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Supplemental information

CAR T cells targeting tumor-associated exons of glypican 2 regress neuroblastoma in mice

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Table S1. The detailed information of tissue specimens shown in Figure S1B. Related to Figure 1.

Organ (Anatomic Site)	Age	Sex	Pathology diagnosis	Type
Cerebrum	2	F	Gray matter tissue	Normal
Cerebellum	24	F	Cerebellum tissue	Normal
Hypophysis	27	F	Hypophysis tissue	Normal
Adrenal gland	18	F	Adrenal gland tissue	Normal
Ovary	36	F	Ovary tissue	Normal
Pancreas	16	F	Pancreas tissue	Normal
Prostate	31	M	Prostate tissue	Normal
Nerve	31	M	Peripheral nerve tissue	Normal
Parathyroid gland	50	M	Thyroid gland tissue	Normal
Thymus gland	15	F	Thymus gland tissue	Normal
Pleural	19	F	Pleural mesothelium tissue	Normal
Thyroid gland	18	F	Thyroid gland tissue	Normal
Breast	21	F	Breast tissue	Normal
Spleen	21	F	Spleen tissue	Normal
Uterine cervix	46	F	Adjacent normal cervix tissue	NAT
Eye	47	F	Cancer adjacent choroid tissue	NAT
Tonsil	23	M	Tonsil tissue	Normal
Small intestine	30	M	Small intestine tissue	Normal
Bone marrow	21	F	Bone marrow tissue	Normal
Lung	24	M	Lung tissue	Normal
Heart	35	M	Cardiac muscle tissue	Normal
Esophagus	45	M	Esophagus tissue	Normal
Striated muscle	40	M	Skeletal muscle tissue	Normal
Larynx	62	M	Submucosal gland tissue	NAT
Stomach	29	M	Stomach tissue	Normal
Uterus	40	F	Adjacent normal endometrium tissue	NAT
Colon	29	M	Colon tissue	Normal
Liver	38	M	Liver tissue	Normal

Tongue	15	F	Salivary gland tissue	Normal
Kidney	16	M	Kidney tissue	Normal
Skin	50	F	Skin tissue	Normal
Testis	30	M	Testis tissue	Normal

Table S2. The identified GPC2 mRNA transcripts. Related to Figure 2.

Name	Transcript ID	BP	Exons	Protein	Biotype	UniProt	RefSeq
GPC2-201	ENST00000292377.4	2532	1-10	579aa	Protein coding	Q8N158	NM_152742 NP_689955
GPC2-202	ENST00000471717.1	2129		No protein	Retained intron	-	-
GPC2-203	ENST00000480087.5	767	1-2, 4-6	134aa	Nonsense mediated decay	A0A0J9YXG7	-
GPC2-204	ENST00000486702.1	787		No protein	Retained intron	-	-
GPC2-205	ENST00000490629.5	798		No protein	Processed transcript	-	-

Table S3. The amino acid sequence of GPC2 exon 3 and the comprising 12 peptides. Related to Figure 3. Each peptide is 18 amino acids long and has 9 overlapped amino acids with adjacent peptide. The predicted epitope containing region in exon 3 is highlighted with red. The overlapped sequences in peptides 10 and 11, peptides 14 and 15 are bolded.

Assigned name	Sequence
GPC2 Exon 3	EFFLEMLSVAQHSLTQLFSHSYGRLYAQHALIFNGLFSR **LRDFYGESGEGLDDT**LADFWAQLLERVFPLLHPQYSF PPDYLLCLSRLASSTDGSLQPFGDSPRRLRLQ
Peptide 10	RKFDEFFLE**MLSVAQHSL**
Peptide 11	**MLSVAQHSL**TQLFSHSYG
Peptide 12	TQLFSHSYGRLYAQHALI
Peptide 13	RLYAQHALIFNGLFSRLR
Peptide 14	FNGLFSRLR**DFYGESGEG**
Peptide 15	**DFYGESGEG**LDDTLADFW
Peptide 16	LDDTLADFWAQLLERVFP
Peptide 17	AQLLERVFPLLHPQYSFP
Peptide 18	LLHPQYSFPPDYLLCLSR

Peptide 19	PDYLLCLSRLASSTDGSL
Peptide 20	LASSTDGSLQPFGDSPRR
Peptide 21	QPFGDSPRRLRLQITRTL

Table S4. The detailed information of tissue specimens shown in Figure S4. Related to Figure 4.

Panel	Position	Age	Sex	Organ/ Anatomic Site	Pathology diagnosis	Type
A	A1	3	F	Retroperitoneum	Neuroblastoma	Malignant
	A2	8	F	Retroperitoneum	Neuroblastoma	Malignant
	A3	1	F	Retroperitoneum	Neuroblastoma	Malignant
	A4	7	M	Retroperitoneum	Neuroblastoma	Malignant
	A5	4	F	Retroperitoneum	Neuroblastoma	Malignant
	B1	6	M	Retroperitoneum	Neuroblastoma	Malignant
	B2	3	F	Retroperitoneum	Neuroblastoma	Malignant
	B3	4	F	Retroperitoneum	Neuroblastoma	Malignant
	B4	1	F	Retroperitoneum	Neuroblastoma	Malignant
	B5	2	F	Retroperitoneum	Neuroblastoma	Malignant
	C1	5	M	Retroperitoneum	Neuroblastoma	Malignant
	C2	4	F	Retroperitoneum	Neuroblastoma	Malignant
	C3	8 Mon.	M	Retroperitoneum	Neuroblastoma	Malignant
	C4	1	M	Retroperitoneum	Neuroblastoma	Malignant
	C5	4	M	Retroperitoneum	Neuroblastoma	Malignant
	D1	1	F	Mediastinum	Neuroblastoma	Malignant
	D2	2	F	Mediastinum	Neuroblastoma	Malignant
	D3	6	M	Adrenal gland	Neuroblastoma	Malignant
	D4	6	M	Adrenal gland	Neuroblastoma (low differentiation type)	Malignant
	D5	5	F	Adrenal gland	Neuroblastoma with necrosis (low differentiation type)	Malignant
	E1	31	M	Nerve	Peripheral nerve tissue	Normal
	E2	36	M	Nerve	Peripheral nerve tissue	Normal
	E3	33	M	Nerve	Peripheral nerve tissue	Normal
	E4	25	M	Nerve	Peripheral nerve tissue	Normal

