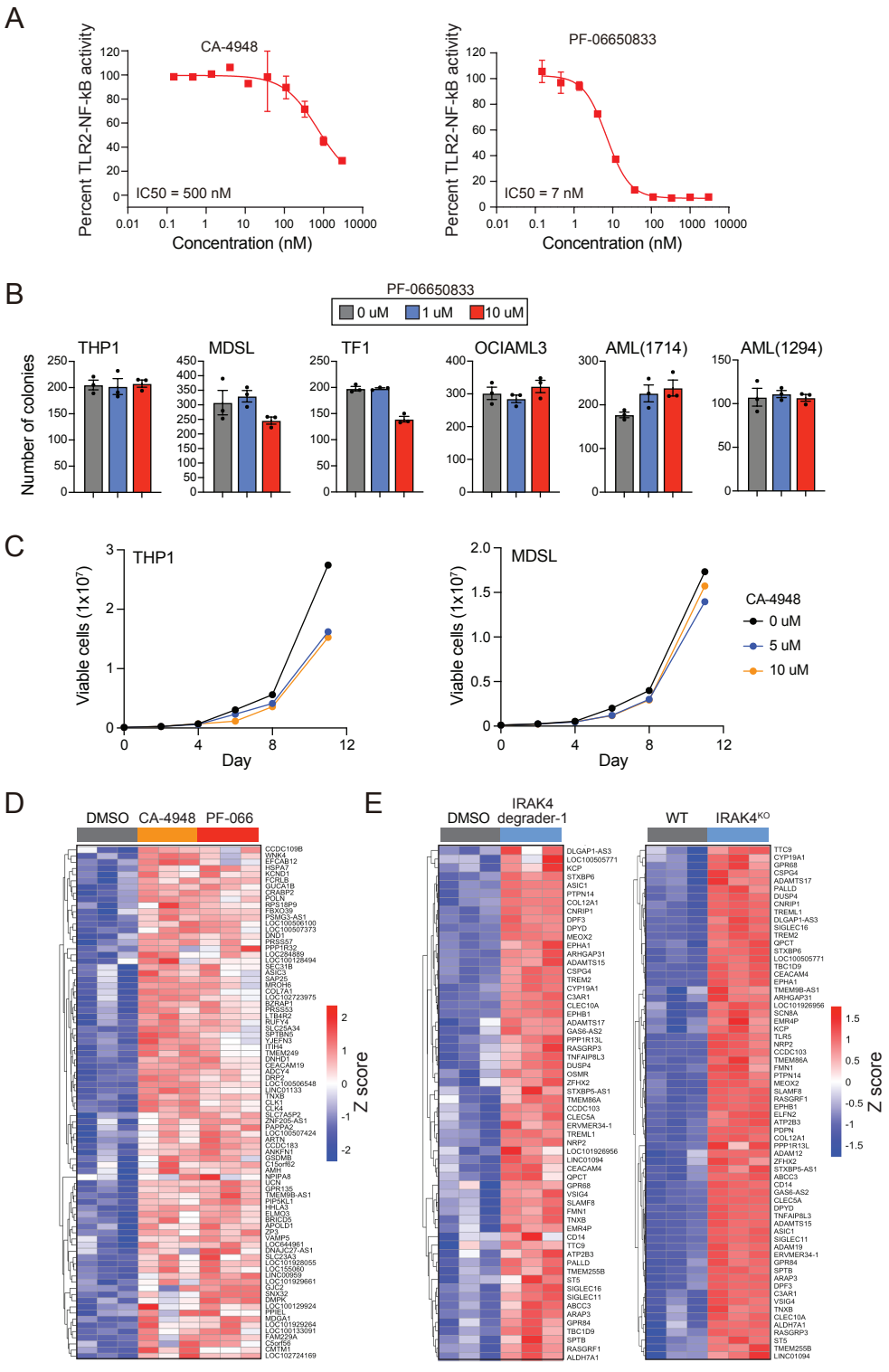
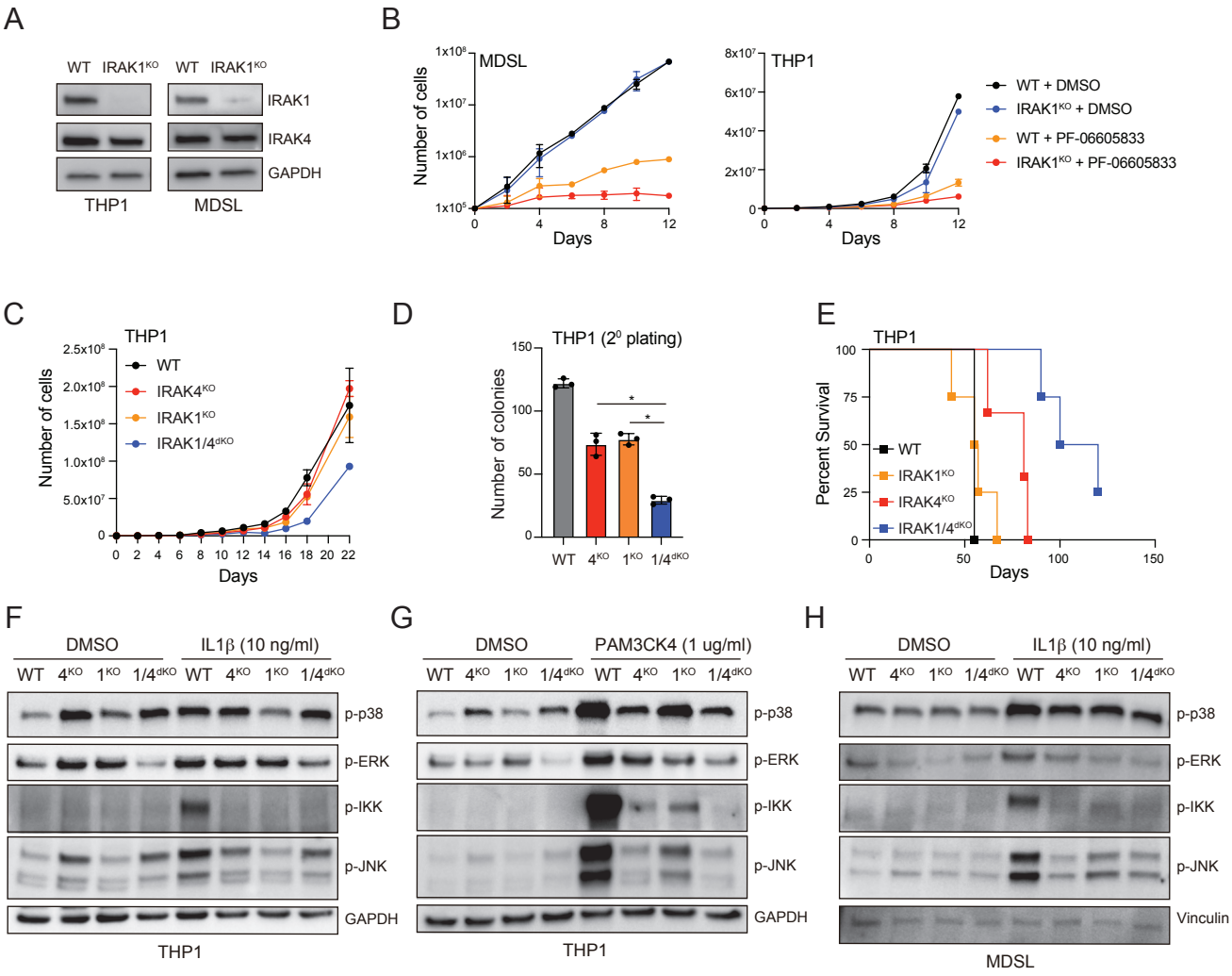
Supplemental Figure 1



