

## Micro-ribonucleic acid-155 is a direct target of Meis1, but not a driver in acute myeloid leukemia

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## **Supplemental Information**

### **Supplemental Methods**

#### **Generation of cell lines and cell culture**

293T and GP-E86 cells were obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures and maintained in Dulbecco's Modified Eagle Medium 1x (DMEM, Life Technologies) containing 10% Fetal Bovine Serum (FBS, Biochrom), 1% Penicillin/Streptomycin 100x and 1% GlutaMAX™ 100x (PSG, Life Technologies).

C57BL/6J mice were purchased from Charles River or bred and maintained at the University Hospital Ulm Animal Facility or University of Gothenburg Animal Facility. B6.CgMir-155tm1.1Rsky/J (miR-155<sup>-/-</sup>) mice were obtained from the Jackson Laboratory and established as colonies at the University Hospital Ulm Animal Facility or University of Gothenburg Animal Facility. Bone marrow cells were harvested from mice treated 4 days previously with 150 mg 5-FU/kg and stimulated for 48 hours in DMEM supplemented 15% FBS (Stemcell Technologies), 1% PSG, 10 ng/ml human interleukin 6 (hIL6, Peprotech), 6 ng/ml mIL3 and 100 ng/ml murine stem cell factor (mSCF, Peprotech). Cells were infected by cocultivation with irradiated (4000 cGy) GP-E86 cells for MSCV-based retroviral vectors carrying expression cassettes consisting of *Hoxa9*<sup>1</sup>, *Meis1*<sup>2</sup>,  $\Delta$ *HDMeis1*<sup>3</sup> and miR-155<sup>4</sup> in the presence of 5 mg/mL protamine sulfate (Sigma) for 48 hours followed by sorting and further cell culture for at least 12 days to establish immortalized cell lines. All the cell lines were routinely tested to confirm the absence of Mycoplasma.

The proliferation rate of transduced bone marrow (bm) cells was determined by seeding of  $5 \times 10^4$  cells/ml in bm medium in technical duplicates at day 12 post transduction. Cell numbers were determined every 24h via Vi-Cell XR Viability Analyzer (Beckmann Coulter) for 6 days and proliferation rates were expressed relative to seeding cell number. Clonogenic assays were performed by plating  $0.1-1 \times 10^3$  cells/ml in technical duplicates in methylcellulose-containing medium (Methocult M3434, Stemcell Technologies) at day 12 post transduction. After 7 days of culture, colonies were counted and pooled and  $0.1-1 \times 10^3$  cells/ml were re-plated in duplicates in methylcellulose media.

#### **Engraftment analysis and workup of moribund mice**

Donor derived engraftment and reconstitution of transplanted mice were monitored by flow cytometry for GFP<sup>+</sup> and/or YFP<sup>+</sup> expression in the peripheral blood (pb) of the transplanted animals. The health and

viability of mice were monitored daily and sick mice were euthanized and analyzed as previously described<sup>5</sup>. Pb cell counts were determined using a Hemavet 950 (Drew Scientific Group). Cytospin images were visualized using a Zeiss Axioskop microscope with a 50 × numerical aperture objective and with Zeiss Immersol medium (Zeiss). A Zeiss axioCam MRc5 camera and AxioVision 4.6.3 software were used to capture images.

### **Limiting dilution and homing assay**

Limiting dilution assays were performed as described before with transplantation of decreasing numbers of transduced cells as shown in Figure 4B. For *in vivo* homing assay, non-irradiated recipient C57BL/6J mice were transplanted by tail vein injection with  $1 \times 10^7$  YFP<sup>+</sup> Hoxa9/Meis1<sup>155+/+</sup> wildtype or Hoxa9/Meis1<sup>155-/-</sup> bm cells. Mice were sacrificed 12 hours post transplantation and the femoral bm was extracted and processed as previously described<sup>6</sup>. Percentage of YFP<sup>+</sup> cells was determined by flow cytometry.

