Supplemental Methods

Tumor growth assay

Mice were intradermally inoculated with Meth A, MO5 tumor cells (2×10^5 in 0.2 mL), 3LL tumor cells (1.5×10^5 in 0.2 mL), and B16 fucci and B16 fucci δIC tumor cells (2×10^5 in 0.2 mL) on the right side of the back using a 27-gauge needle. From day 7 after tumor inoculation, the mice were orally administrated 5 mg/mL metformin hydrochloride (Tokyo Chemical Industry) and 2 mg/mL D-(+)-glucose (Fujifilm Wako Pure Chemical Corporation), dissolved in the free drinking water. 10 mg/kg anti-PD-1 antibody (4H2; Ono Pharmaceutical Co., Ltd.) was intraperitoneally injected from day 7 and at six-day intervals throughout the experiments. MitoTEMPO (Sigma-Aldrich) was intraperitoneally injected 0.7 mg/kg every day from day 6 after tumor inoculation. The long (*a*) and short (*b*) tumor axes were measured using Vernier calipers and used to calculate the mean diameter, whereas tumor volume (*V*) was calculated according to the following equation: $V = ab^2/2$. Animal survival was monitored for 80 days after tumor challenge.

Measurement for glucose level of tumors

Tumor masses were dissected from the mice and minced using a Medimachine system (AS ONE), followed by centrifugation (230 x *g*, 5 min). The supernatant was collected for the measurement. Glucose levels of the supernatant were measured using a Glucose Pilot device (IWAI CHEMICALS COMPANY).

Collection of tumor-infiltrating lymphocytes

Tumor tissues were dissected from the mice and minced into small pieces in RPMI 1640 medium (Thermo Fisher Scientific). TILs were harvested from the minced tumor tissues using a Medimachine system. All cells, including TILs and tumor cells, were stained with the indicated fluorescently-labeled antibodies and subjected to flow cytometric analysis.

Intracellular cytokine staining

Cells were stimulated with 1.25 ng/mL of Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) and 50 nM Ionomycin (Sigma-Aldrich) at 37 ℃ for 6 h in the presence of Protein Transport Inhibitor (containing Monensin; BD Bioscience) followed by staining for cell surface CD3, CD8 and Glut-1 and intracellular IFNγ (XMG1.2; BioLegend).

Measurement of mitochondrial ROS

Mitochondrial ROS production was detected using MitoSOX™ Red Mitochondrial Superoxide Indicator for live-cell imaging (Thermo Fisher Scientific). Cells were incubated in warm PBS, pH 7.4, followed by incubation with MitoSOX Red (5 μM) at 37℃ for 20 min. After washing, cells were stained for surface markers as indicated. The fluorescence of MitoSOX Red in CD8 TILs was measured using a FACS Canto II.

Glucose uptake

Cells were incubated with 1 ml of PBS containing 100 μM 2-NBDG (2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) Amino)-2-Deoxyglucose; Thermo Fisher Scientific) at 37℃ for 2h. After staining as indicated, the fluorescence of 2-NBDG in CD8 T cells were measured using a FACS Canto II.

Metabolic Assay

Cells were treated with recombinant Murine IFN-γ (10 ng/mL: PEPROTECH) for 2 days, and
OCRs and ECARs were measured in XF medium (non-buffered RPMI 1640 containing 25 mM glucose, 2 mM L-glutamine, and 1 mM sodium pyruvate) in response to 4 μM oligomycin (Sigma–Aldrich), 8 μM FCCP (Sigma–Aldrich), and 1 μM rotenone (Sigma–Aldrich) + 1 μM antimycin A (Sigma–Aldrich), 100 mM 2-DG (Sigma–Aldrich) using the XFe96 Extracellular Flux Analyzer (Agilent Technologies). Further, cells were normalized by Cytation1 Cell Imaging Multi-Mode Reader (BioTek).

CD8 T cell separation and expansion

CD8 T cells were isolated from WT and p62 KO mouse spleen by magnetic separation (Miltenyi Biotec). CD8 T cells were then stimulated with the immobilized anti-CD3 mAb (1.0 μg/mL) (eBioscience) and soluble anti-CD28 mAb (2.0 μg/mL) (eBioscience). On day 2 after the stimulation with the antibodies, the cells were collected and used for analysis.

Anti-IFNγ treatment *in vivo*

Mice received intraperitoneal injections of 500 μg anti-IFNγ mAb (clone XMG1.2; BioXCell) or rat IgG_1 isotype control (BioXCell) on day 7.

