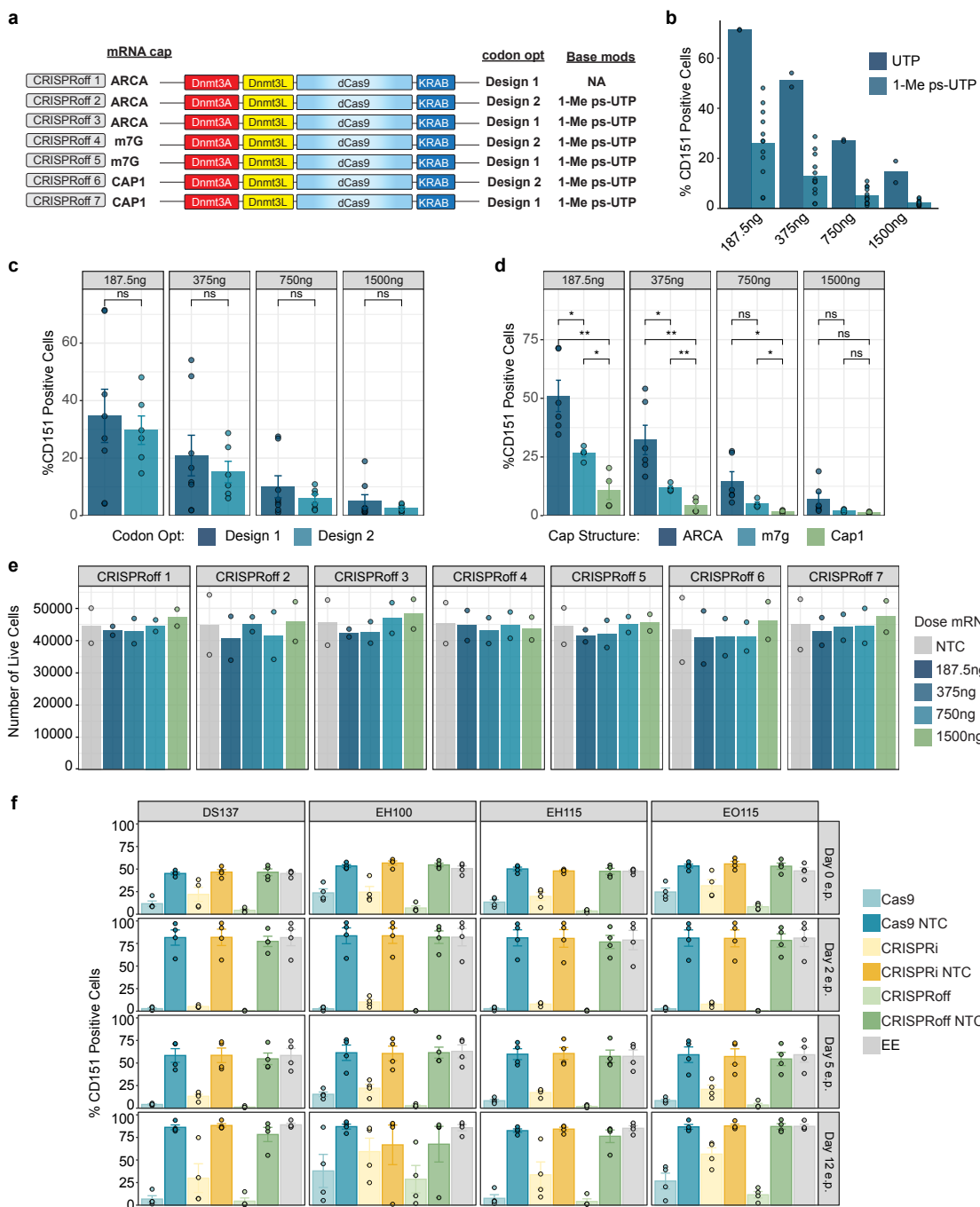
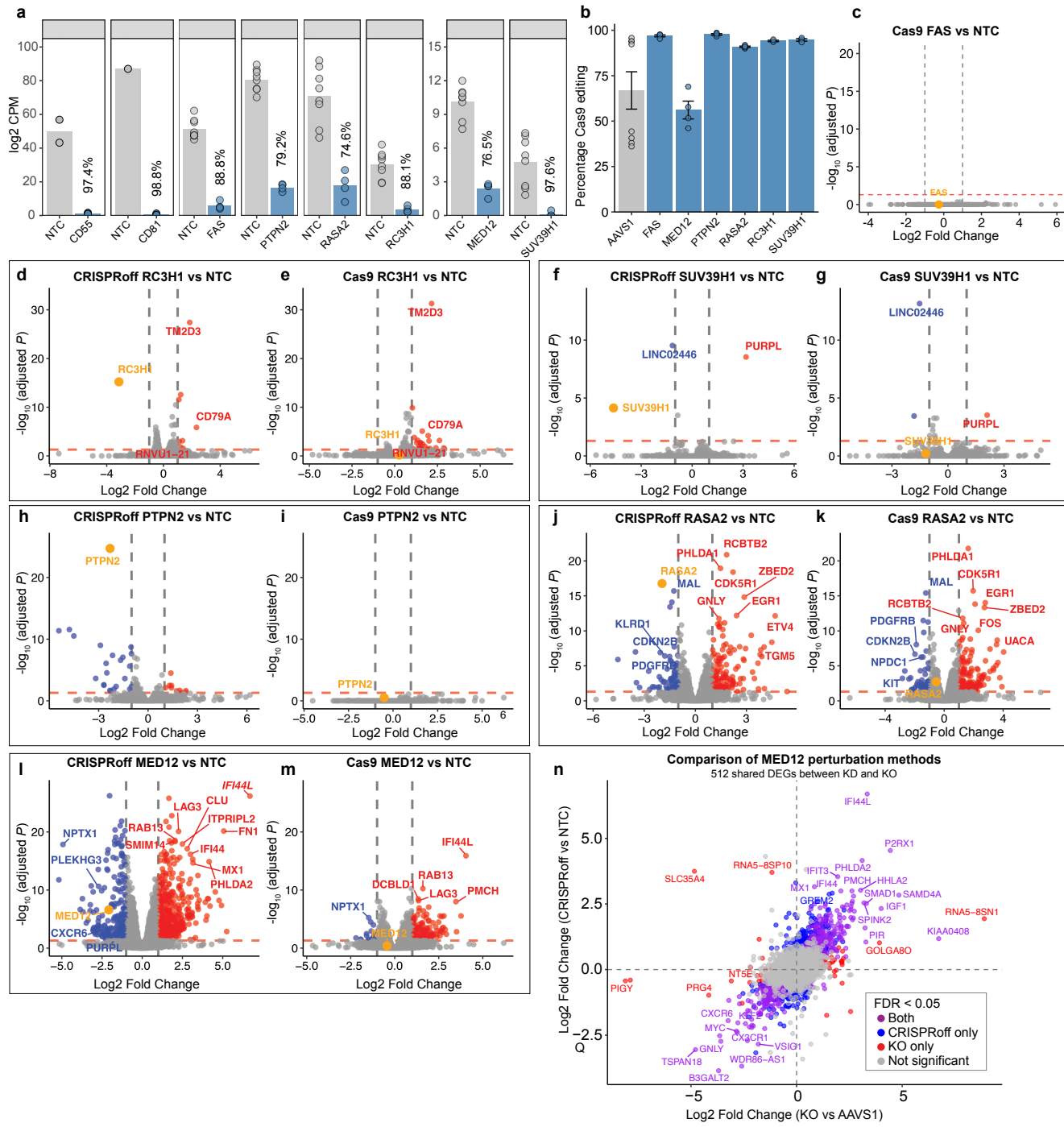

