SUPPLEMENTARY APPENDIX

Targeted therapy for a subset of acute myeloid leukemias that lack expression of aldehyde dehydrogenase 1A1

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Supplemental Figure 1

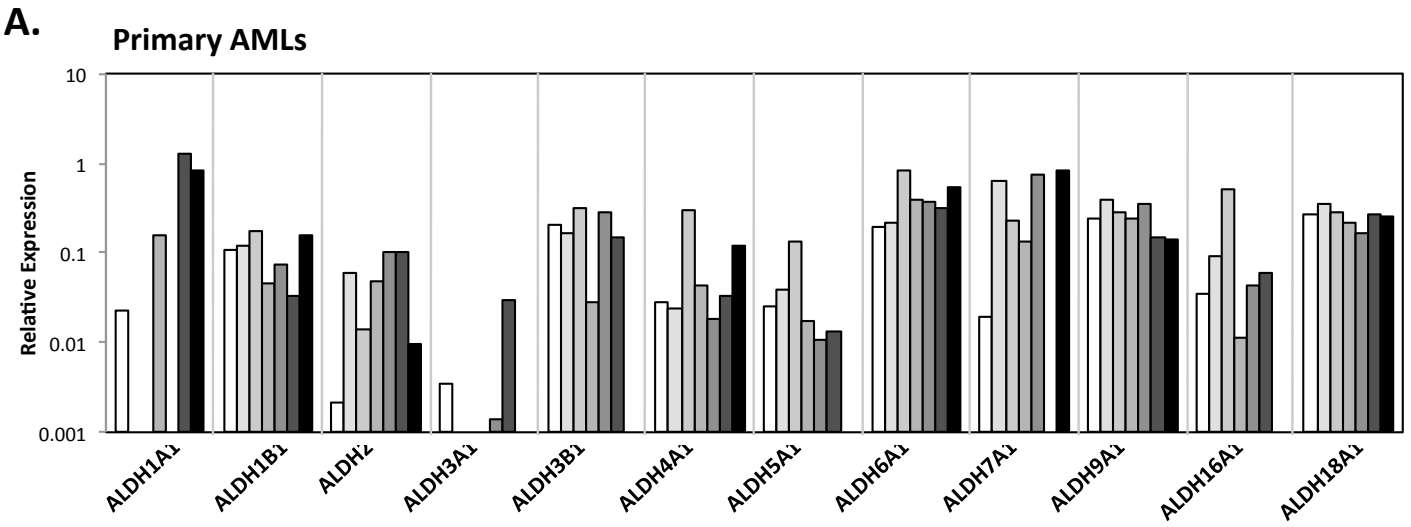


Figure 1A. qPCR for ALDH isoforms on 7 primary AML samples.

