SUPPLEMENTARY APPENDIX

The gene signature in CCAAT-enhancer-binding protein α dysfunctional acute myeloid leukemia predicts responsiveness to histone deacetylase inhibitors

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ONLINE SUPPLEMENTARY DESIGN AND METHODS SECTION

Cell lines. K562 cells stably expressing p42-C/EBPα-ER were maintained in phenol red-free RPMI medium supplemented with 10% charcoal-stripped FBS (1). Induction of nuclear localization of C/EBPα-ER fusion protein was achieved by addition of 1 μM β-estradiol as indicated into the culture medium.

AML gene expression profile data analysis. The expression data of 525 AMLs were downloaded from Gene Expression Omnibus (GSE14468) and were preprocessed using the MAS5 algorithm (Affymetrix) available in BRB-ArrayTools. Pathway activation scoring (2) was used to identify CEBPα activation status in AML patients based on the CEBPα signature. Positive scores indicate that the CEBPα pathway is active, while negative scores indicate that the CEBPα pathway is dysfunctional.

Connection to Compounds in the Connectivity Map. The Connectivity Map (3) was queried using the CEBPα signature (with housekeeping probeset AFFX-BioB-3_at designated as the 'dummy' downregulated portion, since the Connectivity Map O2 web query program (http://www.broadinstitute.org/cmap/) requires both upregulated and downregulated portions of input query signature). Enrichment values for each small molecule were calculated using permutation test (which combines different treatment entries of the same compound). Compounds with positive enrichment values were deemed to have positive connections to CEBPα activation, and thus were candidates for targeting CEBPα dysfunctional AMLs.

Flow cytometry analysis. AML samples were incubated with PBS + 10% FBS for 15 minutes on ice, and stained with pacific blue-conjugated anti-human CD15, and phycoerythrin-conjugated anti-human CD11b. All antibodies were from eBioscience. Exclusion of dead cells was achieved by addition of DAPI. Flow cytometry analysis was

performed on a LSRII flow cytometer (BD Biosciences, San Jose, CA). Data were analyzed with FlowJo software (Treestar, Inc.).

Quantitative RT-PCR. RNA was isolated by TriReagent (MRC Inc, Cincinnati, OH), treated with DNAseI, and reverse-transcribed into cDNA (Invitrogen, Carlsbad, CA). Quantitative RT-PCR was performed using iQ Sybr Green supermix (Biorad, Hercules, CA). Amplification was done with a Corbett Rotor Gene 6000 (Qiagen, Valencia, CA) using the following parameters: 95°C (10 min), 45 cycles of 95°C (15s) and 60°C (1 min). Data was analyzed using the 2^-delta delta Ct method. Primer sequences are depicted below.

Quantitative RT-PCR oligonucleotides, F: Forward and R: Reverse.

GAPDH F set 1: 5'-CCACATCGCTCAGACACCAT-3'

GAPDH R set 1: 5'-CCAGGCGCCCAATACG-3'

GAPDH F set 2: 5'-GGAAGGTGAAGGTCGGAGT-3' (validation microarray data)

GAPDH R set 2: 5'-CCATGGGTGGAATCATATTGGA-3' (validation microarray data)

TRIB1 F: 5'-GAAGTTCGTCTTCTCCACGG-3'

TRIB1 R: 5'-AGGATCTCAGGGCTCACGTA-3'

GPR109B F: 5'-GCCCTGAACAAGATCTCCAA-3'

GPR109B R: 5'-TGATGCACACATTTGCAGTG-3'

ID1 F: 5'-GTCTGTCTGAGCAGAGCGTG-3'

ID1 R: 5'-AGCTCCTTGAGGCGTGAGTA-3'

FOS F: 5'-CCGGGGATAGCCTCTCTTAC-3'

FOS R: 5'-GTGGGAATGAAGTTGGCACT-3'

MOSC2 F: 5'-ATGCCTCCCTGGTAGATTTG-3'

MOSC2 R: 5'-TCATCCCAGGTATCCTCCTC-3'

IL18RAP F: 5'-TGGGAGCACTGGCTCTATTT-3'

