## Supplemental Methods

**mRNA Hybridization and Gene-expression Profiling.** OCI-AML3 and REH cells were exposed to 1% and 21% $O_2$ for 48 hours and total RNA was extracted. After confirmation of RNA quality using a Bioanalyzer 2100 instrument (Agilent Technologies, Inc.), 300 ng of total RNA was amplified and biotin-labeled through an Eberwine procedure using an Illumina TotalPrep RNA Amplification kit (Life Technologies) and hybridized to Illumina HT12 version 4 human whole-genome arrays. Each of these arrays has an average of 15 beads for each of > 48,000 probes measuring > 25,000 annotated genes and additional transcripts. Bead-level data were processed by methods previously described(1). In brief, outlier-filtered bead values underwent model-based background correction,(2) quantile normalization, filtering for probe quality,(3) and log2 transformation. Candidate differentially-expressed probes (DEPs) were then determined for each of 3 independent experiments, comparing cells cultured under hypoxia or normoxia. The significantly deferentially expressed genes in relation to hypoxia were determined using strict statistical criteria with *P* value < 0.01, false discovery rate *q* statistic < 0.1(4). The 1158 genes were significantly upregulated in the OCI-AML3 cells and 69 in REH with respect to hypoxia. Among those genes 41 genes were upregulated in both sets. Final DEPs were those candidates found in all 3 experiments, and their fold-change was determined from the average of mean bead values for each experiment.

**Gene expression analysis by Nanostring technology.** Mononuclear cells from BM and peripheral blood samples from patients with acute leukemia or healthy subjects were lysed to isolate total mRNA. For each sample, 100 ng of mRNA was used to set

up Nanostring codeset; the genes included in the analysis are listed in Supplementary Table S3. The samples were processed per the manufacturer's instructions. For analysis, each sample was normalized to the codeset-positive controls and then to the housekeeping genes. Samples were normalized to each other by comparing to the average of all samples. Genes with less than 30 counts were considered non-expressed and eliminated from further analysis.

**Hyperpolarized Magnetic Resonance Spectroscopy.**

For hyperpolarized $^{13}C$ acquisition, a 15-mm axial slice was prescribed to cover the full extent of the mouse femur (Fig. 2A). A slice-selective pulse-acquisition sequence (TR = 1,500 ms; 15° flip angle; 5 KHz spectral bandwidth; 2048 spectral points; 100 repetitions) was triggered by the HyperSense. Shortly after scan initiation, 200 µL of the hyperpolarized [1-$^{13}C$]pyruvate solution was administered over a ~12-s period. Data were processed to generate dynamic curves characterizing the arrival of hyperpolarized pyruvate and its chemical conversion into lactate. Normalized lactate, defined as the ratio of total cumulative lactate to the total HP carbon signal, was calculated for each $^{13}C$ scan.

***In Vitro* 1-$^{13}$C Pyruvic Acid Feeding Studies and High Resolution Nuclear Magnetic Resonance (NMR) Experiments**

AML/ETO cells were co-cultured with human mesenchymal stromal cells (MSC) cells in MEM media supplemented with 10% FBS until a cell density a 0.5 x $10^6$ cells/ml was reached. When that concentration was achieved, half of the culture was incubated with media containing 1 mM of 1-$^{13}C$ pyruvic acid and the other half not (control). Three 150

μl aliquots were taken at time of addition of the media ($t_o$ time points), 1 hour into the incubation ($t_1$ time points), and 8 hours into the incubation ($t_8$ time points). After 8 hours, the media was removed.

*Sample Preparation*: NMR standard solution was added directly to the media aliquots (final concentration, 5 mM 3-(trimethylsilyl)-1-propanesulfonic acid-$d_6$ sodium salt (DSS-$d_6$) (613150, Sigma-Aldrich, St. Louis, MO), 5 mM potassium phosphate pH 8).

*Analysis of Samples:* Nuclear magnetic resonance (NMR) spectroscopy was performed at MD Anderson's NMR facility. All one dimensional $^{13}C$ spectroscopy were performed on a 500 MHz Bruker Avance III HD NMR equipped with a Prodigy BBO CryoProbe (Billerica, MA).

