The central nervous system microenvironment influences the leukemia transcriptome and enhances leukemia chemo-resistance

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Supplementary Information

Materials and Methods

Reagents and Cell Culture: Leukemia cell lines and HS-5 cells were obtained from ATCC and DSMZ. The leukemia cell lines were cultured in RPMI media supplemented with Fetal Bovine Serum (FBS; Seradigm, Radnor, PA) 10% and Penicillin-Streptomycin. HS-5 cells were cultured in DMEM media supplemented with FBS 10% and Penicillin-Streptomycin. Z310 choroid plexus cells were provided by Dr. Wei Zheng (Purdue University) and cultured in DMEM media supplemented with FBS 10% and Penicillin-Streptomycin. Human cerebral spinal fluid (CSF) from healthy donors was obtained from Lee Biosolutions (Maryland Heights, MO).

Xenograft studies: NSG (NOD.Cg-*Prkdcscid, Il2rgtm1Wjl*/SzJ; Jackson Labs, Bar Harbor, ME) mice were housed under aseptic conditions and received autoclaved cages, bedding material, water, bottles, and irradiated food. Mouse care and experiments were in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of Minnesota. 4-7 week old mice were injected intravenously via the tail vein with ~$2x10^6$ leukemia cells. At signs of systemic leukemia development, including hunched posture, ruffled fur, reduced mobility, labored breathing, loss of motor function, hind limb paralysis, lethargy, and poor feeding/weight loss, the mice were euthanized and perfused with PBS. To isolate leukemia cells from the CNS, the cranial vault was gently scraped to retrieve leukemia cells associated with the leptomeninges and then the whole brain was removed, gently washed once in PBS, and then vortexed at low speeds for 2-3 minutes to collect leukemia cells. To isolate bone marrow leukemia cells, femurs were removed, crushed with mortar and pestle, and red cell lysed with RBC Lysis Buffer (eBioscience, San Diego, CA). Leukemia cells from both the CNS and bone marrow of typically 3 mice were combined and further purified using human CD19 magnetic MicroBeads (Militenyi) according to the manufacturer's instructions. For

immunohistochemistry experiments, femurs and brains were removed, fixed in paraformaldehyde 4%, embedded in paraffin, processed, and stained with a human CD10 antibody.

Nanostring & quantitative RT-PCR: In triplicate, CD19 microbead purified leukemia cells from the bone marrow and CNS of ~3 mice were combined for RNA extraction and processing. Total RNA was extracted from leukemia cells using the RNeasy Mini kit (Qiagen, Valencia, CA). Gene expression on the NanoString® platform (NanoString Technologies, Seattle, WA) was measured with the NanoString PanCancer Pathways Panel consisting of 730 cancer-associated genes and 40 reference or housekeeping genes. nSolver Analysis Software v3.0 was used for data quality control, normalization using positive and negative controls as well as housekeeping reference genes, and analysis.

For quantitative RT-PCR, total RNA was reverse transcribed using qScript cDNA Synthesis Kit (Quanta Bio; Beverly, MA). Gene-specific primers were designed using PrimerBank software (https://pga.mgh.harvard.edu/primerbank/). The primers used were 5′-GACAACTCAGTGGAGCATTCA-3′ (forward) and 5′- CTCTCGCAGGAGATTCATCAC-3′ (reverse) for *PBX1* and 5′-CTCCATCATGAAGTGTGACGTGGA-3′ (forward) and 5′-CAGGAAAGACACCCACCTTGATCT-3′ (reverse) for *GAPDH*. Quantitative PCR was performed using PerfeCTa SYBR Green Fastmix (Quanta Bio; Beverly, MA) on an ABI 7500 Fast Real Time PCR system. All reactions were run in triplicate, and the relative expression of *PBX1* was calculated by normalizing *PBX1* mRNA expression to *GAPDH* mRNA expression.

Proliferation, apoptosis, and colony assays: In 96-well plate format, leukemia cells were treated as described for 48 hours and then viability assessed with the CellTiter-Glo Luminescent Cell Viability Assay (Promega; Madison, WI). All experiments were performed in triplicate with at

least 3 wells per condition. To assess apoptosis, leukemia cells were treated as described for 48 hours, stained with annexin-V, and analyzed by flow cytometry. For colony-formation assays, 10^3 NALM-6, NALM6-GFP, or NALM6-PBX1 cells were plated in methylcellulose (Methocult 4100; StemCell Technologies; Vancouver, BC) supplemented with FCS 10% and colonies were counted 10 days after plating.

