**Supplemental Methods & Figures**

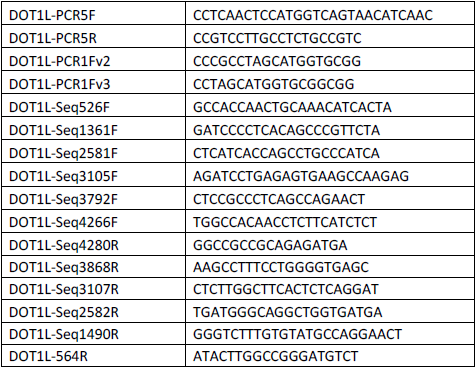
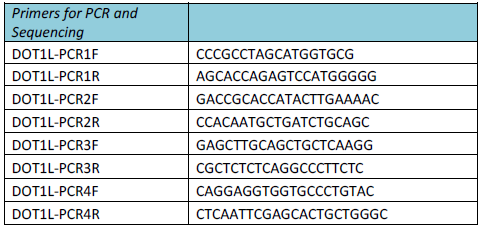
Mechanisms of Pinometostat (EPZ-5676) Treatment Emergent Resistance in *MLL* Rearranged Leukemia

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**Supplemental Methods**

**Sanger Sequencing**

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PCRs for sample KOPN were set up using primers pairs: PCR1F / PCR1R; PCR2F / PCR2R; PCR3F / PCR3R; PCR4F / PCR4R; PCR5F / PCR5R. PCRs for sample NOMO were set up using primer pairs: PCR1Fv2 / PCR2R; PCR2F / PCR2R; PCR3F / PCR3R; PCR4F / PCR4R; PCR5F / PCR5R.

**RNA-seq Library Preparation and Sequencing**

Total RNA samples were converted into cDNA libraries using the TruSeq Stranded mRNA Sample Prep Kit (Illumina, #RS-122-2103). Starting with 100 ng of total RNA, polyadenylated RNA (primarily mRNA) was selected and purified using oligo-dT conjugated magnetic beads. This mRNA was chemically fragmented and converted into single-stranded cDNA using reverse transcriptase and random hexamer primers, with the addition of Actinomycin D to suppress DNA-dependent synthesis of the second strand. Double-stranded cDNA was created by removing the RNA template and synthesizing the second strand in the presence of dUTP in place of dTTP. A single A base was added to the 3’ end to facilitate ligation of sequencing adapters, which contain a single T base overhang. Adapter-ligated cDNA was amplified by polymerase chain reaction to increase the amount of sequence-ready library. During this amplification the polymerase stalls when it encounters a U base, rendering the second strand a poor template. Accordingly, amplified material used the first strand as a template, thereby preserving the strand information. Final cDNA libraries were analyzed for size distribution and using an Agilent Bioanalyzer (DNA 1000 kit, Agilent # 5067-1504), quantitated by qPCR (KAPA Library Quant Kit, KAPA Biosystems # KK4824), then normalized to 2 nM in preparation for sequencing. cDNA libraries were bound to the surface of a flow cell and each bound template molecule clonally amplified up to 1000-fold to create individual clusters.  Four fluorescently labeled nucleotides were then flowed over the surface of the flow cell and incorporated into each nucleic acid chain.  Each nucleotide label acts as a terminator for polymerization, thereby ensuring that a single base is added to each nascent chain during each cycle.  Fluorescence was measured for each cluster during each cycle to identify the base that was added to each cluster. The dye was then enzymatically removed to allow incorporation of the next nucleotide during the next cycle.

**ChIP-Seq Immunoprecipitation and Sequencing**

Chromatin was isolated by the addition of lysis buffer, followed by disruption with a Dounce homogenizer. Lysates were sonicated and the DNA sheared to an average length of 300-500 bp. Genomic DNA (Input) was prepared by treating aliquots of chromatin with RNase, proteinase K and heat for de-crosslinking, followed by ethanol precipitation. Pellets were resuspended and the resulting DNA was quantified on a NanoDrop spectrophotometer. Extrapolation to the original chromatin volume allowed quantitation of the total chromatin yield.

