Supplemental information

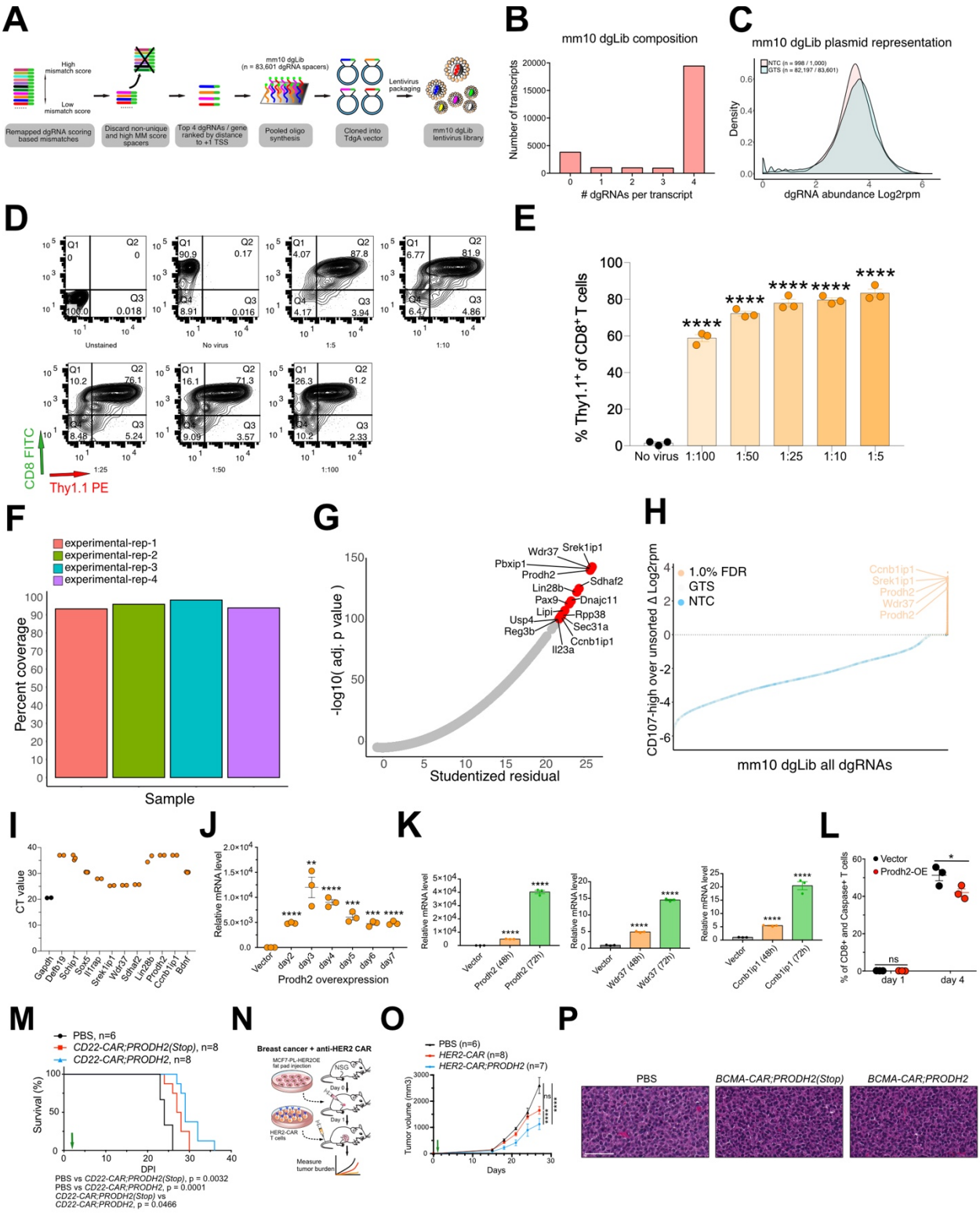

Figure S1. Additional experiments for dgRNA-based T cell CRISPRa GOF screening (Related to: Figures 1, 2, 3)

(A) Schematics of detailed mm10dgLib design process, library cloning, and lentivirus production following Figure 1B.

(**B**) mm10dgLib designed composition. Most coding transcripts (19,440 / 26,205) have 4 dgRNAs. Small portions of coding transcripts only have ≤ 3 dgRNAs that pass the same criteria (3,814, 1,010, 992 and 949 transcripts have 0, 1, 2, 3 dgRNAs, respectively).

(**C**) mm10dgLib plasmid library representation analysis by Illumina sequencing of dgRNA spacers from plasmid. 82,197 / 83,601 (98.3%) of gene-targeting spacers (GTSs) and 988/1,000 (98.8%) of NTCs were successfully cloned. Both GTSs and NTCs in the mm10dgLib showed a log-normal distribution.

