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Perturb-seq characterizing regulators of T cell function

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Andrea R Daniel: This protocol was adapted from the work of Sean McCutcheon and colleagues in the Gersbach lab at Duke University.

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ABSTRACT

This protocol describes methods for a Perturb-seq assay characterizing transcriptional regulators of T cell function.

MATERIALS

Addgene: pLV hU6-gRNA hUbC-dSaCas9-KRAB-T2A-Thy1.1 (Addgene 194278) and pLV hU6-gRNA hUbC-VP64-dSaCas9-VP64-T2A-Thy1.1 (Addgene 194279).

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MANUSCRIPT CITATION: McCutcheon, S.R., Swartz, A.M., Brown, M.C. *et al.* Transcriptional and epigenetic regulators of human $CD8^+$ T cell function identified through orthogonal CRISPR screens. *Nat Genet* (2023). https://doi.org/10.1038/s41588-023-01554-0

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We use this protocol and it's working

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gRNA library cloning

1 Oligonucleotide pools containing 40-gRNA sequences and constant regions for polymerase chain reaction (PCR) amplification were synthesized by Twist Bioscience.

2 gRNA amplicons were gel extracted, PCR purified and input into 20 µl Gibson reactions (5:1 molar ratio of insert to backbone) with 200 ng of Esp3I digested and 1 × solid-phase reversible immobilization (SPRI)-selected (Beckman Coulter) plasmid backbone.

2.1 Addgene: pLV hU6-gRNA hUbC-dSaCas9-KRAB-T2A-Thy1.1 (Addgene 194278) and pLV hU6-gRNA hUbC-VP64-dSaCas9-VP64-T2A-Thy1.1 (Addgene 194279).

3 Gibson reactions were purified using ethanol precipitation and transformed into Lucigen’s Endura ElectroCompetent Cells.

4 Transformed cells were cultured overnight and plasmids were isolated using Qiagen Midi Kits.

Transfections for high-titer lentiviral production

5 Plate 1.2 x 106 or 7 x 106 HEK293T cells in a 6 well plate or 10 cm dish in the afternoon with 2 mL or 12 mL of complete opti-MEM (Opti-MEM‰ I Reduced Serum Medium supplemented with 1x Glutamax, 5% FBS, 1 mM Sodium Pyruvate, and 1x MEM Non-Essential Amino Acids).

6 The next morning, transfect HEK293T cells with 0.5 µg pMD2.G, 1.5 µg psPAX2, and 0.5 µg transgene for 6 well plates or 3.25 µg pMD2.G, 9.75 µg psPAX2, and 4.3 µg transgene for 10 cm dishes using Lipofectamine 3000.

7 Exchanged media 6 hours after transfection and collect and pool lentiviral supernatant at 24 hours and 48 hours after transfection.

Transduction of primary human T cells

8 Centrifuged lentiviral supernatant at 600g for 10 min to remove cellular debris.

9 Concentrate lentivirus to 50–100× the initial concentration using Lenti-X Concentrator (Takara Bio).

10 Transduce T cells at 5–10% v/v of concentrated lentivirus at 24 h post-activation. For dual transduction experiments, T cells were serially transduced at 24 h and 48 h.

scRNAseq

11 $CD8^+CCR7^+$ T cells from three donors were transduced with CRISPRi and CRISPRa mini-TF gRNA libraries.

12 T cells were expanded for 10 days and then stained and sorted for $Thy1.1^+$ cells (a marker to identify transduced cells).

12.1 Antibody: Thy1.1-PE, clone OX-7, Mouse IgG1, κ, 1:300, Flow cytometry, StemCell Technologies, 60024PE

13 An SH800 FACS Cell Sorter (Sony Biotechnology) was used for cell sorting and analysis.

14 For antibody staining of Thy1.1 cells were collected, spun down at 300g for 5 min, resuspended in flow buffer (1× phosphate-buffered saline (PBS), 2 mM ethylenediaminetetraacetic acid and 0.5% bovine serum albumin) with the appropriate antibody dilutions and incubated for 30 min at 4 °C on a rocker.

15 Cells were then washed with flow buffer, spun down at 300g for 5 min and resuspended in flow buffer for cell sorting.

16 Sorted cells were loaded into the Chromium X for a targeted recovery of 2×10^4 cells per donor and treatment according to the Single Cell 5'-High-Throughput Reagent Kit v2 protocol (10x Genomics).

17 SaCas9 gRNA sequences were captured by spiking in 2 µM of a custom primer into the reverse transcription master mix, as previously done for SpCas9 gRNA capture[36]. The custom primer was designed to bind to the constant region of SaCas9's gRNA scaffold.

18 5'-Gene Expression (GEX) and gRNA libraries were separated using double-sided SPRI bead selection in the initial cDNA clean-up step. 5'-GEX libraries were constructed according to manufacturer's protocol.

19 gRNA libraries were constructed using two sequential PCRs (PCR 1: 10 cycles, PCR 2: 25 cycles). The PCR 1 product was purified using double-sided SPRI bead selection at 0.6 × and 2 ×. Twenty percent of the purified PCR 1 product was input into PCR 2. The PCR2 product was purified using double-sided SPRI bead selection at 0.6 × and 1 ×.

20 All libraries were run on a High Sensitivity D1000 tape to measure the average amplicon size and quantified using Qubit's dsDNA High Sensitivity assay.

21 Libraries were individually diluted to 20 nM, pooled together at desired ratios and sequenced on an Illumina NovaSeq S4 Full Flow Cell (200 cycles) with the following read allocation: Read 1, 26; i7 index, 10; Read 2, 90.

22 All oligos are available in Supplementary Table 5 of McCutcheon et al. Nature Genetics, 2023. https://doi.org/10.1038/s41588-023-01554-0

Processing and analyzing scRNA-seq

23 CellRanger v6.0.1 was used to process, demultiplex and generate UMI counts for each transcript and gRNA per cell barcode.

24 UMI counts tables were extracted and used for subsequent analyses in R using the Seurat[69] v4.1.0 package.

25 Low-quality cells with <200 detected genes, >20% mitochondrial reads or <5% ribosomal reads were discarded.

26 DoubletFinder[70] was used to identify and remove predicted doublets. All remaining high-quality cells across donors for each treatment (CRISPRi or CRISPRa) were aggregated for further analyses.

27 gRNAs were assigned to cells if they met the threshold (gRNA UMI >4). Cells were then grouped on the basis of gRNA identity.

28 For differential gene expression analysis, we compared the transcriptomic profiles of cells sharing a gRNA to cells with only NT gRNAs using Seurat's FindMarkers function to test for DEGs with the hurdle model implemented in model-based analysis of single-cell transcriptomics (MAST).

29 All significant gRNA–gene links are available in Supplementary Table 3 of McCutcheon et al. Nature

Genetics, 2023. https://doi.org/10.1038/s41588-023-01554-0

30 Upregulated DEGs were input into EnrichR's GO Biological Process 2021 database[71] for functional annotation.