### **Chromatin immunoprecipitation and sequencing (ChIP-seq)**

ChIP of HA-tagged Meis1 was performed using an anti-HA antibody (Abcam ab9110) and the following antibodies were used in native ChIP for histone modifications: H3K4me1 (Diagenode BP140), H3K4me3 (Diagenode BP1), and H3K36me3 (Abcam BP41) H3K27ac (Abcam ab4729). Illumina sequencing libraries were generated as previously described<sup>7</sup>. Libraries were PCR amplified and sequenced on Illumina HiSeq 2000/2500 sequencing platforms following the manufacturer's protocols (Illumina). Sequence reads were aligned to GRCm38/mm10 and converted to bam format by SAMtools (version 0.1.13).

### **Analysis of miRNA and mRNA expression arrays**

The miRNA was harvested using the miRNeasy Kit (Qiagen) and the mRNA was isolated using the RNeasy Mini Kit (Qiagen). Expression analysis of miRNA and mRNA was done on the Affymetrix GeneChip miRNA 3.0 array and Affymetrix GeneChip Mouse Exon 1.0 ST Array respectively (Affymetrix). All samples were processed and analyzed at the SCIBLU genomic core facility at Lund University. Data analysis was performed using the GeneSpring version 13.1 software. Data in .CEL files were transformed with the ExonRMA16 summarization algorithm to generate RNA expression measures and were normalized using the median of all samples (quantile normalization). Genes below the 20.0th percentile in the raw data were typically filtered away. A difference in expression was considered statistically significant if the fold

change was  $>1.5$  or  $>1.2$  for the *Hoxa9/Meis1*<sup>155/-</sup> array and with a *P* value  $>0.05$  in oneway ANOVA and Benjamini-Hochberg multiple testing correction was used when applicable. Pathway analysis of differentially expressed genes was performed using Onto Pathway Express tool <sup>8</sup>, <http://vortex.cs.wayne.edu/index.html>.

### **Real-time PCR**

RNA was isolated using miRNeasy Mini Kit (Qiagen) and converted to cDNA using PrimeScript RT Reagent Kit (TaKaRa Bio Europe). Reverse transcription of miRNAs via TaqMan<sup>®</sup> microRNA assay (Life Technologies) was performed as previously described <sup>6,9</sup>. Primers used for detection of murine genes with SYBR Premix Ex Taq II Kit (TaKaRa Bio Europe) are available upon request. For human AML samples TaqMan<sup>®</sup> Assays were used in accordance with TaqMan<sup>®</sup> Universal Master Mix II (Life Technologies). For human AML samples TaqMan<sup>®</sup> Assays for HOXA9 (Hs00266821\_m1) and MEIS1 (Hs01017441\_m1) were used and normalized to ABL1 (Hs01104728\_m1). TaqMan<sup>®</sup> miRNA probes for human and murine samples were as follows: mmu-miR-155 (002571), has-miR-155 (002623) and has-miR-708 (002341). MiRNA expression was normalized to that of hsa-RNU6B (001093) (human samples), or snoRNA202 (001232) (transduced cells). qPCR analysis was performed in technical triplicates using an Applied Biosystems 7900HT Fast Real-Time PCR system (Applied Biosystems). All signals were quantified using the  $\Delta\Delta CT$  method.

For microfluidics analysis obtained Ct values were converted into expression thresholds (Et) using the formula  $Et = Ct_{max} - Ct$ , where  $Ct_{max}$  was 40. To remove technical variations, we quantile normalized the Et values.