(**D**) Representative flow cytometry results of the mm10dgLib lentivirus titration by detecting Thy1.1 expression at day 3 after lentivirus transduction (n = 3 biological replicates). The results showed that the mm10dgLib lentivirus was successfully produced with high titer.

(**E**) Quantitative analysis of flow cytometry results.

(F) A barplot of mm10dgLib library coverage in CD8 T cell pool after lentivirus transduction in four independent experiments, at experimental replicate level.

(G) A volcano plot of -log10pvalue vs. residual for mm10dgLib library based on outlier test against log-linear regression between the CD107a$^+$-high FACS sorted CD8$^+$ T cells and the unsorted T cells. The points were shown at the individual gRNA level. Representative top scoring genes targeted by specific sgRNAs were shown.

(H) A dotted waterfall plot of mm10dgLib library differentials between the CD107a$^+$-high FACS sorted CD8$^+$ T cells and the unsorted T cells (delta). Blue dots are NTCs; yellow dots are scoring GTSs that passed FDR 1% cutoff; grey dots are remaining GTSs. The points were shown at the individual gRNA level. Representative top scoring genes targeted by specific sgRNAs were shown.

(I) RT-qPCR for baseline mRNA level of high-ranked hit genes from dgTKS primary screen (n = 2 - 3 biological replicates, variable between genes, see figure for datapoints). Higher CT values indicate the candidates' mRNA levels as lower than house-keeping gene *Gapdh* in primary murine CD8$^+$ T cells.

(**J**) Time-course qPCR detection of *Prodh2* mRNA level in lentiviral-Prodh2-OE mouse CD8 T cells (n = 3 biological replicates).

(**K**) RT-qPCR for overexpression of *Prodh2, Wdr37* and *Ccnb1ip1* after lentiviral transduction of mouse CD8 T cells (n = 3 biological replicates).

(L) Quantification of mouse T cell apoptosis in an IL-2 withdrawal assay. Cleaved caspase 3 was detected by flow cytometry at day 1 and day 4 after IL-2 withdrawal (n = 3 biological replicates).

(M) Overall survival of PRODH2 CAR22 in a leukemia model. Green arrow indicated CAR-T injection.

(**N**) A schematic of breast cancer model PRODH2 knock-in CAR-T efficacy testing, showing tumor induction, HER2-CAR T cell intravenously injection, and survival.

(**O**) Tumor growth curve of breast cancer bearing NSG mice after HER2-CAR;PRODH2 or HER2-CAR T therapy. Green arrow indicated CAR-T injection.

(P) H&E staining of myeloma tumor tissues from end-point mice after CAR-T therapies. Scale bar = 50 μm.

*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, ns = not significant by unpaired *t* tests (E, J, K), multiple *t* tests (L), two-way ANOVA (with multiple comparisons test) (O) or log-rank (Mantel-Cox) tests (M).

