MLL partial tandem duplication leukemia cells are sensitive to small molecule DOT1L inhibition

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SUPPLEMENTAL METHODS, TABLES, RESULTS, AND FIGURE LEGENDS

MLL-PTD leukemia cells are sensitive to small molecule DOT1L inhibition

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Supplemental methods

Cell lines

The *MLL*-PTD positive EOL-1 cell line was obtained from Sigma-Aldrich (94042252-1VL). The *MLL*-PTD positive KOPM-88 cell line was a gift from Dr. Gang Huang (Cincinnati Children's Hospital Medical Center, OH, USA)

Cell proliferation assay

Each cell line was plated in 150 µL per well in a 96-well plate. To account for differences in proliferation rates, EOL-1 and MOLM-13 cells were plated at 2 x 10^4 cells per well, while HL-60 and KOPM-88 cells were plated at 3 x 10^4 cells per well. Cells were treated with 10 µM or serially decreasing concentrations of EPZ00477, or DMSO vehicle control at the same concentration. Viable cells were counted by flow cytometry using Sytox Blue (S34857, Invitrogen, Carlsbad, CA, USA) to stain non-viable cells and debris. At each time point, media and EPZ004777 were replaced and cells were split to the original cell density. Results were plotted as the split-adjusted viable cells in the presence of EPZ004777 compared to DMSO vehicle control.

Western blotting

Histones were quantified using the BCA protein quantification kit (23227, Thermo Scientific/Pierce, Rockford, IL, USA) and 1.5 µg of histones was used. Western blotting was performed using a 10 % Bis-Tris Gel with MES Running Buffer (Nupage, Invitrogen, Carlsbad, CA, USA) and transferred onto a PVDF

membrane with the iBlot Gel Transfer Device (IB1001, Invitrogen, Carlsbad, CA, USA). H3K79 methylation was assessed using the anit-H3K79me2 antibody ab3594 (Abcam, Cambridge, MA, USA). The anti-total H3 antibody ab1791 (Abcam, Cambridge, MA, USA) was used as the loading control. The secondary antibody used was donkey anti-rabbit ECL horseradish peroxidase linked NA934V (GE healthcare UK limited, Little Chalfont Buckinghamshire, UK).

Chromatin IP-sequencing (ChIP-Seq) and analysis (detailed protocol)

Crosslinking was performed with 1% formalin, the cells were lysed in SDS buffer, and DNA was fragmented by sonication. ChIP for H3K79me2 was performed using anti-H3K79me2 antibody ab3594 on human leukemia cell lines EOL-1 and KOPM-88. Eluted DNA fragments were analyzed by qPCR with the following primer sets (5'-3'): *HOXA9*, F: ACGTAGTAGTTGCCCAGGGCC, R: TGCAGTTTCATAATTTCCGTGG; *HOXA10*, F: CCCGAGCTGATGAGCGAGTC, R: GCCAAATTATCCCACAACAATGTC; *HOXB7*, F: AGGTTTTCTTCCCCTCTGGA, R: AACAACAGGTCCCTCCACAA; and *ACTB,* F: AGCGCGGCTACAGCTTCA, R: CGTAGCACAGCTTCTCCTTAATGTC.

For EOL-1, eluted DNA fragments were subjected to sequencing using HiSeq2000 platform. Sequence reads were aligned to human genome assembly hg19 using Bowtie(1). Genome wide ChIP-Seq data was visualized using the integrated genome viewer (IGV) version 2.1. Reads that aligned to multiple loci in the genome were discarded. The ChIP-seq signal was quantified as total number of reads per million in the region 1 Kb upstream of transcription start site (TSS) to

2Kb downstream of TSS for H3K79me2. An empirical background distribution model of reads was constructed to find the significance level of signal at a gene. ChIP sequencing data will be deposited at the NCBI gene expression omnibus portal (accession number pending).