Leukemia co-culture: 10 x 10^6 leukemia cells (NALM-6 or SEM) were plated onto 80% confluent Z310 choroid plexus cells (10 cm plate) and grown in a 1:1 mixture of RPMI and CNS cell media for 3 hours and 48 hours. The media was then aspirated and the co-cultures were gently washed with ice cold PBS. Cells were then trypsinized and leukemia cells were isolated from choroid plexus cells by CD19 MACS positive selection (Miltenyi; San Diego, CA). In a separate experiment, NALM-6 cells were labeled with CellTrace Violet (Invitrogen; Carlsbad, CA) and grown on GFP expressing Z310 cells. The leukemia cells were then isolated by CD19 MACS positive selection as described, and the degree of contaminating Z310 cells in the purified NALM-6 cells was measured by flow cytometry (FACSCanto) (Supplementary Figure 1B).

Western Blotting: Whole cell lysis was performed at 4 °C for 15 minutes in RIPA buffer (Rockland; Limerick, PA) supplemented with protease and phosphatase inhibitors (Roche; Basel, Switzerland). Protein content was estimated by bicinchoninic acid assay (BCA) and equivalent amounts were resolved by SDS-PAGE on 8-16% gradient gels (Novex). Proteins were then transferred to nitrocellulose or PDVF membranes and probed with primary antibodies against PBX1 (Cell Signaling Technologies; Danvers, MA) and beta actin (Thermo Scientific; Waltham, MA), followed by HRP conjugated secondary antibody (Cell Signaling Technologies). Protein bands were detected by enhanced chemiluminescence (Pierce) and captured onto auto-radiographic film.

Retrovirus production and titration. Retroviral vector supernatants were generated by co-transfecting 10 μg of retroviral transfer vector, 10 μg of gag-pol, and 3 μg of VSV-G packaging plasmids into HEK293T (ATCC) cells in a 10 cm culture dish using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA). Viral supernatants were collected 24 and 48 hours after transfection and filtered through a 0.4 μm membrane (Corning, NY). To determine the retroviral titer, NIH3T3 cells were infected with the virus in the presence of polybrene (8 μg/ml) and analyzed 48 hours post-transduction for GFP expression by flow cytometry.

Viral transduction: Leukemia cells adhered to retronectin-coated tissue culture plates were transduced with virus for 48 hours. Transduced cells were subsequently sorted for GFP expression using flow cytometry (BD FACS Aria II) or selected by puromycin. Post-sort purity check confirmed >98% of cells expressed GFP.

CP Conditioned Media and CSF experiments: Z310 choroid plexus cells were cultured until confluent. After an additional 48 hours the conditioned media was collected, centrifuged at 2000 rpm for 10 minutes to remove any cellular debris, and then filtered through as 0.45 μm filter. The conditioned media was then concentrated on a 10K molecular weight cutoff filter (Millipore) and then reconstituted to the original volume with complete DMEM (antibiotic/mycotic, 10% FCS). NALM-6 cells (500,000 per milliliter) were then seeded into the Z310 conditioned media and cultured for 48 hours prior to protein lysate preparation. Pooled human CSF was obtained from Lee Biosolutions (Maryland Heights, MO). NALM-6 cells were grown in either Z310 conditioned media, human CSF, or regular media for 48 hours before the leukemia cells were lysed and assessed for PBX1 by western blot.

shRNA experiments: shRNA lentiviral particles targeting PBX1, or control shRNA lentiviral particles, were obtained from Santa Cruz Biotechnology (Dallas, TX). SEM leukemia cells were transduced and puromycin selected as described above. Leukemia cells expressing shControl and shPBX1 were then labeled with CellTrace Violet and adhered to Z310 cells, or cultured in suspension as a control, for 36 hours prior to the addition of cyatarbine 500 nM or methotrexate 500 nM. After 36 hours of drug treatment, cells were stained with annexin-V and analyzed by flow cytometry after gating on the violet leukemia cells

Quantitation of leukemia cells in the CNS: As described above, mice were injected intravenously via the tail vein with ~$2x10^6$ NALM-6, NALM6-GFP, or NALM6-PBX1 leukemia cells. The mice were euthanized 21 days after injection and perfused with PBS. To isolate leukemia cells from the CNS, the cranial vault was gently scraped to retrieve leukemia cells associated with the leptomeninges and then the whole brain was removed and vortexed at low speeds for 2-3 minutes to collect leukemia cells. Leukemia cells were stained for CD19 (eBioscience; Anti-Human CD19 APC, clone HIB19) and counted using CountBright™ Absolute Counting Beads per the manufacturer's instructions by flow cytometry using a BD FACSCanto™ II Cell Analyzer.