All media aliquot samples were analyzed using 1D proton-decoupled 13C spectroscopy (256 scans, 29761-Hz spectral width, 10 second relaxation delay, 30-degree flip angle). The data was analyzed using MestreNova software package. All 13C spectra were processed with a line broadening of 1 Hz, magnitude phasing, baseline correction, and referenced to trimethylsilyl moiety of DSS-d6 at 0 ppm. The chemical shifts for the metabolites were determined using the Biological Magnetic Resonance Data Bank (University of Wisconsin). Integration values were determined for the NMR standard (DSS-d6), 1-13C pyruvate, and 1-13C lactate. The ratio of the integration for the metabolite resonance over the integration value for DSS-d6 resonance was used to normalize samples (Supplemental Table 4).

**Three-dimensional spheroids**. Cell monolayers were maintained in log-phase. To create three-dimensional (3D) spheroids, cells were dissociated and centrifuged, and the resulting cell pellet was first resuspended in medium at $50\times10^6$ cells/mL and this

suspension was mixed 50/50 with Matrigel. To generate mesenchymal stromal cell (MSC)–leukemic cell spheroids, stromal cells were mixed with leukemic cells at a ratio of 2:1. Next, 1.0 µL of the Matrigel/cell mixture was dropped into 3 mL of medium and incubated overnight. The next day, spheroids were transferred to 15-mL tubes. A spheroid pellet was formed after 30 min incubation at room temperature. The medium was removed and spheroids were mixed 50/50 with collagen I (BD Biosciences) on ice. Final collagen I concentration in the culture was 2 mg/mL. Next, 100-µL aliquots of the spheroid/collagen mixture were transferred to the bottom chambers of a Chambered Coverglass System (Thermo Scientific). After 20 min of polymerization at 37°C, 400 µL of medium was added to each chamber. The resulting spheroid suspensions were incubated at 37°C, 96% humidity, and 5% $CO_2$ for several days; they were then analyzed by confocal microscopy (Olympus FluoView FV1000, Olympus America).

To assess TH-302 cytotoxicity, spheroids were set up directly in 96-well plates. Briefly, 1.0 µL of the Matrigel-cell mixture was dropped into each well, which contained 50 µL of medium containing TH-302 at different concentrations; this was followed by addition of 50 µL of medium containing collagen I. Four wells were used for each condition. After 72 h of incubation, viability was determined by WST-1 assay (Roche Applied Sciences) with a SAFIRE microplate fluorescence spectrometry system (Tecan). Data were analyzed, EC-50 determined, and graphs prepared by GraphPad Prism-4 software (GraphPad).

**Murine tumor models**. All animal work was done in accordance with a protocol approved by the institutional animal care and use committee of MD Anderson Cancer Center. For the syngeneic AML model, 7- to 9-week-old C57Bl/6 mice were

transplanted via tail vein injection with murine BM cells ($1\times10^6$ viable cells per mouse) expressing the AML1/ETO oncoprotein and green fluorescent protein (GFP) (cells provided by Dr Scott Lowe, Cold Spring Harbor, NY; (5)). When at least 5% of GFP-positive cells were detected in peripheral blood by FACS, mice were randomized to receive vehicle (control) or chemotherapy (N=7 or 12, respectively) as follows: vehicle or cytarabine 100 mg/kg intraperitoneally for 5 days or vehicle or doxorubicin 3 mg/kg for 3 days intravenously. One week after finishing treatment, mice from the chemotherapy group were randomized again to receive vehicle or TH-302 (N=6/group). TH-302 was administered at 50 mg/kg intraperitoneally 5 days a week for 2 weeks. Leukemia burden was monitored periodically by noninvasive *in vivo* optical imaging; mice were anesthetized and placed in the IVIS-200 imaging system (Xenogen) after injection of the luciferase substrate colenterazine (Biotium).