IL18RAP R: 5'-ATCCACTACGATTCGGTTGC-3'

ANXA1 F: 5’-GAGATTTTCGGAACGCTTTG-3’

ANXA1 R: 5’-CACGTTTACGTCTGTCCCCT-3’

SAT1 F: 5’-TTTGGAGAGCACCCCTTTTA-3’

SAT1 R: 5’-AATAACTTGCCAATCCACGG-3’

TNFSF10 F: 5’-TTCACAGTGCTCCTGCAGTC-3’

TNFSF10 R: 5’-AAGCAATGCCACTTTTGGAG-3’

GBP2 F: 5’-CTCAGCCATTCCAATGTCAA-3’

GBP2 R: 5’-TCTCCATGCAGGGTAGATCC-3’

HVCN1 F: 5’-GCTCATCCTGGACCTGAAGA-3’

HVCN1 R: 5’-CTCCAGGCGGAAGACAAATA-3’

EPAS1 F: 5’-ATGTGTGAACCAATCCAGCA-3’

EPAS1 R: 5’-CAGCTCCTCAGGGTGGTAAC-3’

C1orf38 F: 5’-GTCTGCTCGGGGGTCTACTT-3’

C1orf38 R: 5’-GGTCTTCGGGTTCTCACAGA-3’

ADD3 F: 5’-TTTGACCGCATCAATGAAAA-3’

ADD3 R: 5’-CATTCCAAGTCTTCCCGAAA-3’

ACSL1 F: 5’-CCTGTTGCTCAGGTGTTTGT-3’

ACSL1 R: 5’-CAAACGACCCTTCAAATCCT-3’

ADFP F: 5’-ACAACCGAGTGTGGTGACTC-3’

ADFP R: 5’-TCTCTGCCATCTCACACACA-3’

LIPG F: 5’-CTGGAAATAGTGGAGCGGAT-3’

LIPG R: 5’-CAGGTGAGCTGGATCTTCAA-3’

MEX3 F: 5’-GTACCCAGTTCTGAGCATGTC-3’

MEX3 R: 5’-TAAGTATTGGTCTTCGCCCG-3’

Evi2b F: 5’-CAGTTTGCTAGAGGAACATTTTAAATC-3’

Evi2b R: 5’-ATCCATTTCAGAATATTTCCTCGTTATC-3’

G-CSF-R F: 5’-TTTCAGGAACTTCTCTTGACGAGAA-3’

G-CSF-R R: 5’-CGAGCCGAGCCTCAGTTTC-3’

C/EBPε F: 5’-CTCCGATCTCTTTGCCGTGAA-3’

C/EBPε R: 5’-TGGGCCGAAGGTATGTGGA-3

Gelatinase A F: 5’-GTGGGACAAGAACCAGATCACAT-3’

Gelatinase A R: 5’-GTCTGCCTCTCCATCATGGATT-3’

Myeloperoxidase F: 5’-AGACCTGCTGGAGAGGAA-3’

Myeloperoxidase R: 5’-CGCAGCCGCTTGACTTG-3’

Lysozyme F: 5′-GCTGCAAGATAACATCGCT-3′

Lysozyme R: 5′-CCCATGCTCTAATGCCTTG-3′

FOS promoter F: 5’-CAAATGTCTTCGCACGTAGG-3’

FOS promoter R: 5’-CCATGTGTGGGAATATTAAGGA-3’

TRIB1 promoter F: 5’-AGCCACCTCATTGCACAAC-3’

TRIB1 promoter R: 5’-TCACGCTGTATACACACACACTC-3’

C1orf38 promoter F: 5’-GAGTTCGAGGCCAACGTAGA-3’

C1orf38 promoter R: 5’-GGGTTTGAGGAGGGAGATGT-3’

ID1 promoter F: 5’-GTCCGAGAAGCATCTTCCAA-3’

ID1 promoter R: 5’-AGGAAGCTAAGCAGGCATTG-3’