Integrated epigenetic and genetic programming of primary human T cells

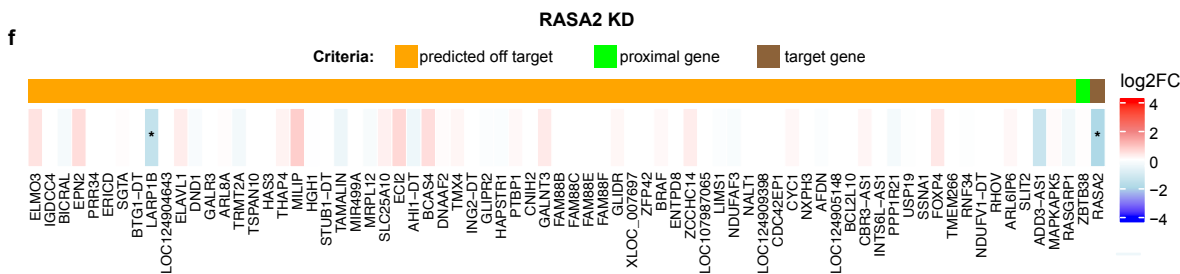
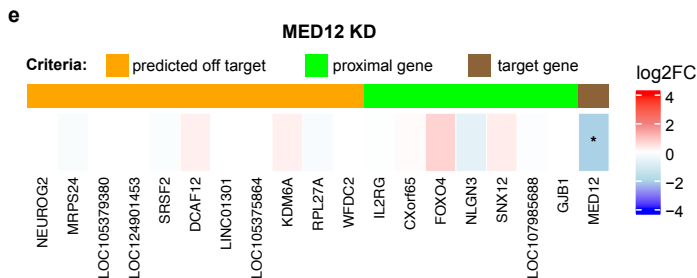
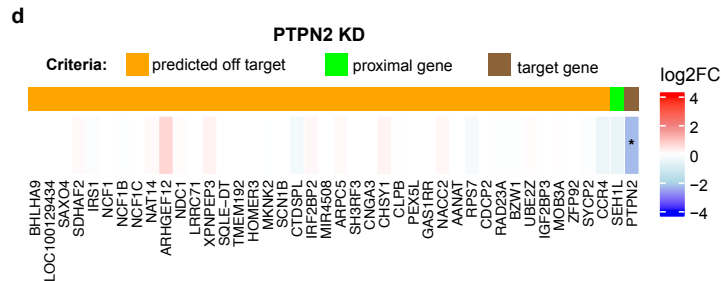
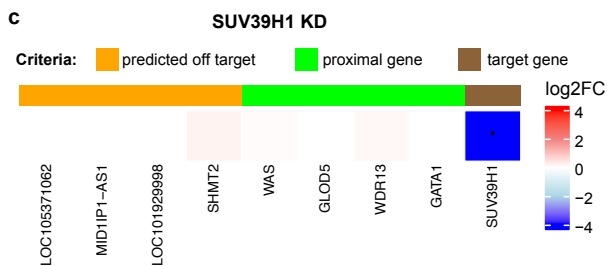
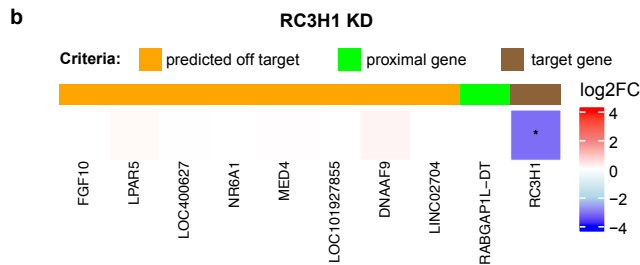
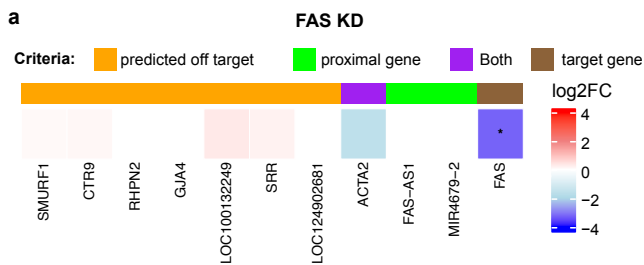
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Supplemental Figure 1: Optimization of CRISPRoff mRNA for durable silencing in primary human T cells. (A) Schematic of seven CRISPRoff mRNA variants tested in primary human T cells. (B) Comparison of CD151 KD efficiency at 4 days post-electroporation between CRISPRoff mRNAs that incorporated base modifications (1-Me ps-UTP) versus standard UTP at 4 doses of mRNA. Each dot represents an mRNA variant across two donors. (C) Comparison of CD151 KD efficiency at 4 days post-electroporation between codon optimization strategies for dCas9 (Design 1 versus Design 2) at four doses of mRNA. Each dot represents an mRNA variant across two donors ($n = 2$ donors, mean $\pm$ s.e.m.; two-sided Welch's t -test). (D) Comparison of CD151 KD efficiency at 4 days post-electroporation between mRNA cap structures (ARCA, m7G, Cap1). Quantification of CD151 KD is depicted as fold change relative to the unoptimized mRNA design (CRISPRoff 1). Each dot represents an mRNA variant across two donors (mean $\pm$ s.e.m.; * $P < 0.05$ and ** $P < 0.01$, two-sided Welch's t -test) (E) Live cell counts collected at four days post-electroporation for each of the CRISPRoff mRNA variants ($n = 2$ donors). (F) Four individual Lonza pulse codes (DS137, EH100, EH115, and EO115) were tested for mRNA electroporation of either Cas9, CRISPRi, or CRISPRoff mRNA at four separate time points, either before initial activation (Day 0) or after initial activation (2 days post-activation, 5 days post-activation, or 12 days post-activation) with anti-CD3/CD28 Dynabeads. Cells were harvested five days after each electroporation timepoint and CD151 protein expression was quantified by flow cytometry ($n = 4$ donors, mean $\pm$ s.e.m.).

