Supplementary Methods

AAV production

AAV-SB100x plasmid cloned with library or single sgRNA was packaged similarly to our previously described approach [1]. Low-passage HEK293FT cells were used for AAV production. Briefly, 2 h before transfection, D10 medium (DMEM (Gibico) medium supplemented with 10% FBS (Sigma) and 200 U/mL penicillin–streptomycin (Gibico)) was replaced by pre-warmed DMEM (FBS-free). For each 15 cm-plate, HEK293FT cells were transiently transfected with 5.2 μg transfer, 8.9 μg serotype (AAV6/9) and 10.4 μg packaging (pDF6) plasmids using 130 μL PEI. After 6-12 h of transfection, DMEM was replaced with 20 mL pre-warmed D10 medium. Cells were dislodged and transferred to 50 mL Falcon tubes after 72 h post-transfection. For AAV purification, 1/10 volume of pure chloroform was added and incubated at 37 °C with vigorously shake for 1 h. NaCl was added to a final concentration of 1 M, shaking the mixture until all NaCl was dissolved, then pelleted at 20,000 x *g* at 4 °C for 15 min. The aqueous layer was gently transferred to another clean tube and discarded the chloroform layer. 10% (w/v) of PEG8000 (Promega) was added and shaken the tubes until dissolved. The mixture was incubated on the ice for 1 h followed by centrifugation at 20,000 x *g* at 4 °C for 15 min. Supernatant was discarded and pellet was resuspended with 5-15 mL PBS including $MgCl_2$ and benzonase (Sigma), incubated at 37 °C for at least 30 min. One volume of chloroform was added, shaken vigorously and spun down at 15,000 x *g* at 4 °C for 15 min. The aqueous layer was collected carefully and concentrated using AmiconUltra 100 kD ultracentrifugation units (Millipore). Virus was aliquoted and stored in -80 °C. To measure virus titer, RT-qPCR was performed using Taqman assays (ThermoFisher) targeted to the human U6 promoter engineered in the AAV vector.

Cell culture for cell lines and primary T cells

HEK293FT, U87, GL261, and E0771 cell lines were cultured in D10 medium. Cells were typically passaged every 1-2 d at a split ratio of 1:2 or 1:4; we usually passaged cells when the confluency reached at 80%.

Naïve $CD8^+$ T cell isolation and culture

Mesenteric lymph nodes (mLNs) and spleens were dissected from OT-I;Cas9β or Cas9β mice, then placed into ice-cold PBS supplemented with 2% FBS. Organs were mashed through a 100 μm filter, lymphocytes were re-suspended with 2% FBS. Red blood cells (RBCs) were lysed with 2 mL ACK lysis buffer

(Lonza) per 5 spleens for 1-2 min at room temperature, then stopping lysis by adding 48 mL 2% FBS PBS. RBC-lysed lymphocyte solution was filtered with 40 μm filters to remove cell debris. Naïve $CD8a^+$ T cell purification was performed using Naïve $CD8a^+$ T cell Isolation Kits according to the Miltenyi Biotec's standard procedures. Naïve $CD8a^+$ T cells were cultured at 1-2 x 10^6 cells/mL density in 2 μg/mL anti-CD3ε (BioLegend) treated plates or dishes, and cRPMI medium was supplemented with 2 ng/mL IL-2, 1 μg/mL anti-CD28, 2.5 ng/mL IL-7, and 50 ng/mL IL-15 cytokines or antibody. For *in vivo* experiments, complete RPMI-1640 medium was supplemented with 2 ng/mL IL-2, 1 μg/mL anti-CD28, 2.5 ng/mL IL-7 and 50 ng/mL IL-15 cytokines or antibodies. For *in vitro* experiments, media was supplemented with 2 ng/mL IL-2, 1 μg/mL anti-CD28 and 2 ng/mL IL-12p70. Cytokines and antibodies were purchased from BioLegend.

