Supporting Information

Orlovsky et al. 10.1073/pnas.1103154108

SI Materials and Methods

Preparation of shRNA Viruses. The human leukemic pro-B-cell line RS4;11 was purchased from DKFZ. For shRNA knockdown experiments, the PLL 3.7 lentilox vector was obtained from L. Van Parijs, and contains a CMV–GFP expression cassette. shRNAs to silence MEISI, HOXA7, HOXA9, and HOXA10 genes were designed using a Hannon algorithm (http://katahdin.cshl.org/siRNA/RNAi.cgi?type=shRNA).

Lentiviral supernatants were obtained from 293T cells co-transfected with the shRNA plasmid, together with packaging plasmids. Lentiviral infection of the RS4;11 leukemic cell line was done at multiplicity of infection of 5–10 transducing units/cell (titers of the stocks, concentrated by ultracentrifugation, were ~1×10^8 transducing units/mL). GFP-positive cells were sorted using FACSVantage cell sorter (BD Biosciences).

Antibodies. Abs included polyclonal anti-HOXA9 Ab (Millipore, 07–178), polyclonal anti–HOXA7 Ab (Millipore, 09–086), polyclonal anti-HOXA10 Ab[22], guinea pig anti-MEIS1 Ab[22], polyclonal rabbit anti-MEIS1 Ab, and polyclonal histone H3 Ab (Millipore, 06–755). Anti-human SDF-1/CXCL12 antibodies for immunohistochemistry were purchased from R & D (MAB350). CXCR4 expression on cells' surface was examined with anti-human CXCR4-PE clone12G5 (R & D, FAB170P). Integrin alfa-4 (Vla-4) and integrin alfa-5 (Vla-5) expression was examined with anti-human VLA4-PE (23933) and VLA5-PE (13547) Santa Cruz antibodies, respectively. Anti-CD44 and anti-SCF R/C-kit Abs were purchased from R & D (BBA10 and AF332, respectively).

siRNA Nucleofection and nCounter Analysis. Pools of MEIS1, HOXA7, HOXA10, or scramble siRNAs (ON-TARGET plus SMART pools from Dharmacon) were nucleofected (Amaxa Instrument) into RS4;11 and SEM cultured cells in portions of 5 micrograms per 5 million cells (total, 25 million cells). Two days later, the nucleofection was repeated, and after 48 h the cells were subjected to RNA extraction with trizole. RNA aliquots of 100 ng biological replicates were analyzed for expression of MEIS1, HOXA, and other genes by hybridization to color-coded oligonucleotides specific to each of the above-mentioned gene transcripts. Synthesis of the oligonucleotides was done by NanoString Technologies, and hybridization and analysis was done using the Prep Station and Digital Analyzer purchased from the company.

Engraftment Experiments. All mouse experiments were approved by the Animal Care and Use Committee of the Weizmann Institute of Science according to National Institutes of Health guidelines. NOD/SCID mice were bred and maintained under defined flora conditions at the Weizmann Institute of Science. RS4;11 cells infected with viruses expressing shRNAs were injected i.v. (1×10^7 cells per mouse) 24 h after sublethal irradiation. One day (16 h) and 1–5 wk later, single cell suspensions were prepared from the BM of the transplanted mice, and engraftment was assayed by flow cytometry acquiring 1×10^6 cells using human-specific anti-CD45–APC (Dako, C7230) mAbs. Cells obtained from mice that did not undergo transplantation, or cells labeled with mouse isotype control antibodies, were used as negative controls.

Flow Cytometry. Cell surface expression of CD45 was assessed by flow cytometry using mouse anti-human CD45 monoclonal antibody (mAb)-APC (Dako). Human plasma and mouse IgG were used to block Fc receptors. All CD45-positive human cells were also positive for GFP encoded by lentiviral vector which harbored the shRNA cassette. BrdU labeling was analyzed with PE mouse anti-human BrdU set (BD Biosciences, 556029). Cells stained with the secondary Ab alone or with IgG2a-PE (BD Biosciences) served as controls.

Analysis of Cell Proliferation, Cell Cycle, and Apoptosis. Identical numbers of cells (5×10^6) were taken from growing cultures, transferred into flasks, and grown in the absence or presence of 200 ng/mL SDF-1-alpha; the total number of cells in the culture was determined every 3–4 d, and cultures were split 1:3 every 3 d. Cell cycle analysis was performed by flow cytometry analysis of propidium iodide-stained cells, or by BrdU incorporation analysis. Cultured cells were exposed to 10 μM BrdU for 4 h. For in vivo analysis, BrdU (BrdU flow kit, BD Biosciences), 2 mg per mouse, was injected i.v. 3 wk after transplantation, and the BM was recovered 12 h later. BrdU incorporation was assayed by flow cytometry analysis. Apoptosis was assayed by flow cytometry analysis after Annexin V-PE (BD Biosciences) staining.