Gene expression analysis quantitative PCR

Total RNA was isolated using the RNeasy Mini Kit (74104, Qiagen, Ambion, Inc, Austin, TX, USA) and hybridized to Affymetrix GeneChip Human Genome U133 Plus 2.0 Array in accordance with the manufacturer's recommendations. Data analysis was performed using Gene Pattern v3.6.1 (http://www.broad.mit.edu/tools/software.html) and Gene Set Enrichement Analysis (GSEA) software tools (www.broadinstitute.org/gsea) as previously described(2, 3). Data preprocessing was performed using the following filter settings: Floor: 10; ceiling: 20000; min fold change: 1.2; min delta: 20. For comparisons of gene expression between two groups, the expression levels correlated with a particular class was determined by t-test. To determine the 100 most significantly downregulated genes within the EPZ004777 treated group, genes with a fold-change <1.5 and/or a FDR >0.05 were filtered out. The remaining genes were ranked according to t-statistics (Table T1).

cDNA was generated using the Tetro cDNA Synthesis Kit (65043, Bioline USA Inc, USA) using oligo-dT primers and quantitative PCR was performed using SYBR Green PCR Master Mix on the ABI 7700 Sequence Detection

System (Applied Biosystems). Average Ct values were normalized to the housekeeping gene *ACTB*.

The following primer sets were used (5'-3'): *HOXA9*, F: CACCAGACGAACAGTGAGGA, R: TGGTCAGTAGGCCTTGAGGT; *HOXA10*, F: CCTACACGAAGCACCAGACA, R: GATCCGGTTTTCTCGATTCA; *ACTB*, F: GCACAGAGCCTCGCCTT, R: CCTTGCACATGCCGGAG; and *GAPDH*, F: GAAGGTGAAGGTCGGAGT, R: GAAGATGGTGATGGGATTTC.

Gene expression array data were deposited at the NCBI gene expression omnibus portal (accession number pending).

Cell differentiation and apoptosis assays

EOL-1 and KOPM-88 cells were plated in triplicates at 4 x 10^4 cells per mL and 2 x 10^5 cells per mL of cell culture media, respectively. Due to differences in proliferation rates between cell lines and the effects of EPZ004777 treatments, cells were plated in either 3 mL in a 6-well plate, 2 mL in a 12-well plate, or 1 mL in a 24-well plate and incubated with 10 μM EPZ00477 or DMSO vehicle control. On days 4, 7, and 11, media with drug was replaced and cell lines were adjusted to the starting density. CD11b expression was assessed on days 4, 7, 11, and 14. Cells treated with either EPZ00477 or DMSO were harvested and washed twice with PBS, stained for 20 minutes with CD11b-PE (555388, BD Pharmingen, San Jose, CA, USA), washed again with PBS and counterstained with Sytox Blue before flow cytometric analysis. Data was acquired with a BD FACS ARIA flow cytometer and analyzed with FlowJo software (TreeStar Inc., CA). To

quantify CD11b expression, mean PE signal of Sytox Blue negative viable cells was calculated in FlowJo.

Annexin V apoptosis assays were also performed on days 4, 7, 11, and 14 in the same experiment. Cells treated with either EPZ00477 or DMSO were harvested and washed twice with PBS, stained with Annexin V-APC (550474, BD Pharmingen, San Jose, CA, USA) for 20 minutes in BD Annexin binding buffer, and counterstained with Sytox Blue before flow cytometric analysis. Data was acquired with a BD FACS ARIA flow cytometer and analyzed with FlowJo software (TreeStar Inc., CA). The percentage of Sytox Blue negative cells was calculated in FlowJo to determine the percent viability. The percentage of Annexin V positive cells was calculated within the Sytox Blue negative population.

Subcutaneous rat xenograft model

In vivo studies were conducted after review by the appropriate animal care and use committee at Charles River Discovery Research Services (Morrisville, North Carolina). 1 x 10^7 EOL-1 cells were resuspended in 100% Matrigel (BD Biosciences) and implanted subcutaneously in the right flank of female athymic nude rats (*rnu/rnu*, Harlan). EPZ-5676 or vehicle (5% hydroxypropyl-β-cyclodextrin (HPBCD) in saline) was delivered by IV infusion into the femoral vein continuously (24 hrs/day) as previously described (4). For the efficacy study, EPZ-5676 infusion was initiated when tumors reached 300 – 450 mm^3. Animals (n = 10 per group) were weighed and tumors callipered twice weekly until the end

of the study. Each test animal was euthanized when its neoplasm reached the predetermined endpoint volume or on the last day of the study, whichever came first. Tumor growth inhibition values were calculated as the percentage reduction in mean tumor size for each dose group relative to the mean size of vehicle treated controls on day 23 of the efficacy experiment. EPZ-5676 blood plasma levels were determined for all animals in each dose group of the efficacy study on day 7, 15 and 21 of EPZ-5676 infusion as described(4). For the study to confirm in vivo target engagement, 5 animals were used in each group and EPZ-5676 infusion was initiated when tumors reached 800 – 1200 mm3. Tumor and bone marrow tissue isolation, histone extraction, mRNA isolation, H3K79me2 ELISA assay and *HOXA9* qRT-PCR was performed as previously described(4).