Generation of stable cell lines

For GBM studies, GL261 cancer cells were infected with Firefly Luciferase (FLuc or Luc for short) expressing lentivirus (with puromycin resistance), after 24 h of virus transduction, cells were selected with 6 μg/mL puromycin, until all cells died in the control group. GL261-FLuc-mCh-cOVA clonal cell lines were generated based on the GL261-FLuc cell line, where GL261-FLuc cells were transduced with mCherry-cOVA (mCh-cOVA) lentivirus, then cultured individually in 96-well plates. 2-3 weeks later, positively expanded clones were identified using fluorescence microscopy. For breast cancer studies, E0771 cancer cells were infected with mCherry-cOVA (mCh-cOVA) lentivirus, and then cultured individually in 96-well plates. 2-3 weeks later, positively expanded clones were identified using fluorescence microscopy. At least two clones from each stable cell lines were established with high purity and used in the study. For the U87-GFP-Luc-EGFRvIII (U87-GLEvIII) and U87-GFP-Luc (U87-GL) cell line establishment, U87 cells were transduced with lentivirus which packaged GFP, Luciferase (Luc), and EGFRvIII cassettes, or lentivirus packaged GFP and Luc genes, respectively. GFP-positive cells were sorted by FACS. Flow cytometry was performed again after stable cell lines were established to ensure purity.

Splinkerette PCR

Sleeping beauty transposon integration was detected by splinkerette PCR [2]. Mouse OT-I;Cas9β $CD8^+$ T cells transduced with AAV-SB-CRISPR were collected for genomic DNA extraction using QIAamp Fast DNA Tissue Kit. A total of 1 μg genomic DNA was digested with *Sau3*AI (NEB) for 4h, then incubated at 65 ℃ to inactivate enzymes for 20 min. Splinkerette adaptors were generated by mixing long-strand

adaptors and short-strand adaptors **(Table S10)**, then denatured and annealed by heating to 95 ℃ for 5 min and then cooled at room temperature. Annealed Splinkerette adaptors were used for ligation immediately or stored at -20 ℃. ~150 ng digested genomic DNA was ligated with 25 μM adaptor at 4 ℃ overnight using T4 ligase (NEB). A nested-PCR reaction was used to amplify transposon arm and its junction genomic DNA sequence. Splink 1 and SB-Right1 primers **(Table S10)** were used for 1st round PCR, Splink 2 and SB-Right 2 primers **(Table S10)** were used for 2nd round PCR. PCR products were run on 2 % gels, and gel purified PCR products were prepared using a Nextera kit (Illumina) before sequencing.

Splinkerette data processing and analysis

Forward and reverse FASTQ reads and their reverse complements from Splinkerette samples were concatenated to obtain pooled reads for processing. BBDuk was used for quality trimming with the following settings trimq = 27 minlen = 80 maq = 30 qtrim = rl. Cutadapt was used with the following settings -e 0.1 --overlap 15 to discard reads outside of the integrating transposon arms (and therefore corresponding to the vector), using the sequences CGCACGCGTTCTAGACTATA, TATAGGCATGCGGTAACCAC, and their reverse complements. Cutadapt was also used to trim the transposon arms using the sequence CAGTTGAAGTCGGAAGTTTA and the following parameters -e 0.1 -m 15 --overlap 15. The resulting filtered reads were then mapped to the mouse genome (mm10) using BWA MEM to determine transposon integration sites. Mapped reads were converted to the BED format and intersected with reference annotations obtained from UCSC Table Browser to determine associated functional regions of integration sites.

Estimation of functional MOI of AAV-SB-CRISPR screen using single cell RT-qPCR

Mouse $CD8^+$ T cells were transduced with AAV-Surf library, with the same parameters as in the screen. T cells were cultured for 5 days, then diluted as single cells (one cell per well) in a 96-well PCR plate. Untransduced T cells (PBS group) were used as negative control. To detect sgRNA expression as a proxy for functional MOI, a Single Cell-to-CT™ Kit (Ambion) was used for quantification of sgRNA expression at a single-cell level. The fraction of single cells expressing sgRNAs out of total cells was used to estimate functional MOI. The detailed qPCR protocol was provided by the manufacturer.