For the primary AML xenograft model, BM cells from NSG mice engrafted with a human AML sample were injected intravenously into irradiated (300 cGy) NSG secondary recipients ($0.3\times10^6$ cells/mouse). Starting on day 37, TH-302 at 75 mg/kg or vehicle (PBS) was administered intraperitoneally 3 times a week for 2 weeks (N=10/group). Leukemia burden was monitored weekly by determining the number of circulating human CD45+ cells. For secondary transplant experiments, BM cells from control or TH-302–treated mice (collected after 2 weeks of therapy) were serially diluted and injected intravenously into secondary NSG recipient mice at 0.01 cells/mouse (N=5 mice/dilution).

For the MOLM-13 FLT3ITD model, NSG mice were injected intravenously with $1\times10^6$ MOLM-13 cells labeled with GFP/luciferase. On day 3 after cell injection, mice were

randomized into four groups and treated according to the following schema: vehicle; TH-302 50 mg/kg 5 times a week intraperitoneally for 3 weeks; sorafenib 5 mg/kg by daily oral gavage for 2 weeks; or a combination of TH-302 and sorafenib at the same doses and schedules. Leukemia progression was evaluated periodically by luciferase imaging as already described.

In all models, overall survival of the mice in each group was estimated by the Kaplan-Meier method.

**Immunohistochemistry.** Mice were euthanized and their organs were harvested and fixed by immersion in 4% paraformaldehyde. Immunostaining for hypoxia marker PIMO and hematoxylin and eosin staining were performed as previously described(6).

## References


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**Supplementary Table 1.** Cell line characteristics

| Cell type | Cell line | Cytogenetics/molecular characteristics | Source |
|---|---|---|---|
| B-ALL | Nalm-6 | Pre-B ALL | ATCC |
| | REH | Pre-B ALL | ATCC |
| AML | OCI-AML3 | NPM1, DNMT3A, N-Ras-mutant | Dr Mark Minden |
| | MOLM-13 | FLT3-ITD | DSMZ |
| | KG-1 | p53-mutant | ATCC |
| CML | KBM-5 | BCR-ABL | Dr Miloslav Beran (MDACC) |

**Supplementary Table S2.** Patient characteristics

| Patient # | Diagnosis | Source | Cytogenetics | Molecular diagnostic | % of blasts | Assay |
|---|---|---|---|---|---|---|
| 1 | B-ALL | BM | Pseudodiploid Clone 46,X,-Y,add(1)(q25),+21[20] | No monoclonal immunoglobulin heavy chain gene rearrangement | 97 | Cytotoxicity |
| 2 | B-ALL | PB | Pseudodiploid clone46,XX,t(1;4)(q21;q35),t(5;9)(q23;q34),+der(5)t(5;9),-20[12]; Hyperdiploid metaphases 47,XX,t(1;4)(q21;q35),t(5;9)(q23;p24),+der(5)t(5;9),+8,-20[1] | Monoclonal immunoglobulin heavy chain gene rearrangements , Monoclonal T-cell receptor gamma chain gene rearrangements | 39 | Cytotoxicity |
| 3 | AML | BM | 47,XY,inv(16)(p13.1q22),+22[20] | CBFB gene rearrangement with a deletion of the 3' gene locus | 83 | Cytotoxicity |
| 4 | AML | PB | del(11)(p13p15)[6]; del(7)(q32q34),del(11)(p13p15)[12] | FLT3 ITD | 48 | Nanostring |
| 5 | AML | PB | 39~45,X,del(X)(p11.2p22.1),+1,del(1)(q32q42),del(3)(p21p25),add(5)(p15),-6,-7,-9,-10,del(11)(q23q24),-12,-13,-14,-15,-15,-17,-20,add(21)(p11.2),-22,+3~6mar[cp20] | No FLT3-ITD, RAS or NPM1 mutations | 99 | Nanostring |
| 7 | AML | PB | 46,X,-Y,+8[6] | e1a2 BCR-ABL | 31 | Nanostring |
| 8 | AML | PB | t(9;11)(p22;q13)[3] | No CEBPA, FLT3-ITD, KIT, RAS or NPM1 mutations | 24 | Nanostring |
| 9 | T-ALL | BM | Diploid male karyotype 46,XY[20] | oligoclonal pattern of T-cell receptor gamma and beta chain gene rearrangements | 98 | Nanostring |
| 10 | B-ALL | PB | 8,XY,del(4)(p14),t(9;22)(q34;q11.2),der(14)t(1;14)(q12;q32),der(15)(q22),+21,+der(22)t(9;22)[5].ish t(9;22)(ABL1-;BCR+,ABL1+),der(22)(BCR+,ABL1+)[2] | b3a2 BCR-ABL | 89 | Nanostring |