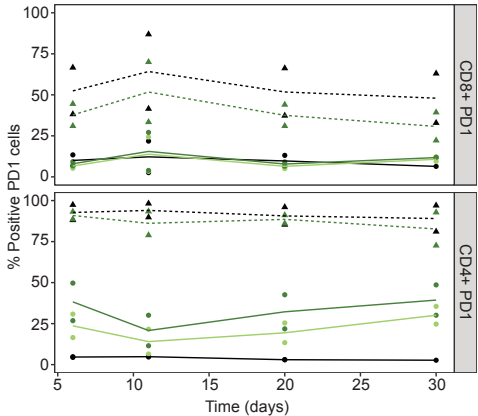


Supplemental Figure 2: Durable at specific CRISPRoff silencing at therapeutically relevant genes in T cells. **(A)** An RNA-sequencing log₂ CPM (normalized counts per million) plot showing cells electroporated with CRISPRoff and sgRNAs targeting *CD55*, *CD81*, *FAS*, *PTPN2*, *RASA2*, *RC3H1*, *MED12*, or *SUV39H1* as compared to NTC treated cells. The percent KD is shown for each gene. (*CD55* and *CD81* were collected 27 days post-electroporation; *n* = 2 donors) (*FAS*, *PTPN2*, *RASA2*, *RC3H1*, *MED12*, and *SUV39H1* were collected on day 7 post-electroporation; *n* = 4 donors). **(B)** Nuclease-active Cas9 editing efficiency at each of the therapeutically relevant target loci collected for RNA-seq as measured by TIDE indel analysis. **(C-M)** Transcriptomic assessment by RNA-seq of CRISPRoff or Cas9 activity upon silencing or KO, respectively, of **(C)** *FAS*, **(D-E)** *RC3H1*, **(F-G)** *SUV39H1*, **(H-I)** *PTPN2* or **(J-K)** *RASA2* or **(L-M)** *MED12* relative to NTC. For CRISPRoff treated samples, cells were electroporated with the most potent guide identified from RT-qPCR for each gene in panel Figure 1H. For both Cas9 and CRISPRoff treated samples, cells were harvested for RNA extraction and bulk RNA sequencing seven days post-electroporation (*n* = 4 donors). The target gene for each panel is labeled in yellow, blue dots indicate significantly downregulated DEGs, red dots indicate significantly upregulated DEGs, and grey dots have no significance (Empirical-Bayes moderated statistics with Benjamini–Hochberg FDR control, adjusted *P* < 0.05). **(N)** Comparison of gene log₂-fold change between CRISPRoff and CRISPR KO targeting *MED12*. CRISPR KO for *MED12* RNA-sequencing data is from Arce et al ⁴⁰. Non-significant hits are shown in grey and significant hits are colored by direction of effect between experiments (FDR < 0.05) (CRISPR KO data: *n* = 2 donors. CRISPRoff data: *n* = 4 donors).