Organs isolation and genomic DNA extraction

Each mouse was dissected after being euthanized. Whole brains, dissected brain tumors, or pre-injected

cell pellets were isolated for genomic DNA extraction. The genomic DNA extraction method follows our previously study [3]. Briefly, each sample was put in a 15 mL Falcon tube, 6 mL NK Lysis Buffer (50 mM Tris, 50 mM EDTA, 1% SDS, pH adjusted to 8.0), and 30 μL of 20 mg/mL Proteinase K (Qiagen) were added to the tissue, and incubated at 55 °C overnight. After tissue disappeared, 30 μL of 10 mg/mL RNase A (Qiagen) was added to the lysed sample, and then tubes were inverted tubes 20 times and incubated at 37 °C for 30 min. Digested tissues were cooled on ice before adding 2 mL cold 7.5 M ammonium acetate (Sigma) to precipitate proteins. Samples were mixed thoroughly after adding ammonium acetate and vortexing for 10 s, followed by centrifuging at 4,000 x *g* at 4 °C for 15 min. After the spin, supernatant was removed to a new 15 mL Falcon tube and pellet discarded, 6 mL 100% isopropanol was added to the tube and inverted tubes until flocculent DNA was observed, centrifuged samples at 4,000 x *g* at 4 °C for 10 min. Genomic DNA pellets was washed one time with 70% ethanol, and then centrifuged at 4,000 x *g* at 4 °C for 5 min. The supernatant was discarded and removed remaining ethanol using a pipette. Air dry genomic DNA for 30-60 min, then added 0.5-1 mL nuclease-free H_2O, resuspended DNA overnight at room temperature. The next day, gDNA solution was transferred to Eppendof tubes and measured concentration using a Nanodrop (Thermo Scientific). For cell pellets, 100-200 μL QuickExtract solution (Epicentre) was directly added to cells and incubated at 65 °C for 30 min. For mouse lymph nodes, QIAmp Fast DNA Tissue Kit (Qiagen) was used for gDNA extraction following the manufacturer's protocol.

SgRNA readout and deep sequencing

Two rounds of PCR reactions were used for the sgRNA library readout. The first PCR used genomic DNA (~2 μg per reaction, three reactions per sample, ~6 μg total per sample) to ensure capturing sufficient representation of the screen, and the second PCR used 1 μL pooled PCR#1 product and barcoded primers. Each sample was amplified with different barcoded primers and pooled with equal quantity PCR products for Illumina sequencing. Primers for PCR#1:

Forward: 5'-aatggactatcatatgcttaccgtaacttgaaagtatttcg-3'

Reverse: 5'-ctttagtttgtatgtctgttgctattatgtctactattctttccc-3'

were used to amplify sgRNA cassette under cycling condition: 98 °C for 1 min, 25 cycles of (98 °C for 1 s, 62 °C for 5 s, 72 °C for 15 s), and 72 °C 2 min for the final extension. All PCR reactions were performed using Phusion Flash High Fidelity Master Mix or DreamTaq Green DNA Polymerase (ThermoFisher).

PCR#1 products for each biological sample were pooled and used for amplification with barcoded second

PCR primers (**Table S1**). The cycling condition of PCR #2 was: 98 °C for 30 s, 30-35 cycles of (98 °C for 1 s, 62 °C for 5 s, 72 °C for 15 s), and 72 °C 2 min for the final extension. Second PCR products were pooled and then normalized for each biological sample before combining uniquely barcoded separate biological samples. The pooled product was then gel purified from a 2% E-gel EX (Life Technologies) using the QiaQuick Gel Extraction kit (Qiagen). The purified pooled library was then quantified with a gel-based method using the Low-Range Quantitative Ladder (Life Technologies), dsDNA High-Sensitivity Qubit (Life Technologies), BioAnalyzer (Agilent) and/or qPCR. Diluted libraries with 5-20% PhiX were sequenced with MiSeq, HiSeq 2500 or HiSeq 4000 systems (Illumina).