Inhibitor treatment

Cells were treated at 37 ℃ for 6 h with 50 μM MitoTEMPO (Sigma-Aldrich), 25 μM Ochratoxin A

(Sigma-Aldrich), 5 μM Rapamycin (Rapa) (FUJIFILM Wako Pure Chemical Corporation), 50 μM Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl) ethyl sulfide (BPTES; Sigma-Aldrich), 50 μM K67 (which was kindly provided by M. Komatsu, University of Juntendo), 5 mM 3-Methyladenine (3MA) (Sigma‐Aldrich), 50 mM 2-Deoxy-D-glucose (2DG) (Sigma‐Aldrich), 40 μM 6-Diazo-5-oxo-L-norleucine (DON) (FUJIFILM Wako Pure Chemical Corporation), 50 μM epigallocatechin gallate (EGCG) (Tokyo Chemical Industry), 50 μM chloroquine (Sigma–Aldrich), 500μM Disodium 2-Oxoglutarate (αKG) (Tokyo Chemical Industry) or 500μM Dimethyl 2-oxoglutarate (DMKG) (Sigma–Aldrich). To detect Nrf2 protein of T cells, the cells were treated at 37 ℃ for 4 h with 10 μM MG132 (Sigma‐Aldrich) and prepared for cell lysates.

Collection of CD8$^+$ cells, including CD8$^+$T cells and CD8$^+$DCs from tumor tissues for RNA sequencing

To prevent RNA degradation, tumor tissues were minced into small pieces in Cell Cover reagent (Anacyte Laboratories) using a Medimachine system. The collected cells, including tumor cells, were stained with fluorescently labeled antibodies to CD45.2 and CD8, processed with a Cell Sorter SH800S (Sony Corporation) to sort 100 CD8$^+$ cells per well containing SingleCellProtect™ (Avidin), diluted 5-fold with RNase free water (QIAGEN) in 12 wells of a 96 well plate. The samples were stored at -80 ℃ until needed for RNA sequencing.

Collection of tumor cells from tumor tissues for RNA sequencing

Tumor tissues were minced into small pieces using a Medimachine system. After centrifugation, the cells were suspended in 20% Percoll™ (GE Healthcare), and then layered onto 65% Percoll™, followed by centrifugation (1000 x g, 20 min). The supernatants were discarded and the cell pellets suspended in Cell Cover reagent for 5 minutes. The collected cells were stained with fluorescently labeled antibodies against CD45.2 and the CD45.2$^-$ population was sorted in the same manner as for CD8$^+$ cell collection.

RNA sequencing and data analysis

Libraries from one-hundred cells (1,2) were prepared for digital RNA sequencing as described previously (3). A total of six libraries were sequenced together in a single MiSeq run (150 cycles, Illumina kit) with different sample indices, and three independent libraries were analyzed for each sample (none, Met, anti-PD-1 and Met plus anti-PD-1). Sequencing data were mapped against the mouse genome (mm10 assembly from the UCSC Genome Browser) with mouse gene annotation

(refFlat from the UCSC Genome Browser) using STAR ver.2.5.4b (4). Differential gene expression of digital RNA-seq was determined using DESeq2 (5). Enrichment analysis was based on the KEGG functional hierarchy (6). P values for the enrichment test were calculated using the GAGE algorithm (7) and the FDR was calculated from the p value for multiple testing using the Benjamini-Hochberg procedure. Enrichment analysis to evaluate the effects of transcription factors on their binding target genes (wPGSA) was performed as described previously (8). The RNA-seq data reported in this paper have been deposited in the NCBI Gene Expression Omnibus (GEO) database under accession number GSE134191.