	E5	32	M	Nerve	Peripheral nerve tissue	Normal
B	A1	8	M	Cerebellum	Medulloblastoma	Malignant
	A2	16	M	Cerebrum	Medulloblastoma	Malignant
	A3	14	M	Cerebrum	Medulloblastoma	Malignant
	A4	9	M	Cerebrum	Medulloblastoma	Malignant
	A5	7	M	Cerebellum	Medulloblastoma	Malignant
	B1	12	F	Cerebrum	Medulloblastoma	Malignant
	B2	8	F	Cerebellum	Medulloblastoma	Malignant
	B3	14	F	Cerebellum	Medulloblastoma of vermis cerebellum	Malignant
	B4	4	F	Cerebrum	Medulloblastoma of vermis cerebellum	Malignant
	B5	6	M	Cerebellum	Medulloblastoma of vermis cerebellum	Malignant
	C1	6	F	Cerebellum	Medulloblastoma	Malignant
	C2	35	M	Cerebrum	Normal cerebrum tissue	Normal
	C3	48	M	Cerebrum	Normal cerebrum tissue	Normal
	C4	24	F	Cerebellum	Normal cerebellum tissue	Normal
C	A1	15	F	Eyeball	Retinoblastoma	Malignant
	A2	2	F	Eye	Retinoblastoma	Malignant
	A3	11	F	Eye	Retinoblastoma	Malignant
	A4	3	M	Eye	Retinoblastoma	Malignant
	B1	2	M	Eye	Retinoblastoma	Malignant
	B2	3	M	Eye	Retinoblastoma	Malignant
	B3	2	M	Eye	Retinoblastoma (choroid tissue)	Malignant
	B4	8 Mon.	M	Eye	Retinoblastoma with necrosis	Malignant
	C1	4	M	Eye	Retinoblastoma (sparse)	Malignant
	C2	63	M	Eye	Cancer adjacent normal retina and choroid tissue	NAT
	C3	1	M	Eye	Cancer adjacent normal cornea tissue	NAT
	C4	2	F	Eye	Cancer adjacent normal sclera and choroid tissue	NAT

Table S5. The shared integration sites in spleens from mice received CT3 CAR T cells from week 1 to week 5. Related to Figure 6. The genes found in the memory T cell subsets shown in Table S5 are bolded and underlined.

Gene	Integration site	Strand	Gene description
PLCB1	chr20:8839536	-	Phospholipase C, β1
STX5	chr11: 62578465	+	Syntaxin 5
GRB2	chr17: 73327158	-	Growth factor receptor bound protein 2
PACS1	chr11: 65884798	+	Phosphofurin acidic cluster sorting protein 1
CLASRP	chr19: 45550055	-	CLK4-associating serine/arginine rich protein
KDM2A	chr11: 66971958	-	Lysine demethylase 2A
IP6K2	chr3: 48743907	-	Inositol hexakisphosphate kinase 2
SEPW1	chr19: 48288693	+	Selenoprotein W
PITPNA	chr17: 1447968	-	Phosphatidylinositol transfer protein alpha
BRD1	chr22: 50213836	-	Bromodomain-containing protein 1
BCL2L12	chr4: 48388789	+	BCL2 like 12
NSD1	chr19: 57807300	-	Nuclear receptor binding SET domain protein 1
BRIP1	chr17: 59883538	-	BRCA1 interacting protein C-terminal helicase 1
TBC1D20	chr20: 427307	+	TBC1 domain family member 20
XRCC5	chr2: 216994979	+	X-ray repair cross-complementing protein 5
BCLAF1	chr6: 136596651	-	BCL2 associated transcription factor 1

Table S6. The shared integration sites in various tissues from mice infused with CT3 CAR T cells. Related to Figure 6. The genes found in the memory T cell subsets shown in Table S5 are bolded and underlined.

Gene	Integration site	Strand	Gene description
SEC16A	chr9: 139346426	+	SEC16 homolog A, endoplasmic reticulum export factor
IP6K1	chr3: 49807052	+	Inositol hexakisphosphate kinase 1
RBM4	chr11: 66409814	-	RNA binding motif protein 4
GIMAP7	chr7: 150218051	-	GTPase, IMAP family member 7
SNX12	chrX: 70251385	-	Sorting nexin 12
NUMA1	chr11: 66971958	+	Nuclear mitotic apparatus protein 1
KIF1B	chr1: 10335597	+	Kinesin family member 1B
PDIA4	chr7: 148715371	+	Protein disulfide isomerase family A member 4

GFER	chr16: 2036134	+	Growth factor, augmenter of liver regeneration
ANKRD11	chr16: 89524020	-	Ankyrin repeat domain 11
NT5C2	chr10: 104940883	-	5'-Nucleotidase, cytosolic II
UHRF1	chr19: 4946341	-	Ubiquitin like with PHD and ring finger domains 1
RIF1	chr2: 152333245	-	Replication timing regulatory factor 1
UBE2E3	chr2: 182040950	+	Ubiquitin conjugating enzyme E2 E3
HORMAD2	chr22: 30520531	-	HORMA domain containing 2
DUS1L	chr17: 80026496	-	Dihydrouridine synthase 1 like
VPREB1	chr22: 22493168	+	V-Set pre-B cell surrogate light chain 1
CTCF	chr16: 67612199	+	CCCTC-binding factor
YTHDC2	chr5: 112883205	+	YTH domain containing 2
PPP6R2	chr22: 50827467	+	Protein phosphatase 6 regulatory subunit 2
LRBA	chr4: 151412815	-	LPS responsive beige-like anchor protein
ARNT	chr1: 150857347	-	Aryl hydrocarbon receptor nuclear translocator
POLRMT	chr19: 619348	+	RNA polymerase mitochondrial
C17orf70	chr17: 79508608	-	FA core complex associated protein 100
LGALS14	chr19: 40201907	+	galectin 14
ZMYND11	chr10: 244271	-	Zinc finger MYND-type containing 11
CHD3	chr17: 7781348	-	Chromodomain helicase DNA binding protein 3
C9orf86	chr9: 139706625	-	Rab-like protein 6
PTPRA	chr20: 2930744	+	Receptor-type tyrosine-protein phosphatase alpha
KIAA0753	chr17: 6531444	+	KIAA0753

Table S7. The 22 shared integrated genes identified from in vivo studies shown in Tables S5 and S6 were found in different cultured memory T cell subsets. Related to Figure 6. The times of each integrated gene appeared were counted. NA: not applicable.