Culture of primary human AML cells

Primary human AML cells were co-cultured with Hs27 (CRL-1634) human stromal cells in an assay designed by Klco and co-workers(5) with one modification: To optimize the maintenance of the leukemic phenotype of the primary AML cells *in vitro*, we used a modified serum-free culture medium (StemSpan SFEM, Stemcell Technologies) supplemented with cytokines (beta-Mercaptoethanol, 0,1mM; SCF, 100ng/ml; IL3, 20ng/ml; G-CSF, 20ng/ml; FLT3L 50ng/ml) and the small molecule SR-1 (1µM) as suggested by Pabst and co-workers(6). Samples originated from the biobank at Memorial Sloan Kettering Cancer Center. Written informed consent and local ethics committee approval was obtained in accordance with the Declaration of Helsinki for both samples.

Supplemental tables and figures

Figure S1: Downregulation of *HOXA9* in response to 7 days continuous infusion of EPZ-5676 in the EOL-1 xenograft tumors implanted subcutaneously (SC) in immunocompromised rats. *HOXA9* transcript levels were measured by quantitative real-time PCR. Transcript levels are plotted as a percent of the mean transcript level in tumors from the vehicle treated group which is set at 100%. Horizontal lines represent the mean percent transcript level in each group.

Figure S2: (A) Co-Culture assays with primary human AML cells from two different patients. Cells were co-cultured with the Hs27 human stromal cell line and plated in cytokine-supplemented serum-free media in the presence of vehicle (DMSO) or EPZ004777 (10μM). These show a marked reduction of cell growth in the presence of EPZ004777 after 10 days of treatment. (B) Cells treated for 10 days with EPZ004777 showed morphological changes consistent with monocytic and neutrophilic differentiation compared to vehicle control.

Supplemental Table T1: 100 most significantly downregulated genes in EOL-1 cells after 7 days of treatment with 10μM EPZ004777.

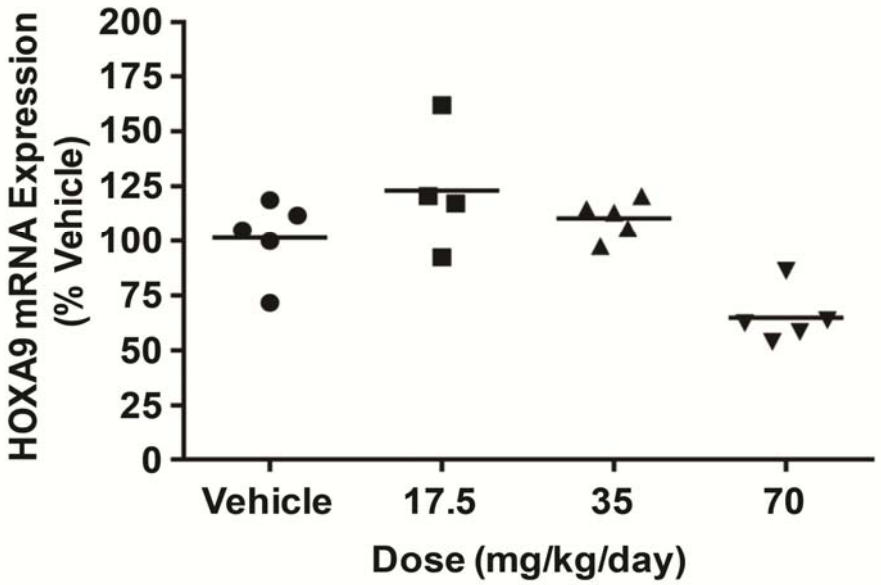
Rank	Gene	T-Statistic-Score	P-value	Probe
1	*RHOH*	85.49	3.04E-06	204951_at
2	*HOXA10 /// HOXA9*	73.496	2.12E-07	209905_at
3	*C12orf75*	48.82	2.38E-06	225105_at
4	*C12orf73*	47.649	4.64E-06	226943_at
5	*CDK6*	46.618	3.18E-06	224847_at
6	*BCL2*	43.264	2.19E-05	232210_at
7	*LOC339862*	42.539	1.00E-05	1557534_at
8	n.d.	35.869	5.18E-04	239963_at
9	*CCL5*	30.612	1.51E-04	204655_at
10	*MLC1*	29.914	3.54E-04	213395_at
11	*PARP8*	29.635	7.75E-06	244008_at
12	*RASGRP2*	29.314	2.01E-05	208206_s_at
13	*WT1*	28.329	9.06E-04	206067_s_at
14	*SOX4*	27.351	2.34E-04	201417_at
15	*RAB27A*	26.808	3.09E-05	210951_x_at
16	*KDM5B*	26.678	2.09E-05	201548_s_at
17	*ADK*	26.401	1.59E-04	204119_s_at
18	*PTK2*	25.873	1.30E-04	208820_at
19	*DUSP4*	25.774	9.63E-05	204014_at
20	*C1orf106*	25.055	2.25E-04	219010_at
21	*ANKRD27*	24.605	1.62E-05	221522_at
22	*MAD2L2*	24.238	7.12E-04	223234_at
23	*SCLT1*	23.697	9.58E-05	1569190_at
24	*CDK6*	23.538	2.30E-04	224851_at
25	*C5orf13*	23.272	3.01E-05	201310_s_at
26	*ALDH5A1*	23.18	6.69E-04	203608_at
27	*FAM46C*	23.096	6.64E-04	226811_at
28	n.d.	22.951	2.43E-04	228487_s_at
29	*ONECUT2*	22.692	2.26E-05	239911_at
30	*JUB*	22.548	2.96E-04	225806_at
31	*UBASH3B*	22.363	2.93E-04	238462_at
32	*SNRPN*	22.346	1.32E-04	226591_at
33	*DUSP4*	22.312	4.94E-05	204015_s_at
34	*RAB27A*	21.963	6.67E-04	209514_s_at

