Perez et al. *RARA* Is a Druggable Dependency in Pediatric AML Supplemental Methods

Cell culture

AML cell lines MV4;11, THP-1, and Kasumi1 and APL cell line NB4 were graciously shared by Dr. Stevens at Texas Children's Hospital. Cell lines were incubated at 37°C under 5% CO2, in RPMI-1640 plus 10% FBS and 1% Penicillin/Streptomycin. Mycoplasma testing was performed using the LookOut Mycoplasma qPCR Detection Kit (Sigma) at entry into the lab. Cell line identity was confirmed annually using STR fingerprinting at the Cytogenetics and Cell Authentication Core at MD Anderson Cancer Center. Cells used for experiments were within <20 passages from thawing.

ChIPmentation

1-3 million pAML cell lines or samples were fixed in 11% formaldehyde solution (1M HEPES-KOH pH7.5, 0.5M EDTA pH8.0, 0.5M EGTA pH8.0, 5M NaCl, 37% formaldehyde) at room temperature for 10min, followed by quenching (125mM glycine). Cells were washed in PBS centrifuged at 1,350g for 5min at 4 °C, washed with PBS, and centrifuged again at 1,350g for 5min at 4 °C. If necessary, cell pellets were flash frozen and stored at −80 °C until further processing. Cells were sonicated and chromatin sheared to an average length of 200 – 700 bp using a LE220 Covaris Ultrasonicator. Genomic DNA regions of interest were isolated using 5µg of H3K27Ac antibody (8173S, Cell Signaling Technology) pre-incubated with protein G beads (88847, Thermofisher). ChIP complexes were washed, eluted from the beads with SDS buffer, and subjected to RNase and proteinase K treatment. Crosslinks were reversed by incubation overnight at 65°C, and ChIP DNA was purified using Zymo DNA Clean & Concentrator-5 (D4013, Zymo research). Quantitative PCR (qPCR) reactions were carried out in triplicate on specific genomic regions using SYBR Green (S7563, Invitrogen). The resulting signals were normalized for primer efficiency by carrying out qPCR for each primer pair using Input DNA. Illumina sequencing libraries were prepared from the ChIP and Input DNAs by the standard consecutive enzymatic steps of end-polishing, dA-addition, and adaptor ligation. After a final PCR amplification step, the resulting DNA libraries were quantified and sequenced on the Illumina Nextseq 500 sequencer platform with single end, 75bp reads.

Cell viability and differentiation assays

Cell viability assays was performed by seeding cells in cells in 24/48-well plates at 250,000 cells/mL for cell lines, 1-1.5 million for patient samples, followed by treatment with DMSO and tamibarotene (S4260, Selleck Chemicals). There were at least 3 biologic replicates for each condition, and experiments were independently repeated at least once to confirm similar results. Viability cell counts over time were done using trypan blue on a TC10 automated cell counter. The cell numbers of tamibarotene-treated cells were normalized to the DMSO-treated cells measured at the same timepoints. Tamibarotene dose-response of cell lines were conducted on 20,000-40,000 cells/mL plated in 96/384-well plates with 4 ten-fold dilutions of tamibarotene and read out by ATPlite (PerkinElmer) at 120 hours of treatment per manufacturer instructions. There were at least 3 biologic replicates for each condition, and experiments were independently repeated at least once to confirm similar results. Analysis was done using GraphPad Prism normalizing to the ATPlite signal of DMSO-treated samples as indicated.

Flow cytometry
DMSO- or tamibarotene-treated cells were harvested at 72 hours and incubated with flow buffer (1% BSA in PBS) along with anti-CD38-FITC antibody (1:20 dilution, 555459, BD Bioscience). After 30 minutes on ice, samples were diluted with 1ml of PBS and centrifuged at 500g for 5 minutes. The samples were decanted and resuspended in flow buffer then collected on the BD canto II flow cytometer. N=3 biologic replicates for each condition, and experiments were all repeated at least once to confirm results. Results were analyzed using FlowJo v10 and plotted in GraphPad Prism using an unpaired t-test with Welch's correction to compare statistical significance.

RNA-seq library preparation
Total RNA was extracted from p401 cells treated for 24 hours with DMSO or Tamibarotene (n=4 biologic replicates), using the Zymo Direct-zol RNA miniprep kit according to manufacturer's instructions. External RNA spike-ins (ERCC, Ambion) were added just before the library preparation was done. Library preparation was performed using the NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina and NEBNext® Ultra™ II Directional RNA Second Strand Synthesis Module. Paired-end sequencing was performed on the Illumina Nextseq 500 sequencer.