Shared integrated genes from mouse studies	CT3 CAR T cells			
	T_{cm}	T_{scm}	T_{emra}	T_{em}
PLCB1	NA	NA	1	NA
STX5	NA	NA	1	NA
GRB2	NA	1	NA	NA
PACS1	2	1	2	3
KDM2A	NA	2	1	NA
IP6K2	NA	NA	1	NA
NSD1	NA	NA	NA	2
XRCC5	NA	NA	1	NA
SEC16A	2	NA	NA	2
IP6K1	NA	1	1	NA
GIMAP7	NA	1	1	NA
NUMA1	NA	1	2	NA
ANKRD11	NA	1	1	NA
HORMAD2	NA	1	NA	1
CTCF	1	1	1	NA
PPP6R2	NA	NA	1	NA
LRBA	1	NA	1	1
ARNT	NA	1	NA	NA
POLRMT	1	NA	NA	1
CHD3	1	2	1	NA
PTPRA	NA	NA	NA	1
KIAA0753	NA	2	NA	NA

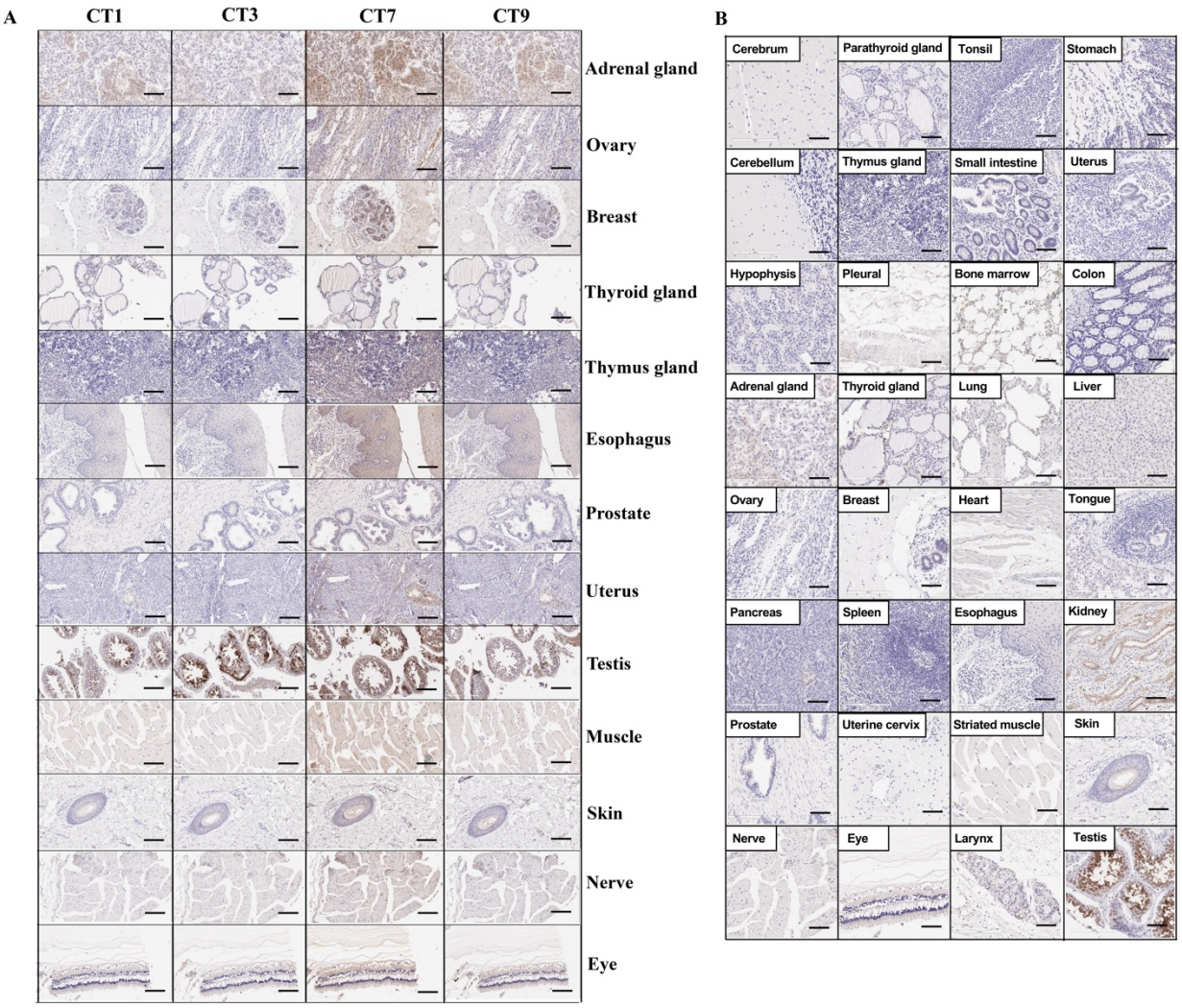

Figure S1. GPC2 expression in normal human tissues as determined by immunohistochemistry. Related to Figure 1. (A) The GPC2 immunostaining by several antibodies including CT1, CT3, CT7 and CT9. (B) The GPC2 immunostaining by CT3 antibody on 32 normal human tissues. The tissues were labeled with 1 μg/ml antibody. Scale bar = 200 μm. The information of each specimen can be found in Table S1.