ONLINE SUPPLEMENTARY REFERENCES

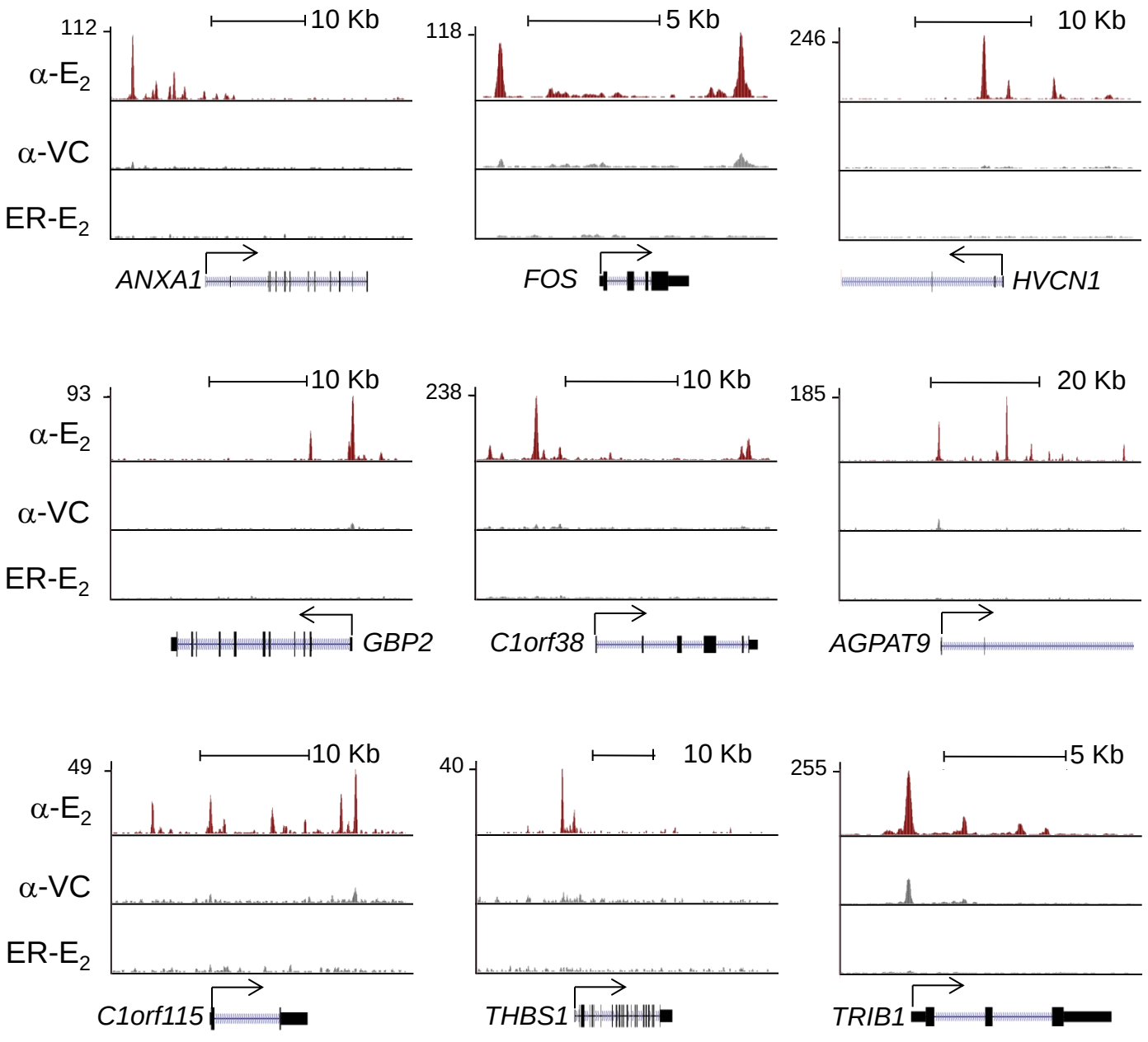
1. D'Alo F, Johansen LM, Nelson EA, Radomska HS, Evans EK, Zhang P, et al. The amino terminal and E2F interaction domains are critical for C/EBP alpha-mediated induction of granulopoietic development of hematopoietic cells. Blood. 2003;102(9):3163-71.