Rank	Gene	T-Statistic-Score	P-value	Probe
35	*PRDX1*	21.769	3.57E-05	208680_at
36	*IL15RA*	21.521	8.35E-04	207375_s_at
37	*C5orf13*	21.361	1.72E-04	201309_x_at
38	*IRX5*	21.16	1.51E-03	210239_at
39	*XBP1*	20.881	9.63E-05	200670_at
40	*SOX4*	20.588	6.66E-05	201418_s_at
41	*HOXA3*	20.38	2.31E-03	235521_at
42	*FOXN3*	20.345	1.56E-03	205022_s_at
43	*MTO1*	20.267	8.85E-04	218716_x_at
44	*TTC28*	20.234	4.95E-05	213058_at
45	*PVT1*	20.223	5.21E-05	1558290_a_at
46	*STX1A*	19.581	5.45E-05	204729_s_at
47	*C10orf47*	19.417	2.86E-04	230051_at
48	*RAB33A*	19.399	1.02E-04	206039_at
49	*NLE1*	19.118	1.28E-04	203867_s_at
50	*CHST11*	18.717	4.80E-05	226372_at
51	*ESYT2*	18.676	2.26E-03	224699_s_at
52	*ZNRF1*	18.457	2.13E-04	223382_s_at
53	*ESYT2*	18.446	5.10E-05	224698_at
54	*BCL11A*	18.277	5.29E-05	222891_s_at
55	*EMB*	18.175	9.13E-05	226789_at
56	*MIR155HG*	18.104	1.14E-04	229437_at
57	*KDM5B*	18.08	1.40E-03	201549_x_at
58	*KDM5B*	17.928	6.51E-04	211202_s_at
59	*HOXA5*	17.776	1.92E-03	213844_at
60	*ONECUT2*	17.678	3.89E-04	230271_at
61	*CHD7*	17.628	9.24E-05	218829_s_at
62	*ACSL1*	17.591	8.63E-05	201963_at
63	*ADA*	17.531	1.46E-03	216705_s_at
64	*KDM1A*	17.507	8.45E-04	212348_s_at
65	*DYSFIP1*	17.465	1.66E-03	1557480_a_at
66	*MTO1*	17.092	3.07E-03	224430_s_at
67	*FAM60A*	16.99	5.94E-04	220147_s_at
68	*CENPV*	16.909	2.77E-03	226610_at
69	*NEXN*	16.895	2.29E-03	226103_at
70	*RAB27A*	16.593	1.74E-04	209515_s_at
71	*CCL5*	16.508	2.98E-03	1405_i_at
72	*SOX4*	16.372	7.58E-04	201416_at