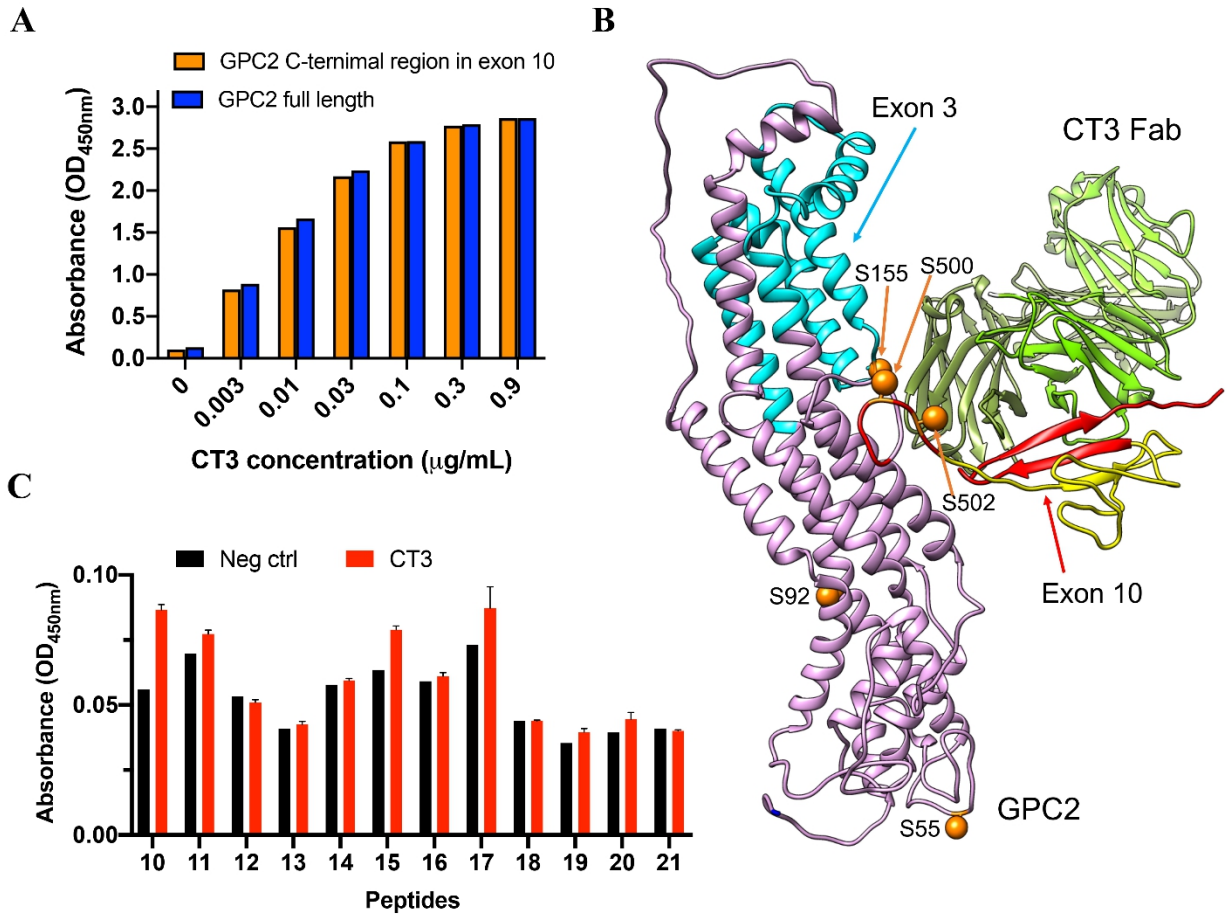

Figure S2. CT3 binds to both exon 3 and exon 10 of GPC2. Related to Figure 3. (A) CT3 reacts to the C-terminal region in exon 10 of GPC2 and full length GPC2 equally well. Various concentrations of CT3 were used for ELISA. (B) A ribbon diagram in which GPC2 and CT3 Fab are placed together. 5 predicted HS attachment sites are labelled at S55, S92, S155, S500 and S502. The CT3 light chain, heavy chain, GPC2 exon 3, and GPC2 C-terminal peptide in exon 10 are colored in light green, olive green, cyan and yellow, respectively. The full exon 10 is colored in both red and yellow. (C) CT3 shows no binding to single linear peptide in exon 3 of GPC2. 1 μg/ml of CT3 was used for ELISA.