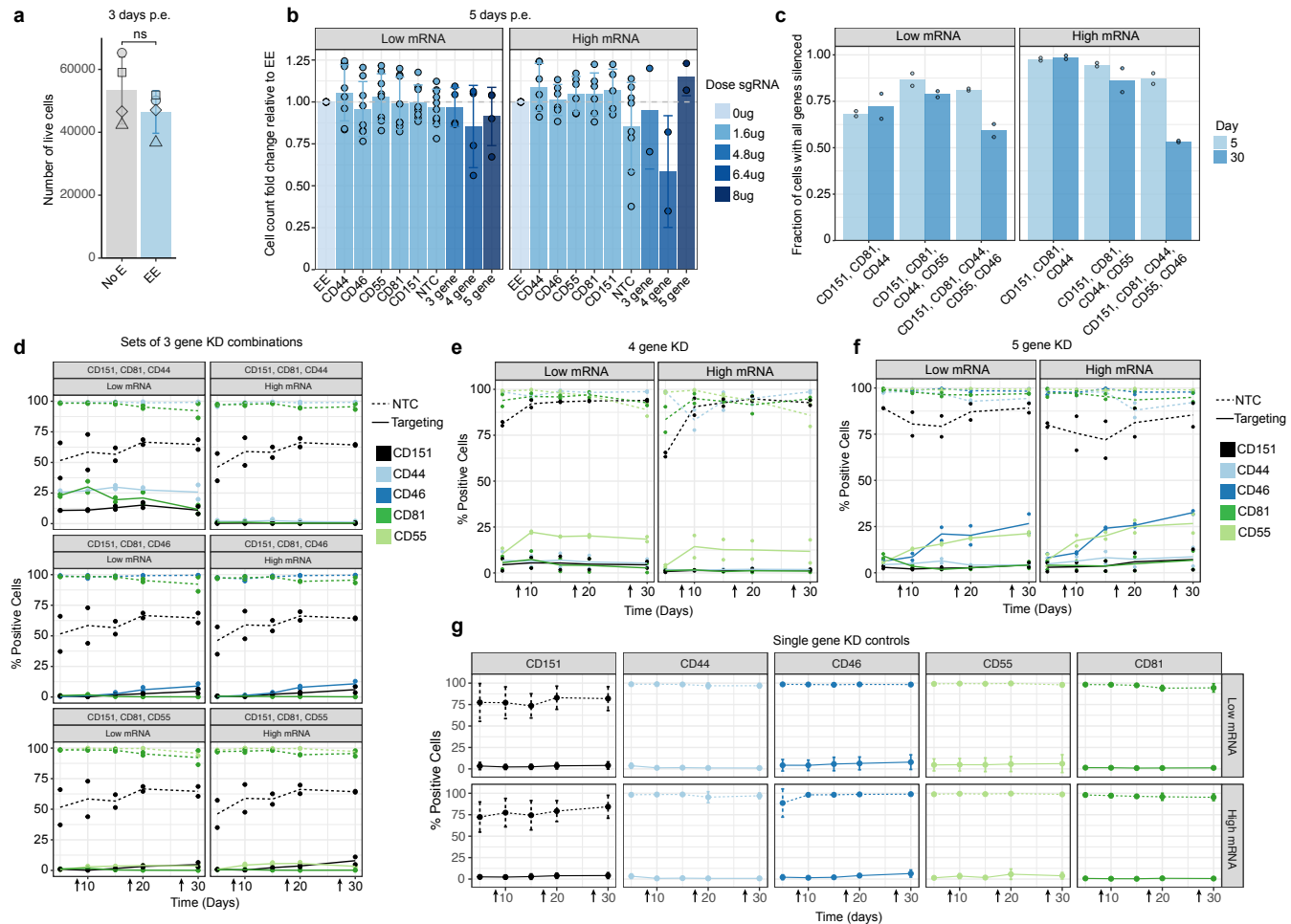
Supplemental Figure 3: Off-target assessment of CRISPRoff. (A-F) Off-target assessment of CRISPRoff silencing as measured by RNA-seq when targeting **(A)** *FAS*, **(B)** *RC3H1*, or **(C)** *SUV39H1*, **(D)** *PTPN2*, **(E)** *MED12*, or **(F)** *RASA2*. Predicted off-target sites were generated through IDT's CRISPR-Cas9 guide RNA design checker for each of the CRISPRoff KD guides, and loci were filtered for ± 1 kb around a gene promoter. Proximal genes within 100kb of the target gene were also assessed. Genes are ordered by left to right by highest to lowest off-target score generated from IDT. Predicted off-target genes that met significance thresholds (adjusted $P > 0.05$ and absolute log2 fold change > 1) are denoted with a star ($n = 4$ donors). Predicted off-targets are annotated with an orange box, proximal genes are annotated with a green box, and the target gene is annotated with a brown box.