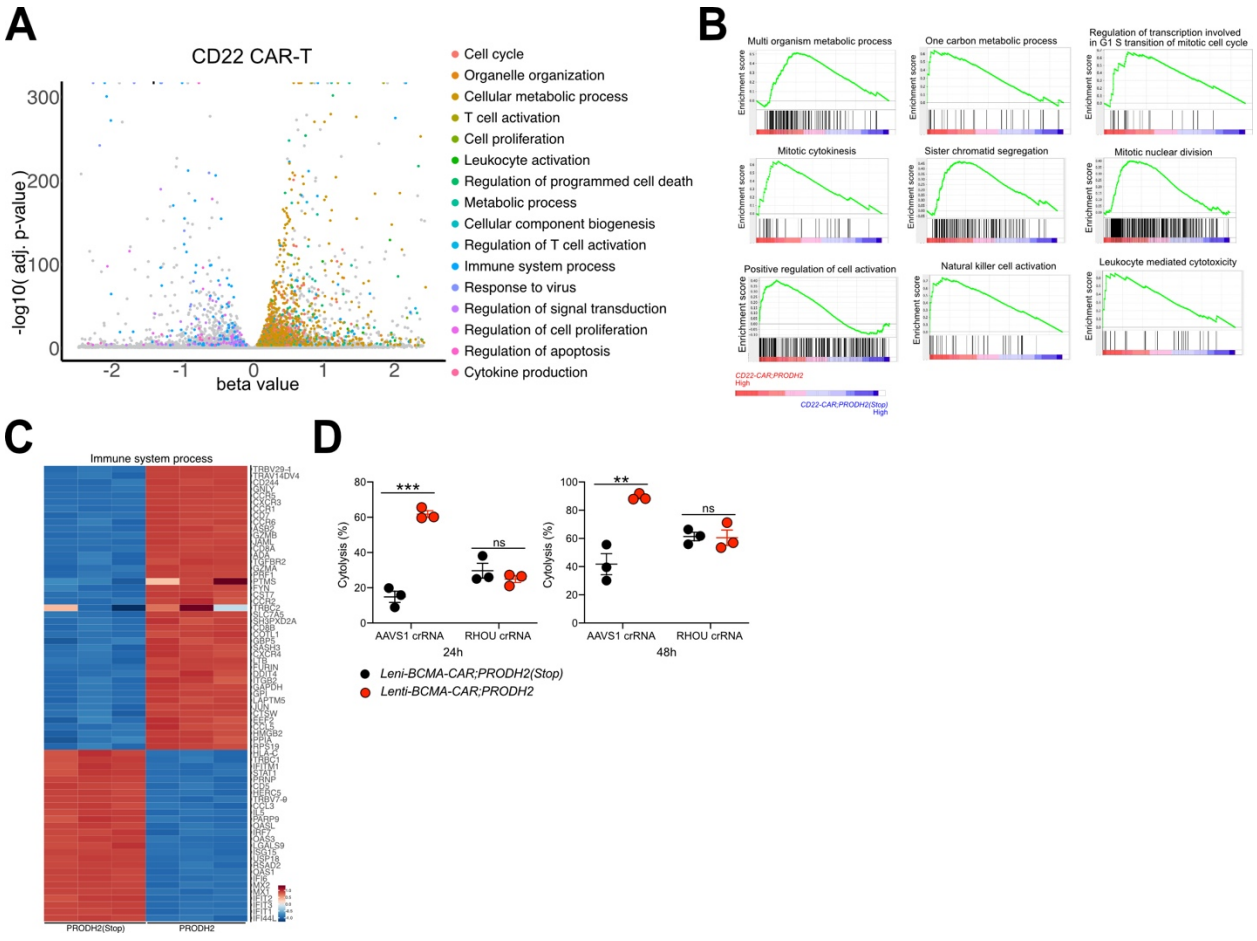

Figure S2. Additional transcriptome profiling and downstream analysis of PRODH2 knock-in CD22-CAR-T cells (Related to: Figure 4)

(**A**) Color-coded volcano plots of representative top enriched pathways between *CD22-CAR;PRODH2(Stop)* and *CD22-CAR;PRODH2* cells. Upregulated and downregulated pathways were annotated from DAVID analysis of the biological processes of upregulated and downregulated genes, respectively (n = 3 biological replicates).

(**B**) GSEA individual pathways for mRNA-seq of PRODH2 knock-in CD22-CAR-T cells. GSEA plots of individual pathways from the representative up- and down-regulated gene sets between *CD22-CAR;PRODH2* and *CD22-CAR;PRODH2(Stop)* T cell groups. (Cut-off criteria: p-value < 0.001)

(**C**) Heatmaps of differentially expressed genes in immune system process.

(**D**) Genetic testing of knockout effect of representative upregulated genes on PRODH2 overexpressed CAR-T cells. Cytolysis of PRODH2 or PRODH2(Stop) knock-in CD22-CAR-T

cells, with or without *RHOU* knockout, at two time points (left panel, 24h; right panel, 48h) (n = 3).