30 ug of chromatin was precleared with protein A (G) agarose beads (Invitrogen). Genomic DNA regions of interest were isolated using 4 ug of antibody against H3K79me2 (Abcam, ab3594). 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 by phenol-chloroform extraction and ethanol precipitation.

Quantitative PCR (QPCR) reactions were carried out in triplicate on specific genomic regions using SYBR Green Supermix (Bio-Rad). 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 Illumina’s NextSeq 500 (75 nt reads, single end). Reads were aligned to the human genome (hg19) using the BWA algorithm (default settings). Duplicate reads were removed and only uniquely mapped reads (mapping quality >= 25) were used for further analysis. Alignments were extended in silico at their 3’-ends to a length of 200 bp, which is the average genomic fragment length in the size-selected library, and assigned to 32-nt bins along the genome. The resulting histograms (genomic “signal maps”) were stored in bigWig files. H3K79me2 enriched regions were identified using the SICER algorithm. Signal maps and peak locations were used as input data to Active Motifs proprietary analysis program, which creates Excel tables containing detailed information on sample comparison, peak metrics, peak locations and gene annotations.

**ABCB1 Transporter Study**

ABCB1 transporter studies were performed at Optivia Biotechnology (Menlo Park, CA). Caco-2 cells were grown as a monolayer in Hank’s Balanced Salt Solution (HBSS) on a permeable support (1 µM PET membrane) in 24-well cell culture plates maintained at 37ºC in 5% CO2. The apical and basal sides of all cells were washed with HBSS. HBSS solutions were added to the wells of the culture plate (apical compartment) containing either blank vehicle or verapamil. A 24-well receiver plate serving as the basal compartment was prepared with a buffer solution corresponding with the apical well. The plates were assembled and incubated at 37ºC for 30 minutes, followed by aspiration of the solution from the apical compartment. The 24-well plate (donor plate) was removed from the receiver plate, and both plates were prepared as follows:

|  |  |  |
| --- | --- | --- |
| **Assay Type** | **HBSS contains** | **Well** |
| Probe Substrate Transport Assay | 100 nM digoxin | Donor |
| Pinometostat Assay | 3 µM pinometostat | Donor |
| Inhibition Assay | 10 µM verapamil | Donor and Receiver |
| 100 nM digoxin or 3 µM pinometostat | Donor |

For flux measurements, the substrate was placed in the basal or apical compartment only (B->A and A->B, respectively). An aliquot of the dosing solution was reserved for mass balance, and the donor and receiver plates were reassembled. After incubation at 37ºC with orbital shaking at 60 RPM for 120 minutes, 100 µL samples were collected from each receiver and donor well, combined with 700 µL scintillation fluid, and counted on a 1450 Microbeta (Perkin-Elmer). Apparent permeability (Papp) was calculated using the formula where Vr is the volume of the receiver compartment (mL), C0 is the initial donor solution concentration, A is the area of the cell monolayer (cm2), Cr is the receiver well concentration at the end of the incubation, and ∆t is the change in time (seconds). Net basal (B) to apical (A) flux (B->A) was calculated by subtracting A->B flux from B->A flux.

**Western Blot Analysis**

Whole cell lysates were prepared by sonicating cell pellets in 10x volume of modified RIPA lysis buffer (1x RIPA Buffer (Millipore, 20-188), 0.1% SDS, PhosSTOP (Sigma-Aldrich, 4906845001), Complete mini protease inhibitor (Sigma-Aldrich, 4693124001)). Lysates were incubated at 4 degrees Celsius for 10 minutes, followed by centrifugation for 15 minutes at 14,000 rpm at 4 degrees Celsius. Protein concentration of the clarified supernatant was determined by BCA assay (Pierce, 23227). 10 µg of each lysate was fractionated on a 4-12% Bis-Tris gel (ThermoFisher, NP0322BOX) and transferred to a nitrocellulose membrane using P3 of the iBlot (ThermoFisher, IB23001). The following primary antibodies were prepared in Odyssey blocking buffer (Licor, 927-40000) diluted 1:2 in PBS: Phospho p38 MAPK (CST 4511, 1:1000 dilution), p38 MAPK (CST 8690, 1:1000 dilution), Phospho p44/42 MAPK (CST 4370, 1:1000 dilution), p44/42 MAPK (CST 4695, 1:1000 dilution), Phospho MEK 1/2 (CST 9154, 1:1000 dilution), MEK 1/2 (CST 9122, 1:1000 dilution), Total H3 (CST 3638, 1:5000 dilution). Membranes were probed with primary antibodies overnight, followed by incubation in secondary antibodies IRDye 800CW donkey-anti-mouse IgG (Licor, 92632212) or Alexa Fluor 680 goat-anti-rabbit IgG (ThermoFisher, A-21076) and imaged with the Licor Odyssey system.