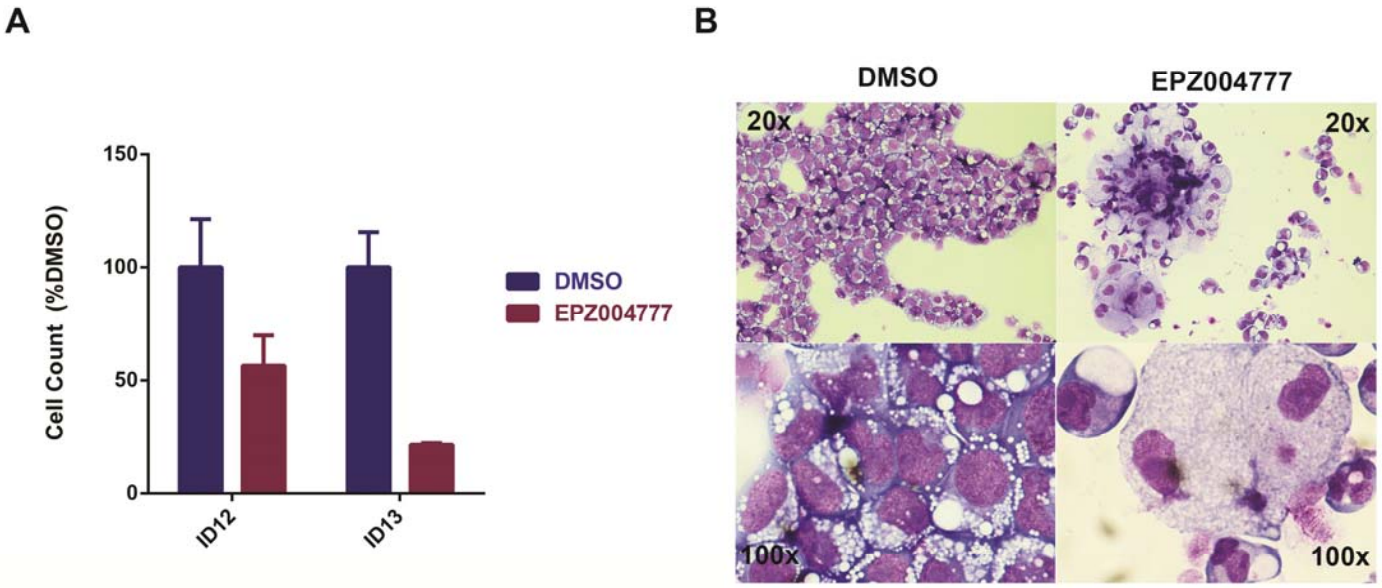
Rank	*Gene*	T-Statistic-Score	P-value	Probe
73	*HOXA10 /// HOXA9*	16.368	1.55E-03	214651_s_at
74	*CDK6*	16.293	6.01E-04	243000_at
75	*TGFBR1*	16.291	2.52E-03	224793_s_at
76	*PHKA2*	16.275	1.09E-04	209439_s_at
77	*UBASH3B*	16.23	8.80E-05	238587_at
78	*MEIS2*	16.217	3.68E-03	207480_s_at
79	*NUCB2*	16.12	2.30E-04	229838_at
80	*F3*	16.042	9.82E-05	204363_at
81	*ZBTB44*	15.976	2.91E-03	225845_at
82	*PITPNC1*	15.896	2.65E-03	219155_at
83	*DCUN1D5*	15.79	1.73E-03	223151_at
84	*LTB*	15.702	2.13E-04	207339_s_at
85	*DYNC2LI1*	15.676	4.18E-04	203762_s_at
86	*CFLAR*	15.668	3.97E-04	209939_x_at
87	*MYB*	15.558	6.67E-04	204798_at
88	*SPIN4*	15.543	4.59E-04	228654_at
89	*ZNRF1*	15.298	1.20E-04	223383_at
90	*ZNF84*	15.274	7.59E-04	204453_at
91	*MTO1*	15.247	4.23E-03	233665_x_at
92	*GOLGA8H*	15.209	1.13E-03	213737_x_at
93	*KIF9*	15.19	1.43E-03	231319_x_at
94	*NOG*	15.186	2.02E-03	231798_at
95	*DNMT3A*	15.115	1.37E-04	222640_at
96	*FAM108B1*	15.082	4.25E-03	227551_at
97	*CMAH*	15.082	4.12E-03	210571_s_at
98	*IRF1*	14.933	5.00E-04	202531_at
99	*BCAT1*	14.927	1.65E-04	225285_at
100	*MST4*	14.843	1.96E-03	218499_at

n.d.= not determined

Supplemental Figure S1



Supplemental Figure S2



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