Quantitative RT-PCR analysis
Expression of RARA and DHRS3 were determined using real-time quantitative reverse transcriptase-polymerase chain reaction (qPCR). N=3 technical replicates per sample and the experiment was performed at least once to confirm results on the same extracted RNA. SuperScript VILO cDNA Synthesis Kit (11754050, Thermofisher) was used to generate the first cDNA strand. RARA (4331182, Hs00940446_m1c, Thermofisher) and DHRS3 (4331182, Hs01044021_m1, Thermofisher) primers followed by using TaqMan™ Gene Expression Master Mix (4369514, Thermofisher) were used along with GAPDH (4331182, Hs02786624_g1, Thermofisher) as housekeeping control. Relative expression was calculated using the comparative $\Delta\Delta$Ct method normalizing to GAPDH and DMSO, and for RARA analysis to the *RARA* SE- cell line (Kasumi1) or sample (p163). All results were plotted in GraphPad Prism using an unpaired t-test with Welch's correction to determine statistical significance.

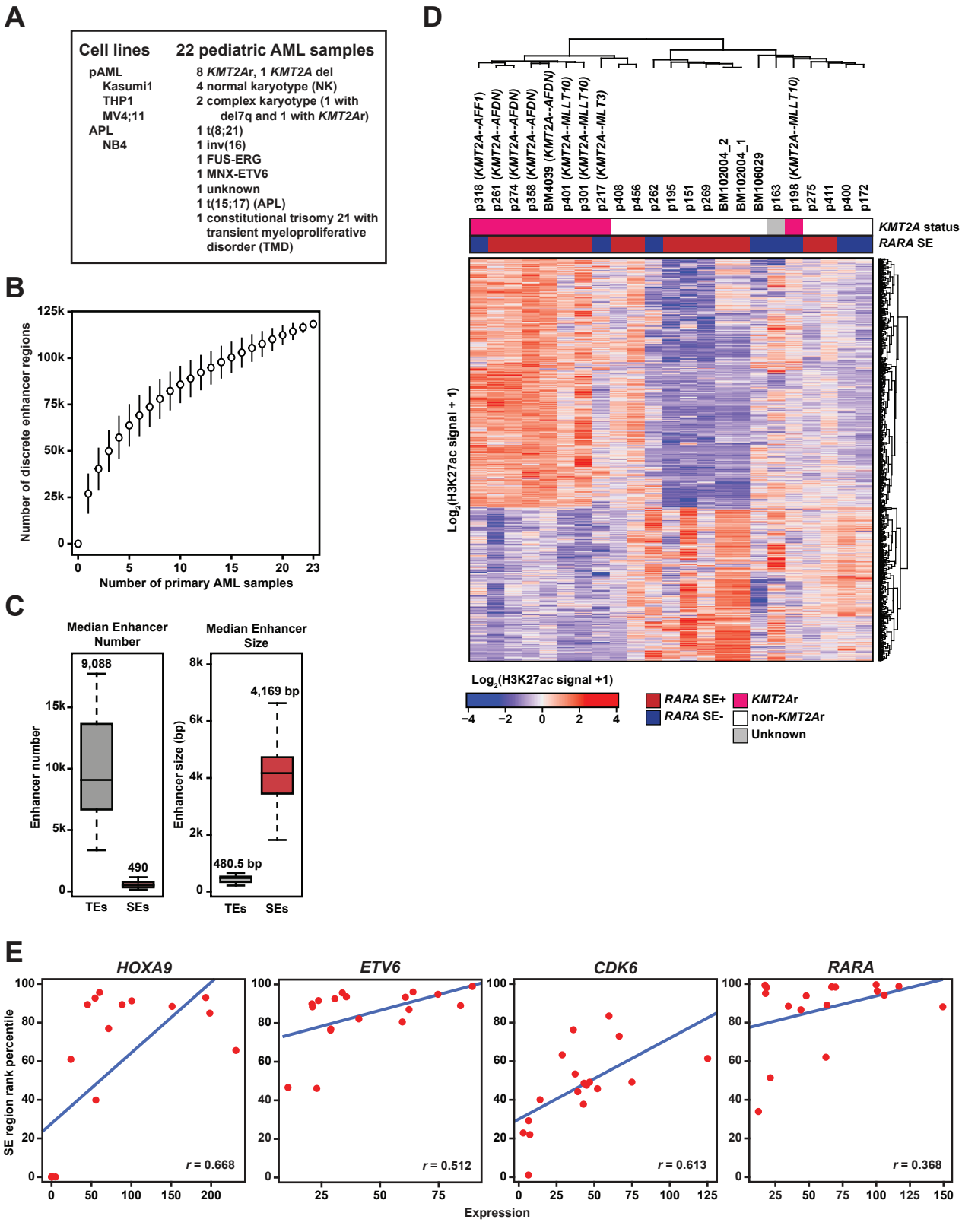
Enhancer saturation analysis
To better understand whether our enhancer profiling adequately captured the primary pediatric acute myeloid leukemia enhancer landscape, we performed a saturation analysis. We measured the total number of discreet regions identified by increasing sample number. This was performed across 500 permutations of the 22 primary pediatric samples (and 1 replicate) to establish 95% confidence intervals (supplemental Figure 1B).