**Bioinformatics Analysis**

**PCA and hierarchical clustering:** log2 transformed gene counts are used for PCA and clustering, analysis and plots use R packages: “FactoMineR” and “WGCNA”. 3D PCA plot was produced by ArrayStudio (<http://www.omicsoft.com/array-studio/>).

**Differentiation analysis:** RSEM quantified raw gene counts are used for differentiation analysis via DESeq2 R package. Lowly expressed genes (average count < 50) are removed before analysis. A gene with adj. pvalue < 0.01 (“BH”) and fold.change >2 is considered significant.

Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics2011 Aug 04;12:323.

Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol2014;15(12):550.

**Pathway analysis:**  For hypergeometric analysis, R package “clusterProfiler” is used; GO terms of “Biological process” and “Molecular function”, MSigDB C2.CP ( <http://software.broadinstitute.org/gsea/msigdb/genesets.jsp?collection=CP>) collections are included the analysis. For Gene Set Enrichment Analysis, java desktop software GSEA 2.2 is used. Fold changes of 22509 quantified genes are the inputs, gene sets with size < 15 or > 500 are removed, this results 1036 gene sets for the analysis. For both analyses, a pathway with an adj. pvalue < 0.05 is considered significant.

Yu G, Wang LG, Han Y, He QY. clusterProfiler: an R package for comparing biological themes among gene clusters. OMICS2012 May;16(5):284-7.

Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A2005 Oct 25;102(43):15545-50.

**Supplemental Figures**

**Figure S1.** **ABCB1 Expression and Resensitization in Pinometostat Treatment Emergent Resistant *MLL*-r Cell Lines**

**[A]**

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**[B]**

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**Figure S1.** **ABCB1 Expression and Resensitization in Pinometostat Treatment Emergent Resistant *MLL*-r Cell Lines** [A] RNA-seq (MV4-11, MOLM-13) and quantitative real-time PCR (SEM) analysis of *ABCB1* expression in cell lines made resistant to pinometostat. Fold-change was calculated using either RNA-seq counts (MV4-11, MOLM-13) or relative expression (SEM). [B] Resensitization of resistant MV4-11 and SEM cell lines following combination with 1 μM valspodar and pinometostat at indicated concentrations.

**Figure S2. *DOT1L* sequence from the KOPN-8 cell line (both parental and resistant) and NOMO-1 cell line (both parental and resistant)**