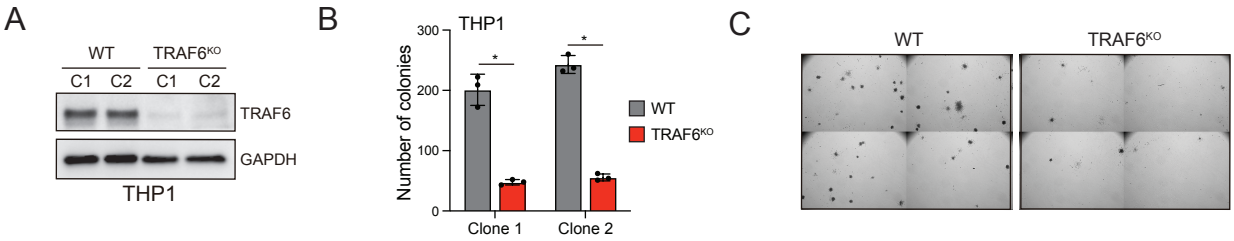
Supplemental Figure 1. Evaluation of IRAK4 inhibitors on AML cells. (**A**) IC50 curves for CA-4948 and PF-06650833 in an assay measuring NF-kB activity upon TLR2 stimulation with PAM3CSK4 in THP1 NF-kB reporter cells. (**B**) Colony formation counts of cells treated with PF-06650833 or vehicle control. (**C**) Growth curves of THP1 and MDSL cells treated with CA-4948 or vehicle control. (**D**) Heatmap of differentially expressed genes upon treatment with IRAK4 inhibitors (fold-change > 1.5; $P < 0.05$). (**E**) Heatmap of differentially expressed genes upon treatment with IRAK4 degrader-1 (fold-change > 1.5; $P < 0.05$) or upon IRAK4 deletion (fold-change > 2.0; $P < 0.05$).

Supplemental Figure 2



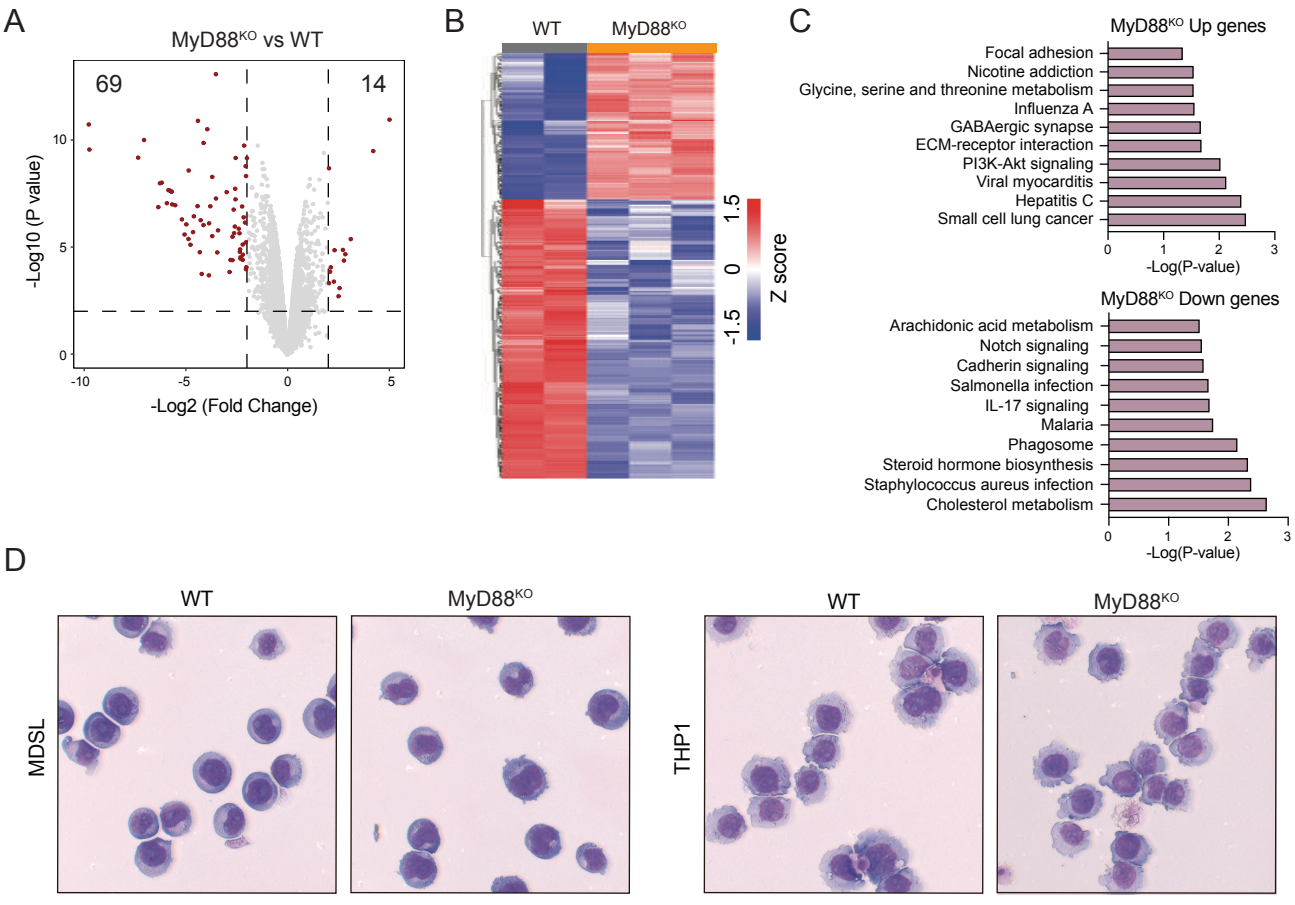
Supplemental Figure 2. Evaluation of IRAK1/4-deficient AML cells. (**A**) Immunoblots for IRAK1 and IRAK4 in WT and IRAK1^{KO} THP1 and MDSL cells. (**B**) Growth curves of WT and IRAK1^{KO} MDSL (10 uM) and THP1 (20 uM) cells treated with PF-06650833 or vehicle control (two independent experiments). (**C**) Growth curve of WT, IRAK4^{KO}, IRAK1^{KO}, and IRAK1/4^{dKO} THP1 cells. (**D**) Colony formation following a secondary replating of isogenic THP1 cells isolated from the primary colony plating (see Figure 2H). (**E**) Kaplan Meier survival analysis of NSGS mice (n = 4 mice/group) engrafted with WT, IRAK4^{KO}, IRAK1^{KO}, and IRAK1/4^{dKO} THP1 cells. (**F**) Immunoblots for phospho-p38 MAPK, phospho-ERK1/2, phospho-IKKa/b, and phospho-JNK in WT, IRAK4^{KO}, IRAK1^{KO}, and IRAK1/4^{dKO} THP1 cells treated with IL-1β (10 ng/ml) as compared to vehicle control. (**G**) Immunoblots for phospho-p38, phospho-ERK1/2, phospho-IKKa/b, and phospho-JNK in WT, IRAK4^{KO}, IRAK1^{KO}, and IRAK1/4^{dKO} THP1 cells treated with PAM3CSK4 (1 ug/ml) as compared to DMSO. (**H**) Immunoblots for phospho-p38 MAPK, phospho-ERK1/2, phospho-IKKa/b, and phospho-JNK in WT, IRAK4^{KO}, IRAK1^{KO}, and IRAK1/4^{dKO} MDSL cells treated with IL-1β (10 ng/ml) as compared to vehicle control.