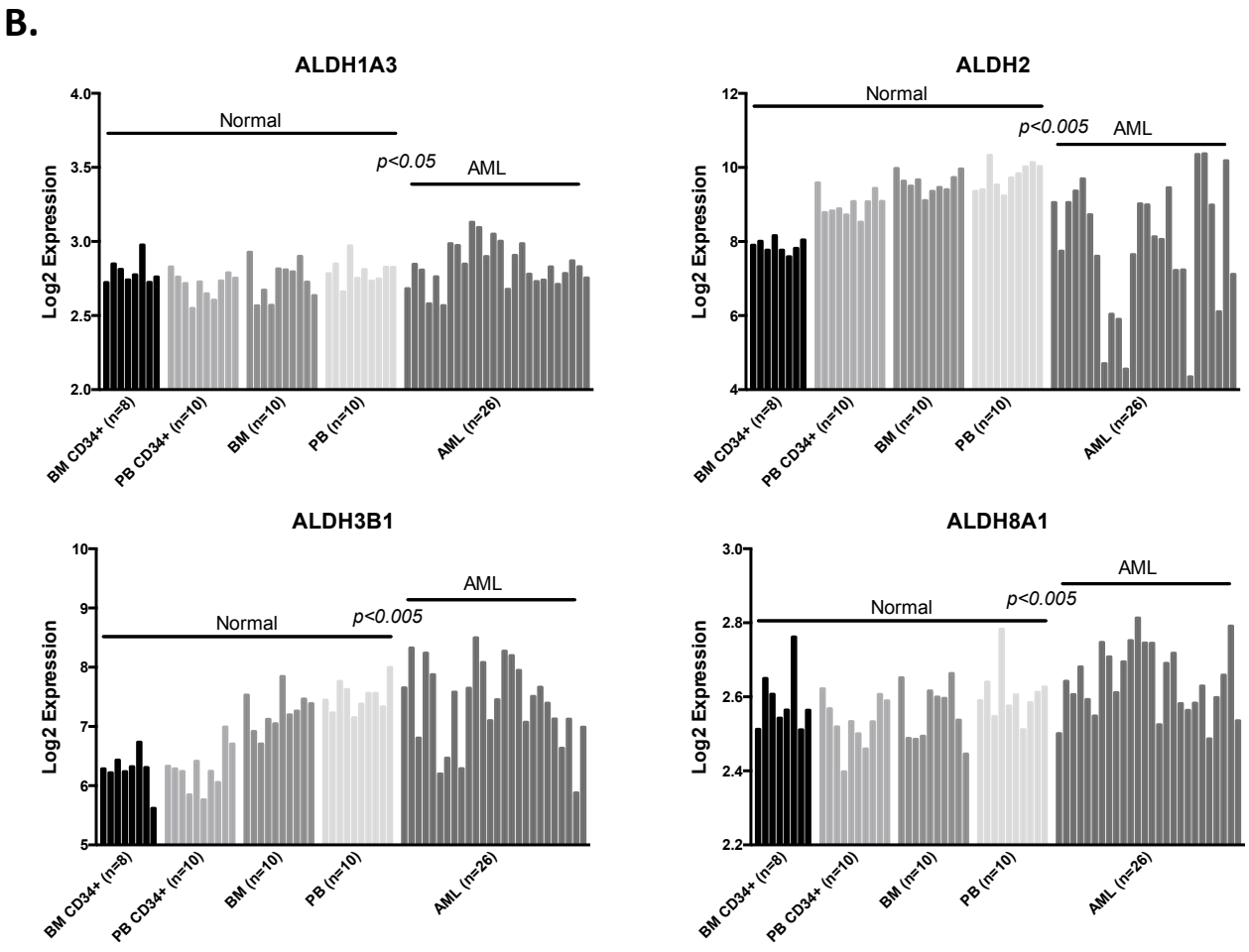
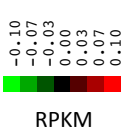
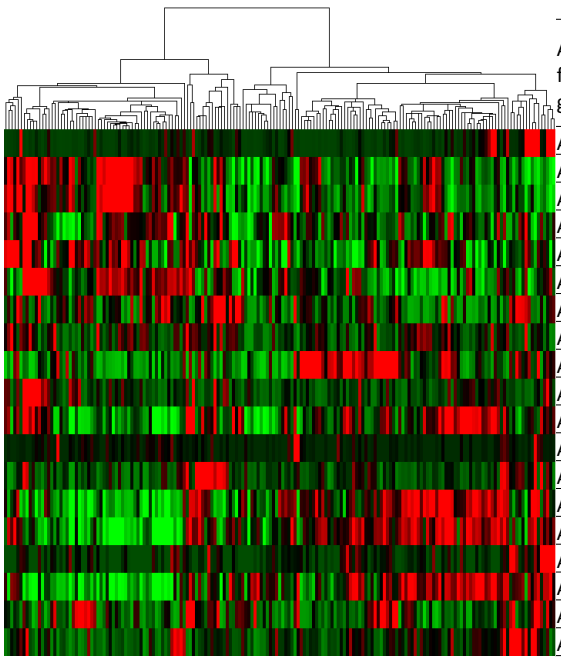


Figure 1B. Differential expression between normal CD34⁺ cells and AML of several ALDH isoforms. Data is from the GSE9476 data set.

Supplemental Figure 1, cont

C.





ALDH family genes	Correlation coefficient (r) to ALDH1A1	p value
ALDH1A1	N/A	N/A
ALDH3B1	-0.241	0.001
ALDH3B2	-0.177	0.018
ALDH9A1	-0.126	0.092
ALDH16A1	-0.100	0.185
ALDH3A2	-0.093	0.216
ALDH2	-0.026	0.728
ALDH4A1	-0.018	0.810
ALDH7A1	-0.012	0.874
ALDH3A1	-0.006	0.938
ALDH1B1	0.048	0.524
ALDH1A2	0.051	0.494
ALDH1L2	0.061	0.414
ALDH6A1	0.177	0.018
ALDH18A1	0.191	0.011
ALDH1L1	0.199	0.008
ALDH5A1	0.202	0.007
ALDH8A1	0.271	0.000
ALDH1A3	0.536	< 0.0001

Figure 1C. Heat map representation of expression of ALDH isoforms within AMLs expressing low to absent levels of ALDH1A1. Data is from the TCGA AML data set.

D.