**KOPN Consensus (4,614bp)**

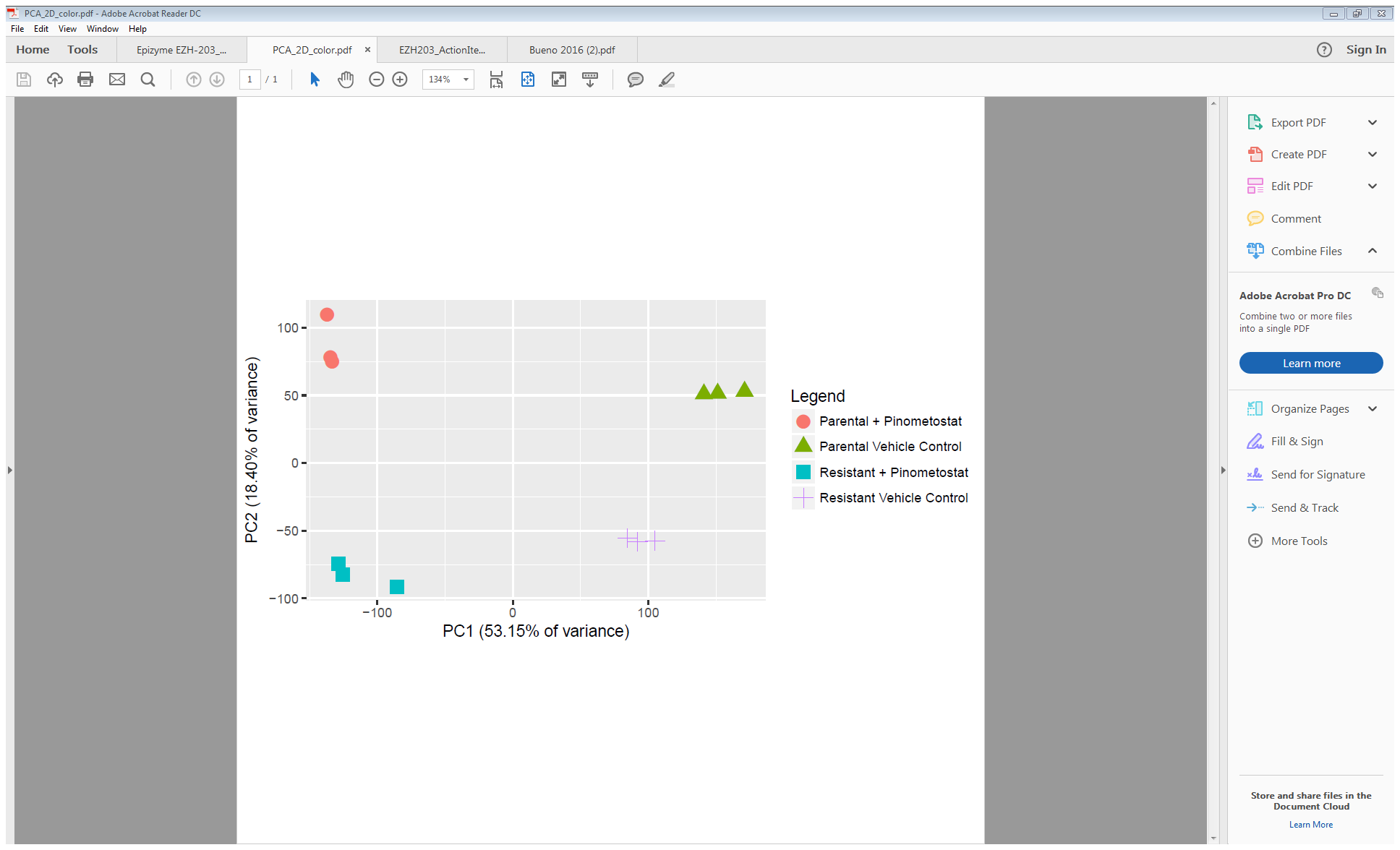
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**NOMO Resistant Consensus (4,614bp)**