**P<0.01, ***P<0.001, ns = not significant by multiple *t* tests (D).

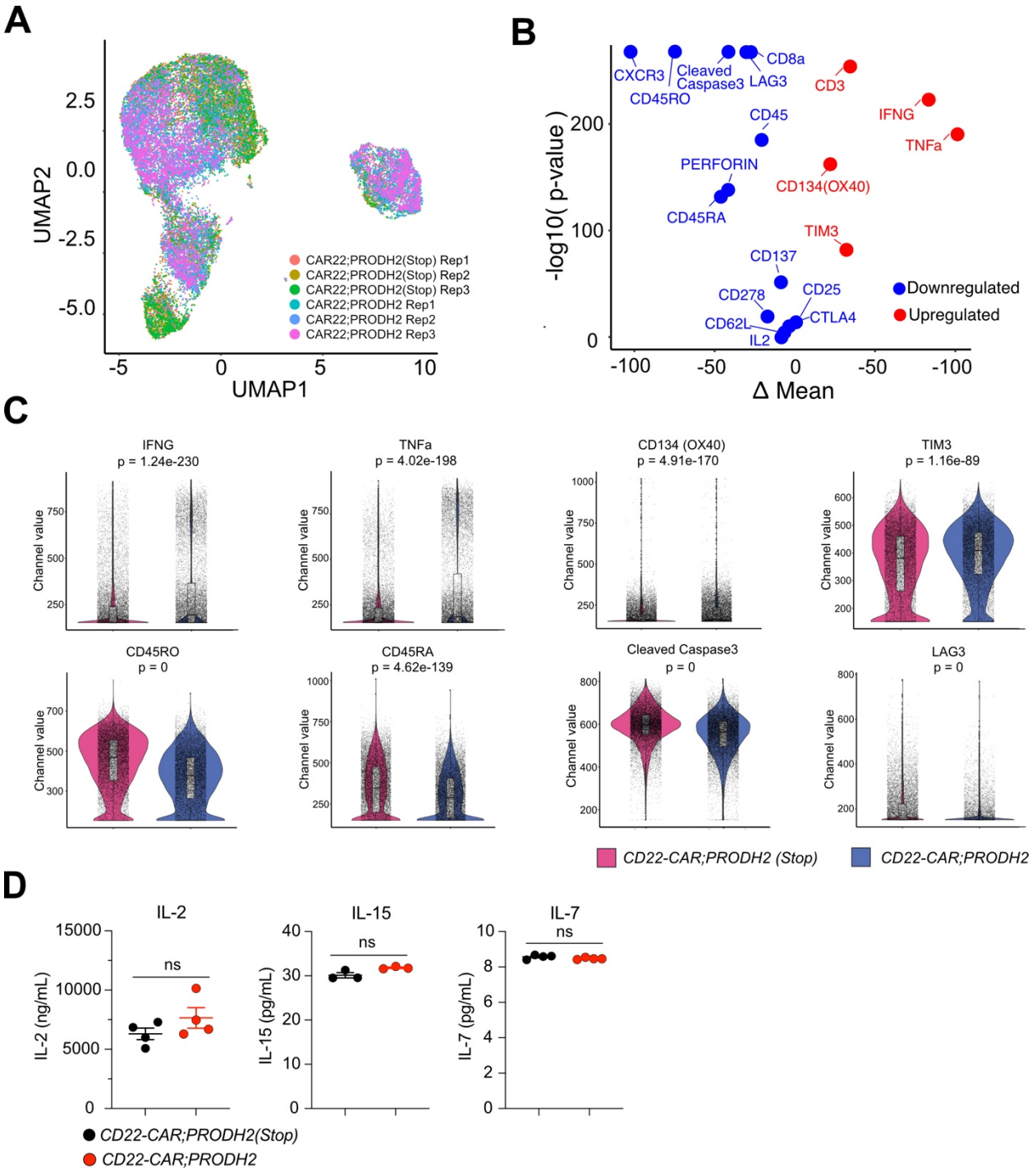

Figure S3. Baseline CyTOF analysis of CD22-CAR T cells with PRODH2 overexpression (Related to: Figure 4)

(**A**) UMAP plots labeled by individual biological samples. All T cells are at baseline without cancer stimulation in this assay.

(**B**) Volcano plot of CyTOF data of *CD22-CAR;PRODH2* vs. *CD22-CAR;PRODH2(Stop)* T cells.

(**C**) Violin plots of CyTOF data of *CD22-CAR;PRODH2* vs. *CD22-CAR;PRODH2(Stop)* T cells. Each panel represents one individual marker, including IFNG, TNFa, CD134 (OX40), TIM3, CD45RO, CD45RA, Cleaved Caspase 3, and LAG3. Violin plots were used for quantification of gene expression level in single cell level. Wilcoxon Rank Sum test adjusted by Benjamini & Hochberg method was used to assess the significance.

(D) ELISA analysis of IL-2, IL-15, and IL-7 for *PRODH2* knock-in and control CAR-T cells. (n = 4 biological replicates)

ns = not significant by unpaired *t* tests (D).