Microscopy: Leukemia colony images were taken with an EVOS FL Auto Imaging System (Life Technologies).

Statistical analysis: Results are shown as the mean plus or minus the SEM of the results of at least 3 experiments. The Student's *t*-test or ANOVA were used for statistical comparisons between groups and were calculated using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA). *P*-values less than 0.05 were considered statistically significant.

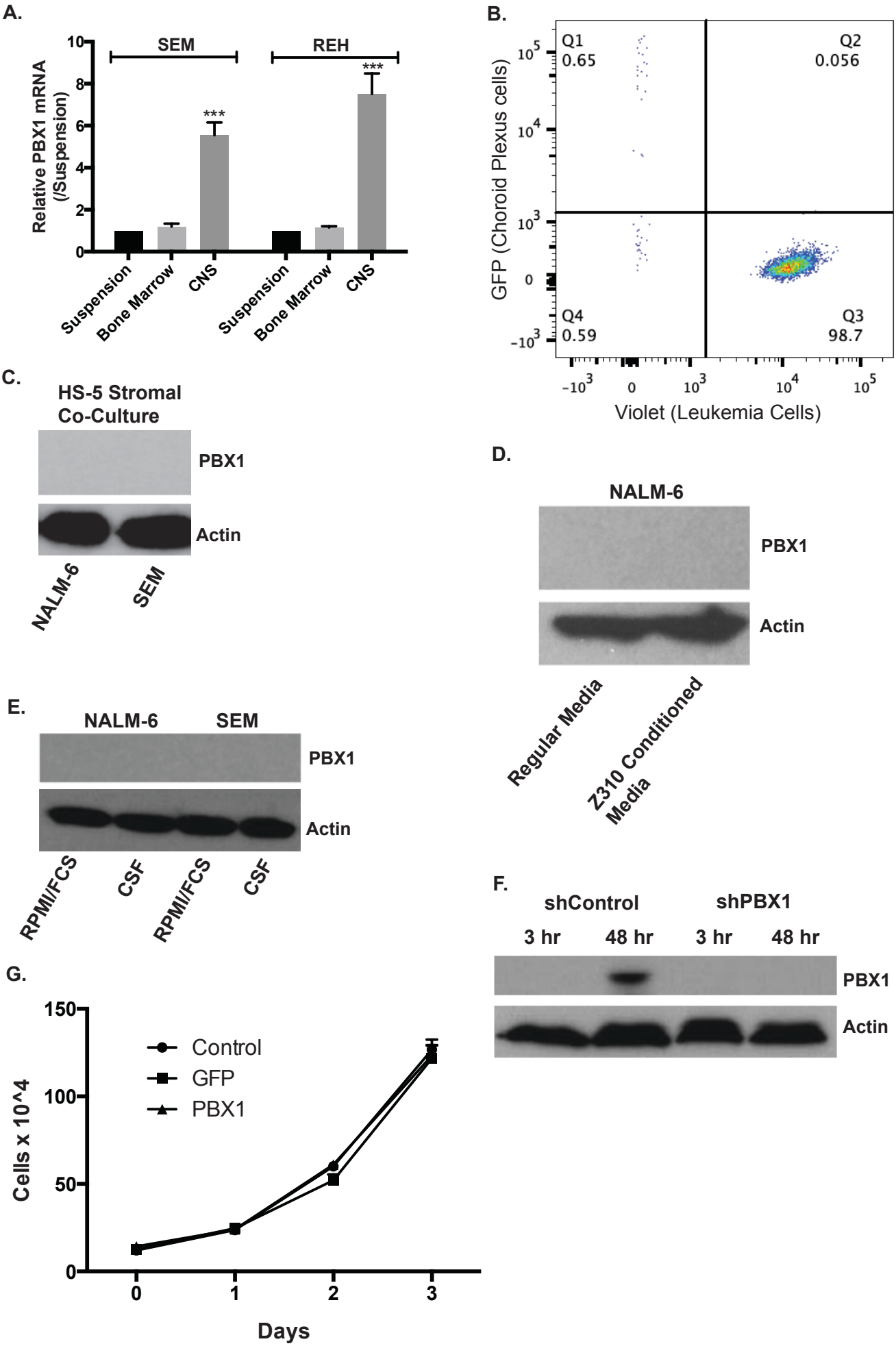
Supplementary Figure Legends

Supplementary Figure 1: A. *PBX1 is up-regulated in additional leukemia cell lines in the CNS niche.* Expression of *PBX1* in REH and SEM leukemia cells isolated from the mouse bone marrow or CNS relative to leukemia cells in suspension as determined by quantitative RT-PCR. *PBX1* mRNA levels were normalized to *GAPDH* expression for each sample. Reactions were performed in triplicate and the data are the mean +/- SEM. from three independent experiments. ***, $P<0.0005$ when compared to leukemia cells grown in suspension. **B.** *Purity of leukemia cells isolated with CD19 microbeads.* Leukemia cells, labeled with CellTrace Violet, were co-cultured with Z310 cells expressing GFP. After 48 hours, the leukemia cells were purified using CD19 microbeads and purity assessed by flow cytometry. A representative flow cytometry plot, showing percent of cells in each quadrant, illustrates minimal GFP positive (Z310) cells present after CD19 microbead isolation of leukemia cells. **C-D.** PBX1 *is not up-regulated in 1) leukemia cells cultured adherent to HS-5 bone marrow stromal cells (**C**), 2) choroid plexus conditioned media (**D**), or 3) cerebral spinal fluid (CSF;* **E***).* Western blots show PBX1 and beta actin protein levels in leukemia cells cultured for 48 hours in the described conditions. **F:** *shRNA targeting PBX1 blocks PBX1 up-regulation in leukemia cells co-cultured with choroid plexus cells*. Western blot showing PBX1 and beta actin protein levels in SEM leukemia cells expressing either shControl or shPBX1 after being co-cultured adherent to Z310 choroid plexus cells and then isolated using CD19 microbeads. **G:** *Effect of ectopic PBX1 expression on NALM-6 leukemia cell proliferation.* The proliferation of NALM-6 cells expressing GFP, PBX1, or control cells was measured by determining cell number using trypan blue exclusion at 0, 24, 48, and 72 hours. The data are the mean +/- SEM from three independent experiments.