Survival analysis

Mice with brain tumors rapidly deteriorated in their body condition score. Mice with observed macrocephaly and body condition score < 2 were euthanized and the euthanasia date was recorded as the last survival date. Occasionally, mice died unexpectedly because brain tumors progressed faster than anticipated, in which cases the dates of death were recorded as the last survival date. For the subcutaneous and fat pad tumor modeling, once tumor volume was over 2500 mm^3, the mouse was euthanized and the euthanasia date was recorded as the last survival date.

T cell adoptive transfer with a subcutaneous glioma tumor model in *Rag1*$^{-/-}$ mice

4 x 10^6 GL261-FLuc-mCh-rOVA cells were subcutaneously injected into male *Rag1*$^{-/-}$ mice. 7 days post-transplantation, OT-I;Cas9β $CD8^+$ T cells were isolated and transduced with AAV-sgPdia3, 3 days later, 1 x 10^6 of $CD8^+$ T cells were intravenously injected in tumor-bearing *Rag1*$^{-/-}$ mice. Tumour size was measured in a blinded fashion approximately every 3-5 days after adoptive T cell transfer. Tumor volume was calculated as π/6 x (length x width x height) of the tumour.

T cell adoptive transfer with a triple-negative breast cancer tumor model in *Rag1*$^{-/-}$ mice

For triple-negative breast cancer modeling, 3 x 10^6 E0771-mCh-rOVA cells were injected into fat pad at day 1. On day 2, naïve $CD8^+$ T cells were isolated from OT-I;Cas9β mice, activated, transduced with single AAVs on day 3 and cultured for another 3-4 days. 1.5 x 10^6 and 0.5 x 10^6 T cells were intravenously injected on day 7 and day 17 post tumor implantation. Two rounds of ACT were performed due to the aggressive nature of this model. Tumor sizes were measured every 2-3 days after T cell ACT. Tumor volume was calculated as π/6 x (length x width x height) of the tumour.

Mouse brain dissection and histology

Mice were euthanized by cervical dislocation or carbon dioxide asphyxiation. Mouse brains were carefully dissected then fixed in 4% PFA for 2-3 days. Brains were embedded in paraffin, sectioned at 4 μm and stained with hematoxylin and eosin (H&E), slides were scanned using an Aperio digital slide scanner (Leica) to quantify tumor size.

Brain tumor monitoring and IVIS imaging

Mice were monitored for brain tumor development by observation of macrocephaly, as well as by *in vivo* luciferase imaging where GL261-FLuc cells were used. Mice were euthanized as poor body condition and/or macrocephaly was developed. Mouse IVIS imaging was performed by intraperitoneally injecting 150 mg/kg D-Luciferin (PerkinElmer), bioluminescence signal intensity was measured by bioluminescent measurement, and quantified with a region of interest over the brain region after 10 min of Luciferin injection.

Flow cytometry

T cells were collected and washed once time using MACS buffer (0.2% BSA and 5 mM EDTA in PBS) before doing staining. T cells were stained on ice for 15-30 min after adding antibodies (1:200 dilution). Samples were run on a BD FACSAria flow cytometer, and analysis was performed using FlowJo software 9.9.4 (Threestar, Ashland, OR).

Intracellular flow cytometry

Intracellular flow cytometry was performed to detect the expression level of IFNγ. Briefly, naïve $CD8^+$ T cells were transduced with AAV after isolation, 5 days after infection T cells were transferred into a new 6-well plate without anti-CD3ε incubation (rest T cells), and supplemented with the new media including IL-2 and IL-12p70. After a 12 h rest, T cells were re-stimulated with different concentration anti-CD3ε, and media was supplied with brefeldin A, 2 ng/mL IL-2, 1 μg/mL anti-CD28, and 2 ng/mL IL-12p70. T cells were re-stimulated for 4 h in the incubator. T cells were collected and stained with anti-CD3 PE/Cy7 and anti-CD8 FITC, after membrane protein staining, cells were fixed and permeabilized, then anti-IFNγ APC antibody was used for intracellular staining. For the human $CD8^+$ T cell IFNγ staining, the same protocol was used as for mouse IFNγ staining.