- Cas9
- Targeting
- CRISPRoff single
- Control
- CRISPRoff pool



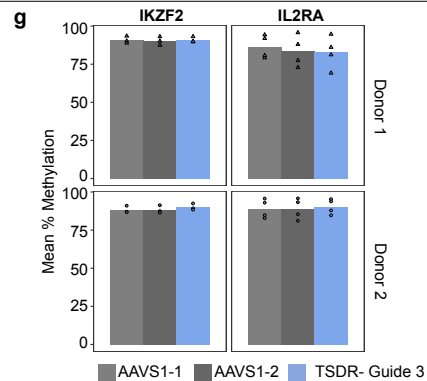
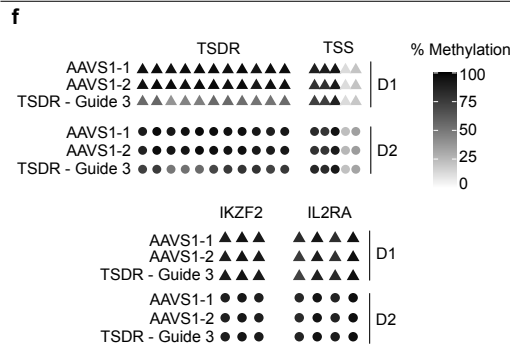
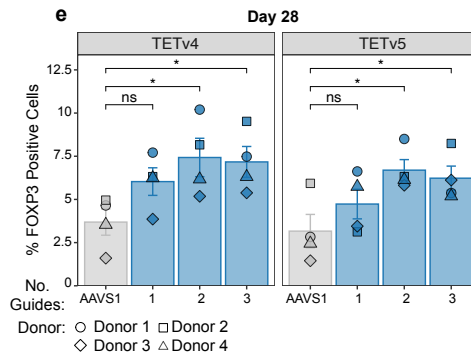
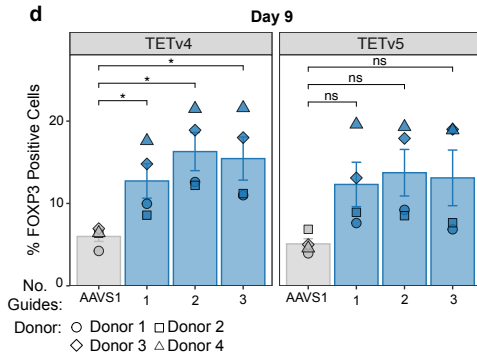
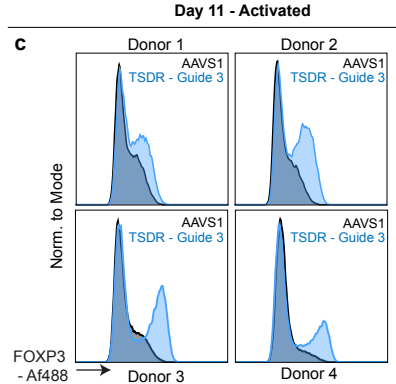
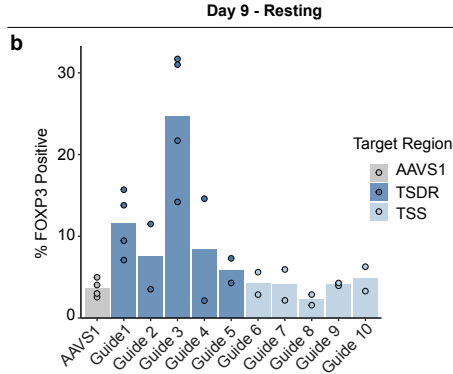
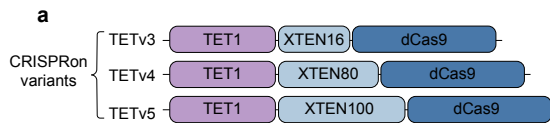
Supplemental Figure 4: CRISPRoff silencing of PD1 in CD8⁺ and CD4⁺ T cells.

Comparison of Cas9 KO (black) or CRISPRoff KD activity with either a single sgRNA (light green) or a pool of three sgRNAs (dark green) at *PDCD1* over a time course of 30 days post-electroporation. CD8⁺ T cells are shown in the top graph, and CD4⁺ T cells are shown in the bottom graph. Cells were restimulated with anti-CD2/CD3/CD28 at 24 hours prior to each flow cytometry timepoint ($n = 2$ donors).

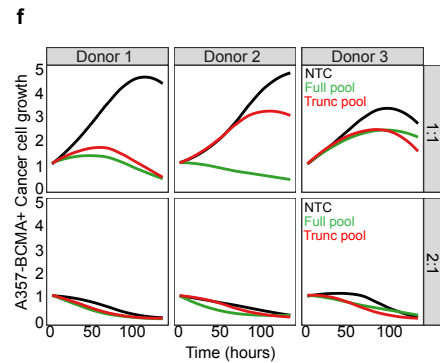


Supplemental Figure 5: Durable multiplexed gene silencing with CRISPRoff. (A)