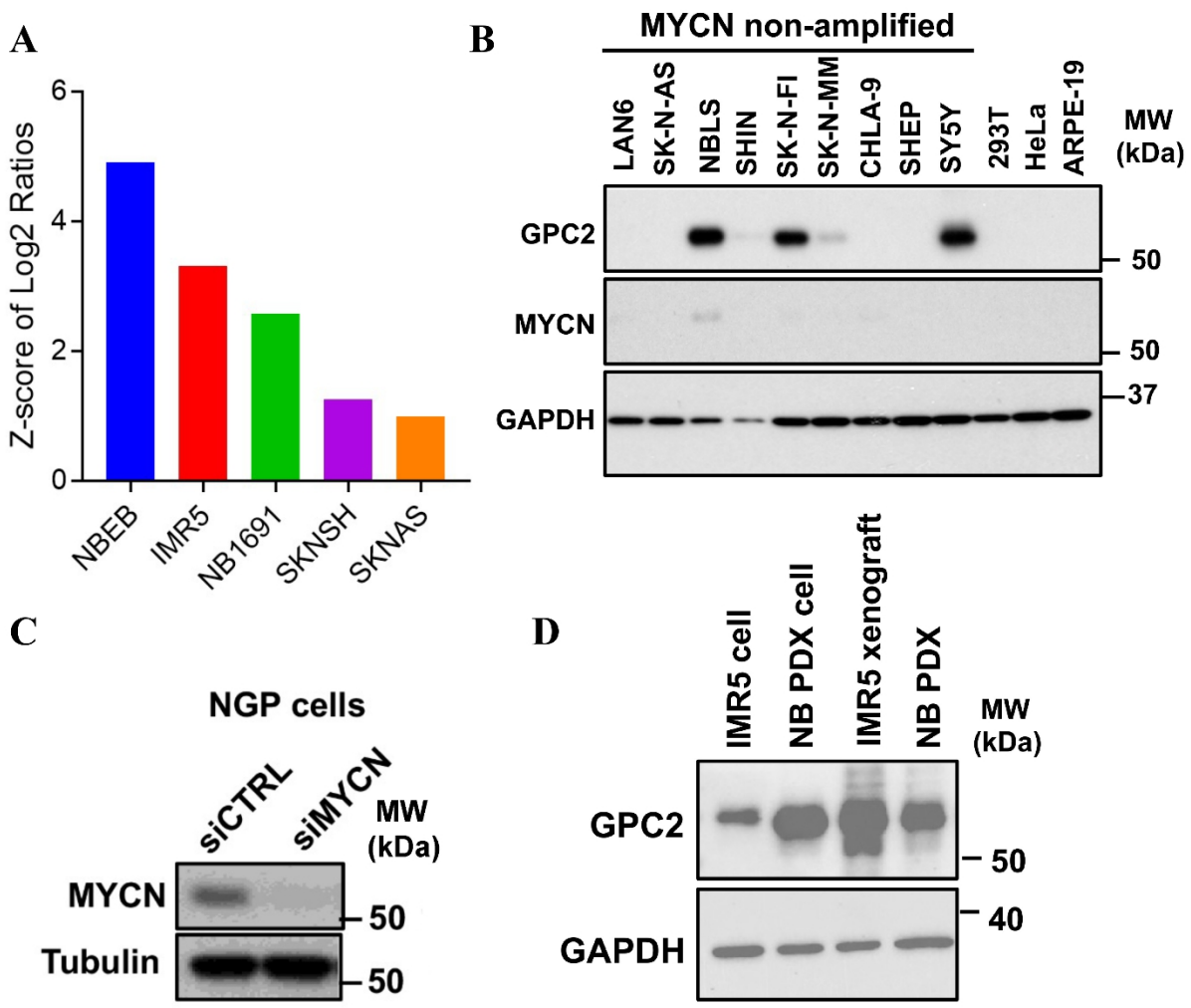

Figure S3. GPC2 expression in neuroblastoma cell lines and PDX. Related to Figure 4. (A) *GPC2* mRNA expression in neuroblastoma cell lines. (B) Western blotting with CT3 was performed to detect GPC2 protein expression in 9 *MYCN* non-amplified neuroblastoma cell lines (LAN6, SK-N-AS, NBLS, SHIN, SK-N-FI, SK-N-MM, CHLA-9, SHEP, SY5Y), as well as non-neuroblastoma cell lines (293T, HeLa, and ARPE-19). (C) The specificity of the anti-N-Myc antibody was demonstrated in knockdown of *MYCN* in NGP cells. (D) GPC2 protein expression is appreciably higher in neuroblastoma PDX and IMR5 xenograft than IMR5 cells cultured as monolayers *in vitro*. Neuroblastoma PDX: SJNBL012407