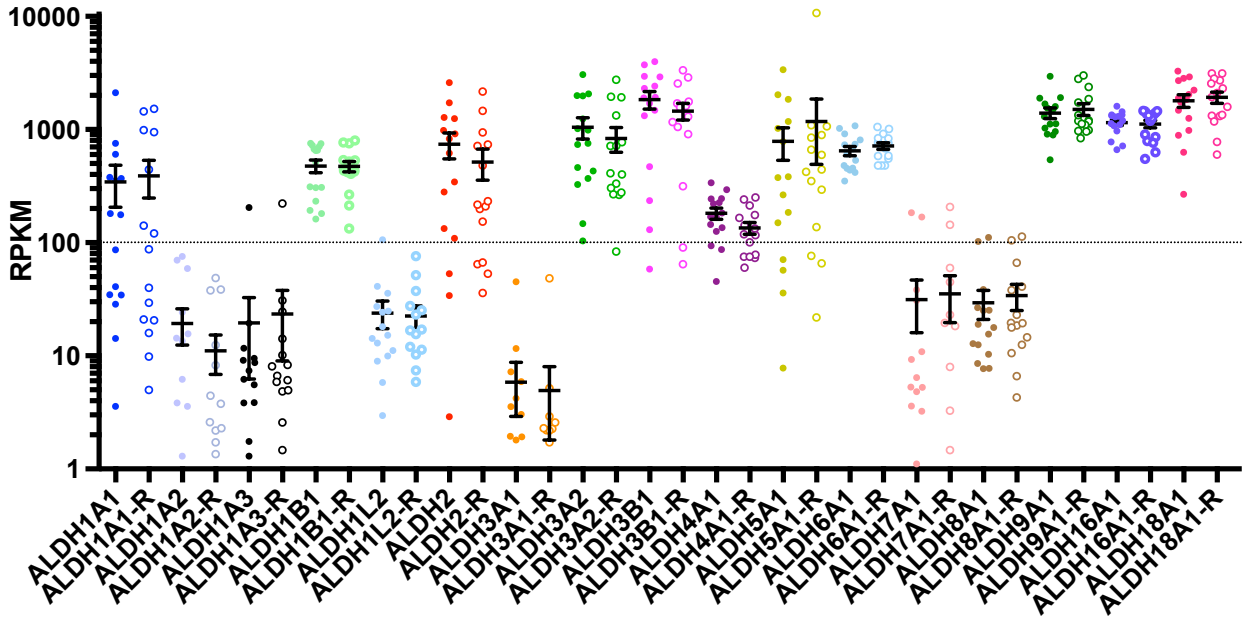


Figure 1D. Diagnosis and relapse (-R) AML subclones (LSC and Non-LSC) from 6 patients were analyzed for differential expression of ALDH isoforms.

Supplemental Figure 1, cont

E.

