group, which were set to 100%.

Table S1 - Multivariate analysis of independent prognostic factors for patient survival in the three independent datasets

	Dataset 1 (From NTUH)		**Dataset 2** (From the Shedden cohort)		**Dataset 3** (From the KM-plotter database)	
Variables	Hazard Ratio (95% CI)	P value	Hazard Ratio (95% CI)	P value	Hazard Ratio (95% CI)	P value
Gender	1.57 (0.90-2.74)	0.112	1.37 (1.06-1.70)	0.018	1.40 (1.14-1.73)	0.001
Stage	1.78 (1.29-2.45)	< 0.001	2.4 (2.05-2.8)	< 0.001	1.53 (1.35-1.75)	< 0.001
MCT4	1.72 (1.01-2.91)	0.045	1.21 (1.03-1.30)	0.003	1.81 (1.47-2.23)	< 0.001

Supplemental Materials and Methods

Reagents Roswell Park Memorial Institute (RPMI) 1640 medium, Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were obtained from GIBCO/BRL Life Technologies (Grand Island, NY). Antibodies against MCT1 (SC-365501), MCT4 (SC-50329), Sp1 (SC-17824), and anti-mouse (SC-2005) and anti-rabbit (SC-2004) IgGs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against MCT2 (GTX129600) was purchased from GeneTex (Hsinchu, Taiwan). Antibodies against poly(ADPribose) polymerase-1 (PARP-1) (9546), caspase-9 (9502) and caspase-3 (9662) were purchased from Cell Signaling Technologies (Boston, MA). Antibody against β-actin (A5441) was obtained from Merck Millipore (Billerica, MA). Antibody against MCT3 (ab60333) and TAp63 (ab124762) was obtained from Abcam (Cambridge, UK). Antibody against ΔNp63 (619001) was obtained from BioLegend (San Diego, CA). Antibody against Sp1 (39058) used for chromatin immunoprecipitation was obtained from BioLegend (San Diego, CA). Alexa Fluor 488-conjugate goat anti-mouse IgM antibody (A21042) was obtained from Thermo Fisher Scientific (Waltham, MA). Dimethylsulfoxide (DMSO), crystal violet, ammonium persulfate (APS), N,N,N′,N′-tetramethylenediamine (TEMED), sodium dodecyl sulfate (SDS), glycine, TRIS hydrochloride, TRIS base, formaldehyde, penicillin, streptomycin, sodium bicarbonate, glucose, 2-deoxyglucose, trichloroacetic acid, sulforhodamine B (SRB), acetic acid, Na-deoxycholate, Nonidet P-40, sodium chloride, and sodium phosphate were obtained from Merck Millipore.

Immunoblotting The cells were harvested and lysed in RIPA lysis buffer (1% Nonidet P-40, 1% Na-deoxycholate, 0.1% SDS solution, 0.15 M NaCl, and 0.01 M sodium phosphate, pH 7.2). Protein concentration was then measured by using the BCA assay (Thermo Fisher Scientific). Equivalent amounts of cell extracts were separated using SDS–PAGE with protein loading dye and transferred to polyvinylidene membranes (Merck Millipore). The membranes were blocked with 5% non-fat milk in PBS containing 0.1% Tween-20 (Merck Millipore) for 1 hour and incubated with respective primary antibodies overnight at 4ºC followed by incubation with appropriate HRP-conjugated secondary antibodies for 1 hour at room temperature. The signal was visualized with an ECL detection system.

Quantitative PCR analysis Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific). *SLC16A3* (Hs02758991_g1), the region between -253 to -14 of *SLC16A3* promoter, and the endogenous control GAPDH (Hs00358829_m1) were analyzed using Applied Biosystems TaqMan Gene Expression Assay systems (Thermo Fisher Scientific).

Construction of *SLC16A3* knockdown lentivirus Two *SLC16A3* shRNAs (shMCT4 A & shMCT4 C) and control shRNA plasmid were purchased from the National RNAi Core Facility (Taipei, Taiwan). The sequences were as follows: shMCT4 A: 5'-CCGGCGTCTACATGTACGTGTTCATCTCGAGATGAACACGTACATGTAGACGT

TTTTG-3'; shMCT4 C: 5'-CCGGGCTCATCATGCTGAACCGCTACTCGAGTAGCGGTTCAGCATGATGAGC

TTTTTG-3'; control shLacZ: 5'-CCGGTGTTCGCATTATCCGAACCATCTCGAGATGGTTCGGATAATGCGA

ACATTTTTG-3'. The three shRNAs were cloned into the vector pLKO.1. The lentiviral particles were produced by transfecting the plasmids along with the packaging plasmid pCMV-ΔR8.91 and the envelope plasmid pMD.G into 293T cells.

Luciferase reporter assay Cells were co-transfected with 200 ng of pGL4 vector or pGL4-*SLC16A3* promoter plasmid and the Renilla luciferase pRL-SV40 plasmid (Promega, Fitchburg, WI). Cells were lysed 36 hours after transfection, and luciferase activity was measured using a Dual-Luciferase system (Promega) according to the manufacturer's instructions.

Chromatin immunoprecipitation Chromatin immunoprecipitation was performed using the Magna ChIP A/G Chromatin Immunoprecipitation Kit (Merck Millipore) according to the manufacturer's instructions. Briefly, Cells were fixed with 1% formaldehyde for 10 min and blocked with 125 mM glycine for 5 min. Then, the cells were suspended in cell lysis buffer and spun to pellet the nuclear fraction. The nuclear fraction was next suspended in nuclear lysis buffer and sonicated to shear the DNA. The sheared and cross-linked chromatin was then incubated with anti-Sp1 or control rabbit IgG antibody overnight at 4 °C. The DNA–protein complexes were then incubated with protein G agarose beads at 4 °C for 2 hours. Following immunoprecipitation, the beads were washed with four different buffers according to the manufacturer's protocol. The immunoprecipitated complexes were then treated with proteinase K at 62 °C for 2 hours and eluted by incubating at 95 °C for 10 min. The DNA was purified using spin columns and used as templates for qPCR amplification with primers specific to the *SLC16A3* promoter region.