Cell Migration Assay. RS4;11 cells (2×10^5 cells/well) were allowed to migrate toward a gradient of SDF-1. Briefly, 125 ng/mL SDF-1 (PeproTech) was added to the lower chamber of a Costar 24-well 5 μM pore transwell (Corning Incorporated Life Sciences). Four hours later, the percentage of migrated cells was evaluated by FACS.

Immunohistochemistry of BM Sections. Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded mouse BM sections. Samples were deparaffinized and dehydrated. Endogenous peroxidase activity was quenched in 3% H_2O_2 in double-distilled water for 20 min. Staining was performed following a standard indirect avidin-biotin HRP method using the LSAB2-HRP detection kit (DAKO) according to the manufacturer's instructions. Tissue was blocked for 30 min with 1% BSA, washed with Tris-buffered saline, and incubated overnight at 4 °C with 7–10 μg/mL CD45 Ab (Dako LCA 2B11+PD7/26) followed by biotinylated secondary Ab (Dako LASB kit K0673). Control slides were incubated with biotinylated secondary Ab only. Slides were counterstained with Mayer hematoxylin (DAKO). For CXCL12/SDF-1 expression, serial frozen sections were prepared from bone marrow, sections were fixed by acetone, and evaluation was performed using anti-human CXCL12/SDF-1 antibodies purchased from R&D (MAB350).

Adhesion Assay. Twenty-four–well plates were covered with 50 μg/mL fibronectin (FC010, Chemicon International), 1 μg/mL V-1 (ADP5, R & D) or 150 μg/mL hyaluronan (Sigma) ON at 4 °C. The cells were seeded at concentration of 4×10^5 cells/well. An hour later the cells were detached from the plates and counted by FACS.

Statistical Analysis. Data were analyzed statistically by ANOVA. Single factor or *P* value was calculated using the two-tailed Student *t* test in Excel (Microsoft). SE bars are indicated in Figs. S3, S5, and S6.

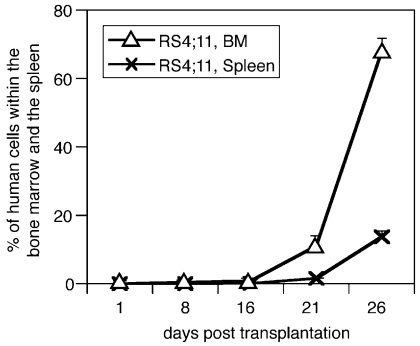

Fig. S1. Most WT RS4;11 cells engraft in the BM of NOD/SCID mice and not in the spleen. RS4;11 intact cells were injected into NOD/SCID mice i.v. 24 h after sublethal irradiation (1×10^7 cells per mouse). Cells were obtained from BM and spleen at different time points after transplantation, and percentage of human cells was assayed by flow cytometry applying human-specific anti-CD45–APC mAbs. Percentage of human cells among mouse cells at the two sites are shown. Results are average ± SD of four independent experiments.