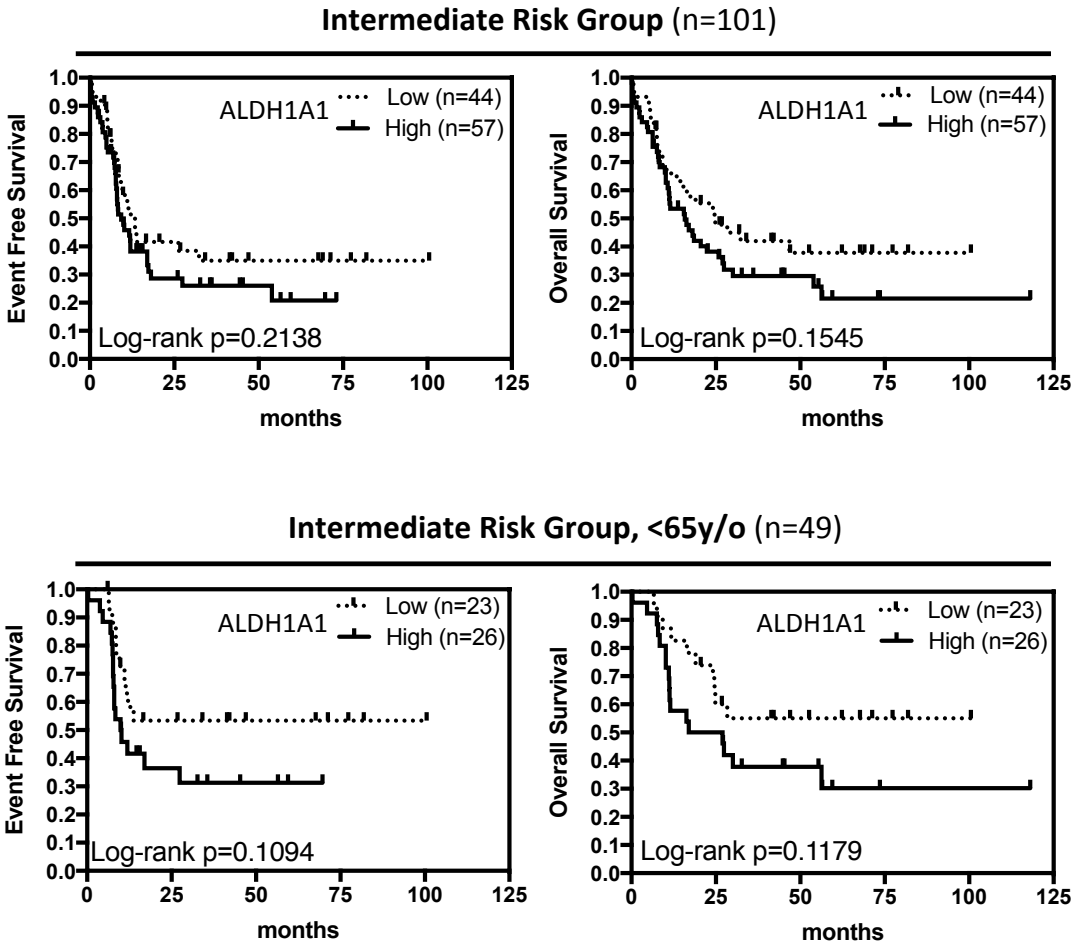


Figure 1E. EFS and OS from all Intermediate risk group patients (n=101) and patients that are < 65 years old and were treated with standard chemotherapy regimens (n=49). A cut-off value of 1.5 RPKM was used to segregate patients into ALDH1A1-low (RPKM<1.5) and ALDH1A1-high (RPKM>1.5) groups**.** Data is from the TCGA AML data set.

Supplemental Figure 2

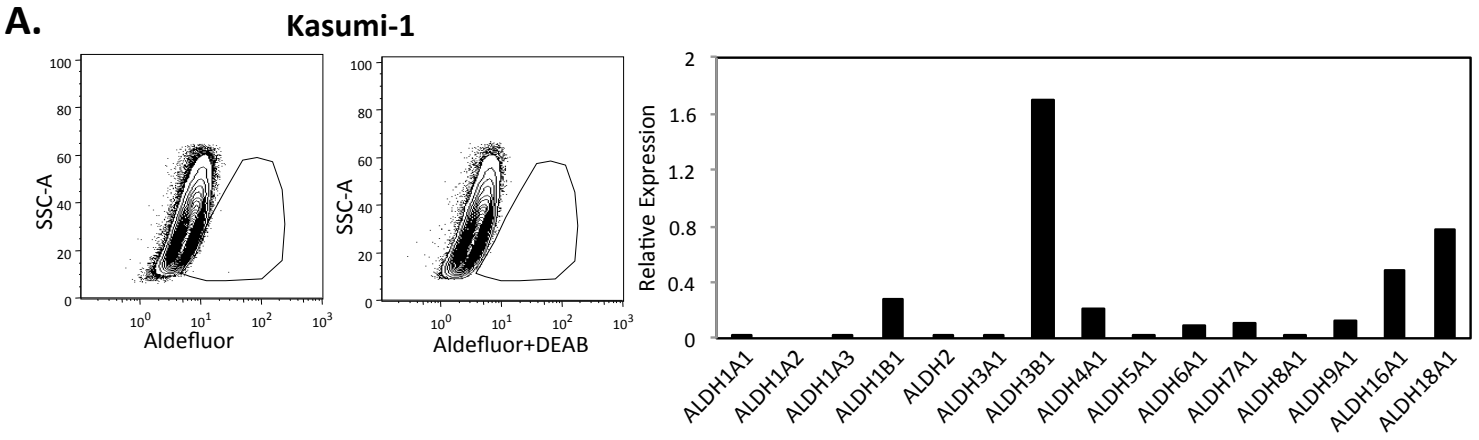


Figure 2A. Aldefluor flow cytometric analysis of Kasumi-1 cells and qPCR for ALDH isoforms.