Flow cytometric analysis

Cells were washed and incubated with antibodies to CD3 (17A2; BioLegend), CD45.2 (104; BioLegend), CD8 (53-6.7; BioLegend), glucose transporter Glut-1 (EPR3915; Abcam), CXCR4 (L276F12; BioLegend), CXCR6 (SA051D1; BioLegend), CXCR3 (CXCR3-173; Invitrogen), CD11c (N418; BioLegend), CD11b (M1/70; BioLegend), CD86 (GL-1; BioLegend), CD80 (16-10A1; BioLegend), I-A/I-E (M5/114.15.2; BioLegend) antibodies for 30 min at 4 ˚C in FACS staining buffer consisting of 5 mM EDTA and 2% fetal calf serum (FCS) in phosphate-buffered saline (PBS). Intracellular cytokine and p-S6 (Ser235/236) (D57.2.2E; Cell Signaling Technology, Japan), HO-1 (HO-1-2; Abcam), p62 (EPR4844; Abcam), LC3B (Polyclonal; Novus Biologicals) Phospho-p62 (SQSTM1) (Ser 351) (5D5; Medical and Biological Laboratories) and Phospho-p62 (SQSTM1) (Ser 403) (4F6; Medical and Biological Laboratories), Phospho-4EBP1 (Thr37, Thr46) (4EB1T37T46-A5; Thermo Fisher Scientific) staining was performed using a Fixation/Permeabilization kit (BD Biosciences). Anti-Phospho-p62 (SQSTM1) (Ser 351) and anti-Phospho-p62 (SQSTM1) (Ser 403) were labeled using FITC Conjugation Kit (Abcam) before use. Staining for transcription factors was performed with a Transcription Factor Buffer Set (BD Pharmingen™) with Ki67 (6A8; BioLegend), NRF2 (D1Z9C; Cell Signaling Technology Japan), IRF8 (V3GYWCH; Invitrogen), and IRF1 (D5E4; Cell Signaling Technology Japan) antibodies. After treatment, the cells were washed, suspended in staining buffer and analyzed on a FACS Canto II flow cytometer (BD Biosciences).

Western Blot Analysis

Cell lysates were prepared by suspending cells in Cell Lysis Buffer M (FUJIFILM Wako Pure Chemical Corporation). The lysates were cleared by centrifugation and subjected to electrophoresis on a sodium dodecyl sulfate–polyacrylamide gel. The proteins were then transferred to nitrocellulose

membranes, blocked with 5 % dry milk (or bovine serum albumin) in TBS-T buffer [TBS (10 mM Tris–HCl, pH 7.5, 135 mM NaCl) + 0.05% Tween-20]. Membranes were incubated with antibodies to anti-Glut1 (1:1000) (EPR3915; Abcam), anti-OCT1 (1:500) (2C5; Novus Biologicals), anti-Nrf2(1:1000) (D1Z9C; Cell Signaling Technology), anti- β actin (1:5000) (13E5; Cell Signaling Technology) antibodies. Antigen–antibody complexes were visualized by chemiluminescence with an enhanced chemiluminescence substrate.

Supplemental References:

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Supplemental Figure Legends

Supplemental figure S1 Individual tumor growth curve

(A, B, C) Individual tumor growth curves of Figures 1A, B, C are shown. The results are from pooled data from two independent experiments (N=5 x 2).

Supplemental figure S2 Glycolysis, mtROS, and IFNγ production of CD8TILs

(A) CD8TILs from tumor bearing mice treated as indicated were stimulated with PMA/ionomycin, and their IFNγ production was determined. (B) CD8TILs from mice treated as indicated were cultured for the indicated hours and their Glut-1 expressions were determined. (C) CD8TILs were cultured in the presence of the indicated glucose concentration, and their Glut-1 expressions were determined. (D) IFNγ production by Glut-1^{-} and Glut-1^{+} CD8TIL populations of mice treated as indicated. (E) CD8TILs, and CD8T lymphocytes from draining lymph node (dLN) or spleen from mice treated as indicated were cultured for 6h, and Glut-1 expressions were analyzed. All flow cytometry data were performed using pooled samples from 5 mice. The experiment was performed twice to four times with similar outcome.

Supplemental figure S3 mtROS dependent Glut-1 elevation of CD8TILs

(A) CD8TILs from mice treated as indicated were cultured for 0, 1, 3h and expressions of Glut-1 and mtROS were analyzed. Tumor infiltrating cells were pool of 5 mice and the results were from two independent experiments.

(B) CD8TILs from mice treated as indicated were cultured for 6h in the presence or absence of MitoTEMPO and expressions of Glut-1 and mtROS were analyzed. Tumor infiltrating cells were pool of 5 mice and the results were from three independent experiments.

Supplemental figure S4 Antitumor effects by glucose supplementation

(A–H) Syngeneic mice inoculated with Meth A (A, H) or MO5(B–G) were treated with Met, anti-PD-1 Ab, and glucose (Glc), as indicated from day 7, after the tumor challenge. (A, H) Individual tumor growth curves of Figures 2A, D are shown. (B–G) Tumor growth curves of MO5 in (B) and (E) are shown as mean tumor volume ± standard error from pooled data of two independent experiments (N=5 x 2). The individual tumor growth curves are shown in (D) and (G), respectively (N=5 x 2). The mice survivals are shown in (C) and (F), respectively. (I) Glucose concentrations in the tumors of mice, three days after treatment as indicated. Data are from tumors (Meth A) pooled from five mice. Experiments were repeated

three times, and the mean glucose concentration is shown with standard deviation. (J) Tumor weights from mice, three days after treatment as indicated. The average individual tumor weight from five mice was calculated. Experiments were repeated three times, as in I, and the average tumor weight is shown with standard deviation. $^{**}P < 0.01$; $^{***}P < 0.001$ by Student's *t*-test.