Mouse brain TIL analysis

8-10 week-old *Rag1*$^{-/-}$ mice were injected with GL261-mCh-rOVA#1 cells, OT-I;Cas9β $CD8^+$ T cells. T cells were isolated and infected with AAV-sgMgat5 and AAV-sgPdia3 virus after luciferase signal was

observed in the mouse brain. 5 x 10^6/mouse OT-I;Cas9β $CD8^+$ T cells were i.v injected. Brain tumors were isolated after 5 days of i.v injection. Mice were sacrificed, and whole brains were quickly isolated and put into cold PBS with 2% FBS. After hindbrain and olfactory bulb removal, the brain tumours were crushed using two glasses with rough surface, then gently mashed into small pieces. Collagenase and dispase (Roche) were used for tissue digest at 37 °C for 1h in the shaking block. Digested samples were quenched by adding cold RPMI-1640, then centrifuging at 500 x *g* for 5 min. Cell pellets were resuspended with 2 mL ACK lysis buffer for 2 min followed by dilution with 2% FBS PBS, filtered with 40 μm filters to remove tissue aggregates. Ficoll density centrifugation was performed to enrich mononuclear cells. Enriched cells were stained with antibodies for 30 min on the ice, then washed with MACS buffer (0.2% BSA and 5 mM EDTA in PBS) before running on a FACS machine.

RT-qPCR

Total RNA was extracted from $CD8^+$ T cells using RNasy Plus Mini Kit (Qiagen). Gene expression was quantified using Taqman Fast Universal PCR Master Mix (Thermo Fisher) and Taqman probes (Invitrogen) specific to each gene. Relative mRNA expression was determined via the $\Delta\Delta C_t$ method.

T7 endonuclease I assay (T7EI)

Mouse $CD8^+$ T cells infected with AAVs, or human $CD8^+$ T cells electroporated with RNPs, were collected for genomic DNA extraction using QIAmp Fast DNA Tissue Kit (Qiagen). PCR was performed using site-specific primers (**Table S7**) with Phusion Flash High Fidelity Master Mix (ThermoFisher) under cycling condition as: 98 °C for 1 min, 35 cycles of (98 °C for 1 s, 60 °C for 5 s, 72 °C for 25 s), and 72 °C 2 min for the final extension. PCR products were gel purified using the QIAquick Gel Extraction Kit (Qiagen). 200 ng of PCR DNA in Buffer 2 (NEB) was annealed on a thermocycler with the following setting: 95 °C, 5 min, 90 °C, 1 min, 85 °C, 1 min, 80 °C, 1 min, 75 °C, 1 min, 70 °C, 1 min, 65 °C, 1 min, 60 °C, 1 min, 55 °C, 1 min, 50 °C, 1 min, 45 °C, 1 min, 40 °C, 1 min, 35 °C, 1 min, 30 °C, 1 min, 25 °C, 1 min, and hold at 4 °C. 10 units of T7 endonuclease I (NEB) was added to digest the re-annealed DNA for 30-60 min at 37 °C, then being loaded into the 2 % E-gel, the gel imaging was performed using Image Lab (Bio-Rad).

Human primary $CD8^+$ T cell endogenous gene knockout

Human primary $CD8^+$ T cells were isolated from health donors. $CD8^+$ T cells were stimulated with anti-CD3 / CD28 beads (Invitrogen). T cells were cultured in X-VIVO™ 15 media (Lonza) supplied with 5 % human serum and IL-2. Before the electroporation, crRNA and tracrRNA were 1:1 ratio mixed (final

concentration was 44 μM), heat at 95 ^{o}C for 5 min, then cooled to room temperature. 0.3 μL Cas9 protein (61 μM) was mixed with 0.2 μL Buffer R (Neon Transfection Systerm Kit, Thermo Fisher), then being mixed with 0.5 μL annealed crRNA:tracrRNA duplex, incubated the mixture at room temperature for 20 min. High viability cells were collected and washed with PBS to completely remove the media. 5 x 10^5 of T cells were resuspended in 9 μL Buffer R per electroporation, then 1 μL RNP complex was added and mixed well using pipette. 10 μL of cell:RNP mixture was loaded into the Neon pipette without any bubbles. The tip of the loaded Neon pipette was inserted into the pipette station. The setup of the electroporation parameter was set at 1600 V, 10 ms for 3 pluses. For the 100 μL reaction, this preparation was scaled up with the same ratio. After electroporation, cells were transferred to a 24-well plate with pre-warmed media, then cultured in a tissue culture incubator.