Supplemental Figure 3



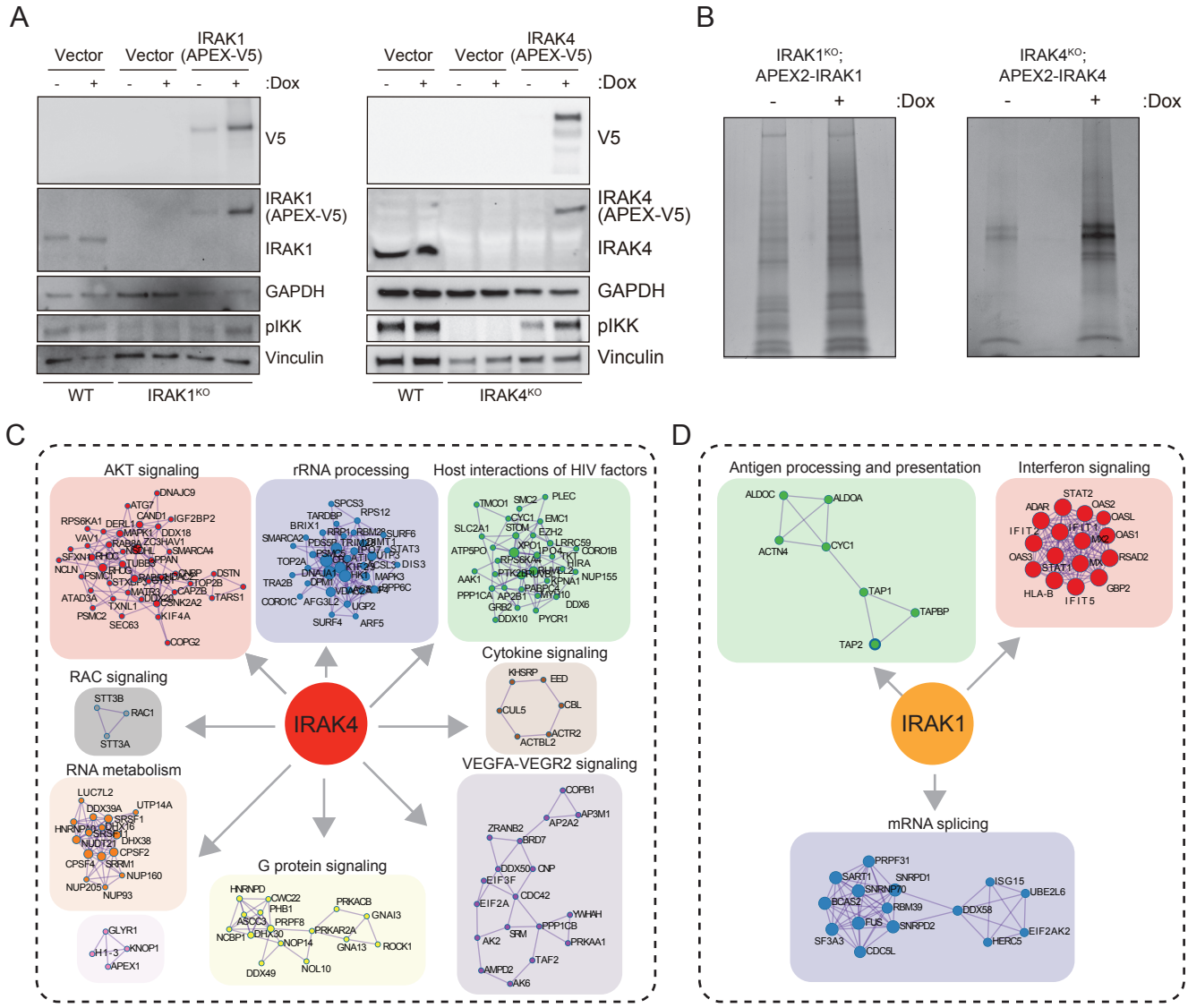
Supplemental Figure 3. Evaluation of TRAF6-deficient AML cells. (**A**) Immunoblots for TRAF6 in WT and TRAF6^{KO} THP1 cells. (**B**) Colony formation of WT and TRAF6^{KO} THP1 cells. (**C**) Representative colony images of WT and TRAF6^{KO} THP1 cells. C, clone.