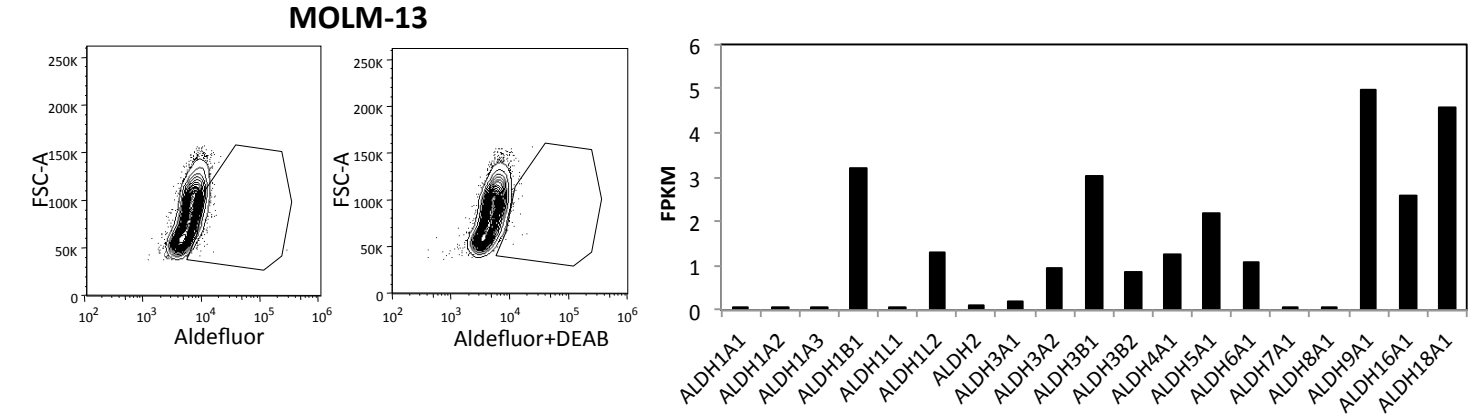


Figure 2B. Aldefluor flow cytometric analysis of MOLM-13 cells and RNA-Seq for ALDH isoforms.

Supplemental Figure 2, cont

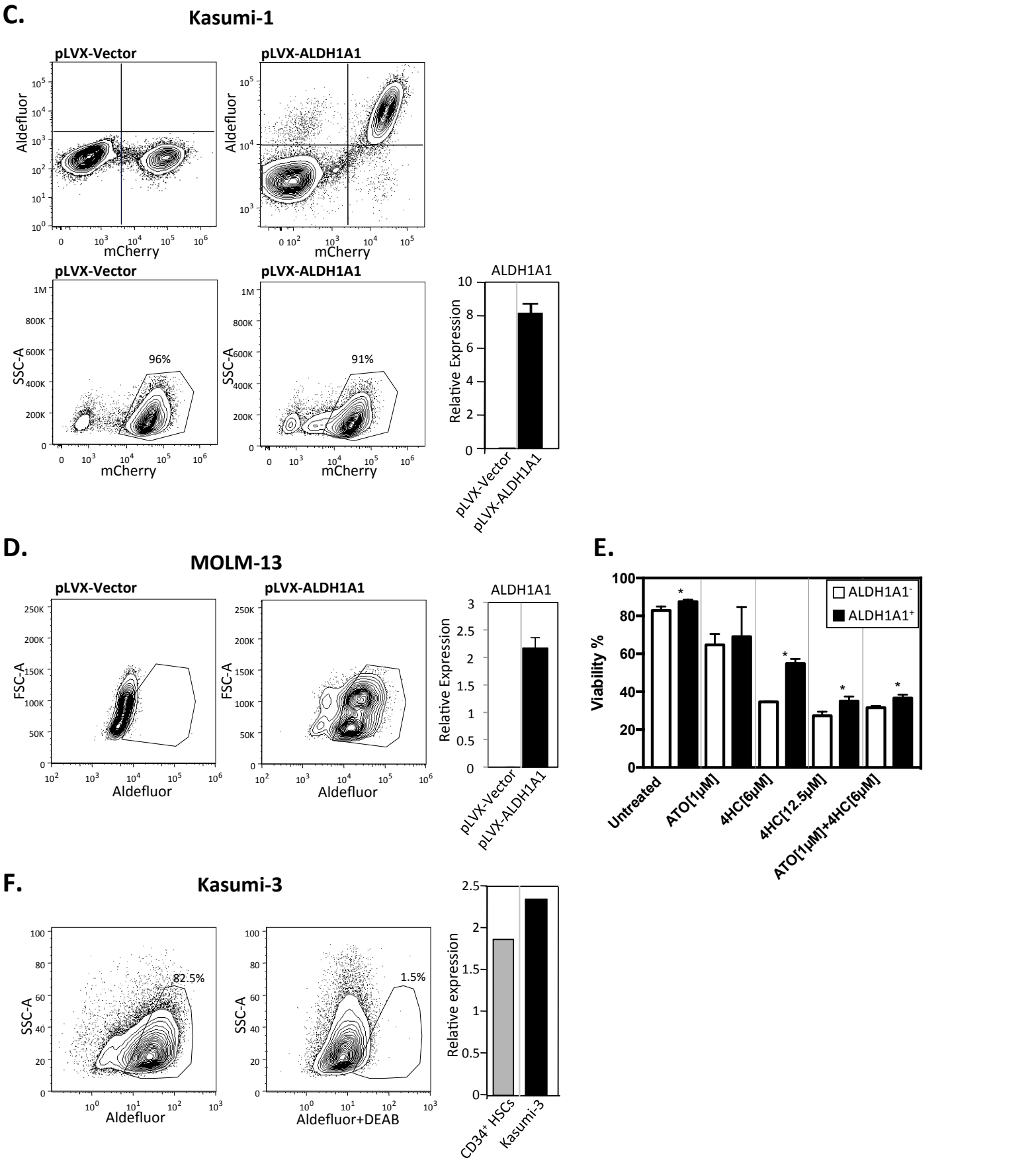


Figure 2C. Restoration of ALDH1A1 in Kasumi-1 cells detected by qPCR and Aldefluor. Flow cytometric analysis of ALDH1A1^{+} and vector control cells showing infection efficiency by m-Cherry and Aldefluor and post-sort enrichment phenotype.