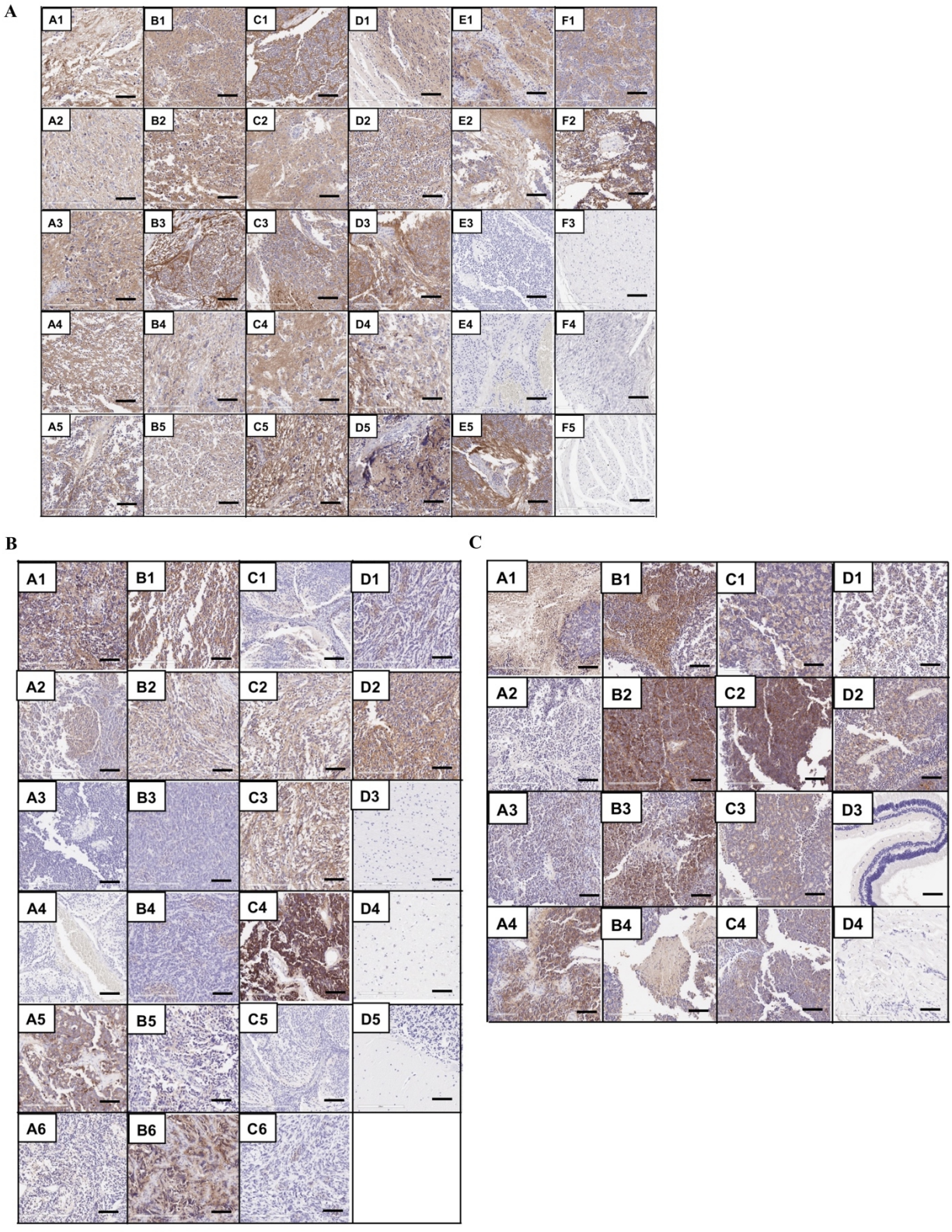

Figure S4. GPC2 expression in pediatric cancers as determined by immunohistochemistry. Related to Figure 4. (A) Strong GPC2 staining was found in 95% (19/20) cases of neuroblastoma tissues (A1 through D5). E1 through

E5 are normal nerve tissues. (B) Strong GPC2 staining was found in 64% (7/11) cases of medulloblastoma tissues (A1 to C1). C2 to C4 are normal brain tissues. (C) Strong GPC2 staining was found in 78% (7/9) cases of retinoblastoma tissues (A1 to C1). C2 to C4 are cancer adjacent normal eye tissues. All tissues were labeled with 1 μg/ml CT3. Scale bar = 200 μm. The information of each specimen can be found in Table S4.

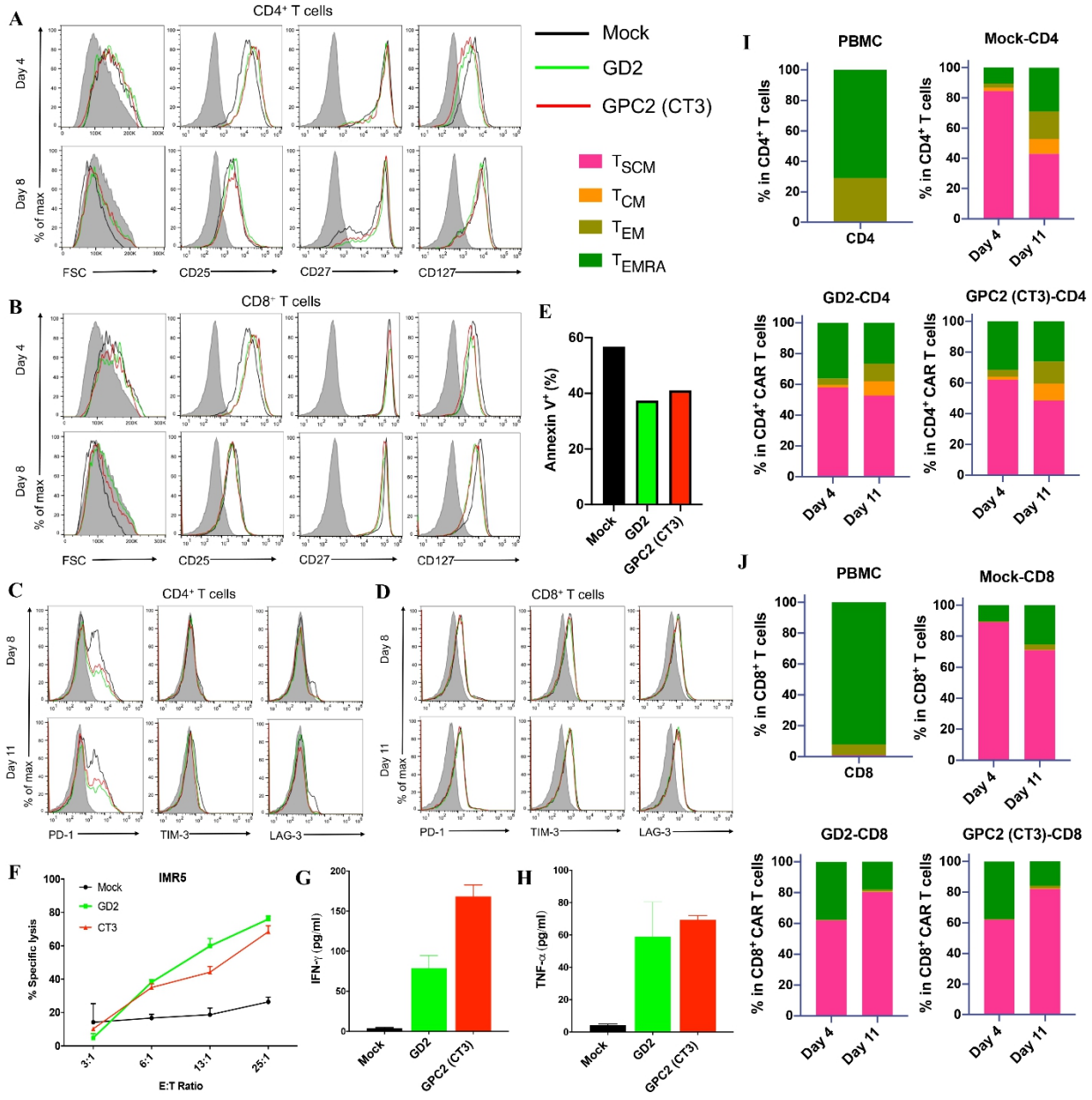

Figure S5. CT3 CAR T cells exhibit low level of tonic signaling during *ex vivo* expansion. Related to Figure 5.