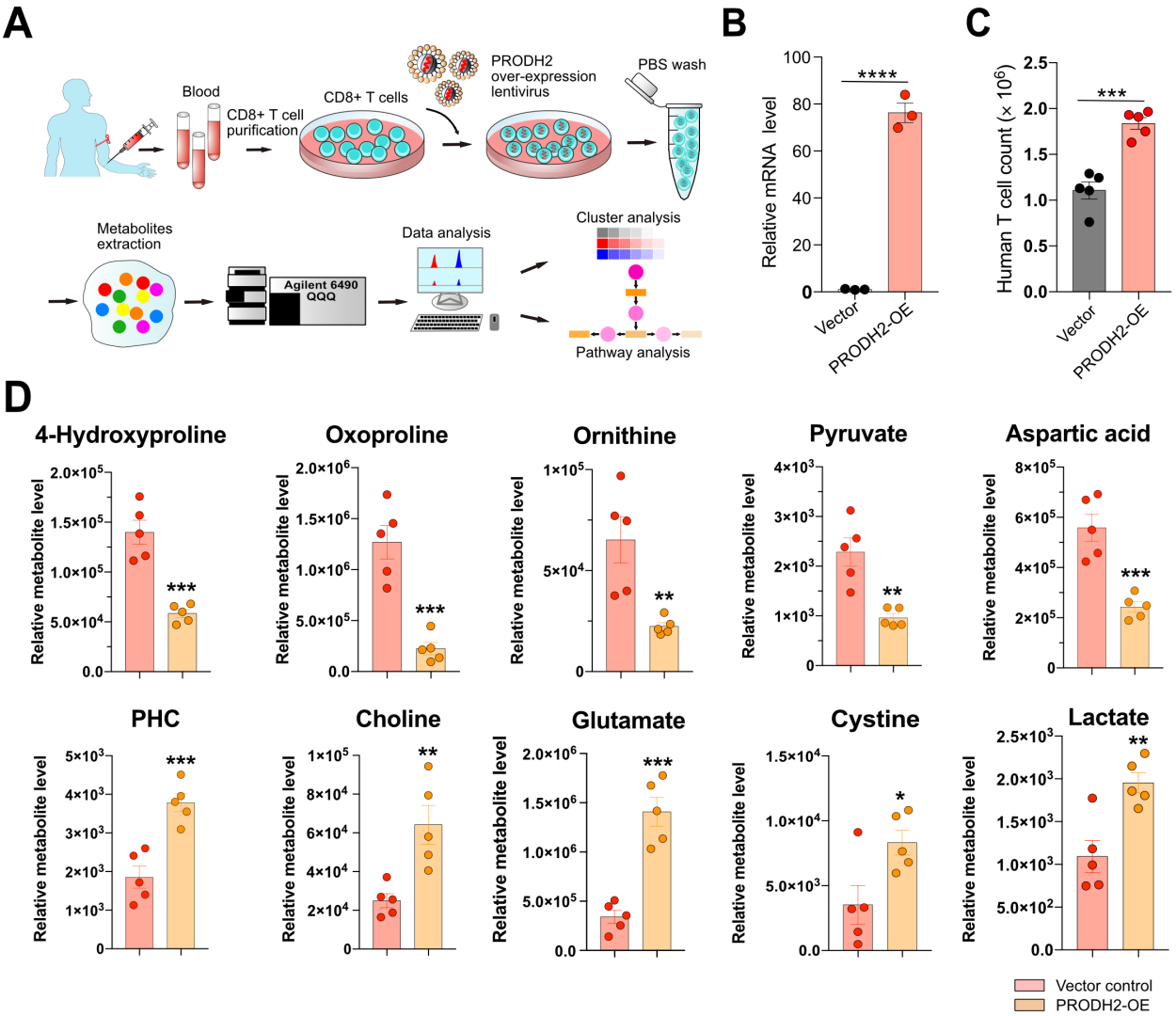

Figure S4. Metabolomic analysis of PRODH2-OE in human primary CD8 T cells (Related to: Figure 5)

(A) A schematic of human CD8 T cell lentivirus transduction, metabolites extraction, LC/MS analysis, and data analysis.

(B) Transcriptional level of human *PRODH2* in primary CD8 T cells (no CAR) after PRODH2-OE lentivirus transduction (n = 3 biological replicates).

(C) Human T cell number quantification after PRODH2-OE lentivirus transduction (n = 5 biological replicates). Representative data from two independent experiments.

(D) Quantification of significantly changed metabolites after PRODH2-OE (n = 5 biological replicates).