### **Flow cytometric analysis**

Single-cell suspensions of cultured cells were stained at a density of  $10^7/ml$  for 30 min at 4°C before analysis. The following antibodies were used for immunophenotype determination of murine cell lines: APC-CY7, V450 or APC conjugated anti-CD11b (Mac-1; clone M1/70), PE or PE-CY7 conjugated anti-Gr-1 (clone RB6-8C5), APC conjugated anti-CD117 (c-kit; clone 2B8) (all from eBioscience) and 7 aminoactinomycin D (7AAD, Invitrogen) or Sytox (Life Technologies) for determination of cell viability. For sorting of hematopoietic populations, murine bm cells were stained with monoclonal antibodies specific for, CD19 (clone 6D5), CD3e (clone 145-2C11), CD4 (clone GK1.5), CD8a (clone 53-6.7), CD45R (clone RA3-6B2), TER-119 (clone TER-119), Mac-1 and Gr-1 as PE conjugates to define the lineage negative population (lin<sup>-</sup>). Antibodies specific for Sca-1 (clone D7), CD34 (clone Ram34) and CD16/CD32 (clone 93) conjugated

to PerCP-Cy5.5, Pacific Blue and PE-Cy7, respectively, were used in combination with c-kit for sorting of LSK ( $\text{lin}^-/\text{Sca-1}^+/\text{c-kit}^+$ ), CMP ( $\text{lin}^-/\text{Sca-1}^-/\text{c-kit}^+/\text{CD34}^+/\text{CD16}^-/\text{CD32}^{\text{lo}}$ ), GMP ( $\text{lin}^-/\text{Sca-1}^-/\text{c-kit}^+/\text{CD34}^+/\text{CD16}^-/\text{CD32}^{\text{high}}$ ), MEP ( $\text{lin}^-/\text{Sca-1}^-/\text{c-kit}^+/\text{CD34}^-/\text{CD16}^-/\text{CD32}^-$ ) and granulocyte ( $\text{Mac-1}^+/\text{Gr-1}^+$ ) populations.

For human AML samples and healthy donor blood samples erythrocytes were lysed using 150 mmol/l  $\text{NH}_4\text{Cl}$ , 10 mmol/l  $\text{KHCO}_3$ , and 0.1 mmol/l EDTA. Cells from AML patients were stained with PerCP-Cy5.5 conjugated anti-CD34 (clone 8G12), PE conjugated anti-CD38 (clone HIT2), Horizon V450 conjugated anti-HLA-DR (clone L243), Horizon V500 conjugated anti-CD45 (clone HI30) antibodies (all from Becton Dickinson Biosciences), and PE-Cy7 conjugated anti-CD117 (clone 104D2D1, Beckman Coulter). Sorted populations were defined as follows, arranged in the order of differentiation:  $\text{CD34}^+\text{CD117}^+\text{CD38}^-$  cells,  $\text{CD34}^+\text{CD117}^+\text{CD38}^+$  cells,  $\text{CD34}^-\text{CD117}^+$  cells, and  $\text{CD34}^-\text{CD117}^-\text{CD45}^{\text{dim}}\text{SSC}^{\text{high}}$  cells. Lysed donor blood cells were stained with APC conjugated anti-CD33 (clone 6C5/2) and FITC conjugated anti-CD15 (clone MC-480) antibodies from Becton Dickinson Biosciences and granulocytes were defined as  $\text{CD15}^+\text{CD33}^+$  cells.

For sorting of CN-AML subpopulations used for microfluidics assay, the following antibodies were used: anti-human CD34 APC (clone 10613, Stemcell Technologies), Human anti C38-PECY7 clone HIT2 (clone 303516, Biolegend) and Human CD19 PE antibody (clone 12-0198-42, eBioscience) and CD3 PE (clone UCHT1, Stemcell Technologies).