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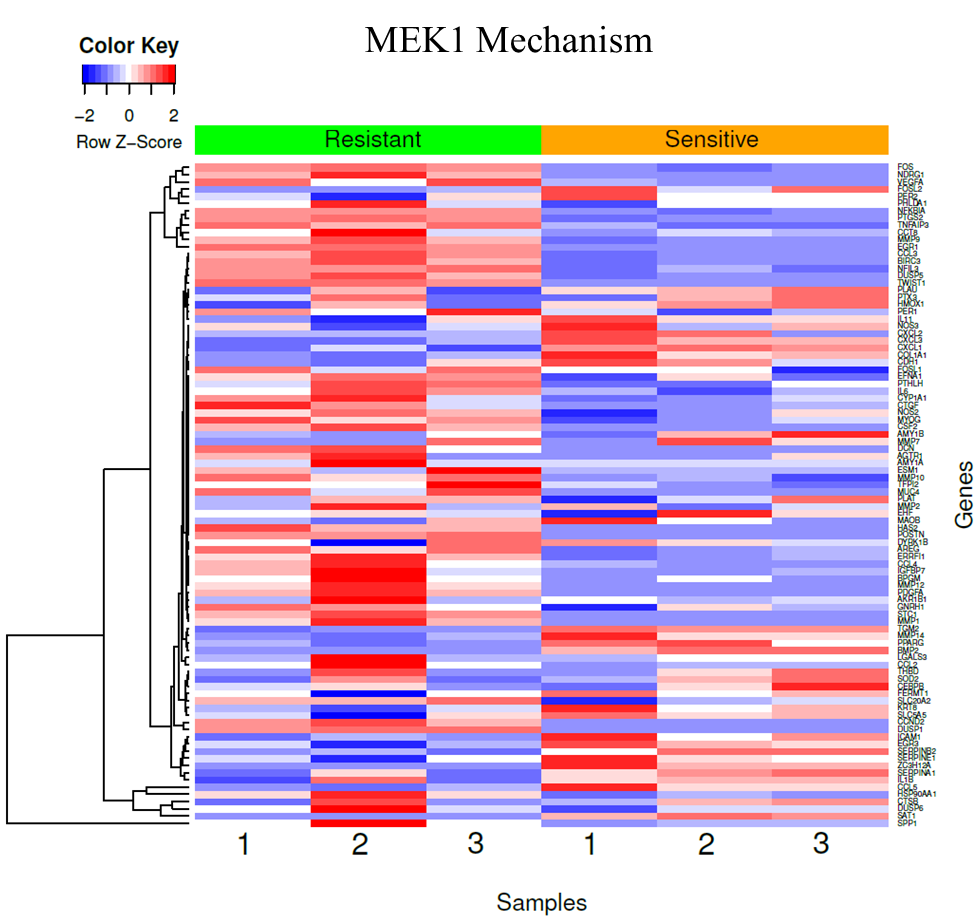
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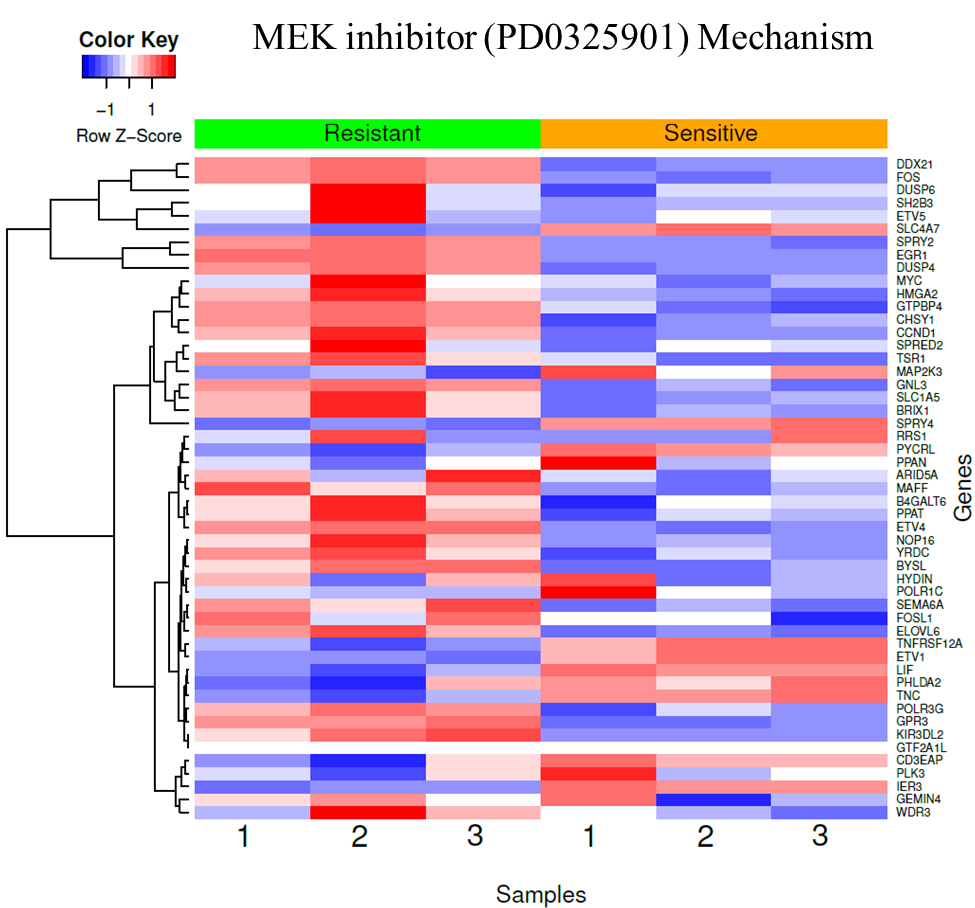
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* The