Measurement of intracellular lactate concentration The concentration of intracellular lactate was measured by the L-Lactate Assay Kit I (Eton Bioscience, San Diego, CA), according to the manufacturer's instructions.

RNA-Sequencing CL1-5 NSCLC cells transduced with shRNA virus targeted *SLC16A3* gene or LacZ control was collected in TRIzol (Thermo Fisher Scientific) for RNA extraction. The RNA samples would then be purified by the RNeasy Mini Kit and DNase I (Qiagen). The mRNA contents were enriched by using oligo(dT)-labeled magnetic beads, followed by fragmentation, conversion into cDNA, ligation of sequencing adaptors, and amplification. Quality-checked library products were subjected to 75-bp paired-end sequencing using a NextSeq 500 sequencer (Illumina Inc) with a throughput of approximately 20 million reads per sample. The sequencing reads were aligned to the Ensembl GRCh37 human reference genome by TopHat2. The HTSeq software was used to count the mapped reads against Ensembl annotated genes (ENSG IDs). Gene-level read counts were normalized and fold change was evaluated.

Gene set enrichment analysis (GSEA) GSEA software was used to identify cellular functions associated with MCT4 knockdown. Briefly, transcriptome-wide genes were ranked based on the significance of differential expression between the shMCT4 and shLacZ groups. Predefined gene ontology gene sets (C5) were downloaded from the MSigDB and tested for enrichment on either side of the ranked list.

Colony formation assay Colonies were fixed with 3.7% formaldehyde and stained with crystal violet. The cell-bound crystal violet was dissolved in 10 % acetic acid and read by ELISA reader (490 nm).

MCT4 monoclonal antibody production and purification BALB/c mice were used to produce MCT4 monoclonal antibody. The hybridoma supernatants and ascites fluid were obtained according to current protocols. Briefly, BALB/c mice (6 weeks old) were given five subcutaneous injections of Freund's adjuvant (Merck Millipore) mixed with the peptide with the sequence pertaining to the extracellular domain of the MCT4 protein (peptide sequence: PLGQLLQDRYGWRGGFLILGGC). Three days after the final injection, 10 μg MCT4 peptide was intraperitoneally injected into the mice. The spleens were removed 3 days after the final intraperitoneal injection. Lymphocytes were isolated and fused with the FO myeloma cell line using a standard polyethylene glycol fusion technique. The hybridoma supernatants were tested for MCT4 inhibition using the lactate assay kit. For antibody ascites production, BALB/c mice (8 weeks old) were intraperitoneally injected with 0.5 ml of pristane (2, 6, 10, 14-tetramethyldecanoic acid) at the first day. After one week, approximately 2×10^6 hybridoma FO cells/ml in PBS were injected into the pristine-treated BALB/c mice. The mice were observed daily for abdominal distension and ascites fluid was collected approximately 7-14 days after cell injections. After the collection of ascites fluid, the fluid was centrifuged at 3000x g for 15 minutes to separate the serum from other blood components. The straw-colored fluid was collected as the ascites fluid. The fluid was then stored at -80°C until the time of purification. The mouse Ig isotype was determined using the Mouse Ig Isotyping Ready-SET-Go! ELISA Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The anti-MCT4 IgM monoclonal antibody was further purified with 1 ml HiTrap IgM Purification HP columns (GE Healthcare Life Sciences, Chicago, IL) according to the manufacturer's instructions.

***In vivo* animal models** Animals were raised in a specific-pathogen-free environment in the animal facility at the Institute of Biomedical Sciences, Academia Sinica.

In the subcutaneous tumor model, CL1-5 (2×10^6/ mouse), Hop62 (2×10^6/ mouse), PC9 (2×10^6/ mouse) or A549 (4×10^6/ mouse) cells were subcutaneously injected into the flanks of 6-week-old male NOD/SCID mice or nude BALB/c nu/nu mice. When the size of subcutaneous tumor reached 50 mm^3, the animals were randomized divided into different groups. For the experiment of metformin treatment, the animals were given intraperitoneal injections of PBS or metformin (300 mg/kg in Hop62 group and 250 mg/kg in A549 group) daily. For the experiment of MCT4 knockdown by inducible shRNA system, 25mg/kg doxycycline hyclate was given daily by oral gavage. For the experiment of anti-MCT4 IgM monoclonal antibody, the animals in the control group were given intratumor injections of elution buffer with control mouse IgM once a week for 3 weeks. The animals in the treatment group were given intratumor injections with 0.5 μg or 0.25 μg anti-MCT4 antibody once a week for 3 weeks. The body weight and tumor size were measured three times per week during the treatment period. The tumor volume was determined by measuring the largest diameters (l) and smallest diameters (s), and the volumes were calculated ($V = 0.5 \times sl^2$).

Supplemental References

1. Wang Q, Gao J, Nikolaus S. Figshare http://doi.org/10.6084/m9.figshare.5330539 (2017).
2. Wang Q, Gao J, Nikolaus S. Figshare http://doi.org/10.6084/m9.figshare.5330575 (2017).
3. Wang Q, Gao J, Nikolaus S. Figshare http://doi.org/10.6084/m9.figshare.5330593 (2017).