Immunoblot and TCR signaling

Human $CD8^+$ T cells electroporated with RNP were collected and washed with PBS to remove media. 3 x 10^6 cells were lysed with RIPA lysis buffer and incubated on the ice for 30 min, followed by centrifuging at 13,000 x *g* for 15 min at 4 ^{o}C. The supernatant was collected for protein quantification. The total protein concentration was measure by using a Bradford protein assay (Bio-Rad), a total of 30 μg protein per sample was loaded into SDS-PAGE gel (Bio-Rad), proteins in the gel were transferred into Amersham Protran 0.45 μm NC Nitrocellulose Blotting membrane (GE Healthcare) after electrophoresis. Membranes were blocked with 5 % BSA in TBST for 1 h at room temperature, followed by the primary antibody incubation at 4 ^{o}C overnight.

Anti-PDIA3 antibody was from Atlas Antibodies (HPA003230). Antibody binding was detected using horseradish peroxidase-conjugated secondary antibody and ECL substrate (Bio-Rad). For the TCR signaling experiments, mouse naïve $CD8^+$ T cells were isolated from OT-I;Cas9β mice, then infected with AAV6 packaged with *Mgat5, Pdia3,* or Vector control sgRNAs. T cells were washed with PBS and cultured with cRPMI media without anti-CD3ε and anti-CD28 antibodies (resting) overnight at day 5 after AAV infection. Following resting, T cells were collected and washed with cold PBS, 3-5 x 10^6 cells per sample were resuspended with cold PBS containing biotin anti-mouse CD3ε (BioLegend) and Streptavidin (BioLegend) and incubated on the ice for 30 min. T cells were then re-stimulated at 37 ^{o}C for 30s after ice incubation. Following stimulation, cells were lysed with RIPA lysis buffer which containing protease and phosphatase inhibitor cocktail (ThermoFisher). The standard immunoblot assay was performed as described above.

Single cell TCR sequencing (TCR-seq)

Mouse naïve CD45.1;Cas9β $CD8^+$ T cells were isolated from spleens and lymph nodes, then transduced with AAV-Surf library with the same method and parameter as in the screen. T cells were collected for 10x single cell TCR-seq library prep at day 5 after AAV transduction, and the rest of the T cells were i.v injected into C57BL/6J mice which brain engrafted with GL261-Luc cancer cells 2 weeks prior. GBM TILs were isolated by sorting $CD45.1^+CD3^+CD8^+$ T cells from Ficoll density gradually centrifugation products (mononuclear cell layer). Sorted T cells were spin down for 10x single cell TCR-seq following the manufacturer's protocols (10x Genomics).

TCR-seq data analysis

Cellranger was used to generate V(D)J sequences and annotations from 10x library preps and sequencing runs. Adjusted clonotype proportions were calculated based on total assigned clonotype frequencies (clonotype freq / sum (all clonotype freqs)). Clonal event distributions and ring plots were generated using ggplot2, with CDR3s of top clones highlighted.

Supplementary References

1. Chow, R.D. et al. AAV-mediated direct in vivo CRISPR screen identifies functional suppressors in glioblastoma. *Nat Neurosci* (2017).
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3. Chen, S. et al. Genome-wide CRISPR screen in a mouse model of tumor growth and metastasis. *Cell* **160**, 1246-1260 (2015).
4. Jiang, P. et al. Signatures of T cell dysfunction and exclusion predict cancer immunotherapy response. *Nat Med* **24**, 1550-1558 (2018).