Supplementary References

1 Chen J, Bardes EE, Aronow BJ, Jegga AG. ToppGene Suite for gene list enrichment analysis and candidate gene prioritization. *Nucleic Acids Res* 2009; **37**: W305–11.

Supplementary Figure 1A-G



	Gene	P value	Mean BM	Mean CNS	Difference	SE	t ratio
Up-Regulated	FGF12	1.3E-07	1.01333	36.8033	-35.79	0.434652	82.3418
	AKT3	1.46E-06	5.66	220.96	-215.3	4.78914	44.9559
	PBX1	2.64E-06	1.00333	154.2	-153.197	3.95175	38.7668
	PRKCB	4.21E-06	7.97333	248.35	-240.377	6.96472	34.5135
	FBXW7	8.07E-06	55.0667	95.99	-40.9233	1.39645	29.3052
	MAPT	1.73E-05	1.00333	65.7133	-64.71	2.67366	24.2027
	RASGRP1	2.1E-05	1.00333	30.1	-29.0967	1.26176	23.0603
	TSPAN7	4.7E-05	5.76667	245.653	-239.887	12.7519	18.8118
	PRKCG	5.59E-05	1.00333	35.13	-34.1267	1.89487	18.01
	FGF13	6.98E-05	1.00333	80.3333	-79.33	4.65943	17.0257
	VEGFA	7.49E-05	95.1967	384.987	-289.79	17.3285	16.7233
	BMP4	9.01E-05	1.29	16.8	-15.51	0.971888	15.9586
	RUNX1T1	0.000118	1.00333	24.68	-23.6767	1.58872	14.9029
	BMP6	0.000138	1.01333	35.28	-34.2667	2.39377	14.315
	BNIP3	0.000247	285.89	788.033	-502.143	40.6555	12.3512
	RASGRF1	0.000266	1.01333	9.30333	-8.29	0.684268	12.1151
	CACNA1G	0.000293	1.29	61.9133	-60.6233	5.1264	11.8257
	MAPK8IP1	0.00044	2.95	168.49	-165.54	15.5433	10.6503
	RELN	0.000508	1.00333	82.2167	-81.2133	7.91341	10.2627
	ETV1	0.000521	1.00333	54.16	-53.1567	5.21156	10.1998
	RAD52	0.000589	44.27	103.427	-59.1567	5.98731	9.88034
	GRIN1	0.000595	1.00333	169.753	-168.75	17.1248	9.85413
	ZBTB16	0.000662	1.00333	30.1967	-29.1933	3.0458	9.58479
	MAP3K12	0.000784	3.35333	27.9033	-24.55	2.67572	9.17512
	COL5A1	0.000794	95.1	255.2	-160.1	17.5119	9.14235
	FGFR2	0.000926	2.67333	19.08	-16.4067	1.86747	8.7855
	DDIT4	0.001383	69.42	150.323	-80.9033	10.2291	7.90916
	PAK3	0.002154	1.00333	10.2733	-9.27	1.31804	7.03319
	IL11RA	0.002203	3.99667	16.9033	-12.9067	1.84625	6.99076
	BMP5	0.002487	3.01667	13.9533	-10.9367	1.61609	6.76735
Down-Regulated	HHEX	0.000238	1091.32	732.953	358.367	28.7578	12.4616
	DUSP6	0.000792	944.017	335.683	608.333	66.4865	9.14973
	ALKBH2	0.002567	44.29	26.8367	17.4533	2.60103	6.71015
	ITGA6	0.00268	642.56	276.487	366.073	55.1878	6.63323
	IL1B	0.002848	41.6267	13.5167	28.11	4.30754	6.52577
	ID2	0.003324	48.58	18.8067	29.7733	4.75711	6.2587