RARA Pearson correlation
The Pearson correlation coefficient was used to determine the pairwise correlation of all consensus SE regions to the *RARA* SE locus by determining the pairwise Pearson correlation between each SE region and the *RARA* SE region as determined by the H3K27ac signal identified across the cohort.

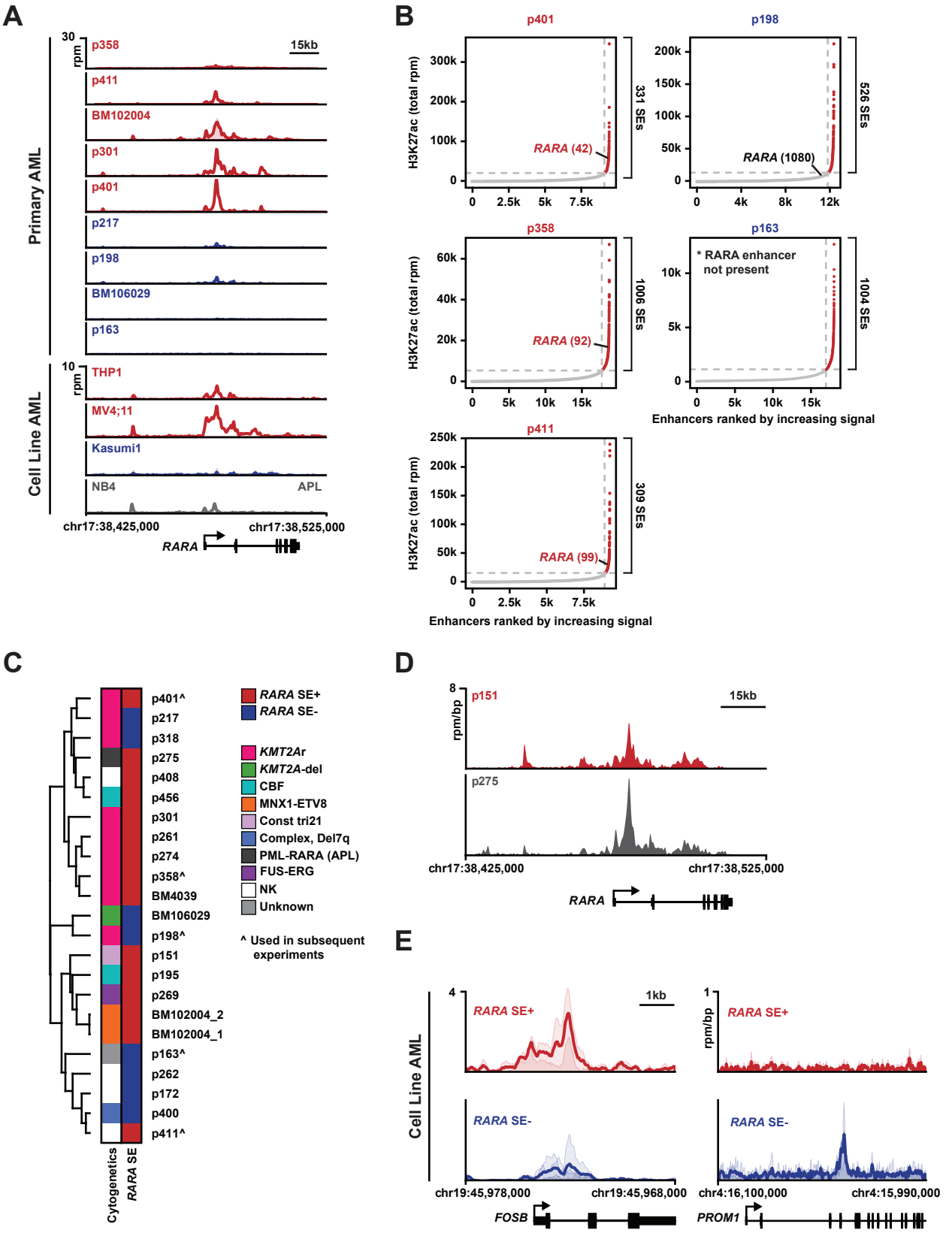
Plotting meta representations of ChIP-seq signal

For all samples within a group, ChIP-seq signal is smoothed using a simple spline function and plotted as a translucent shape in units of rpm per bp. Darker regions indicate regions with signal in more samples. An opaque line is plotted and gives the average signal across all samples in a group.