2. Ooi CH, Ivanova T, Wu J, Lee M, Tan IB, Tao J, et al. Oncogenic pathway combinations predict clinical prognosis in gastric cancer. PLoS Genet. 2009;5(10):e1000676.

3. Lamb J, Crawford ED, Peck D, Modell JW, Blat IC, Wrobel MJ, et al. The Connectivity Map: using gene-expression signatures to connect small molecules, genes, and disease. Science. 2006;313(5795):1929-35.

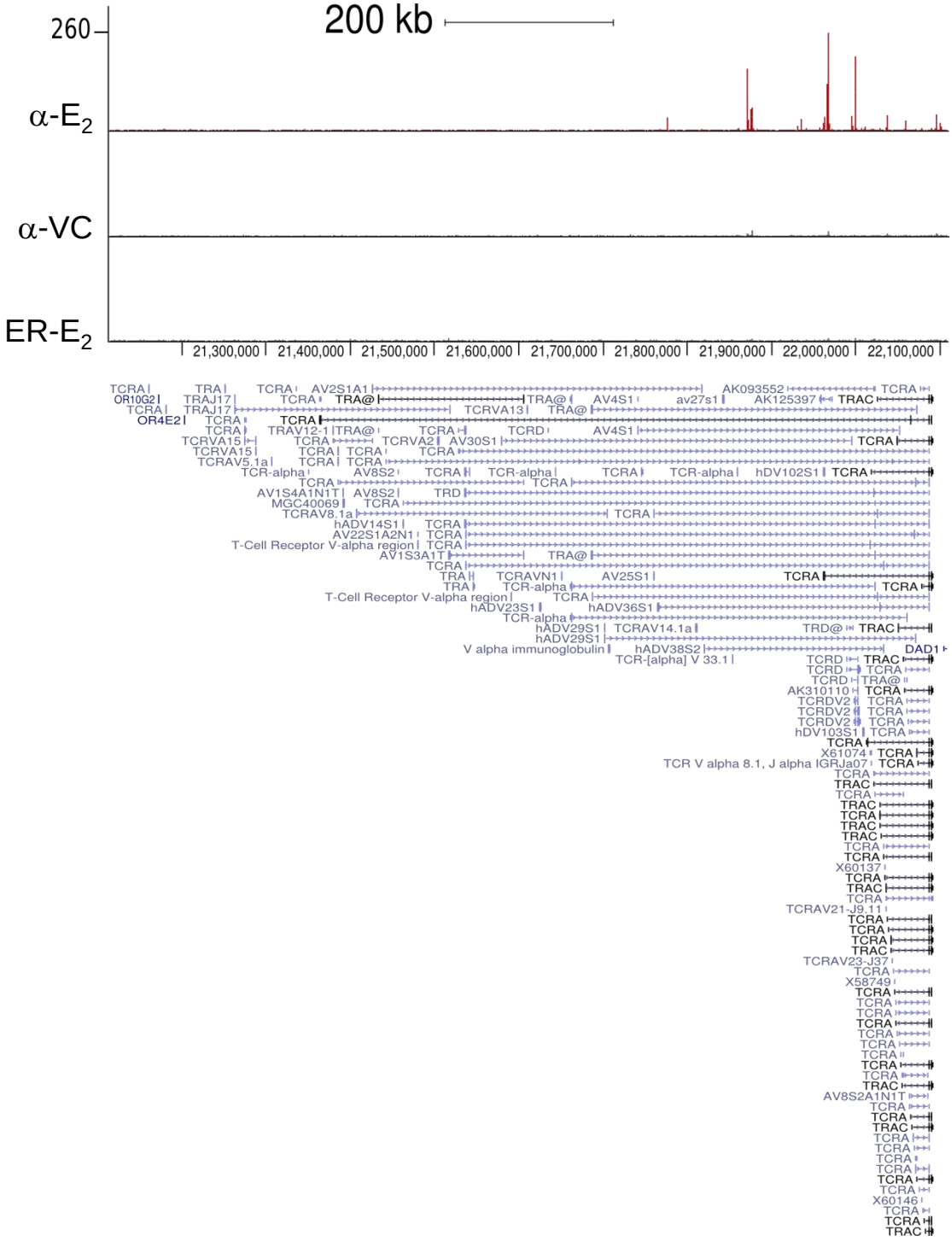