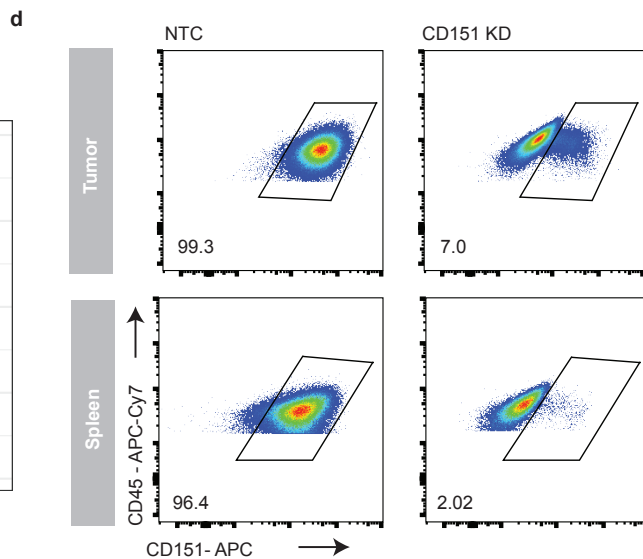
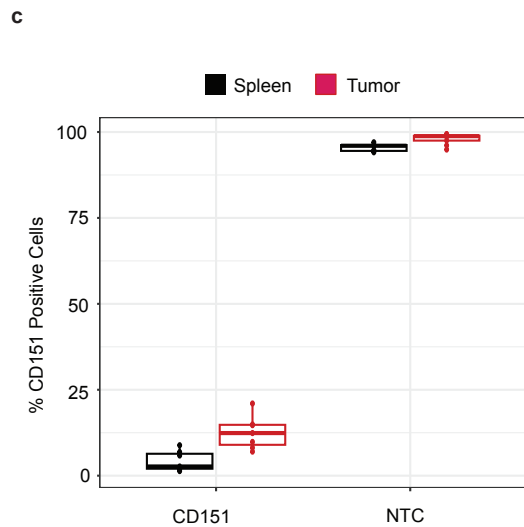
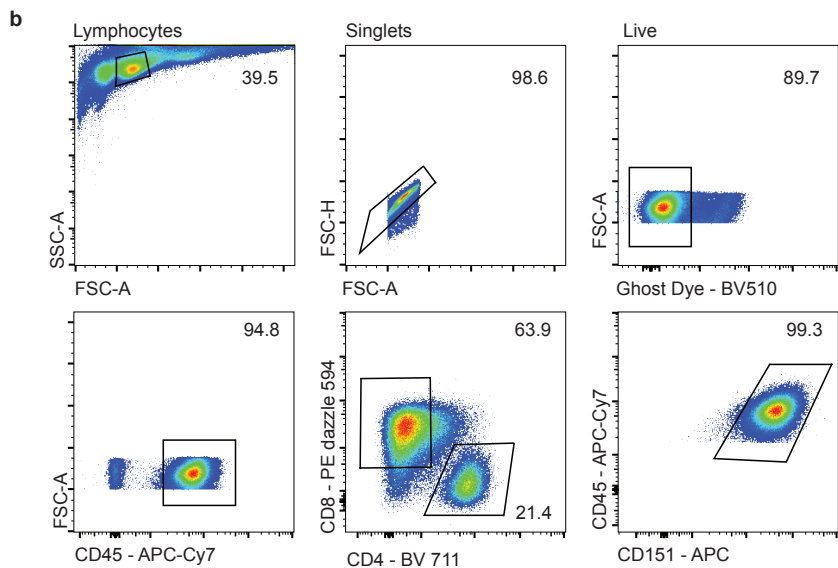
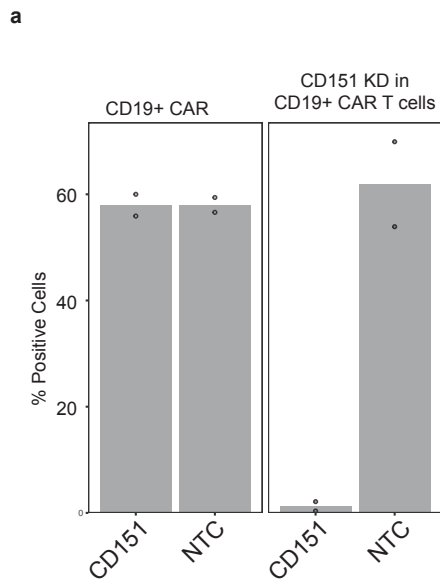
Viability of cells that were either not electroporated (No E; grey bar) or empty electroporated (EE; blue bar). Empty electroporated cells were treated with P3 buffer only. ($n = 4$ donors per condition, mean $\pm$ SD; two-sided Welch's t -test). **(B)** Viability of cells electroporated with CRISPRoff at two doses of mRNA (low = 1.5 μ g, high = 3 μ g). Cells were co-electroporated with either one sgRNA targeting a single gene (sgRNA dose = 1.6 μ g), three genes simultaneously (4.8 μ g sgRNA), four genes simultaneously (6.4 μ g of sgRNA) or five genes simultaneously (8 μ g sgRNA). For multiplexed conditions, each gene target received one sgRNA to the TSS. Live cell counts were collected at 5 days post-electroporation, and cell counts were normalized and displayed as fold-change relative to an empty electroporation condition. ($n = 8$ donors for single gene targeting, NTC, and EE; $n = 4$ donors for multiplex conditions in the low mRNA condition; $n = 2$ donors for multiplex conditions in the high mRNA condition, mean $\pm$ SD) **(C)** Comparison of three-gene, four-gene, and five-gene multiplex silencing with CRISPRoff at either a high or low dose of mRNA. Flow cytometry data were collected at 5 days post-electroporation and 30 days post-electroporation, and the percentage of cells with all genes silenced for a given multiplex combination was calculated (see Methods) ($n = 2$ donors). **(D)** Multiplex analysis of triple-gene CRISPRoff silencing when targeting three distinct combinations of genes (*CD151*, *CD81*, *CD44*), (*CD151*, *CD81*, *CD46*), (*CD151*, *CD81*, *CD55*) over a timecourse of 30 days post-electroporation with CRISPRoff mRNA and the indicated sgRNAs. NTC are shown as dotted lines, and targeting sgRNAs are shown as solid lines. Cells were restimulated every nine days post-initial activation, indicated by black arrows ($n = 2$ donors). **(E,F)** Multiplex analysis of either four-gene **(E)** or five-gene **(F)** CRISPRoff gene silencing over a timecourse of 30 days post-electroporation with CRISPRoff mRNA and the indicated sgRNAs. NTC are shown as dotted lines, and targeting sgRNAs are shown as solid lines. Cells were restimulated every nine days after initial activation, indicated by black arrows ($n = 2$ donors). **(G)** The graphs show CRISPRoff silencing of individual genes (*CD151*, *CD44*, *CD46*, *CD55*, or *CD81*) over a timecourse of 30 days post-electroporation of CRISPRoff mRNA and the indicated sgRNA. Cells were re-stimulated every nine days after initial activation with anti-CD2/CD3/CD28 soluble antibodies. Black arrows indicate restimulation timepoints. NTC are shown as dotted lines and targeting sgRNAs are shown as solid lines (mean $\pm$ SD, $n = 10$ donors).