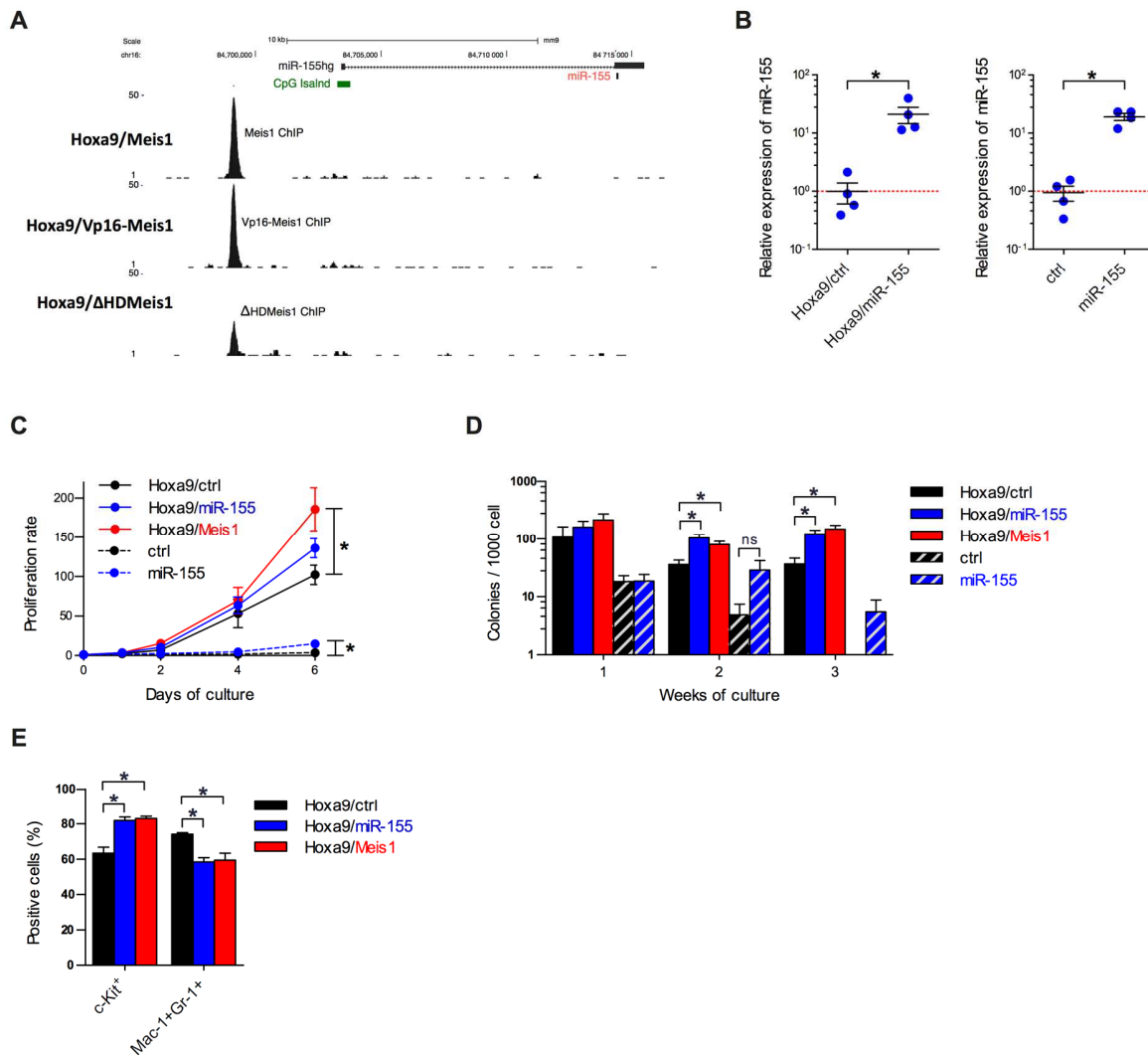
### **Human samples from healthy donors and AML patients**