Supplemental Figure 4



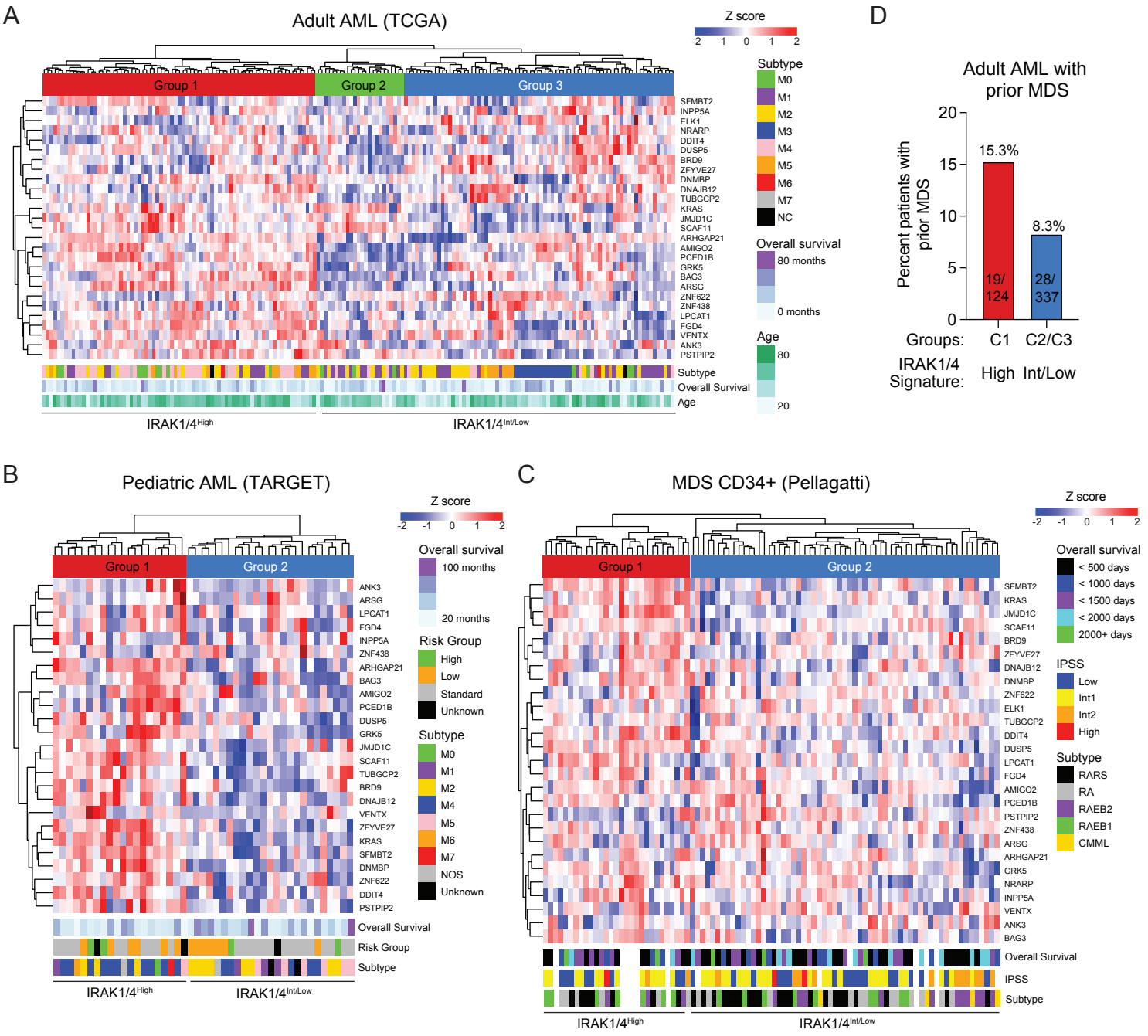
Supplemental Figure 4. Evaluation of MyD88-deficient AML cells. (**A**) Volcano plots showing differentially expressed genes in MyD88^{KO} relative to WT THP1 cells. (**B**) Heatmap of differentially expressed genes in MyD88^{KO} relative to WT THP1 cells. (**C**) Pathway enrichment using KEGG of upregulated and downregulated genes in MyD88^{KO} relative to WT THP1 cells. (**D**) Representative Wright-Giemsa stains of WT and MyD88^{KO} THP1 and MDSL cells.

Supplemental Figure 5



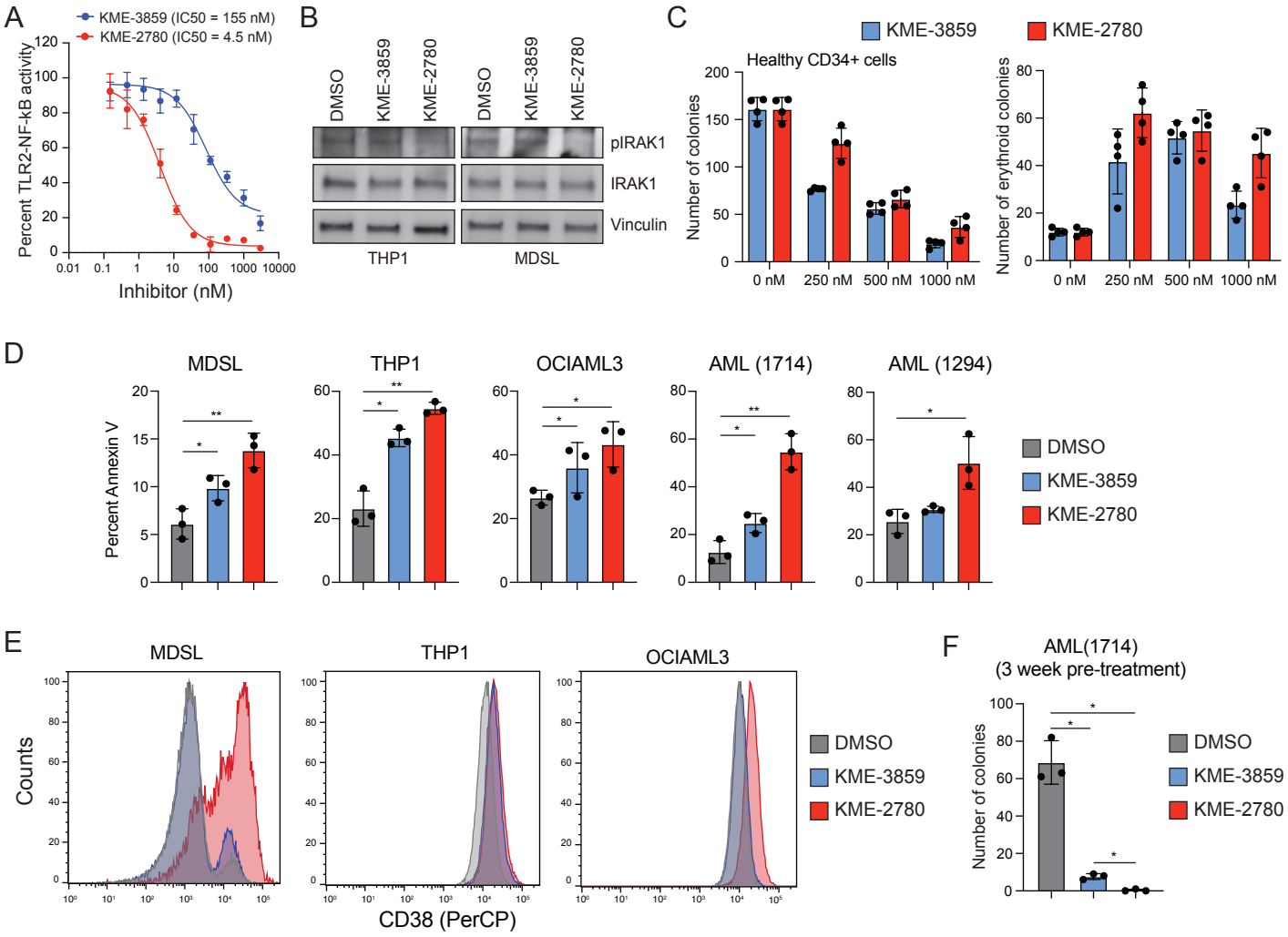
Supplemental Figure 5. IRAK1 and IRAK4 APEX2 proximity labeling in AML cells. (**A**) Left: Immunoblots for V5, IRAK1, and phoshpo-IKKα/β in untreated versus doxycycline (Dox)-treated WT THP1 cells transduced with empty vector and IRAK1^{KO} THP1 transduced with empty vector or inducible IRAK1-APEX2. Righ: Immunoblots for V5, IRAK1, and phosh-po-IKKα/β in untreated versus Dox-treated WT THP1 transduced with empty vector and IRAK4^{KO} THP1 cells transduced with empty vector or the inducible IRAK4-APEX2. For the pho-psho-IKK and vinculin immunoblots, cells were additionally treated with IL-1β (10 ng/ml). (**B**) Imperial stain of biotinylated proteins pulled down with blocked-streptavidin in untreated and Dox-treated IRAK1^{KO} THP1 cells expressing IRAK1-APEX2 (left) or Dox-treated IRAK4^{KO} THP1 cells expressing IRAK4-APEX2 (right) following incubation with biotin phenol and APEX2 activation with hydrogen peroxide. (**C**) Interaction networks of proteins identified as IRAK4 interactors. (**D**) Interaction networks of proteins identified as IRAK1 interactors.