sequence covered with single-strand coverage is shown in lower case
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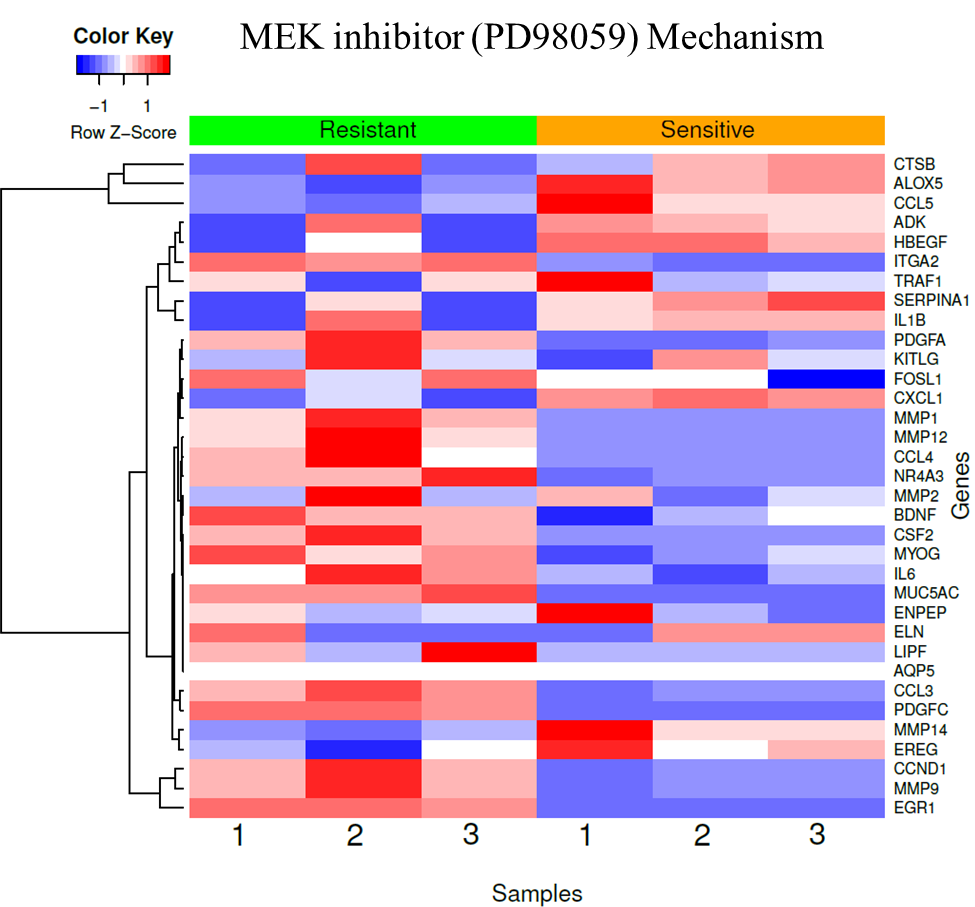
**Figure S3.** **NOMO-1 Principal Component Analysis**