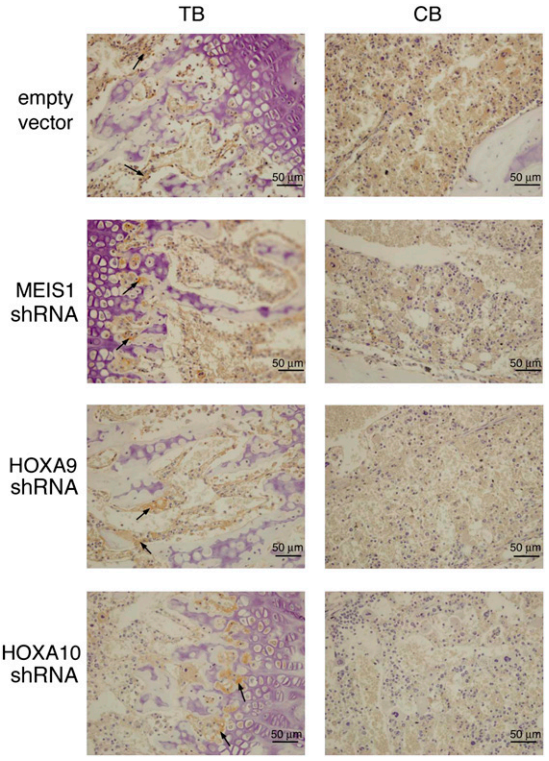

Fig. S2. SDF-1 is produced mostly in the TB of the bone, and is found at similar abundance in BM containing mutant or control RS4;11 cells. BM sections from NOD/SCID mice engrafted with manipulated human RS4;11 cells were subjected to immunohistochemistry, applying anti–SDF-1 mAb. Dako LSAB2 biotinylated Link was used as secondary Ab. Regions expressing SDF1 appear brown and are detected only in TB; some of these regions are indicated by arrows. Constructs as in Fig. 3. CB, compact bone; TB, trabecular bone.

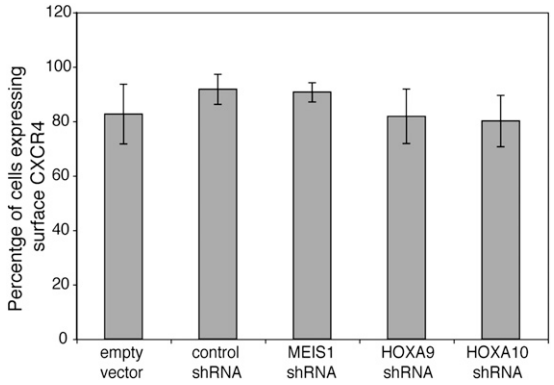

Fig. S3. Similar abundance of surface CXCR4 protein in HOXA and MEIS1 mutants and in control cells. Manipulated RS4;11 cells in culture were examined for surface CXCR4 expression by applying anti-human CXCR4-PE clone 12G5 in flow cytometry analysis. Data are percentages of cells expressing CXCR4. Results are average ± SD of four independent experiments. Mean fluorescent intensity (MFI) of protein in mutant and control cells was highly similar (136, 142, 140, 137, and 135 for empty vector, control shRNA, MEIS1 shRNA, HOXA9 shRNA, and HOXA10 shRNA, respctively). Constructs as in Fig. 3.

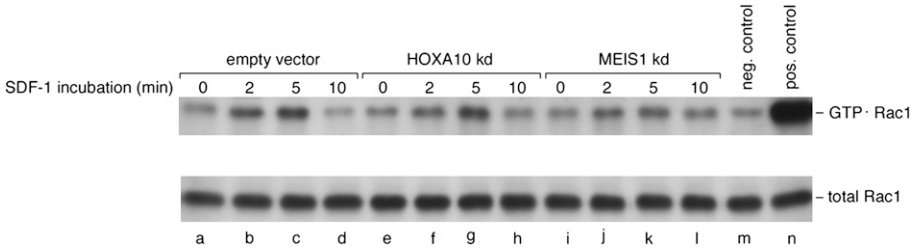

Fig. S4. Comparable kinetics of RAC1 activation (GTP.Rac1) in HOXA10 and MEIS1 knockdown cells and in control cells. RS4;11 cells expressing knockdown (kd) constructs (MEIS1_3a and HOXA10_4a) or empty vector were cultured to ~70% confluency, serum was reduced to 1%, and cells were grown for an additional 6 h. SDF-1 at a concentration of 100 ng/mL was added for 2, 5, or 10 min. Cultures were chilled quickly and thoroughly, and cells were collected by centrifugation at 4 °C. Rac1 activation was assayed using the Rac1/Cdc42 Activation Assay Kit (Millipore, 17–441) according to the manufacturer's instructions. Experiment was repeated five times, yielding similar results.

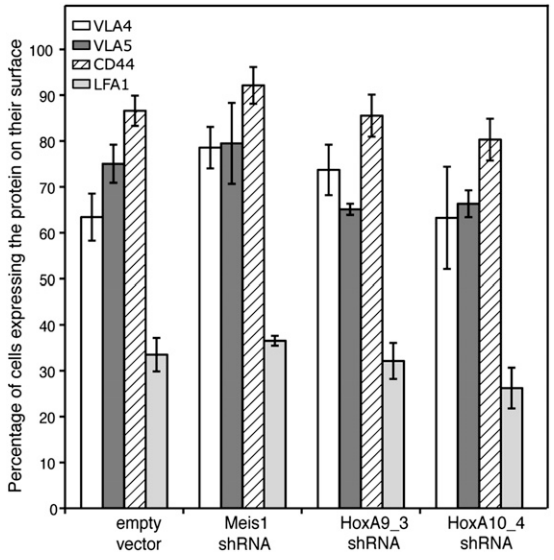

Fig. S5. Similar expression of VLA4, VLA5, CD44, and LFA1 on surface of mutant and control cells. Cultured cells were examined by flow cytometry. Experiments were repeated three or four times. shRNA constructs MEIS1_3a, HOXA9_3 and HOXA10_4a were used.