**P<0.01, ***P<0.001 by unpaired *t* tests (B-D).

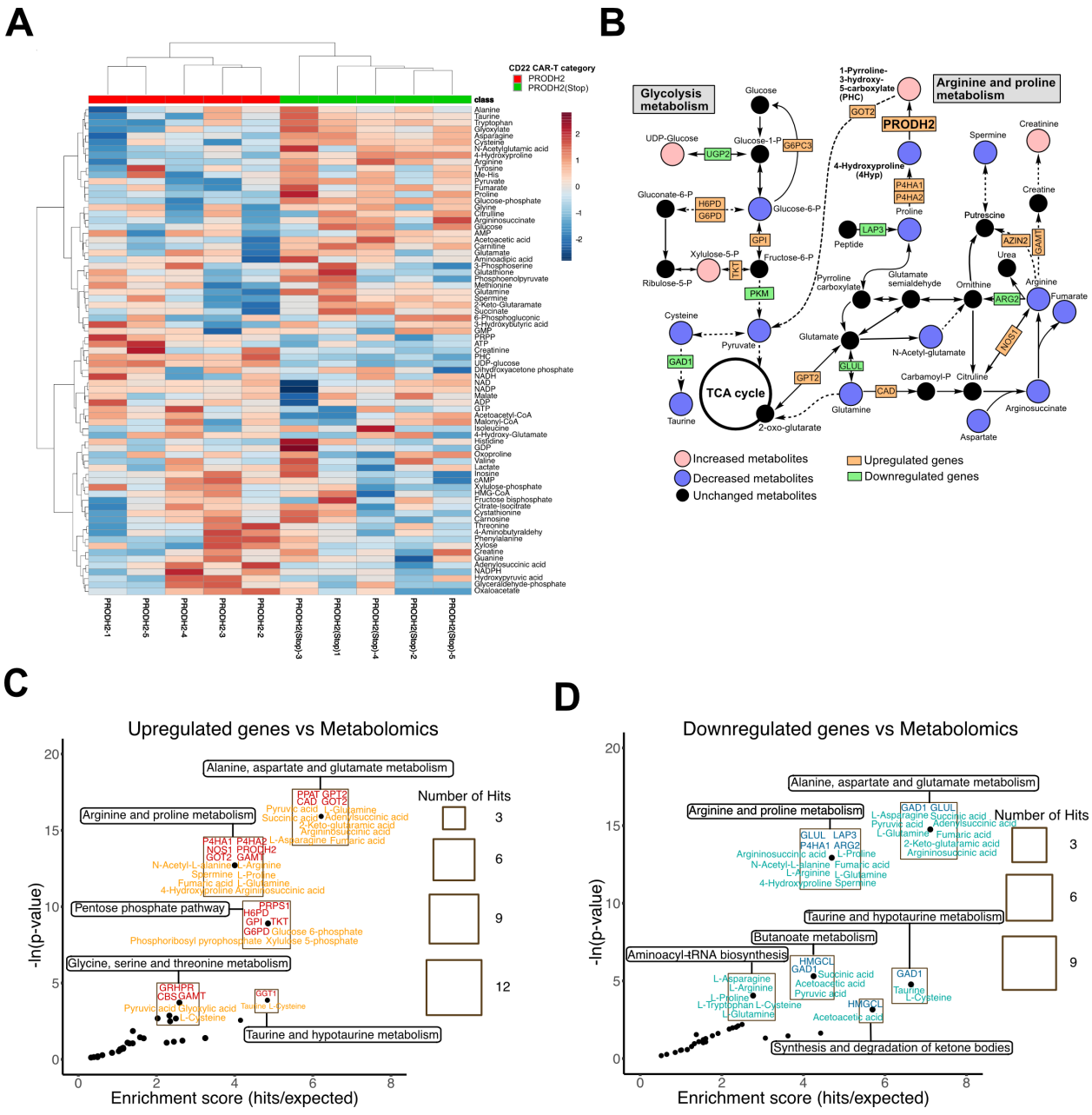

Figure S5. KEGG-guided multi-omics analysis of human *CD22-CAR;PRODH2* T cells (Related to: Figure 5)

(**A**) A summary illustration highlighting representative enzymes encoded by DE genes, as well as DR metabolites on a metabolic map adapted from KEGG metabolic pathways. As in legend, blue circles indicate decreased metabolites; pink circles indicate increased metabolites; green squares indicate enzymes of downregulated genes; orange squares indicate enzymes of upregulated genes.

Solid arrows indicate direct biochemical reactions; dashed arrows indicate indirect or multi-step biochemical reactions; bi-direction arrows indicate reversible reactions. All arrows were drawn according to the known reactions in KEGG. Only reactions related to DE genes or DR metabolites were shown. A simplified version is shown in a main figure panel.

(B) A summary illustration highlighting representative enzymes encoded by DE genes, as well as DR metabolites on a simplified metabolic map adapted from KEGG metabolic pathways. As in legend, blue circles indicate decreased metabolites; pink circles indicate increased metabolites; green squares indicate enzymes of downregulated genes; orange squares indicate enzymes of upregulated genes. Solid arrows indicate direct biochemical reactions; dashed arrows indicate indirect or multi-step biochemical reactions; bi-directional arrows indicate reversible reactions. All arrows were drawn according to the known reactions in KEGG. Only reactions related to DE genes or DR metabolites were shown.

(**C**) Scatter plots of multi-omics analysis of RNA-seq upregulated genes vs. DR metabolomics metabolites (both increased and decreased) between PRODH2 vs. PRODH2(Stop) CD22-CAR-T cells. Each dot represents a metabolic pathway, with those enriched in multi-omics analysis text-labeled. The upregulated genes and the DR metabolites associated with each significant pathway were labeled inside a box scaled to the number of metabolic hits.

(**D**) Scatter plots of multi-omics analysis of RNA-seq downregulated genes vs. DR metabolomics metabolites (both increased and decreased) between PRODH2 vs. PRODH2(Stop) CD22-CAR-T cells.

Each dot represents a metabolic pathway, with those enriched in multi-omics analysis text-labeled. The downregulated genes and the DR metabolites associated with each significant pathway were labeled inside a box scaled to the number of metabolic hits.