Supplemental Figure 6



Supplemental Figure 6. Evaluation of the IRAK1/4 signature in MDS and AML. (**A**) The heatmap represents a subset of genes that are downregulated and associated with loss of chromatin accessibility in IRAK1/4^{dKO} THP1 ("IRAK1/4 gene signature"). Unsupervised hierarchical clustering analysis resolved distinct cohorts of IRAK1/4-high signature (Group 1) and IRAK1/4-low/intermediate signature (Groups 2 and 3) AML patients from the TCGA dataset. (**B**) The heatmap showing relative expression of genes in the IRAK1/4 signaling signature in pediatric AML patients using the TARGET dataset. (**C**) Heatmap showing relative expression of genes in the IRAK1/4 signaling signature in CD34+ cells from IRAK1/4-high signature (Group 1) and IRAK1/4-low/intermediate signature (Group 2) MDS patients (GSE114922, GSE58831). The bottom panels indicate overall survival, International Prognostic Scoring System (IPSS) score, and MDS subtype for each individual patient in the analysis. (**D**) Percent of AML patients (from the BEAT AML dataset) diagnosed with MDS were stratified on the IRAK1/4 signature. The number in the histogram represents the number of patients with prior MDS within each group.

Supplemental Figure 7



Supplemental Figure 7. Evaluation of the dual IRAK1/4 inhibitor (KME-2780) and IRAK4 selective inhibitor (KME-3859) on MDS and AML cells. (**A**) IC50 curves for KME-3859 and KME-2780 in an assay measuring NF-kB activity upon TLR2 stimulation with PAM3CSK4 in THP1-Blue NF-kB reporter cells. (**B**) Immunoblots for phospho-IRAK1 and total IRAK1 in THP1 and MDSL cells treated with vehicle (DMSO), KME-3859 (500 nM), or KME-2780 (500 nM) for 24 hours. (**C**) Myeloid and erythroid colony formation of healthy donor CD34+ cells treated with DMSO, KME-3859, or KME-2780. (**D**) Annexin V staining of cells treated with vehicle (DMSO), KME-3859 (500 nM), or KME-2780 (500 nM) for 48 hours. (**E**) Immunophenotyping for CD38 expression on the indicated cells after treatment with DMSO, KME-3859, or KME-2780 for 12 days. (**F**) Colony formation of AML(1714) cells treated in vitro with DMSO, KME-3859, or KME-2780 for 3 weeks. Following in vitro treatment, 250 live cells were plated in methylcellose to assess colony formation after 10 days.

Supplemental Material and Methods

Cell lines and patient-derived samples

THP1 and TF1 were purchased from the American Type Culture Collection. OCIAML3 was purchased from DSMZ. MDSL cells were provided by K. Tohyama (Kawasaki Medical School, Okayama, Japan). THP1 were cultured in RPMI-1640 medium with 10% FBS and 1% penicillin–streptomycin. MDSL and TF1 cell lines were cultured with complete RPMI supplemented with 10 ng ml^{-1} recombinant human IL-3 (Peprotech). OCIAML3 were cultured with Modified Eagle Medium α (MEMα) with 10% FBS and 1% penicillin–streptomycin. AML(08), MDS(3328), AML(1714), AML(1294), and AML(0169) were obtained from bone marrow (BM) or peripheral blood of patients at initial diagnosis with written informed consent and approval of the institutional review board of Cincinnati Children's Hospital Medical Center and University of Cincinnati, or from the Eastern Cooperative Oncology Group (ECOG). These samples had been obtained within the framework of routine diagnostic BM aspirations after written informed consent in accordance with the Declaration of Helsinki. De-identified leukemic cells from peripheral blood and BM of patients were obtained at CCHMC following consent under the IRB approved Study ID #2008-0021. AML (64519) and MDS(76960) were obtained from the Public Repository of Xenografts (PRoXe)[1]. AML(1714), AML(1294), and AML(0169) samples were RBC lysed and coated with OKT3 antibody (UCHT1, BioXCell). Primary NSGS mice were given a single 30 mg/kg intraperitoneal dose of busulfan 24 hrs prior to intravenous or intrafemoral injection of the OKT3-coated cell preparations. After ~60 days (median 56 days, average 70 days) in xenografted mice, single cell spleen preparations were isolated and cultured in IMDM, 20% FBS, and 10 ng/mL of cytokines (SCF, TPO, FLT3L, IL3, IL-6) (**Supplemental Table 1**).

Generation of CRISPR/Cas9 mutant cells

The THP1 IRAK4^{KO} clone was generated from a pre-derived WT THP1 clone as previously described[2]. To generate the THP1 IRAK1^{KO} and IRAK1/4^{dKO}, the WT and IRAK4^{KO} clone were suspended in buffer R with Cas9-NLS and a modified synthetic gRNA targeting exon 1 of IRAK1 (Synthego) and electroporated (1700 mV x 20 ms x 1 pulse) using the Neon Transfection system (Invitrogen). Transfected cells were recovered for 48 hours in antibiotic-free RPMI-1640 with 1% FBS. Following recovery, transfected cells were plated in 96 well plates at a target density of 0.25 cells/well to isolate single clones. Clones were expanded and screened for IRAK1 deletion by immunoblotting. Deletion was confirmed by PCR amplification of the PAM site for Sanger sequencing. MDSL, TF-1, OCIAML3, AML(1714) and AML(1294) IRAK4^{KO} clones were generated from parental populations following the protocol outlined above using a synthetic gRNA targeting Exon 1 of IRAK4. WT clones were derived in parallel by plating parental cells in single-cell suspensions and expanding clones. MDSL and THP1 MyD88^{KO} and TRAF6^{KO} clones were generated from parental populations following the protocol outlined above using a synthetic gRNA targeting Exon 1 of MyD88 and Exon 1of TRAF6, respectively.

Clonogenic assays

Clonogenic progenitor frequencies were determined by plating cell lines or patient samples in Methocult H4434 (StemCell Technologies, Cat#04434) in SmartDish meniscus-free 6-well plates (StemCellL Technologies, Cat#27371). Plates were kept in humidified chambers and colonies were imaged and manually scored after 9-14 days using the STEMvision counter (StemCell Technologies). For CFC assays with inhibitor treatments, inhibitors were added to H4434 at the indicated concentrations and vortexed immediately prior to use. Cells were incubated at 37ºC and 5% CO2. Colonies were scored after 9-14 days in culture.