For RT-qPCR analysis of miR-155 we received RNA samples of mononuclear cells prepared from diagnostic bm or pb of newly diagnosed AML patients with de novo AML. Ten of these samples were classified as CN-AML with wildtype *NPM1* gene, ten were classified as CN-AML with mutated *NPM1* gene and eight were classified with t(11q23) translocation. As control, we used RNA extracted from whole bm cells of eight healthy donors.  $\text{CD34}^+$  cells were isolated from umbilical cord blood units obtained from the Swedish national cord blood bank. After enrichment of pb mononuclear cells (PBMC) using Lymphoprep™ (Axis-Shield PoC AS),  $\text{CD34}^+$  hematopoietic stem cells were isolated with the CD34 MicroBead Kit Ultrapure using the autoMACS® Pro Separator (both Miltenyi Biotec). The purity of isolated  $\text{CD34}^+$  cells was 95 (88-96) % (median, range) with a viability of 97 (94-98) %. Granulocytes were sorted from peripheral blood of voluntary donors as described in the flow cytometry section.

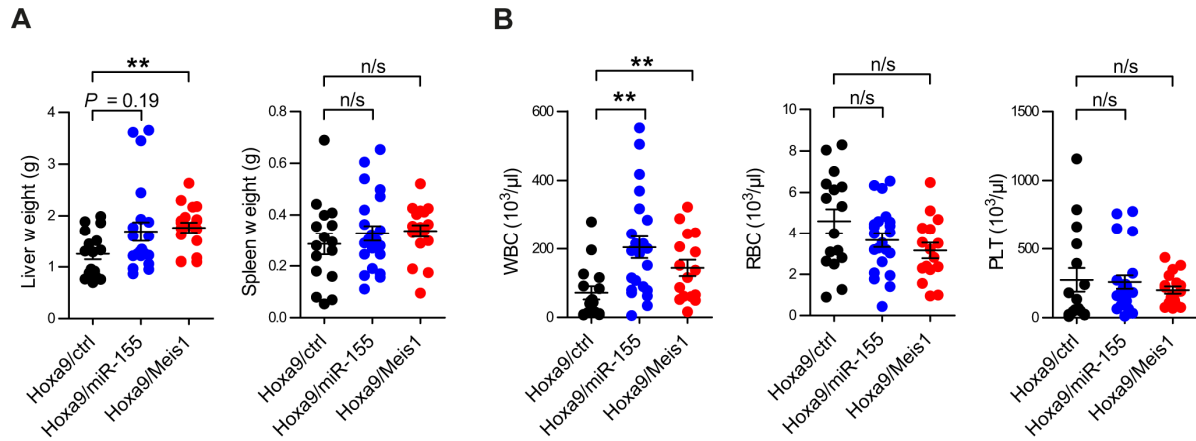
For sorting of defined human AML populations, fresh blood or viably frozen bone marrow cells from diagnostic samples of AML patients were collected according to protocols approved by the Ethics Committee of the Sahlgrenska University Hospital in Gothenburg. AML cases were included based on the

presence of a heterogeneous leukemic cell population in the diagnostic flow cytometry analysis. Diagnosis of the sorted populations was as follows: one case with AML with t(8;21)(q22;q22); *RUNX1T1-RUNX1*, one case with AML with del(16)(q22); *CBFB-MYH11*, two cases with AML with mutated *NPM1*, two cases with AML with maturation, and one case of AML not otherwise specified (NOS). For quantification of *CBFB-MYH11*, *RUNX1-RUNX1T1* and mutated *NPM1* in sorted cell populations, RT-qPCR was performed with published primers and *ABL1* as a reference gene <sup>10</sup>. Total RNA, including miRNA, was isolated using the Qiagen miRNeasy Mini Kit (Qiagen).