**Figure S3.** **NOMO-1 Principal Component Analysis** PCA plot of the pinometostat resistant line, the vehicle control “resistant” cell line, the parental cell line treated with pinometostat for 10 days, and parental cell line treated with DMSO (vehicle) for 10 days.

**Figure S4.** **RAS/RAF/MEK/ERK Mechanisms Implicated with NOMO-1 Resistance**

**[A] **

**[B] **

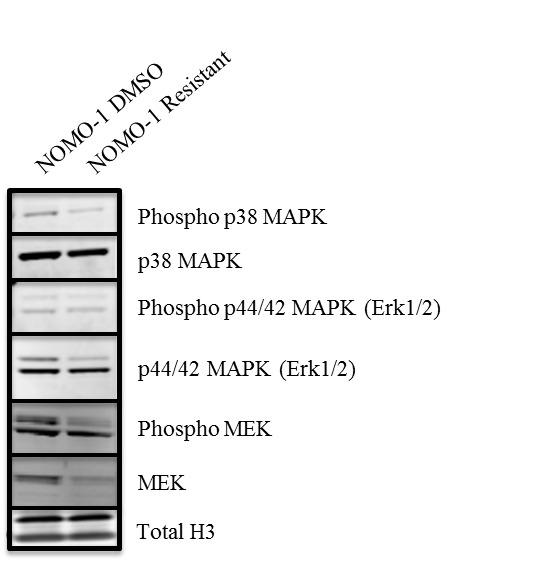
**[C]**

**Figure S4.** **RAS/RAF/MEK/ERK Mechanisms Implicated with NOMO-1 Resistance** Heatmap representations of differentially expressed genes in the strongest three RAS/RAF/MEK/ERK pathway mechanisms: MEK1 [A], MEK inhibitor PD0325901 [B], and MEK inhibitor PD98059 [C], associated with NOMO-1 resistant compared with NOMO-1 sensitive cell lines. The color key in the top left indicates the Z-score of relative expression levels, with red indicating high and blue low gene expression.

**Figure S5.** **Vehicle and Resistant NOMO-1 Cells Treated with RAS/RAF/MEK/ERK pathway inhibitors**

**Figure S5.** **Vehicle and Resistant NOMO-1 Cells Treated with RAS/RAF/MEK/ERK pathway inhibitors** Percent inhibition of NOMO-1 resistant and DMSO vehicle control cells after 14 day treatment with a dose response of trametinib or GDC0994 or 11 day treatment with dabrafenib. Viable cells were counted and split every 3 to 4 days and split adjusted results plotted on a logarithmic scale

**Figure S6.** **RAS/RAF/MEK/ERK Pathway Signaling Unchanged in Resistant NOMO-1 Cells**

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**Figure S6.** **RAS/RAF/MEK/ERK Pathway Signaling Unchanged in Resistant NOMO-1 Cells** Whole cell lysates were prepared from the NOMO-1 DMSO control and NOMO-1 resistant cell lines and probed for phosphorylated proteins in the RAS/RAF/MEK/ERK pathway.

**Supplemental Tables**

**Table S1.** **In vitro data for the assessment of ABCB1-mediated transport of pinometostat at a single concentration of 3 μM**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Transporter | Test Conditions | Papp B->A (nm/s) | Papp A->B (nm/s) | Mean Net B->A flux (pmol/hr/cm2) | Efflux ratio (B->A)/(A->B) | Inhibition (%) |
| ABCB1 | 100 nM digoxin | 10.9 ± 0.261 | 0.234 ± 0.0439 | 3.84 ± 0.0941 | 46.5 ± 1.12 | - |
| 100 nM digoxin + 100 µM verapamil | 2.97 ± 0.109 | 1.74 ± 0.0445 | 0.442 ± 0.0391 | 1.71 ± 0.0624 | 88.5 ± 1.02 |
| 3 µM pinometostat | 1.65 ± 0.142 | 0.227 ± 0.0393 | 15.3 ± 1.54 | 7.27 ± 0.629 | - |
| 3 µM pinometostat + 100 µM verapamil | 1.48 ± 0.0314 | 0.415 ± 0.0325 | 11.5 ± 0.34 | 3.56 ± 0.0757 | 25.1 ± 2.21 |
| *Data represent the mean and standard deviation of triplicate samples* | | |  |  |  |  |

For ABCB1, pinometostat showed an efflux ratio of 7.27, greater than the 2.0 cutoff for ABCB1 substrates, suggesting that pinometostat may be an ABCB1 substrate. Upon the addition of ABCB1 inhibitor verapamil, a decrease in the Papp B->A and an increase in the Papp A->B was observed, resulting in an decrease of efflux ratio from 7.27 to 3.56. These findings suggest that pinometostat may be a substrate of ABCB1. However, since the efflux ratio did not decrease to below 2.0, it also suggests that other mechanisms may be involved in the efflux of pinometostat.