(A-B) activation marker expression after initial activation in $CD4^+$ (A) and $CD8^+$ (B) T cell populations. (C-D) exhaustion marker expression after initial activation in $CD4^+$ (C) and $CD8^+$ (D) T cell populations. (E) Quantification of apoptosis of CAR T cells 11 days after initial activation. (F) cytolytic activity of GD2 and GPC2 (CT3) CAR T cells in IMR5 cells after 24 hours of co-culture. (G-H) production of IFN-γ (G) and TNF-α (H) of CAR T cells co-incubated with IMR5 cells at the E:T ratio of 6:1. (I-J) Memory T cell subsets of unstimulated PBMCs, mock T cells, GD2 and GPC2 (CT3) CAR T cells. (I) relative proportion of stem cell-like memory (T_{SCM}),

central memory (T_{CM}), effector memory (T_{EM}), and terminally differentiated effector memory (T_{EMRA}) subsets defined by CD62L, CD45RA and CD95 expression in the $CD4^+$ T cell population. (J) relative proportion of T_{SCM}, T_{CM}, T_{EM}, and T_{EMRA} subsets in the $CD8^+$ T cell population.

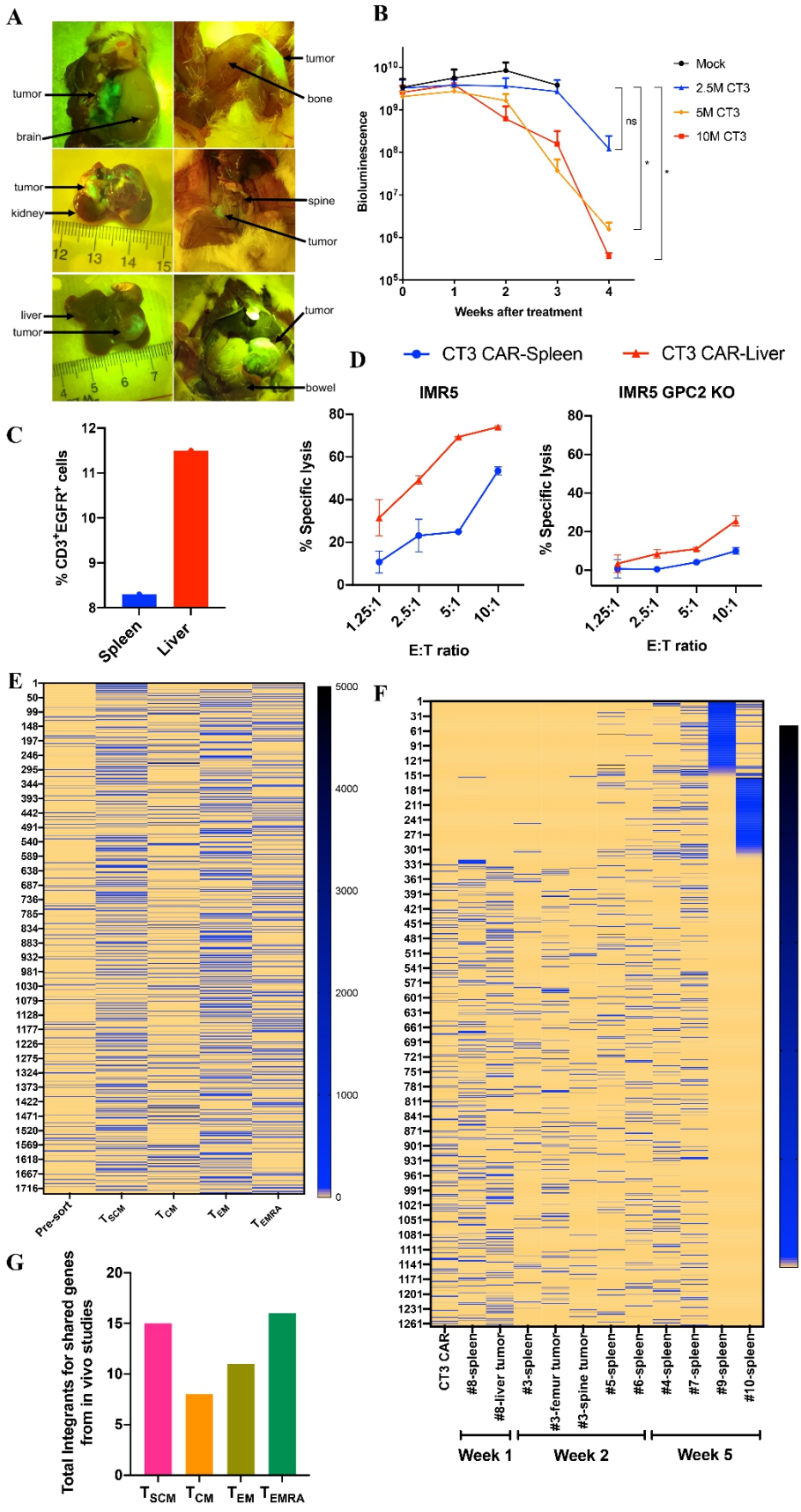
A
tumor
brain
tumor
bone
tumor
kidney
spine
tumor
liver
tumor
tumor
bowel
B
Bioluminescence
Weeks after treatment
Mock
2.5M CT3
5M CT3
10M CT3
ns
*
*
C
% CD3+EGFR+ cells
Spleen
Liver
D
CT3 CAR-Spleen
CT3 CAR-Liver
IMR5
IMR5 GPC2 KO
% Specific lysis
E:T ratio
1.25:1
2.5:1
5:1
10:1
E
Pre-sort
TSCM
TCM
TEM
TEMRA
F
CT3 CAR-
#8-spleen
#8-liver tumor
#3-spleen
#3-femur tumor
#3-spine tumor
#5-spleen
#6-spleen
#4-spleen
#7-spleen
#9-spleen
#10-spleen
Week 1
Week 2
Week 5
G
Total Integrants for shared genes from in vivo studies
TSCM
TCM
TEM
TEMRA