## Supplemental Figures

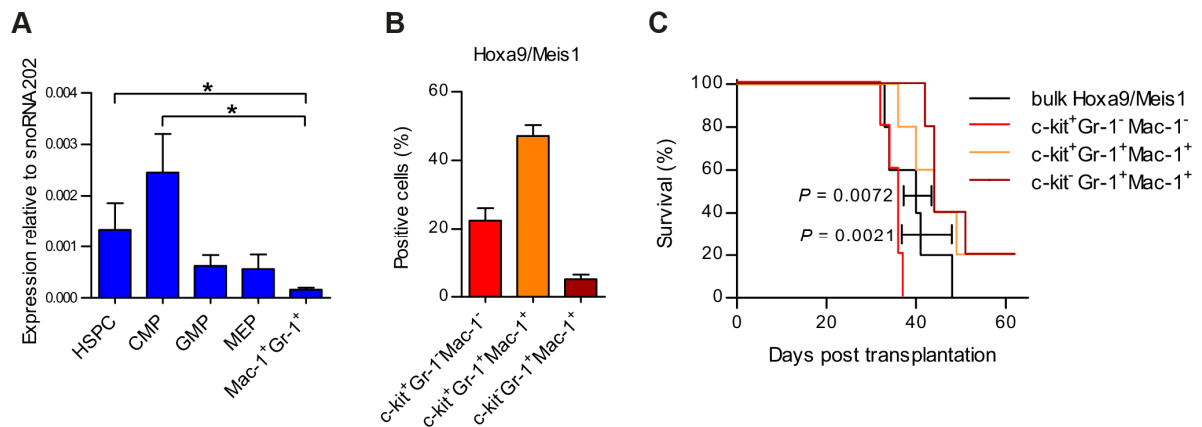


**Figure S1. Co-overexpression of Hoxa9 and miR-155 induces an *in vitro* phenotype similar to Hoxa9/Meis1. Related to Figure 2. (A)** ChIP-seq peaks (normalized to the respective input samples) upstream of the *miR-155hg* locus for Meis1, Vp16-Meis1 and ΔHDMeis1 in Hoxa9/Meis1, Hoxa9/Vp16-Meis1 and Hoxa9/ΔHDMeis1 cell lines respectively. **(B)** Left panel shows the expression of miR-155 in bm cells independently transduced with Hoxa9/miR-155 ( $n=4$ ) relative to expression in cells transduced with Hoxa9/ctrl ( $n=4$ ). Right panel shows the expression of miR-155 in bm cells independently transduced with miR-155 ( $n=4$ ) relative to expression in cells transduced with an empty control vector ( $n=4$ ). **(C)** Growth in liquid culture from  $n=4$  biological replicates of depicted cell lines. The proliferation rate represents the cell counts relative to the seeded cell number. Statistical significance was calculated using the Student's *t*-test (two-tailed). **(D)** Colony forming assay of  $n=4$  biological replicates of described cell lines, respectively. Numbers of colonies per 1000 plated cells are presented for three replates. **(E)** Immunophenotypic analysis of  $n=4$  biological replicates at d15 post transduction, presented as mean percentage of Mac-1<sup>+</sup>Gr-1<sup>+</sup> and c-kit<sup>+</sup> cells.