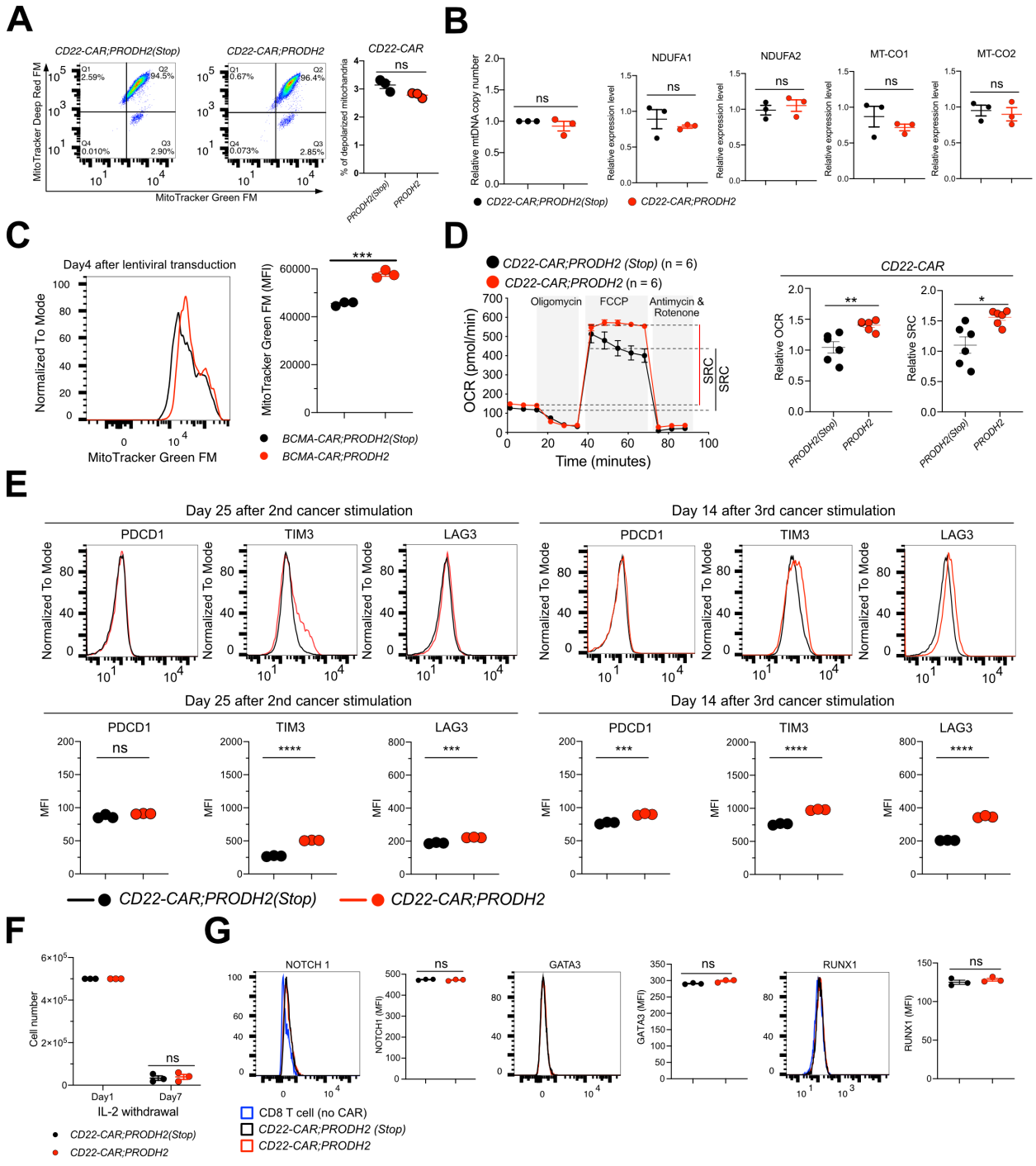

Figure S6. Additional analyses of PRODH2 engineered CAR-T, on mitochondria level, depolarization, metabolic function, Mt DNA copy number, cytokine secretion, T cell exhaustion and oncogenic potential (Related to: Figures 5, 6, 7)

(**A**) Mitochondria depolarization analysis. CAR T cells were stained with MitoTracker Green FM and MitoTracker Deep Red FM dyes to measure mitochondria depolarization (n = 3 biological replicates).

(B) Mt DNA analysis. Mitochondrial DNA copy and representative Mt gene expression of complex I and IV (n = 3 biological replicates).

(C) Mitochondra level analysis of lentiviral BCMA CAR-Ts. MitoTracker staining and quantification of *PRODH2* overexpression and Control CAR-T cells.

(D) Seahorse OCR analysis of Mt metabolic function. Seahorse experiment of *PRODH2* knock-in and control CAR-T cells, with a density of 4e5 CAR-T cells / well. Oxygen consumption rate (OCR) was measured at baseline and in response to oligomycin (Oligo), FCCP, and rotenone plus antimycin A. Data are representative of three independent experiments. Relative maximum OCR and relative spare respiratory capacity (SRC) were quantified (n = 6 biological replicates).