Xenografts

Animals were bred and housed in the Association for Assessment and Accreditation of Laboratory Animal Care-accredited animal facility of Cincinnati Children's Hospital Medical Center (IACUC2019-0072). For the xenograft using isogenic THP1 cells, WT, IRAK1^{KO}, IRAK4^{KO}, and IRAK1/4^{dKO} THP1 cells were suspended in PBS and injected via tail vein into NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl} Tg(CMV-IL3,CSF2,KITLG)1Eav/MloySzJ (NSGS) mice at a dose of 2.5 x 10^5 cells per mouse. Moribund mice were sacrificed and assessed for leukemic burden measurements. Briefly, mice were euthanized with carbon dioxide following the AVMA Guidelines for the Euthanasia of Animals and BM cells were immediately extracted by breaking the femurs with a mortar and pestle. BM cells were frozen in FBS with 10% DMSO until time of analysis. BM was analyzed for huCD45 (BDPharmingen, Cat#555485) and huCD33 (BDPharmingen, Cat#555450) expression by flow cytometry using a BD LSRFortessa (BD Biosciences). For staining, 1x10^6 cells from each BM sample were incubated with antibodies diluted 1:100 in a solution of PBS, 0.2% FBS for 30 minutes on ice in the dark. Cells were washed once with PBS, resuspended in PBS with 0.2% FBS, and immediately analyzed by flow cytometry. For the xenograft using AML cells pre-treated the IRAK1/4 inhibitors, AML(1714) was cultured in vitro for three weeks in the presence of either vehicle (DMSO), KME-3859 (250 nM), or KME-2780 (250 nM). Media and inhibitors were replaced every other day. After 3 weeks, viable cells were injected into NOD.Cg-*Rag1*^{tm1Mom} *Il2rg*^{tm1Wjl} Tg(CMV-IL3,CSF2,KITLG)1Eav/J (NRGS) mice at a dose of 4 x 10^4 cells per mouse. Bone marrow aspirates were collected at day 36 post-injection and analyzed for huCD45 (BDPharmingen, Cat#555482) and huCD33 (BDPharmingen, Cat#555450) expression by flow cytometry using a BD LSRFortessa. For xenografts treated with KME-3859 and KME-2780 in vivo, we first performed pharmacokinetic studies to assess serum uptake, plasma protein binding percent, and clearance of each compound (**Supplemental Table 14**). KME-3859 and KME-2780 were solubilized in a solution of 0.9% saline and delivered to male C.129S2-*Cd1*^{tm1Gru}/J mice by single oral gavage at dosages of 10, 30, and 100 mg/kg. Serial plasma samples were collected at 5 minutes, 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 8 hours, and 24 hours post-gavage for quantitative analysis by LC/MS. The final concentrations of inhibitors used in subsequent xenografts were determined so that similar free-drug levels in the blood were achieved (**Supplemental**

Table 14). For the xenograft models using MDS(76960) and AML(64519), 1 x 10^6 cells per mouse were injected via tail vein into NRGS mice. Peripheral blood was collected on day 33 post-injection to assess pre-treatment engraftment by analyzing huCD45 (BDPharmingen, Cat#555482) and huCD33 (BDPharmingen, Cat#555450) expression by flow cytometry. Starting on day 33, mice were started on a 2-week regimen of once daily (M-F) oral gavage of vehicle (50 mM sodium acetate 10% propylene glycol), KME-3859 (30 mg/kg), or KME-2780 (100 mg/kg). Peripheral blood was collected to assess engraftment post-treatment. For the AML(0169) xenograft, 5 x 10^5 cells were injected by tail vein into NSGS mice. Starting on day 6 post-engraftment and continuing through day 44, mice received daily (M-F) oral gavage of vehicle, KME-3859 (30 mg/kg), or KME-2780 (100 mg/kg). Bone marrow aspirates were collected on day 29 to assess leukemic engraftment by flow cytometry as described above. For survival analysis, time of death was recorded, and Kaplan Meier survival analysis was performed using GraphPad Prism version 9 for Mac (GraphPad Software, www.graphpad.com).

Immunoblotting, **nuclear fractionation and co-immunoprecipitation**

Protein lysates were made by lysing cells in cold RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, and 0.1% sodium dodecyl sulfate (SDS)) in the presence of sodium orthovanadate, phenylmethylsulfonyl fluoride (PMSF), and protease and phosphatase inhibitors. Protein concentration was quantified using bicinchoninic acid (BCA) assay (Pierce, Cat#23225). Protein lysates were separated by SDS-polyacrylamide gel electrophoresis (BIO-RAD), transferred to nitrocellulose membranes (BIO-RAD, Cat#1620112), and immunoblotted. For nuclear/cytoplasmic fractionation assays, 1.5 x 10^6 cells were lysed on ice for 5 minutes in a 75 uL volume of hypotonic lysis buffer (10 mM Hepes pH 7.9, 10 mM KCL, 0.1 mM EGTA, 0.1 mM EDTA, 0.4% IGEPAL) supplemented with 0.1% Triton X-100 1 mM DTT, 0.1 mM PMSF, 10 ug/ml aprotonin, and 1X Halt Protease Inhibitor Cocktail (Thermofisher Cat#78429). Lysates were spun down at 20,000 x g and the supernatant was collected (cytoplasmic fraction) while the pellet was washed and lysed for 20 minutes on ice with 2.5X loading buffer supplemented with benzonase (Millipore Sigma Cat#70746) to produce the nuclear fraction. Nuclear and cytoplasmic fractions were then used for immunoblots as described above. For Co-immunoprecipations, $IRAK4^{KO}$ THP1 were transduced with a FLAG-IRAK4 cDNA and sorted on GFP. To generate the FLAG-IRAK4, IRAK4 cDNAs were created by custom gene synthesis (IDT) and cloned into pMSCV-pGK-GFP (Addgene, #35615) with EcoRI and XhoI. Extracts were obtained by lysing cells in a buffer of 30 mM Tris-HCL 300 mM NaCl 1mM EDTA 1%Triton X-100 0.5% IGEPAL supplemented with protease and phosphatase inhibitors. Extracts were normalized for protein concentration, precleared with protein A/G beads (Santa Cruz, Cat#sc-2003), and rotated overnight at 4 degrees with protein A/G beads and either anti-FLAG antibody (Sigma, Cat#F3165) or mouse IgG1 isotype control (Cell Signaling, Cat#5415). Beads were washed the following morning, eluted with loading buffer, and analyzed by immunoblot as described above. The following antibodies were used for western blot analysis: GAPDH (Cell Signaling, Cat#5174), Vinculin (Cell Signaling, Cat# 13901), IRAK4 (Cell Signaling), IRAK1 (Santa

Cruz, Cat#sc-5288), phospho-IRAK1 (T209) (Assay Biotech, Cat#A1074), phospho-SAPK/JNK (Thr183/Tyr185) (Cell Signaling, Cat#4668, 1:500 BSA), phospho-p38 MAPK (Thr180/Tyr182) (Cell Signaling, Cat#4631), phospho-p44/42 MAPK (ERK1/2. Thr202/Tyr204) (Cell Signaling, Cat#4377), phospho-IKKa/b (Ser176/180) (Cell Signaling, Cat#2697), MyD88 (Cell Signaling, Cat#4283), TRAF6 (Santa Cruz, Cat#sc-7221), IRAK2 (Cell Signaling, Cat#4367), HDAC1 (Cell Signaling, Cat#5356), EZH2 (Cell Signaling, Cat#5246), peroxidase-conjugated AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., Cat#111-035-003), peroxidase-conjugated AffiniPure goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, inc., Cat#115-035-003). Blots were visualized using ECL Western Blotting Substrate (Pierce, Cat#32106) or SuperSignal West Femto Substrate (Thermo Scientific, Cat#34096) and imaged on a BIO-RAD ChemiDoc Touch Imaging system.