| | | | | | | |
|---|---|---|---|---|---|---|
| **11** | **B-ALL** | **PB** | **46,Y,-X,t(5;17)(q13;q21),add(8)(p23),t(9;22)(q34;q11.2),del(12)(p13),del(20) (q11.2),-21,+2mar[20]** | **b2a2 BCR-ABL f** | **96** | **Nanostring** |
| **12** | **B-ALL** | **PB** | **49,XX,+del(X)(q24q26),+8,t(9;22)(q34;q11.2),+der(22)t(9;22)[17]//** | **e1a2 BCR-ABL** | **91** | **Nanostring** |
| **13** | **T-ALL** | **BM** | **47,XY,der(5)t(5;12)(q31;q13),der(9)t(9;9)(p21;q11),-12,add(13)(q34), add(14)(p12),del(17)(p12),+18,-21,+2mar[5]** | **monoclonal T-cell receptor beta chain gene rearrangements, oligoclonal pattern of T-cell receptor gamma chain gene rearrangements** | **98** | **Nanostring** |
| **14** | **T-ALL** | **PB** | **46,XY,del(2)(p21p23),add(7)(q32),del(9)(q22),add(10)(q24),-13,-14,+2mar[1]** | **Monoclonal T-cell receptor beta chain gene rearrangements** | **27** | **Nanostring** |
| **15** | **AML** | **PB** | **6,X,t(X;2)(q22;q21),add(3)(q21),del(5)(q13q33),del(7)(q22q34),del(12)(p11.2p13),del(20)(q11.2q13.1)[18]** | | **61** | **Nanostring** |
| **18** | **AML** | **PB** | **Hyperdiploid clone 47,XY,+9[3]** | **IDH2 (R140Q); KRAS (G12C)** | **50** | **Nanostring** |
| **19** | **AML** | **BM (no PIM)** | **46,XX,t(3;5)(p23;q15)?c[20** | **mutation in exon 12 of NPM1 gene; IDH1 (R132S)** | **40** | **Nanostring** |
| **20** | **AML** | **PB** | **Pseudodiploid clone 46,XY,del(5)(q13q33),i(8)(q10),add(15)(p13),-17,i(21)(q10),+mar[7]; Hyperdiploid clone 47,XY,del(5)(q13q33),i(8)(q10),+i(8)(q10),-17,add(19)(p13),i(21)(q10),+mar[6]** | **No CEBPA, FLT3-ITD, KIT, RAS, IDH1/2 or NPM1 mutations** | **83** | **Nanostring** |
| **21** | **AML** | **PB** | **41~45,Y,del(X)(p11.2p22.1),add(1)(p36.3),add(3)(p25),del(5)(q31q35)del(7)(p14p15),+11,del(11)(p11.1),-12,-15,-17,-18,del(18)(q21.1),psudic(20;12)(q13.3;p11.2),add(21)(q22),+1~4mar[cp20]** | **NRAS(G12D)** | **48** | **Nanostring** |

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; B-ALL, B-cell ALL; BM, bone marrow; PB, peripheral blood; T-ALL, T-cell ALL