Online Supplementary Figure S1

A



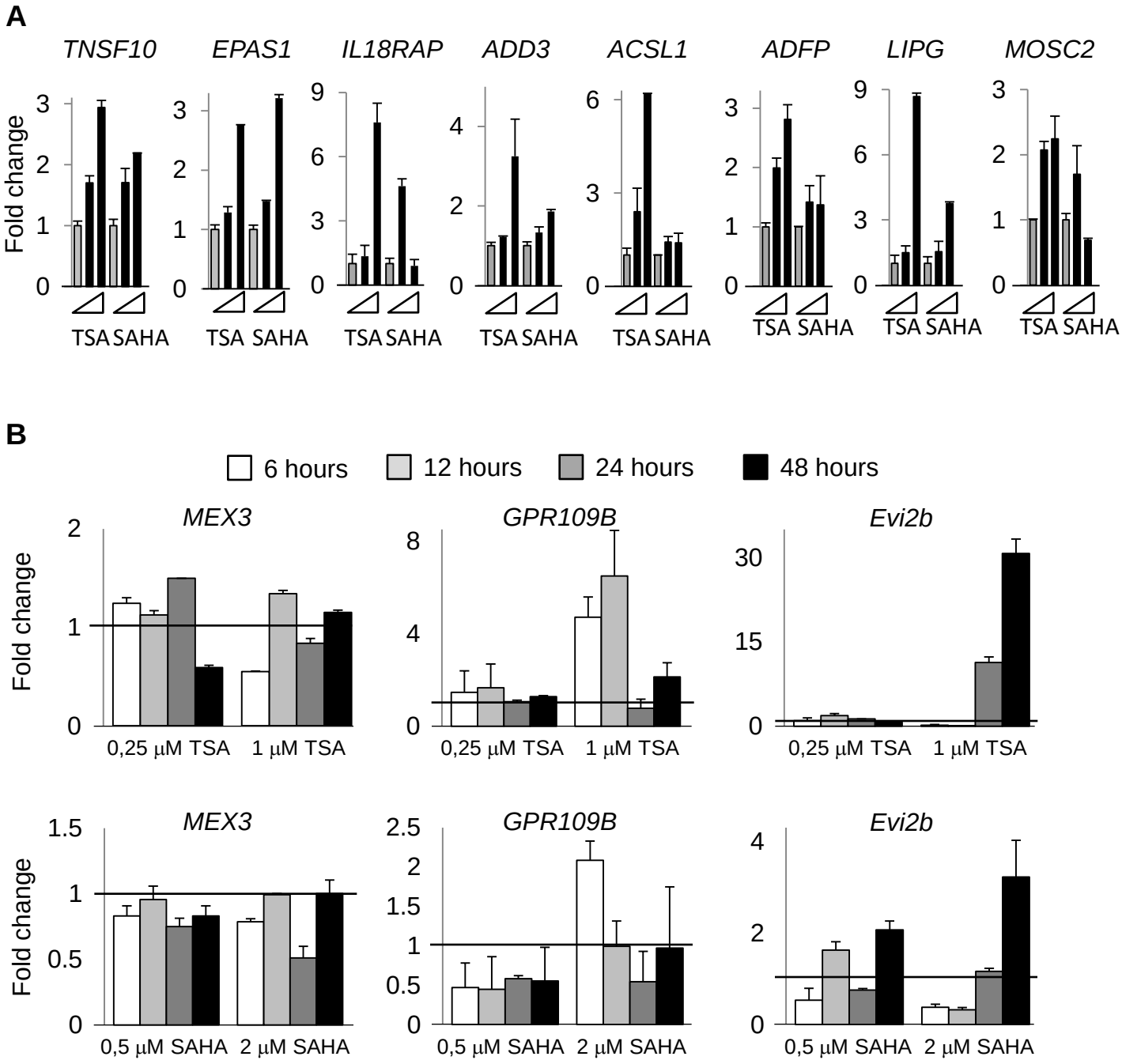
Online Supplementary Figure S1

B



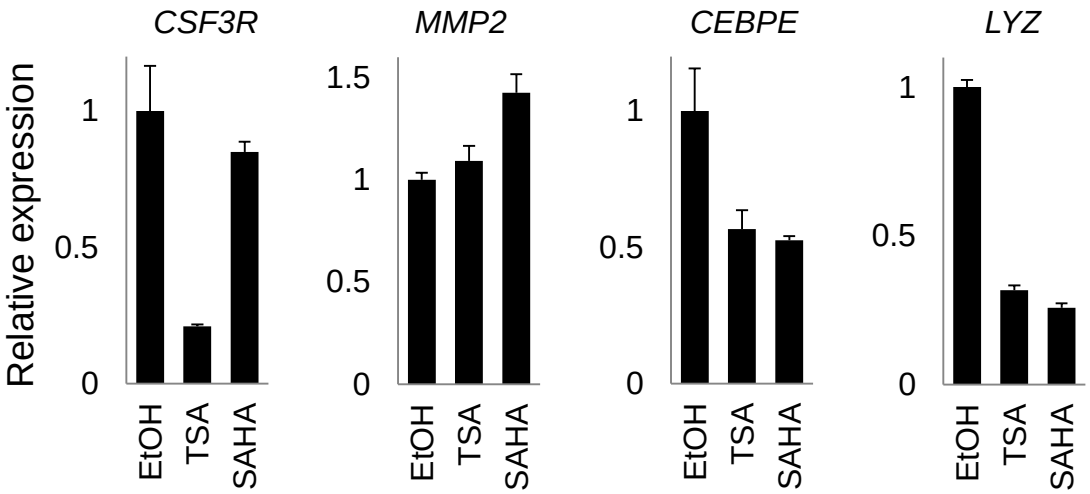
Online Supplementary Figure S1. C/EBPα ChIP seq data in K562 C/EBPα-ER expressing cells treated with 1 μM β-estradiol (α-E_2) or vehicle control (α-VC), and K562 ER control expressing cells treated with 1 μM β-estradiol (ER-E_2). **(A)** C/EBPα binding profiles in the proximity of C/EBPα signature genes: *ANXA1, FOS, HVCN1, GBP2, C1orf38, AGPAT9, C1orf115, THBS1,* and *TRIB1*. Arrows indicate transcription start site and direction. **(B)** C/EBPα binding profile in the T-cell receptor alpha/delta region. Gene segments present in within this region are indicated below. Significant peaks are located at the 3' end of the locus.