Figure 2D. Restoration of ALDH1A1 in MOLM-13 cells detected by qPCR and Aldefluor. Flow cytometric analysis of ALDH1A1^{+} and vector control cells showing infection efficiency by Aldefluor.

Figure 2E. Restoration of ALDH1A1 expression in MOLM-13 partially blocked the effects of 4HC alone and in combination with ATO depending on their concentrations.

Figure 2F. ALDH1A1 activity in Kasumi-3 cell detected by qPCR (compared to CD34^{+}HSCs) and Aldefluor.

Supplemental Figure 3

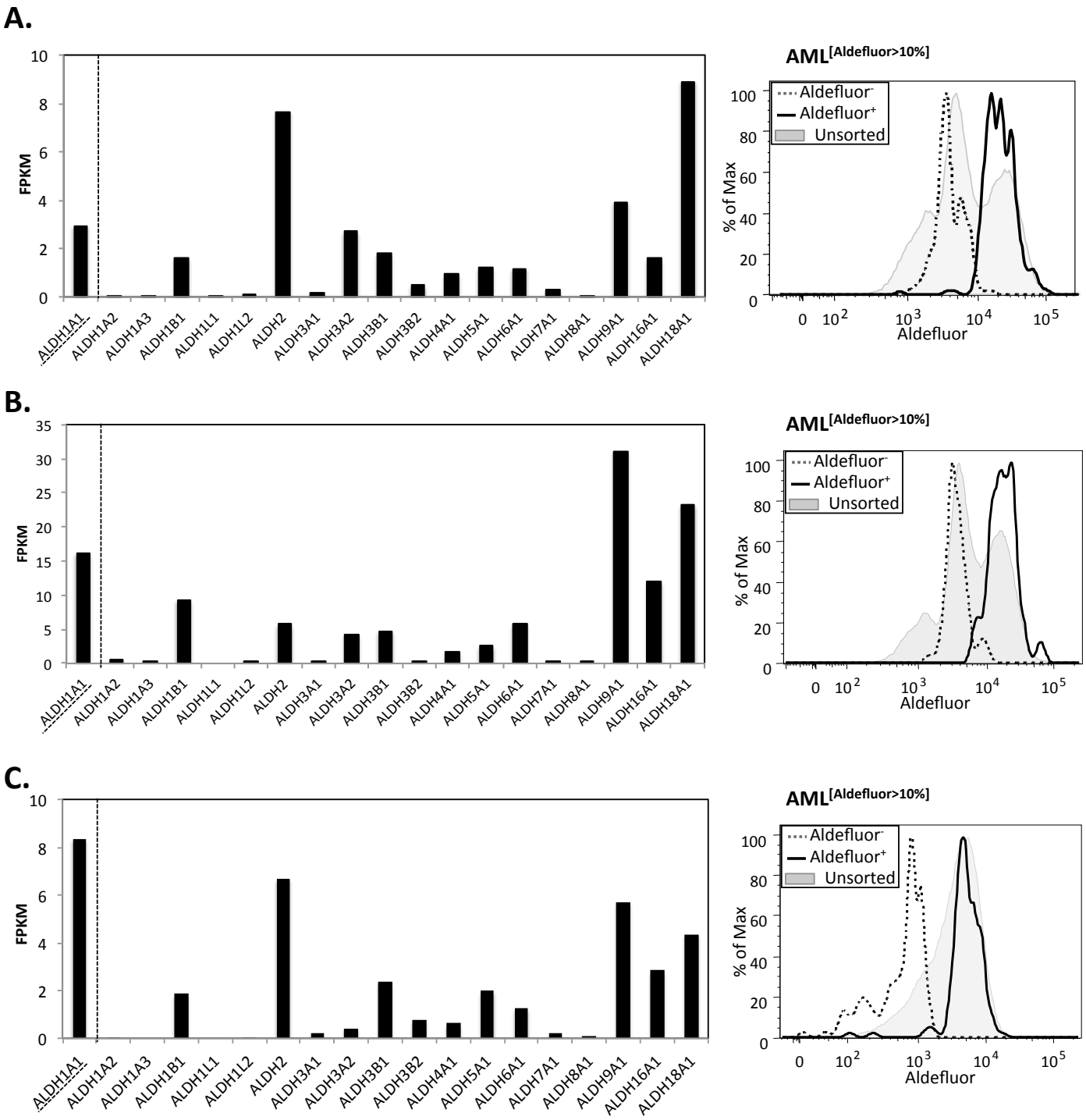


Figure 3A-C. RNA-Seq analysis for ALDH isoforms from 3 AMLs [Aldefluor>10%] and Aldefluor measurements by flow cytometry. Primary AML samples containing both Aldefluor⁺ and Aldefluor⁻ fractions were sorted into the respective subsets for *in vitro* sensitivity studies (3 independent experiments).