(**E**) Long-term T cell exhaustion analysis. Flow measurement of PD-1, TIM-3 and LAG-3 for CD22-CAR;PRODH2 and CD22-CAR;PRODH2 (Stop) T cells after long-term co-culture and cancer stimulation.

(**F-G**) Oncogenic potential analysis of PRODH2 in CAR-T.

(**F**) IL-2 withdrawal assay. Cell count after IL-2 withdrawal assay to evaluate the survival of *PRODH2* knock-in and control CAR-T cells.

(**G**) Flow analysis of key T-ALL oncogenic transformation markers, such as NOTCH1, GATA3, and RUNX1.

*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, ns = not significant by multiple *t* tests (with adjusted P value) (F), and unpaired *t* tests (A-E, G).

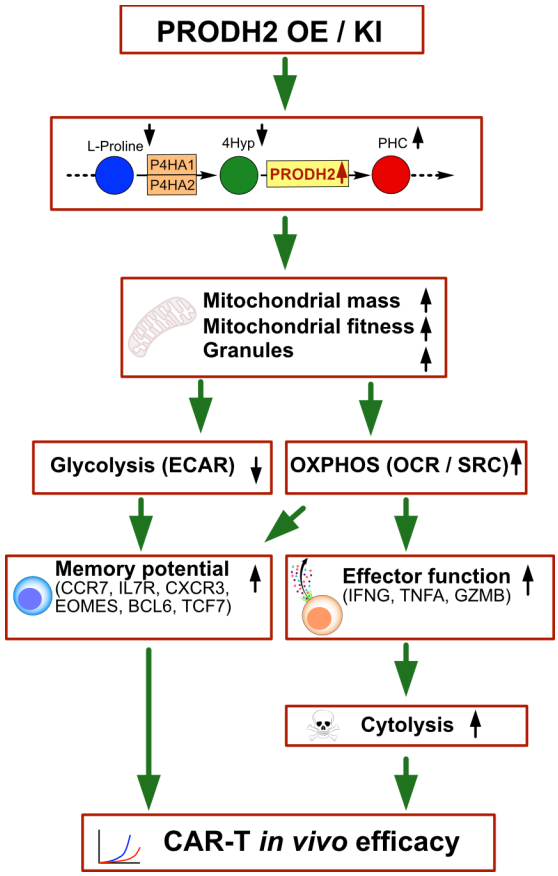

Figure S7. Brief summary of mechanism of action of PRODH2 GOF in CAR-T cell

Supplemental Datasets

Dataset S1. Design of the mm10dgLib library and readout of the dgTKS screen.

Tabs in this dataset:

Composition of the mm10dgLib library.

Readout of mm10dgLib library representation in the dgTKS screen.

Summary analysis of mm10dgLib library complexity / coverage.

dgTKS screen outlier test.

CD107a-high vs unsorted analysis of the dgTKS screen.

Ranked differential analysis of the dgTKS screen.

Dataset S2. mRNA-seq analyses of human PRODH2 knock-in CAR-T cells.

Tabs in this dataset:

Human CD22CAR-T mRNAseq PRODH2 vs. PRODH2(Stop) gene-level differential expression analysis.

Human CD22CAR-T mRNA-seq PRODH2 vs. PRODH2(Stop) upregulated genes (adj. $p < 0.001$) DAVID gene-annotation analysis.

Human CD22CAR-T mRNA-seq PRODH2 vs. PRODH2(Stop) downregulated genes (adj. $p < 0.001$) DAVID gene-annotation analysis.

Human CD22CAR-T mRNA-seq PRODH2 vs. PRODH2(Stop) GSEA analysis (Up-regulated)

Human CD22CAR-T mRNA-seq PRODH2 vs. PRODH2(Stop) GSEA analysis (Down-regulated)

Dataset S3. Metabolomics, multi-omics and CyTOF analyses of human PRODH2 knock-in CAR-T cells.

Tabs in this dataset:

Metabolomics analysis of human CD8 T cells with PRODH2-OE.

Metabolomics – relative abundance of detected metabolites in PRODH2 vs. PRODH2(Stop) CD22CAR-T cells.

MetaboAnalyst joint pathway analysis using consensus upregulated differentially expressed genes and differentially represented metabolites from metabolomics analysis.

MetaboAnalyst joint pathway analysis using consensus downregulated differentially expressed genes and differentially represented metabolites from metabolomics analysis.

CyTOF channel values for sampled CD22-CAR;PRODH2 and CD22-CAR;PRODH2(Stop) T cells.

UMAP coordinates for sampled CD22-CAR;PRODH2 and CD22-CAR;PRODH2(Stop) T cells.

Differential marker expression for sampled CD22-CAR;PRODH2 and CD22-CAR;PRODH2(Stop) T cells.

Supplemental Source Data and Statistics

Original data and statistics for non-high-throughput data used in figure generation.

Supplemental sequences

Additional sequences of probes and oligos were provided in an excel file.