**Supplemental Table S3.** List of upregulated DEPs in hypoxia.

| GENE Abbreviation | Name | PROBE_ID | REFSEQ_ID |
|---|---|---|---|
| GAPDH | glyceraldehyde-3-phosphate dehydrogenase | **ILMN_2038778** | NM_002046.3 |
| ARNT | aryl hydrocarbon receptor nuclear translocator | **ILMN_1697519** | NM_178427.1 |
| BNIP3 | BCL2/adenovirus E1B 19kDa interacting protein 3 | **ILMN_1724658** | NM_004052.2 |
| DDIT4 | DNA-damage-inducible transcript 4 | **ILMN_1661599** | NM_019058.2 |
| EGLN1 | egl-9 family hypoxia-inducible factor 1 | **ILMN_1749892** | NM_022051.1 |
| EPAS1 | endothelial PAS domain protein 1 | **ILMN_1760034** | NM_001430.3 |
| HIF1A | hypoxia inducible factor 1 alpha | **ILMN_1681283** | NM_001530.2 |
| HIF1AN | hypoxia inducible factor 1, alpha subunit inhibitor | **ILMN_1681812** | NM_017902.2 |
| HK2 | hexokinase 2 | **ILMN_1723486** | NM_000189.4 |
| HMOX1 | heme oxygenase (decycling) 1 | **ILMN_1800512** | NM_002133.1 |
| LDHA | lactate dehydrogenase A | **ILMN_1807106** | NM_005566.1 |
| NDRG1 | N-myc downstream regulated 1 | **ILMN_1809931** | NM_006096.2 |
| PDK1 | pyruvate dehydrogenase kinase, isozyme 1 | **ILMN_1670256** | NM_002610.3 |
| PGK1 | phosphoglycerate kinase 1 | **ILMN_1755749** | NM_000291.2 |
| PGM1 | phosphoglucomutase 1 | **ILMN_1800659** | NM_002633.2 |
| SERPINE1 | serpin peptidase inhibitor, clade E, member 1 | **ILMN_1744381** | NM_000602.1 |
| SLC2A1 | solute carrier family 2 (facilitated glucose transporter), member 1 | **ILMN_1809256** | NM_006516.1 |

| | | | |
|---|---|---|---|
| TGFA | transforming growth factor, alpha | **ILMN_1805175** | NM_001099691.1 |
| TGFB1 | transforming growth factor, beta 1 | **ILMN_1665766** | NM_000660.3 |
| VIM | vimentin | **ILMN_1782538** | NM_003380.2 |
| VLDLR | very low density lipoprotein receptor | **ILMN_1675092** | NM_003383.3 |

**Supplemental Table S3.** Differentially-expressed probes (DEPs) were determined for each of 3 independent experiments, comparing cells cultured under 21% $O_2$ or 1% $O_2$ with a significance threshold of p-value < 0.01 and false discovery rate q statistic < 0.1. **ILMN_Gene:** Illumina gene nomenclature.

**Supplemental Table S4.** 1-13C pyruvate uptake and 1-13C lactate production in cell culture.

| $^{13}$C Chemical Shift | Pyruvate at $t_0$ | Pyruvate at $t_1$ | Pyruvate at $t_8$ |
|---|---|---|---|
| 173.0 ppm Pyruvate | 0.623 ± 0.084 | 0.554 ± 0.007 | 0.067 ± 0.021 |
| 185.2 ppm Lactate | 0* | 0.121 ± 0.020 | 0.846 ± 0.039 |

$T_0$ is beginning of the experiment, t1 and t8 refers to 1 and 8 h into the incubation, respectively.

*Peaks above background for lactate were not seen in $t_0$ samples.

Normalized integration values given as Mean + Standard Error of the Mean. Based on these values, approximately 11% of the pyruvate is taken up by the culture in 1 hour and increases to 89% in 8 hours.

**Supplemental Table S5.** TH-302 cytotoxic activity against leukemia cell lines

| Cell line | Normoxic $IC_{50}$(μM) | Hypoxic $IC_{50}$(μM) | HCR |
|---|---|---|---|
| KBM5 | 1.8 | 0.04 | 44 |
| KG-1 | 3.3 | 0.04 | 81 |
| OCI-AML3 | 2.6 | 0.01 | 260 |
| MOLM-13 | 2.5 | 0.17 | 14 |
| REH | 8.7 | 0.8 | 11 |
| Nalm6 | 1.3 | 0.005 | 257 |

HCR, hypoxic cytotoxicity ratio ($IC_{50}$normoxia/$IC_{50}$ hypoxia)