NF-κB activation reporter

THP1-Blue NF-κB SEAP reporter cells were grown in a 96-well plate in triplicate with the indicated inhibitor for 24 hours. In a new 96-well plate, 20 µl of cell supernatant was added to 180 µl of QuantiBlue Reagent (Invivogen, Cat#rep-qbs2) and incubated at 37 °C for 30 minutes. Absorbance was read at 630 nm.

RNA sequencing

WT, MyD88^{KO}, IRAK1^{KO}, IRAK4^{KO}, and IRAK1/4^{dKO} THP1 cells were plated in triplicate at a uniform density of 5 x 10^5 cells per ml of complete media 24 hours prior to RNA collection. For inhibitor studies, cells lines were cultured at a density of 5x10^5 cell/mL in the presence of IRAK4 inhibitors (1 µM) for 24 hours. Inhibitor studies were performed in duplicate. RNA from IRAK KO and inhibitor-treated cell lines was isolated with the RNeasy Mini Kit (Quiagen). RNA quality was assessed with the Agilent 2100 Bioanalyzer. Libraries were sequenced at an average depth of 30M paired-end 100 nucleotide reads. FASTA files were used for alignment to the human genome (GRCh37) with the STAR software to generate BAM files. After the quality of reads was examined using FastQC, paired-end reads were aligned against the UCSC/hg38 genome using HISAT2 (v2.0.5, http://daehwankimlab.github.io/hisat2). The raw gene counts were calculated using featureCounts (v1.5.2, http://subread.sourceforge.net/) and normalized using edgeR. Differentially expressed genes were predicted using limma/voom (v3.30.6, https://bioconductor.org/packages/release/bioc/html/limma.html).

IRAK1/4 gene signature

The gene signature associated with IRAK1/4 signaling in AML cells was established by utilizing the set of MyD88-independent genes that were downregulated and associated with loss of chromatin accessibility in IRAK1/4^{dKO} THP1 cells (n = 116)(**Supplemental Table 11**). We next acquired a raw gene expression count matrix from the BEAT-AML data (http://www.vizome.org/) and normalized gene counts (trimmed mean of M values: TMM) using edgeR (https://bioconductor.org/packages/release/bioc/html/edgeR.html). We then

performed an unsupervised hierarchical clustering analysis of RNA sequencing data from BEAT-AML using the defined IRAK1/4-associated genes. We prioritized the list of genes to ones that were also differentially expressed in AML versus healthy controls (>2-fold change; $P < 0.05$)(n = 51)(**Supplemental Table 12**). The 51 genes were then applied to the BEAT-AML, TCGA-AML, TARGET-AML, and Pellagatti-MDS data sets (GSE114922, GSE58831). Genes that showed uniform expression across all samples were removed from the heatmap.

Assay for Transposase-Accessible Chromatin (ATAC) sequencing

ATAC-sequencing was performed using a slightly modified version of the OMNI protocol. Briefly, nuclei were isolated by collecting 50,000 cells per sample and lysing in 10 mM Tris-HCl, pH 7.5/10 mM NaCl/3 mM $MgCl_2$ supplemented with 0.1% Tween-20, 0.1% NP-40 and 0.01% digitonin. Samples from each condition were collected in triplicate. The Illumina Tagment DNA TDE1 Enzyme kit (Illumina) was used for the transposition and tagmentation of open chromatin regions under the conditions specified by the OMNI protocol. DNA was next purified using the Qiagen MinElute Reaction Clean-up kit, per manufacturers instructions. For library preparation, tagmented DNA was PCR amplified with the NEBNext High-Fidelity 2X PCR Master Mix using the Nextera i5 common adapter and i7 index adaptors. The amplified library was purified by size-selective precipitation with AMPure XP magnetic beads. Library concentrations were determined with the Qubit fluorometer and the dsDNA HS Assay Kit. Library quality was assessed by running a portion of each sample on an agarose gel with SYBR Safe to visualize nucleosome banding. ATAC libraries were paired-end sequenced at 150 nucleotides per end on an Illumina NovaSeq 6000. After the quality of reads was examined using FastQC, adapters in paired-end reads were trimmed using Trim Galore (v0.6.6, https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Trimmed reads were aligned against the UCSC/hg38 genome using Bowtie2 (v2.4.2, https://bowtie-bio.sourceforge.net/bowtie2/). Duplicated and multi-mapped reads (MAPQ cutoff = 30) reads were removed using PICARD (v2.18.22, https://broadinstitute.github.io/picard/) and Samtools (v1.13.0, http://www.htslib.org/), respectively. HOMER (v4.11, http://homer.ucsd.edu/homer/) was used to predict and annotate differential peaks with the following parameters (-size 75 -mDist 50 -style factor -FDR 0.05). We considered differential peaks with FDR < 0.05 as statistically significant.

CRISPR/dCas9-VP64 activation rescue screen

A pooled lentiviral preparation of the Calabrese P65-HSF activation library was purchased from Addgene (Cat#92379-LV). WT and IRAK1/4^{dKO} THP1 were transduced with a lentiviral dCas9 construct (Addgene #61422) and sorted for GFP expression. GFP positive cells were next transduced in duplicate with pooled lentivirus and selected in puromycin for 5 days at an MOI of 0.2-0.5 to achieve a gRNA coverage >30X. Cells were maintained in liquid culture for 4 weeks and periodically split while maintaining gRNA representation of >100X. Genomic DNA was harvested using the DNeasy Blood and Tissue kit (Qiagen

Cat#69504). The genomic library was amplified and indexed following the Broad Institute protocol for PCR of sgRNAs for Illumina sequencing. The barcoded libraries were pooled and sequenced on an Illumina MiSeq at a read depth of ~21X. We used the MAGeCK pipeline for data processing and analysis (v0.5.9; https://sourceforge.net/p/mageck/wiki/Home/). First, original paired-end reads were automatically trimmed using MAGeCK. Next, MAGeCK-count was used to calculate sgRNA read counts using trimmed reads and human CRISPRa sgRNA library (https://media.addgene.org/data/plasmids/92/92379/92379-attachment_IRjBnhb_GMxk.txt). Finally, MAGeCK-test was used to predict gene rescue from the resulting count matrix after considering a control sample. The default FDR threshold for gene test is 0.25.

Growth curves

For growth curves with IRAK4 inhibitors, cells were initially plated at a uniform density in 12-well plates with media containing designated concentrations of drug. Cells were counted every other day, at which point cultures were spun down to completely replace the media with fresh media and drug. Cells were split to maintain appropriate confluency, and dilution factors were recorded to adjust cell counts.