Supplemental Figure 4

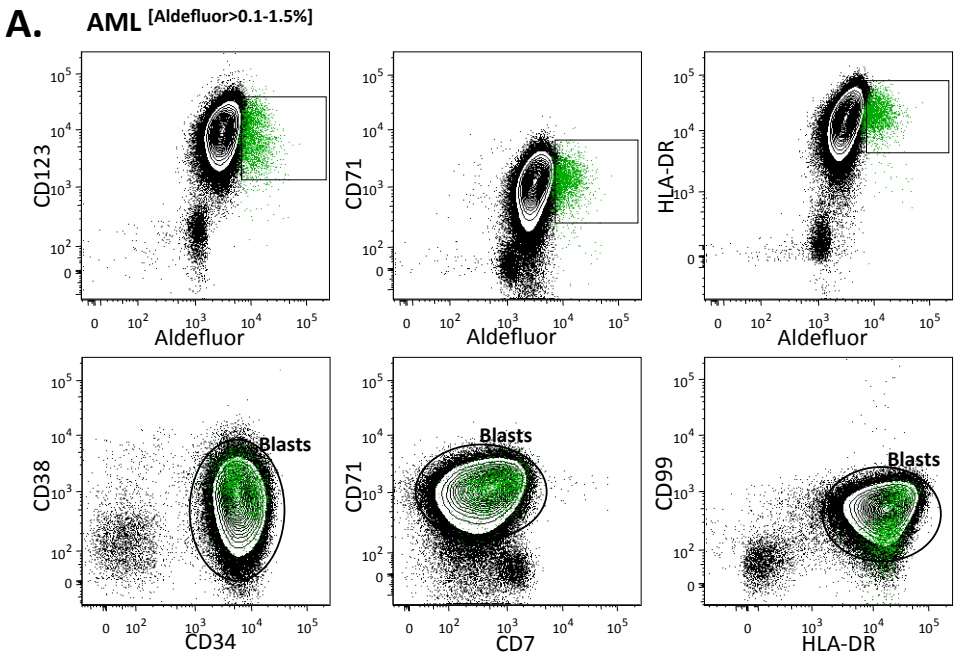


Figure 4A. Flow cytometric analysis of an AML [Aldefluor>0.1-1.5%] with few ALDH⁺ cells (green) that overlay on the blast gates.

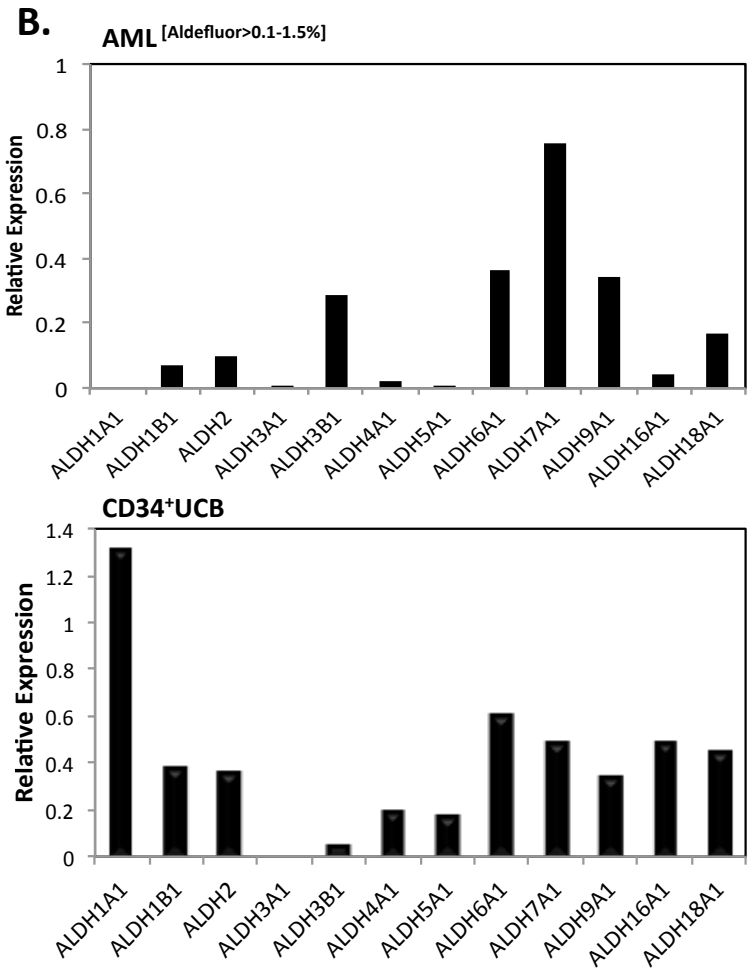


Figure 4B. qPCR for ALDH isoforms from an AML [Aldefluor>0.1-1.5%] and from a CD34⁺UCB normal control.