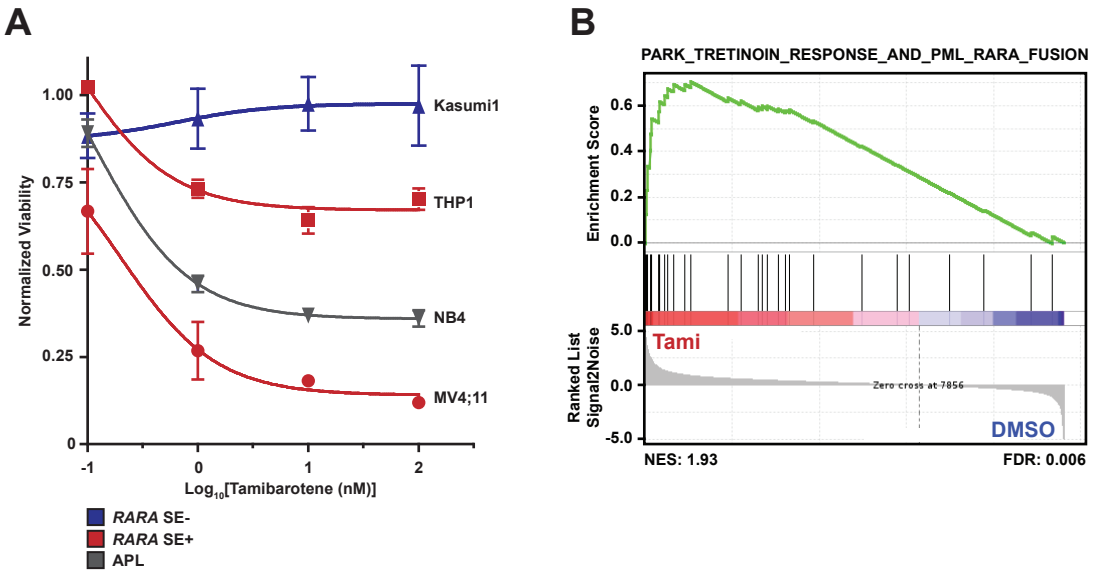
Supp Figure 1



Supp Figure 2



Supp Figure 3



Perez et al. *RARA* Is a Druggable Dependency in Pediatric AML Supplemental Figure Legends

Supplemental Figure 1. (A) Characteristics of cell lines and pediatric AML patient samples profiled by H3K27ac ChIPmentation. (B) Saturation analysis by number of discrete enhancer regions identified with each pAML sample added. (C) Median number and size of typical (gray) and super enhancers (red) within each sample across all primary pAML samples. (D) Hierarchical clustering of significantly different super enhancers ($P < 0.05$) between samples by *KMT2A*-rearrangement status. The heatmap represents log transformed H3K27ac enrichment. (E) Pearson correlation of expression (by RNA-sequencing) and associated SE region rank for select genes across all primary samples. SE regions were ranked by percentile signal within the consensus SE landscape.

Supplemental Figure 2. (A) H3K27ac signal at the *RARA* locus for cell lines and primary samples. Replicate samples merged and represented as a metatrack plot. (B) Enhancers ranked by H3K27ac signal in selected *RARA* SE+ and SE- samples from supplemental Figure 2A. The *RARA* associated enhancer is annotated when present. (C) *RARA* SE status of primary samples relative to cyto/molecular findings when clustered by pattern of H3K27ac signal across consensus SE regions (see Figure 1B). (D) H3K27ac tracks at the *RARA* locus of the patient sample with constitutional trisomy 21 and TMD (p151) on top and of the APL t(15;17) sample (p275) on bottom. (E) H3K27ac metatracks for pAML cell lines at loci of SEs with high and low Pearson correlation to the *RARA* SE as seen in Figure 2C.

Supplemental Figure 3. (A) Tamibarotene dose response after 5 days of treatment in pAML/APL cell lines (n=3 biologic replicates for each dose, normalized to DMSO). Experiment performed two additional times and representative data shown. (B) Running sum for the second most enriched retinoid gene set within the tamibarotene-treated p401 samples.

Supplemental Tables:

Supplemental Table 1. Primary pAML sample information.
Supplemental Table 2. H3K27ac signal at all enhancers across all patient samples.
Supplemental Table 3. FASE-associated genes and gene set enrichments of the core pAML SE-associated genes.
Supplemental Table 4. Genes and gene set analysis of most and least correlated SE-associated genes to the *RARA* SE in pAML.
Supplemental Table 5. Lists of the up- and down-regulated genes with Tamibarotene.
Supplemental Table 6. GSEA of transcriptional changes in response to Tamibarotene compared to the MSigDB C2 curated gene sets.