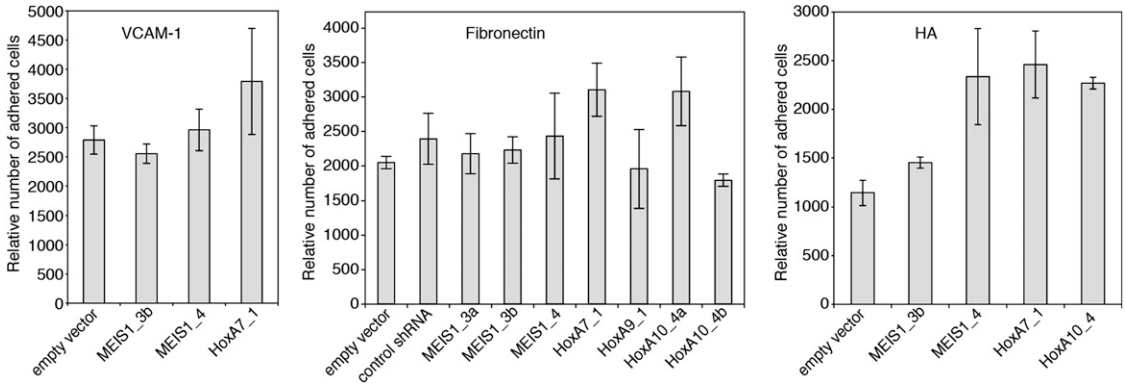

Fig. S6. Comparison between adhesion of mutant and control cells to fibronectin (FN), hyaluronan (HA) and VCAM1. Details of adhesion assay are given in *SI Materials and Methods*. Each experiment was repeated eight times.

Table S1. shRNA constructs

Gene	Accession no.	shRNA construct	Nucleotides and sequence
MEISI	U85707	MEIS1_3	GGCGTGGCTGTTCCAGCATCTA (n. 929–950)
		MEIS1_4	GAGTCATTCAATGAAGATATAG (n. 408–429)
HOXA7	AF032095	HOXA7_1	ACCGCCGCATGAAGTGGAAGAA (n. 2094–2115)
HOXA9	U82759	HOXA9_1	AGCCGGCCTTATGGCATTAAAC (n. 407–428)
		HOXA9_3	TTCTCCTCCAGTTGATAGAGAA (n. 508–529)
HOXA10	AF040714	HOXA10_3	CCGGGAGCTCACAGCCAACTTT (n. 2642–2663)
		HOXA10_4	AGCCAACTGGCTCACGGCAAAG (n. 2423–2444)

n, nucleotides.

Table S2. Impaired engraftment of RS4;11 cells knocked down for *HOXA* or *MEIS1* genes

	1 d	2 wk	3 wk	4 wk	5 wk
Empty vector control	0.10	1.16	24.26	45.01	50.00*
Control shRNA	NM	1.06	23.09	40.22	55.00*
MEIS1_3a	NM	0.17	8.3	NM	23
MEIS1_3b	0.10	0.11	5.17	NM	23
MEIS1_4	0.08	0.16	1.56	NM	NM
HOXA7_1	0.22	NM	7.57	6.52	29.00
HOXA9_1	NM	0.14	4.53	10.36	NM
HOXA9_3	0.16	NM	7.96	3.87	NM
HOXA10_3	0.11	NM	3.67	18.1	NM
HOXA10_4a	0.14	0.24	2.86	4.71	42.00
HOXA10_4b	0.09	0.25	2.02	8.61	NM

Table details engraftment as monitored by percentage of human RS4;11cells in the bone marrow of transplanted mice. Bone marrows were isolated from the transplanted mice at different times after the transplantation, and percentage of human cells in BM was determined by FACS analysis. Numbers 1, 3, and 4 correspond to cells infected with viruses encoding shRNAs directed against different sequences of the gene. a and b correspond to different plasmid clones of the same construct. NM, not monitored.
*More than one-half of transplanted mice died by 5 wk, and analysis is of the surviving animals. These data represent a single experiment out of five independent experiments, each performed in duplicate, and giving similar results.