**Table S2. ELISA analysis of H3K79me2 IC50 values after 4 days of pinometostat treatment in a panel of leukemia cell lines**

|  |  |
| --- | --- |
| **Cell Line** | **H3K79me2 IC50 (µM)** |
| KG-1a | 2.1 |
| Kasumi-1 | 0.589 |
| EOL-1 | 0.027 |
| KOPN-8 | 0.019 |
| SEMK2 | 0.016 |
| RS4-11 | 0.012 |
| REH | 0.008 |
| NOMO-1 | 0.005 |
| HL-60 | 0.004 |
| MOLM-13 | 0.002 |
| MV4-11 | 0.002 |

**Table S3. RAS/RAF/MEK/ERK Mechanisms Implicated with NOMO-1 Resistance**

|  |  |  |  |
| --- | --- | --- | --- |
| **Mechanism** | **Mechanism size** | **Strength** | **Adjusted P-Value** |
| MEK1 Kinase Activity | 79 | 0.670546126 | 1.31E-86 |
| MEK inhibitor (PD0325901) Treatment Schema | 50 | -0.451724477 | 7.84E-79 |
| MEK inhibitor (PD98059) Treatment Schema | 26 | -0.401977624 | 1.35E-16 |
| Abundance KRAS G12V | 109 | 0.357953542 | 7.46E-78 |
| ERK2 Kinase Activity | 134 | 0.349971693 | 4.38E-51 |

Table of the top 5 RAS/RAF/MEK/ERK pathway mechanisms implicated with NOMO-1 resistance. Mechanism size indicates the number of genes in the mechanism. The mechanism footprint gene list is included in Supplemental Table S4. Mechanism strength is the geometric mean of the fold changes corrected for the literature-specified regulatory directions for each gene, further described in Martin et al.

Martin F, Thomson TM, Sewer A, Drubin DA, Mathis C, Weisensee D, Pratt D, Hoeng J, Peitsch MC. Assessment of network perturbation amplitudes by applying high-throughput data to causal biological networks. BMC Syst Biol2012 May 31;6:54.

**Table S4. Mechanism Gene List Footprint**

See supplemental file “Supplemental Table S4” for table.

**Table S5. Pathway Analysis of Resistant vs. Sensitive NOMO-1 Cell Lines**

|  |  |  |  |
| --- | --- | --- | --- |
| Database | Pathway | Adj. P-value | Gene Count |
| KEGG | KEGG\_RIBOSOME | 1.82E-44 | 73 |
| REACTOME\_PEPTIDE\_CHAIN\_ELONGATION | 4.81E-32 | 73 |
| BILANGES\_SERUM\_AND\_RAPAMYCIN\_SENSITIVE\_GENES | 4.47E-30 | 53 |
| HSIAO\_HOUSEKEEPING\_GENES | 3.50E-29 | 146 |
| REACTOME\_INFLUENZA\_VIRAL\_RNA\_TRANSCRIPTION\_AND\_REPLICATION | 6.73E-29 | 75 |
| GO Biological Process | protein targeting to ER | 4.67E-40 | 73 |
| SRP-dependent cotranslational protein targeting to membrane | 8.59E-40 | 70 |
| establishment of protein localization to endoplasmic reticulum | 8.59E-40 | 74 |
| cotranslational protein targeting to membrane | 6.87E-39 | 72 |
| protein localization to endoplasmic reticulum | 2.63E-38 | 80 |
| Database | Pathway | FDR q-value | Gene Count |
| MSigDB | REACTOME\_PEPTIDE\_CHAIN\_ELONGATION | 0 | 85 |
| KEGG\_RIBOSOME | 0 | 86 |
| REACTOME\_3\_UTR\_MEDIATED\_TRANSLATIONAL\_REGULATION | 0 | 105 |
| REACTOME\_SRP\_DEPENDENT\_COTRANSLATIONAL\_PROTEIN\_TARGETING\_TO\_MEMBRANE | 0 | 108 |
| REACTOME\_INFLUENZA\_VIRAL\_RNA\_TRANSCRIPTION\_AND\_REPLICATION | 0 | 101 |

Table of the top 5 most significant pathways implicated with NOMO-1 resistance from the KEGG, GO – Biological Process, and MSigDB databases. Gene count indicates the number of genes in the pathway. The complete listing of all pathways is available in supplemental table S6.

**Table S6. Complete Pathway Analysis of Resistant vs. Sensitive NOMO-1 Cell Lines**

See supplemental file “Supplemental Table S6” for table.