Supplementary Table 1: *Niche-regulated leukemia genes***.** NALM-6 genes dysregulated (fold change ≥ 2 and FDR<0.05) in the central nervous system (CNS) niche relative to the bone marrow (BM) niche were identified with the Nanostring PanCancer Pathway Panel.

		ToppGene Analysis						
		ID	Name	Source	pValue	FDR B&H	FDR B&Y	Bonferroni
Molecular Function	1	GO:0008083	growth factor activity		1.82E-07	4.56E-05	2.78E-04	4.56E-05
	2	GO:0070700	BMP receptor binding		5.52E-07	6.93E-05	4.23E-04	1.39E-04
	3	GO:0046983	protein dimerization activity		2.19E-06	1.58E-04	9.65E-04	5.50E-04
	4	GO:0042802	identical protein binding		2.98E-06	1.58E-04	9.65E-04	7.48E-04
	5	GO:0070696	receptor protein serine/threonine kinase binding		3.22E-06	1.58E-04	9.65E-04	8.07E-04
Biological Process	1	GO:0006468	protein phosphorylation		3.94E-12	9.97E-09	8.38E-08	9.97E-09
	2	GO:0000165	MAPK cascade		3.64E-11	4.61E-08	3.87E-07	9.21E-08
	3	GO:0023014	signal transduction by protein phosphorylation		5.88E-11	4.96E-08	4.17E-07	1.49E-07
	4	GO:0006915	apoptotic process		5.18E-10	3.27E-07	2.75E-06	1.31E-06
	5	GO:0012501	programmed cell death		6.54E-10	3.31E-07	2.79E-06	1.66E-06
Pathway	1	83048	MAPK signaling pathway	BioSystems: KEGG	1.46E-12	7.08E-10	4.78E-09	7.08E-10
	2	868085	Ras signaling pathway	BioSystems: KEGG	9.53E-12	2.31E-09	1.56E-08	4.61E-09
	3	83105	Pathways in cancer	BioSystems: KEGG	8.95E-09	1.44E-06	9.76E-06	4.33E-06
	4	83067	Focal adhesion	BioSystems: KEGG	5.75E-08	6.96E-06	4.71E-05	2.79E-05
	5	868086	Rap1 signaling pathway	BioSystems: KEGG	7.46E-08	7.22E-06	4.88E-05	3.61E-05
Disease	1	umls:C0585442	Osteosarcoma of bone	DisGeNET BeFree	1.46E-07	2.13E-04	1.68E-03	2.18E-04
	2	umls:C0029463	Osteosarcoma	DisGeNET Curated	2.85E-07	2.13E-04	1.68E-03	4.25E-04
	3	umls:C1961102	Precursor Cell Lymphoblastic Leukemia Lympho	DisGeNET Curated	4.59E-07	2.29E-04	1.80E-03	6.86E-04
	4	umls:C0023449	Acute lymphocytic leukemia	DisGeNET Curated	1.16E-06	4.33E-04	3.42E-03	1.73E-03
	5	umls:C1458155	Mammary Neoplasms	DisGeNET Curated	8.41E-06	1.16E-03	9.13E-03	1.26E-02

Supplementary Table 2: *Functional analyses of CNS-niche regulated leukemia genes.* Leukemia genes up-regulated (fold change ≥ 2; FDR<0.05) in the CNS niche relative to the bone marrow identified with the Nanostring PanCancer Pathway Panel were analyzed using the ToppGene Suite[1] (https://toppgene.cchmc.org) to identify putative leukemia cell pathways or processes influenced by the CNS niche.