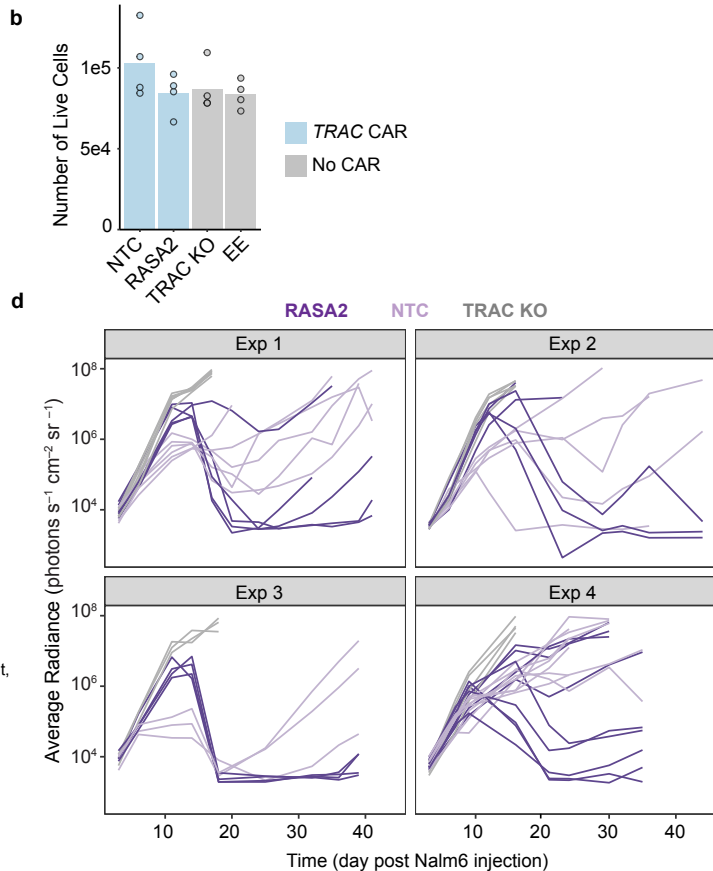
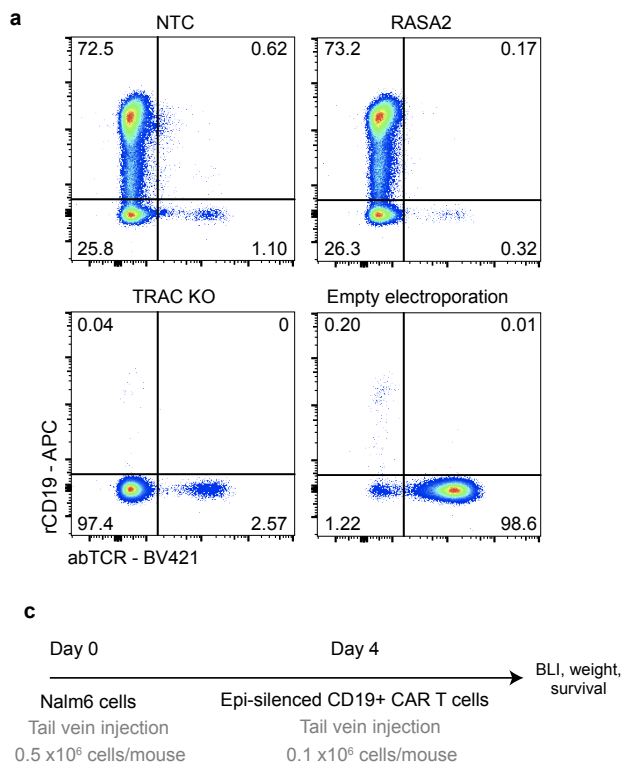
Supplemental Figure 6: Targeting the *FOXP3* TSDR with CRISPRon in primary human T cells. **(A)** Schematic of three CRISPRon variants (TETv3, TETv4, and TETv5), each of which consists of TET1 catalytic domain fused to dCas9 with varying XTEN linker lengths. **(B)** Percentage of FOXP3 expressing cells after Tconv received CRISPRon mRNA-TETv3 and sgRNAs targeting the TSDR (dark blue), the TSS (light blue), or AAVS1 control region (grey) as measured by flow cytometry at nine days post-electroporation (AAVS1 controls and Guides 1 & 3 targeting the TSDR: $n = 4$ donors. For the remaining conditions: $n = 2$ donors). **(C)** Flow cytometry histograms showing FOXP3 expression in cells treated with CRISPRon-TETv3 mRNA and Guide 3 targeting the *FOXP3* TSDR or an AAVS1 control sgRNA at 48 hours after restimulation with anti-human CD2/CD3/CD28 soluble antibodies. **(D-E)** Percentage of FOXP3⁺ Tconv cells after epi-editing with CRISPRon mRNA variants (TETv4, TETv5) and 1-3 sgRNAs targeting the TSDR (blue) or AAVS1 control region (grey) as measured by flow cytometry at 9 days post-electroporation **(D)** or 28 days post-electroporation **(E)**. Cells treated with a single sgRNA received Guide 3, cells treated with a pool of two sgRNAs received Guide 1 and Guide 3, and cells treated with a pool of three sgRNAs received Guide 1, Guide 3, and Guide 4. ($n = 4$ donors per condition, mean $\pm$ s.e.m.; two-sided Welch's *t*-test; For cells collected on day 9 treated with TETv4: 1 guide targeting the TSDR $*P = 0.044$, 2 guides $*P = 0.018$, 3 guides $*P = 0.033$. For cells collected on day 28 treated with TETv4: 2 guides targeting the TSDR $*P = 0.037$, 3 guides $*P = 0.026$. For cells collected on day 28 treated with TETv5: 2 guides targeting the TSDR $*P = 0.027$, 3 guides $*P = 0.046$). **(F)** Targeted bisulfite sequencing analysis indicates the percent methylation of individual CpGs assayed within either the TSDR, the FOXP3 TSS, *IKZF2* TSS or *IL2RA* TSS for cells treated with CRISPRon mRNA and Guide 3 targeting the *FOXP3* TSDR or an AAVS1 control. Each triangle (donor 1) or circle (donor 2) representants an individual CpG. **(G)** The mean percentage methylation across the *IKZF2* or *IL2RA* regions shown in **(F)** when cells were treated with CRISPRon mRNA and Guide 3 targeting the *FOXP3* TSDR or an AAVS1 control. Each triangle (donor 1) or circle (donor 2) represents an individual CpG.