APEX affinity purification

The cDNA sequences for IRAK4 and IRAK1 were optimized using the IDT Codon Optimization tool. To produce N-terminal fusions, gBlocks encoding 5'-APEX2-spacer-V5 tag-IRAK1/4 cDNA-3' with overhangs for Gibson assembly were obtained from IDT. Due to the large size of IRAK1, the IRAK1 sequence was split into two gBlocks. NEBuilder HiFi DNA Assembly master mix was used to Gibson assemble the IRAK4 and IRAK1 gBlocks into the pCW57.1-eGFP plasmid, which was linearized by digestion with NheI and MluI. pCW57.1-eGFP was generated from Addgene (#41393) by substituting the puromycin cassette for eGFP (a gift from Dr. Andrew Volk). Assembled APEX2 vectors were transformed into One Shot™ Stbl3™ E. coli and individual colonies were expanded. APEX2 vectors were maxi-prepped and sequenced to confirm the fidelity of the inserts and then transfected into HEK-293T with 3rd generation lentiviral helper plasmids. IRAK4-APEX2, IRAK1-APEX2, and empty vector viral supernatants were collected and used to transduce IRAK4^{KO} and IRAK1^{KO} THP1. Transduced cells were sorted on GFP expression to obtain pure populations. Inducible expression of fusion proteins and functional rescue of canonical signaling was confirmed by immunoblot. To perform the proximity labeling, 2x10^{7} doxycycline (Dox)-induced (0.5 mg/ml, 48 hrs) and uninduced cells were plated in 1 ml of pre-warmed media in quadruplicate. Cells were preloaded by incubating with 500 uM biotin phenol for 30 minutes at 37^{o}C. The APEX2 enzyme was then activated by adding H_2O_2 at a final concentration of 1 mM and gently agitating for 45 seconds. The reaction was immediately quenched by adding quenching buffer (PBS with 10 mM sodium azide, 10 mM sodium ascorbate, 5 mM Trolox). Cells were washed with quenching buffer two additional times and then lysed and sonicated in 500 uL RIPA containing protease inhibitor and quenchers. Lysates were rotated overnight

with Sera-Mag blocked streptavidin SpeedBeads to precipate biotinylated proteins. Beads were washed and stored at -80^{o}C prior to protein identification by mass spectrometry. Beads from one replicate each of the uninduced controls and dox-induced samples were eluted with sample buffer containing 20 mM DTT. Eluents were run on an SDS gel which was stained with imperial stain to ensure capture of biotinylated proteins above background in the dox-induced samples.

Liquid chromatography coupled mass spectrometry

The biotin-enriched proteins captured on the streptavidin SpeedBeads from 3 replicates each of the IRAK4-APEX2, IRAK1-APEX2 and the corresponding uninduced controls were eluted in 40 uL of Laemmli solubilization buffer contain 2 mM biotin by heated to 100°C for 10 minutes and loaded on a 1.5 mm, 4-12% Bis-Tris gradient SDS-PAGE gel (Invitrogen) using MOPS buffer and electrophoresed at 125 V for 15 min until the samples ran about 2 cm into the gel. Prestained molecular weight protein markers we used in surround lanes for each sample to define the gel region containing the biotin enriched proteins. The entire gel sections were excited, reduced with dithiothreitol, alkylated with iodoacetamine, digested overnight with trypsin, and recovered all as described previously[3]. The extracted peptides were dried, reconstituted in 10 uL of 0.1% formic acid used for nanoLC-MS/MS analysis. Mass spectrometry data were collected on a Thermo Orbitrap Eclipse mass spectrometer coupled to a Dionex Ultimate 3000 RSLCnano system. Samples were injected onto a 5 mm nanoviper µ-Precolumn (i.d. 300 µm, C18 PepMap 100, 5.0 µm, 100 Å) from ThermoFisher Scientific at 5 µl/min in formic acid/H2O 0.1/99.9 (v/v) for 5 min to desalt and concentrate the samples. For the chromatographic separation of peptides, the trap-column was switched to align with the EASY-Spray column PepMap RSLC C18 with a 150 mm column (i.d. 75 µm, C18, 3.0 µm, 100 Å). The peptides were eluted using a variable mobile phase (MP) gradient from 98% phase A (Formic acid/H2O 0.1/99.9, v/v) to 32% phase B (Formic Acid/Acetonitrile 0.1/99.9, v/v) for 90 min at 300 nL/min. MS1 data were collected in the Orbitrap (120,000 resolution; maximum injection time 50 ms; AGC 4x10^{5}). Charge states between 2 and 6 were required for MS2 analysis, and a 20s dynamic exclusion window was used. Cycle time was set at 2.5s. MS2 scans were performed in the ion trap with HCD fragmentation (isolation window 0.8 Da; NCE 30%; maximum injection time 40 ms; AGC 5 × 104). The data was recorded using Xcalibur 4.3 software (ThermoScientific).

Protein identification and Label-free Quantitation (LFQ) was achieved using Proteome Discoverer 2.4 (ThermoScientific) searched against the Uniprot Homo sapiens database (UP000005640 downloaded on 12/11/2020) with the Sequest HT search algorithm and using a modified LFQ standard processing and consensus workflows. The samples were grouped into two categories for pairwise comparations: IRAK4-APEX2 versus uninduced, and IRAK1-APEX2 versus uninduced, with 3 biological replicates each. In the processing workflow, the mass recalibration node (spectrum files RC) along with the standard spectrum selector, minora feature detector, Sequest HT and Percolator nodes were used. The precursor detector node was used to help minimize chimeric spectra. The precursor mass tolerance was 10ppm and the

fragment mass tolerance was set to 0.02 Da, with 2 missed trypsin cleavages and variable peptide modifications including oxidized methionine and carbamidomethyl cysteine. Default variable N-terminal protein modifications of Acetyl, Met loss were also used. FDR tolerances in the Percolator node were set to 0.01 for high confidence (99%) and 0.05 for medium confidence (95%). Protein abundance calculations were based on summed abundance of extracted ion profiles from the collective MS1 profiles of peptides unique to the identified protein with ratios calculated using pairwise comparisons. P values were calculated using t-test. The maximum allowed fold-change was 100.

Kinome screens

Dissociation constants (Kd) were measured at DiscoverX using the KINOME*scan* Profiling Service. Kinase inhibition (IC50s) was measured at Reaction Biology using the Kinase Assay service.

Statistical analysis

The number of animals, cells, and experimental/biological replicates can be found in the figure legends. Differences among multiple groups were assessed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison posttest for all possible combinations. Comparison of two group was performed using the Mann-Whitney test or the Student's *t* test (unpaired, two tailed) when sample size allowed. Unless otherwise specified, results are depicted as the mean ± standard deviation or standard error of the mean. A normal distribution of data was assessed for data sets >30. For correlation analysis, Pearson correlation coefficient (r) was calculated. For Kaplan-Meier analysis, Mantel-Cox test was used. All graphs and analysis were generated using GraphPad Prism software or using the package ggplot2 from R[4].

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