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Flow-cytometry CRISPRi screen

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We use this protocol and it's working

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Abstract

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1 2 Mbp up/down stream of the 10 core TFs were selected for further chromatin accessibility filtering.

2 Only the regions showing accessibility in either ESC stage or DE-48h stage were kept.

3 Regions that overlapped with promoters or exons were removed from the list, resulting in 394 putative enhancers being selected in total.

4 gRNA design was performed by using CHOPCHOP to achieve full-tiled coverage of the selected regions.

5 We further added 3 gRNAs targeting SOX17 promoter, and 1,100 gRNAs targeting safe harbor loci as positive and negative controls, respectively.

6 gRNA oligos were synthesized on-Chip (Agilent).

7 Synthesized oligos were amplified and restriction cloned into lentiGuide-puro (Addgene; 52963) by the MSKCC Gene Editing & Screening Core Facility.

8 Cloned plasmid library was PCR amplified to incorporate adapters for NGS.

9 Samples were purified and sequenced using Illumina HiSeq 2500 platform.

10 FASTQ files were clipped by position and reads were mapped back to the reference library file to show relative abundance of reads per gRNA.

11 Reads within each sample were normalized to total number of mapped reads and library size.

12 The Overall Representation of Library was charted over a one-log fold change to evaluate if any gRNA was over- or under-represented in the final library.

13 A total of 13.6 µg core enhancer perturbation library plasmids with 5.44 µg lentiviral packaging vector psPAX2 and 1.36 µg vesicular stomatitis virus G (VSV-G) envelope expressing plasmid pMD2.G (Addgene plasmid 12260 and 12259) were transfected with the JetPRIME (VMR; 89137972) reagent into 293T cells to produce the lentivirus.

14 Fresh medium was changed 24h after transfection and viral supernatant was collected, filtered, and stored at −80°C 72h after transfection.

15 We aimed for a ~1,000-fold coverage per gRNA to maximize sensitivity. 35 million idCas9-KRAB SOX17eGFP/+ HUES8 hESCs were collected and infected with the lentiviral library at a low MOI of ~0.3 on Day 0 in 15-cm plates.

16 A total of 6 µg/ml protamine sulfate per plate was added during the first 24h of infection to improve the infection efficiency.

17 One day after infection (Day 1), cells were treated with 2 µg/ml doxycycline to induce dCas9-KRAB expression, which continues till the end of the screen at DE-36h.

18 Infected cells were selected with 1 µg/ml puromycin from Day 2-Day 4 and harvested on Day 5 for recovery passage.

19 2 days after recovery passage, 60 million cells were collected and seeded into 15-cm plates for DE differentiation as described above.

20 36h after differentiation, cells were dissociated using 1X TrypLE Select and sorted using FACS Aria according to GFP expression.

21 Cells whose GFP expression levels were in the top or bottom 20% were pelleted individually, with each pellet containing 15 million cells.

22 Genomic DNA from sorted cell pellets was extracted using the QIAGEN Blood & Cell Culture DNA Maxi Kit (QIAGEN; 13362) and quantified by Qubit (ThermoScientific; Q32850) following the manufacturer's guidelines.

23 A quantity of gDNA covering 1000X representation of gRNAs was PCR amplified to add Illumina adapters and multiplexing barcodes.

24 Amplicons were quantified by Qubit and Bioanalyzer (Agilent) and sequenced on the Illumina HiSeq 2500 platform. Sequencing reads were aligned to the gRNA library sequences and counts were obtained for each gRNA.

25 The read counts were normalized to total reads of each sample to offset differences in read depth.

26 To calculate the Z-score of each gRNA, we subtracted the mean log2FC of all negative control gRNAs targeting safe harbors from the log2FC of each gRNA, and then divided the result by the standard deviation of log2FC from the negative control gRNAs. Off-targets of each gRNA were further assessed by CRISPOR.

27 gRNAs with 0 mismatch (MM)=1, 1MM 500 were kept for calculating average Z-score of each putative enhancer region.

28 Putative enhancer regions with less than 3 qualified gRNAs were filtered out.