Supplemental Methods

Analysis of public databases

For analysis of cytogenetic risk groups, overall survival (OS) and event free survival (EFS), 165 AML specimens from The Cancer Genome Atlas (TCGA) with complete RNAseq, cytogenetic risk, and survival outcome were utilized. In other analyses where cytogenetic risk and survival outcome data were not needed, 179 AML specimens with complete RNA-Seq data were analyzed (TCGA).

Flow cytometry analysis

Flow cytometric analysis was performed on a FACSCalibur device, FACSAria or an LSRII (Becton Dickinson (BD); San Jose, CA) and data was analyzed using FlowJo Software (TreeStar; Palo Alto, CA). Cell sorting was performed on FACSAria device equipped with 405nm violet, 488 nm argon and 633 HeNe lasers (BD).

Cytotoxicity studies

Primary AML, Kasumi-1, Kasumi-3 and MOLM-13 cells were incubated overnight at 37°C with 1-5μM ATO and 6-25μM 4HC, or an equivalent volume of vehicle (EtOH or DMSO). Higher concentrations of 4HNE (20-40μM) were used for short-term incubation experiments where indicated in the figure legends. Viability was measured with the LIVE/DEAD® Fixable Near-IR Dead Cell kit (Invitrogen; Carlsbad, CA) for all experiments requiring fixation/permeabilization. For ROS or apoptosis detection, cells were incubated with surface antibodies for 30 min before adding 5μM MitoSOX, CellROX (Invitrogen, Molecular Probes) or 5μl AnnexinV (BD) for an additional 30 minutes at 37°C. Cells were resuspended in serum-free media or AnnexinV buffer, containing the cell viability dye 4',6-diamidino-2-phenylindole (DAPI) at 1μg/ml.

PCR and Quantitative Real Time PCR (qPCR)

Total mRNA was isolated with a PicoPure RNA isolation Kit (Thermo Fisher Scientific; Waltham MA), mRNA samples were reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics USA) and quantitative real-time PCR (qPCR) was performed with LightCycler480 or 96 systems (Roche) using a LightCycler 480 SYBR Green I Master Mix or a Probes Master reaction mix for detection of ALDH isoform RNAs.

Xenotransplant studies

To test the drug sensitivity of the ALDH1A1$^-$ AML cell line MOLM-13 *in vivo*, 12 NSGS mice were treated intraperitoneally (IP) with busulfan (30mg/kg) then injected intravenously (IV) with 7x10^6 AML cells. Five days post injection, 6 control mice were treated IP with saline while 6 mice were treated IP with 5µg/g ATO daily for 4 days together with 150µg/g Cy at day 1 and day 4. The mice were sacrificed by CO2 asphyxiation after 6 more days and analyzed for MOLM-13 content by flow cytometry. To test primary AMLs in the xenotransplant model, first, three different primary ALDH1A1$^-$ AMLs were cultured in IMDM+Ins-Trans-Se+4%FBS+Pen/Strep (STEMCELL Technologies; Vancouver, BC, Canada) at a final concentration of 1x10^6 cells/ml and treated with vehicle control or 4HC (30µM/ml) plus ATO (5µM/ml) for 24 hours. 5x10^6 treated cells were then transplanted per recipient into 8-10 week old NSGS mice; 12-13 weeks later, femora were flushed and human AML engraftment was analyzed by flow cytometry. To assess the sensitivity of primary AML cells *in vivo* to Cy and ATO, 5x10^6 ALDH1A1$^-$ or ALDH1A1$^+$ AML cells were injected intravenously into 10 busulfan-conditioned NSGS mice. After marrow engraftment was confirmed in 2 representative mice, Cy and ATO were administered as described above for the MOLM-13 studies. Fourteen or nineteen days later, marrow was harvested and human AML levels determined by flow cytometry. For studies of sensitivity of normal human HSCs and progenitors to 4HC and ATO, CD34$^+$UCB cells were treated in vitro and transplanted as described above with human engraftment measured in marrow 13 weeks later using flow cytometry.

Study Approval

NSGS mice used for xenotransplantation studies were maintained in the University of Colorado in compliance with the University of Colorado Institutional Animal Care and Use Committee and National Institutes of Health Guidelines. Human bone marrow and peripheral blood samples were obtained from patients diagnosed with AML at the University of Rochester and the University of Colorado after obtaining IRB approved informed consent. Cells typically had a post-thaw viability of >70%.