Online Supplementary Figure S2



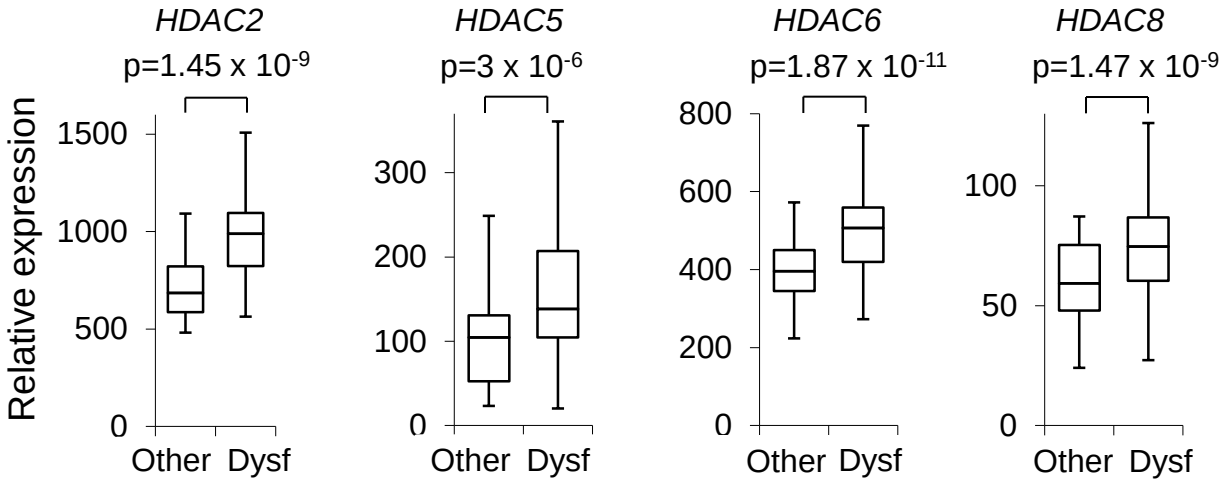
Online Supplementary Figure S2. Quantitative RT-PCR in K562 cells treated with TSA (0.25 μM or 1 μM), SAHA (0,5 μM and 2 μM), or vehicle control (gray). Y-axis indicates fold change relative to vehicle control. This is one representative experiment out of 3. **(A)** K562 cells were stimulated for 12 hours as indicated. **(B)** RNA was isolated from K562 treated cells after 6, 12, 24, and 48 hours upon stimulation. Black line indicates vehicle control expression.

Online Supplementary Figure S3



Online Supplementary Figure S3. HDAC inhibitors do not upregulate the C/EBPα signature in primary AML samples from outside the C/EBPα dysfunctional subset with C/EBPα biallelic mutations. G-CSF-R (*CSF3R)*, gelatinase A (*MMP2*), C/EBPε (*CEBPE*) and lysozyme (*LYZ*) mRNA expression was determined by quantitative RT-PCR in a primary AML sample with C/EBPα biallelic mutations located outside the C/EBPα dysfunctional group (patient H). Analysis was performed after 5 days of EtOH control, TSA (0.25 mM), and SAHA (1 mM) treatment. Y-axis indicates fold change relative to control treatment.

Online Supplementary Figure S4



Online Supplementary Figure S4. HDAC mRNA expression determined by Affymetrix U133A GeneChips in AML patient samples from the dysfunctional C/EBPα subset (Dysf, n=101) and the remainder of AML patient samples (Other, n=415). Y-axis indicates relative expression. P values are indicated above each box-and-whisker plot and were calculated using two-sided unpaired Student's *t*-test.

ONLINE SUPPLEMENTARY TABLE S1

rank	Small molecule name	mean	n	enrichment	p	specificity	percent non-null
1	trichostatin A	0.398	182	0.617	0	0.1185	53
3	vorinostat	0.447	12	0.656	0.00004	0.2412	58
6	valproic acid	0.261	57	0.258	0.00078	0.1053	54
56	parthenolide	-0.506	4	-0.66	0.0301	0.2	75
253	MS-275	0.374	2	0.677	0.2082	0.5758	50
427	naphazoline	0.388	5	0.35	0.47115	0.3359	60
521	sodium phenylbutyrate	0.158	7	0.252	0.67861	0.7107	57
604	depudecin	0.004	2	-0.295	0.98386	0.9868	50
649	scriptaid	0	3	0.64	---	---	0

Online Supplementary Table S1. List of HDAC inhibitors obtained from the Connectivity Map. The first column indicates the ranking of the compounds based on the enrichment p-values. Column n indicates the number of instances (experiments) per compound, notice that the last 6 compounds are under-represented in comparison to trichostatin A, vorinostat, and valproic acid. Enrichment scores and p-values were calculated using the C/EBPα signature (33 genes upregulated in the K562 C/EBPα-ER cells treated with E_2 versus EtOH control). Enrichment values are the result from permutation testing combining different treatment instances of the same compound. Positive enrichment values indicate positive connectivity to C/EBPα-activation and negative connectivity to C/EBPα-NON-activation. The highlighted HDAC inhibitors (trichostatin A, vorinostat, and valproic acid) presented positive enrichment and significant p values.