Figure S6. IMR5 xenograft bearing mice treated with CT3 CAR T cells in Figure 6A-C, and distribution of CT3 CAR integration sites. Related to Figure 6. (A) IMR5 tumor cells metastasized to various sites including brain, spine, femur, etc. (B) the averaged tumor bioluminescence of all groups including mock T cells, 2.5 million CT3 CAR T cells, 5 million CT3 CAR T cells and 10 million CT3 CAR T cells. (C) Frequencies of $CD3^{+}hEGFRt^{+}$ cells representing CT3 CAR T cells in spleen and liver from one treated mouse. (D) cytolytic activity of CT3 CAR T cells recovered from mouse spleen and liver against IMR5 cells as well as GPC2 KO-IMR5 cells after 24 hours of co-culture. ns: not significant; *P<0.05 (E) integration sites were randomly distributed among cultured CT3 CAR T cells. Memory T cells include central memory (T_{CM}), stem cell-like memory (T_{SCM}), terminally differentiated effector memory (T_{EMRA}), and effector memory (T_{EM}) subsets defined by CD62L, CD45RA and CD95 expression. (F) distribution of integration sites in spleen and IMR5 tumors of mice treated with CT3 CAR T cells at week 1, week 2 and week 5 post-infusion seen in Figures 6A-C. (G) The shared integration genes from *in vivo* studies were detected in different memory T cell subsets from *in vitro* culture as seen in Table S5. The times of every founded gene were added together in each subset.

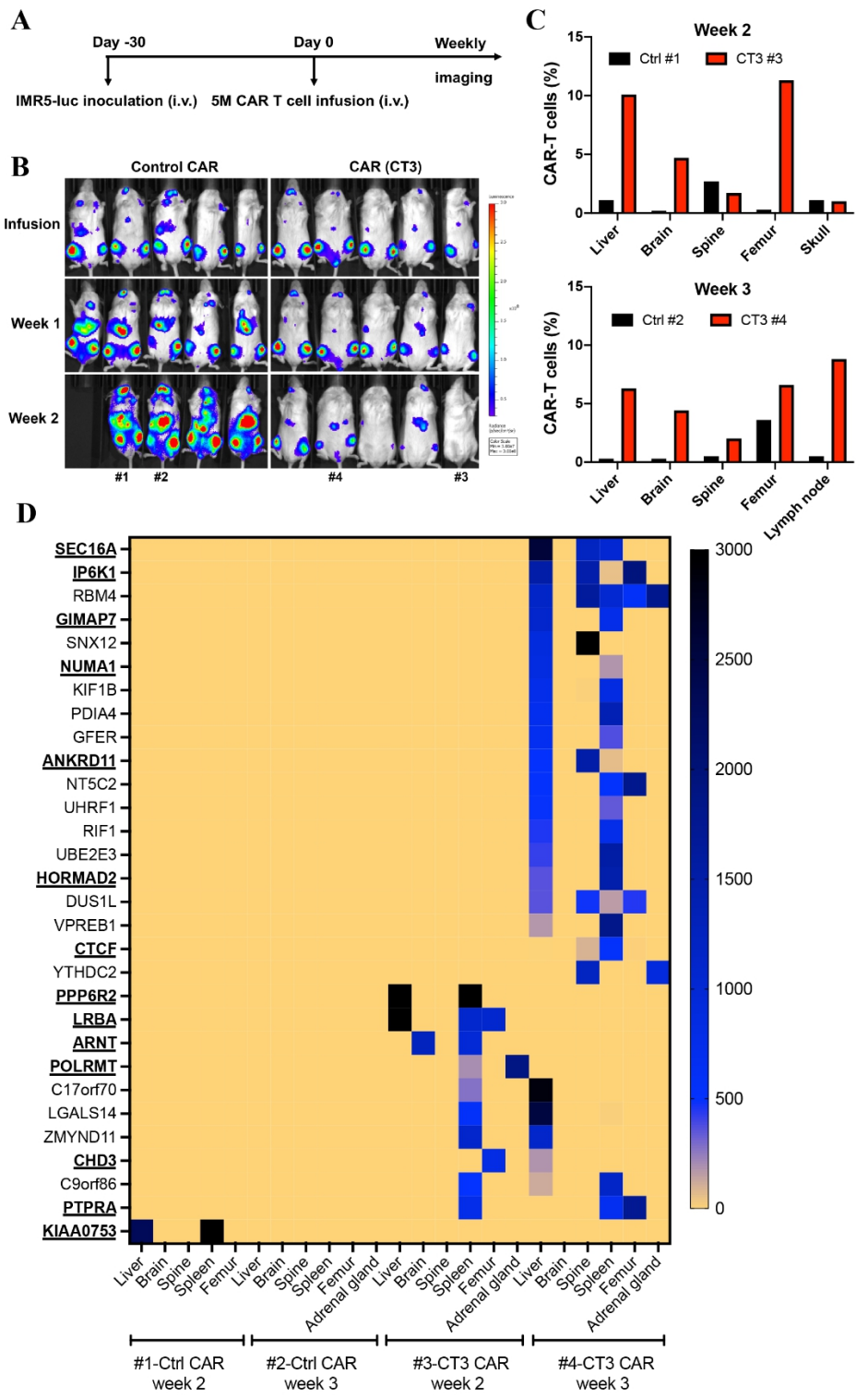

Figure S7. CT3 CAR T cells regress tumor growth in the IMR5 xenograft mouse model. Related to Figure 6.

(A) Schematic of the metastatic IMR5 xenograft mouse model. IMR5 tumor-bearing NSG mice were i.v. injected with 5 million control CAR T cells targeting glypican 3 and CT3 CAR T cells. (B) CT3 CAR T cells reduced tumor

burden in mice 2 weeks post-infusion. (C) CAR vector-positive cells were detected in various tissues from the same mouse receiving CT3 CAR T cells at week 2 and week 3 post-infusion. Minimal CAR vector-positive cells were found in mice infused with control CAR T cells targeting GPC3. (D) Distribution of integration sites in mice treated with control CAR T cells and CT3 CAR T cells. The integrated genes were largely shared in T cells recovered from various tissues of the same mouse, while some overlap was also observed in different mice receiving treatment. None of the shared integrated sites were found in mice from the control CAR group. The genes found in the memory T cell subsets shown in Table S5 are bolded and underlined.