Supplemental figure S5 Generation of Nrf2^{flox} mice.

(A) The murine Nfe2I2 genomic locus and the targeting vector for the conditional KO of the gene, showing the floxed and deleted allele. (B) Southern blot strategy for identifying homologous recombination by the targeting vector. The linearized targeting vector was transfected into B6 ES cells. The ES clone-derived genome was digested with XhoI/KpnI and with BamHI and then probed with the indicated 5' and 3' probes, respectively. (C) The results of Southern blot experiments. Clones 4, 10, 15, 20, 44 and 52 were identified as positive for homologous recombination at both the 5' and 3' sites. (D) Expression of Nrf2 protein was determined in CD8T and CD4T cells of WT or Nrf2 conditional KO mice upon stimulation with anti-CD3mAb plus anti-CD28mAb.

Supplemental figure S6 Individual tumor growth curve in Nrf2 conditional KO mice

(A, B) Individual tumor growth curves of Figures 2G, H, are shown, respectively. The results are from pooled data from two independent experiments (N=5 x 2).

Supplemental figure S7 Glutaminolysis and expressions of p62, p-S6, and Ki67 in CD8TILs

Expression levels of p62, p-S6 and Ki67 in CD8TILs of mice treated as indicated and after incubation for 6h in conventional medium in the presence or absence of inhibitors for glutaminolysis, BPTES (A), DON (B), and EGCG (C). Histogram data show results from CD8TILs pooled from five mice, and the tabulated results are from individual mice (n = 5). The experiment was performed twice with similar outcome. $^{**}P < 0.01$; $^{***}P < 0.001$ using Student's *t* test.

Supplemental figure S8 Individual tumor growth curve in p62 conditional KO mice

Individual tumor growth curves of Figures 5A are shown. The results are from pooled data from two independent experiments (N=5 x 2).

Supplemental figure S9 Expressions of HO-1, p62, p-S6, and Ki67 in CD8TILs of conditional KO mice

Expression levels of HO-1, p62, p-S6 and Ki67 in CD8TILs of WT (A, B) and conditional KO mice for p62 (A) and Nrf2 (B). (C) Expression of phospho-4E-BP1(Thr37, Thr46) in CD8TILs of WT and Nrf2KO mice. The tabulated results are from individual mice (n = 5). The experiment was performed twice with similar outcome. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ using Student's *t* test.

Supplemental figure S10 RNA seq analysis for immune-related genes of tumor cells

(A) Gene expression patterns of tumor cells (MO5) assessed by tSNE scores. (B) Regulatory transcriptional factors in tumor cells obtained from mice treated as indicated and as assessed by weighted Parametric Gene Set Analysis (wPGSA). (C, D) Results of gene-set enrichment analysis of tumor cells based on KEGG and GO databases are shown as GAGE_KEGG (C) and GAGE_GO (D).

Supplemental figure S11 IFNγ response in CD8TILs and dendritic cells (DCs)

(A) Expression of CXCR4, CXCR6, and CXCR3 in CD8TILs from mice, treated as indicated. (B, C) Expression of IRF8 and IRF1 by CD8TILs (B), and CD8DCs (C) from mice, treated as indicated. (D) Expression of CD80, CD86, and MHC II in CD8DCs from mice, treated as indicated. The red vertical line in each panel indicates the right edge of the histogram for the isotype control antibodies. (E) Flow cytometry data for the expression of CD11b and CD11c in the $CD45.2^{+}CD3^{-}$ population of tumor-infiltrated cells from mice, treated as indicated. All flow cytometry data, including the histogram, show results from tumors pooled from five mice. Results in E are representative of four independent experiments. The experiments in A–D were performed twice with similar outcome.

Supplemental figure S12 Individual tumor growth curve of B16-fucci and B16-fucci δIC

(A, B) Individual tumor growth curves of Figures 6D, E are shown, respectively. The results are from pooled data from two independent experiments (N=5 x 2).

Supplemental figure S13 RNA-seq analysis for OxPhos-related genes of tumor cells

OxPhos-related Gene expressions of CD8TILs and tumor cells of mice treated as indicated.