Supplemental Figure 7: GMP-compatible *S. pyogenes* Cas9 for simultaneous KD and CAR KI. (A) The graph depicts translocation frequencies between *RASA2* and *TRAC* loci, measured by ddPCR, following transfection of cells with Cas9 RNPs targeting *TRAC* alone or along with CRISPRoff mRNA and the indicated full-length sgRNAs targeting *RASA2*, truncated sgRNAs targeting *RASA2*, or NTC ($n = 2$ donors). (B) The graph compares BCMA-specific CAR KI efficiency with Cas9 mRNA or CRISPRoff mRNA used in combination with a NTC or sgRNA targeting *RASA2*. CRISPRoff was electroporated with either one sgRNA or a pool of three sgRNAs targeting the *RASA2* TSS. sgRNAs were either delivered as full-length or truncated (mean $\pm$ SD, $n = 6$ donors). (C) Western blot images show *RASA2* KO with Cas9 mRNA, or CRISPRoff *RASA2* KD with either full-length sgRNAs (as one sgRNA or pool of three) or truncated sgRNAs (as one sgRNA or a pool of three). Cells were harvested for protein lysis at 7 days post-electroporation. Data are representative of one donor. (D,E) T cell immunophenotypes on day 7 based on CD45RA and CD62L expression, measured by flow cytometry. Data are representative of one donor. (F) Graphs show CAR-T cell cytotoxicity as measured by Incucyte analysis after five repetitive stimulations with target cancer cells. Green lines indicate *RASA2*-epi-edited CAR-T cells for cells treated with full-length sgRNAs targeting the *RASA2* TSS, red lines indicated *RASA2*-epi-silenced CAR T cells with truncated sgRNAs targeting the *RASA2* TSS, and black lines are control-edited CAR-T cells. The shaded areas depict the 95% confidence interval for technical replicates ($n = 3$ technical replicates per donor). Each row represents an E:T ratio (top: 1:1, bottom: 2:1).



Supplemental Figure 8: Durability of CRISPRoff silencing in CAR-T cells transferred in vivo. (A) NSG mice were engrafted with CD19⁺ A375 melanoma cells via flank injection. Cas12a-compatible CD19-specific TRAC CAR-T cells were generated in combination with CRISPRoff mRNA and a pool of three sgRNAs targeting CD151 or a NTC. The left graph shows the CD19⁺ CAR KI frequency prior to injection and the right graph shows the *CD151* KD prior to injection. CD151-epi-silenced CAR-T cells or control-edited CAR T cells were then transferred into mice via the tail vein 7 days later. (B) Representative gating strategy of tumors harvested from mice at 14 days after CAR-T cell injection (C). At 14 days after CAR-T cell injection, the tumor and spleen were harvested from mice, and CD151 expression among CD45⁺ human T cells was assessed via flow cytometry (tumor: red; spleen: black). (D) Representative flow cytometry graphs for T cells from the tumor or spleen that were treated with a NTC or sgRNAs targeting *CD151*.



Supplemental Figure 9: RASA2 epi-silenced CAR T cells improve tumor control in vivo. (A) Flow cytometry data for levels of CD19-specific CAR positive and TCR α/β KO T cells immediately prior to injection. An equal number of CAR⁺ T cells was injected into each mouse. Data are representative of one donor. **(B)** Live cell counts collected nine days after electroporation between cells that have a *TRAC* CAR KI (blue) or no CAR KI (grey). EE: empty electroporation. (*n* = 4 donors). **(C)** Experimental timeline. **(D)** Bioluminescence images data for tumor growth over time in NSG mice engrafted with 0.5×10^6 Nalm6 cells and treated with 0.1×10^6 CAR⁺ T cells that underwent treatment for *RASA2*-epi-silencing.

Supplemental Figure 7C