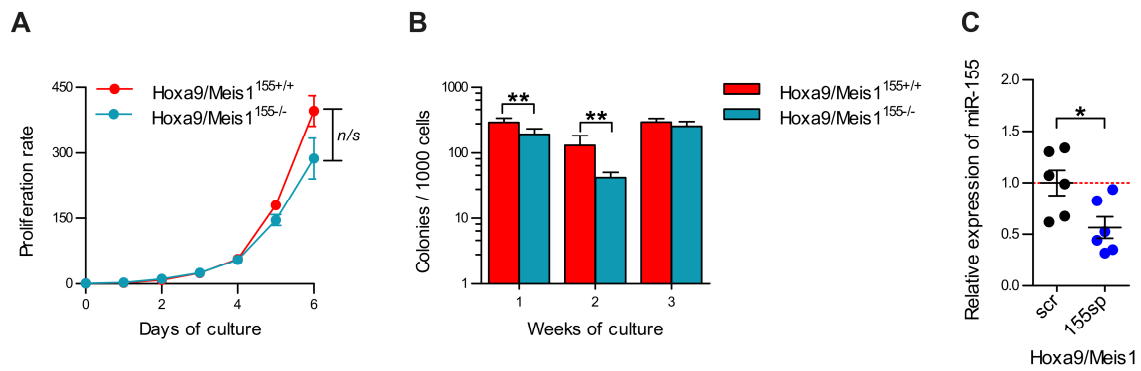


**Figure S2. Hoxa9/miR-155 shows similar phenotype as Hoxa9/Meis1 *in vivo*. Related to Figure 2. (A)** Mean of liver and spleen weights from deceased mice that were transplanted with Hoxa9/ctrl ( $n=16$ ), Hoxa9/miR-155 ( $n=22$ ) and Hoxa9/Meis1 ( $n=19$ ) cells. **(B)** White blood cell (WBC), red blood cell (RBC) and platelet counts in the pb of deceased mice transplanted with Hoxa9/ctrl ( $n=16$ ), Hoxa9/miR-155 ( $n=22$ ) and Hoxa9/Meis1 ( $n=16$ ) transduced cells.

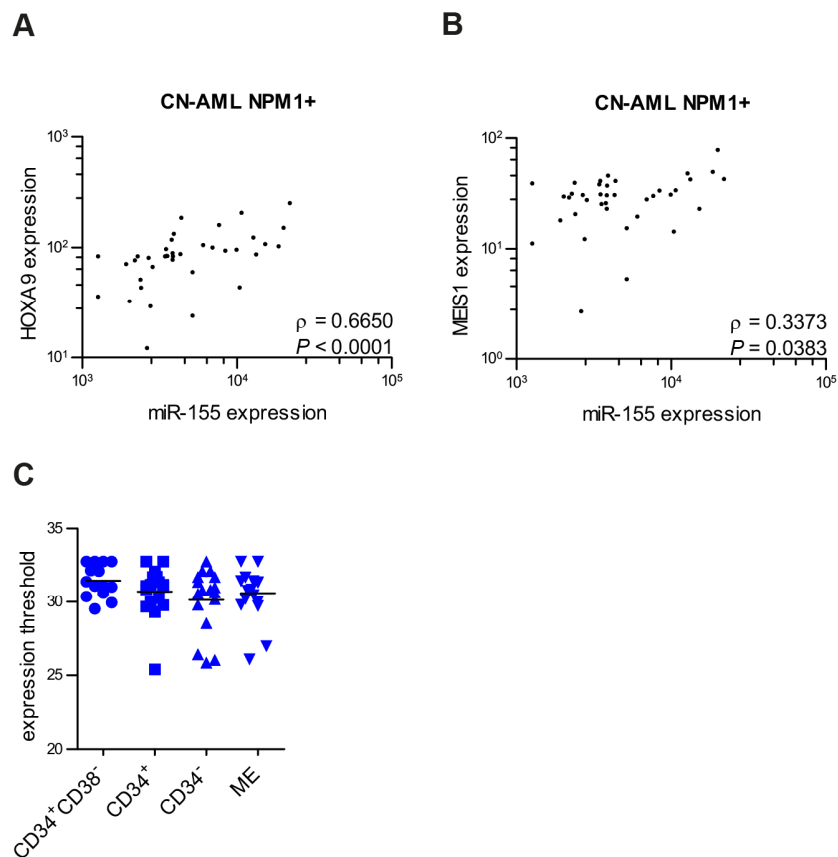




**Figure S3. MiR-155 expression is higher in primitive cells compared to mature myeloid cells. Related to Figure 3.** **(A)** MiR-155 expression in murine bm cells sorted from  $n=4$  individual mice for Lin<sup>-</sup>/Sca-1<sup>+</sup>/cKit<sup>+</sup> (HSPC), common myeloid progenitors (CMP), granulocyte/monocyte progenitor (GMP), megakaryocyte/erythroid progenitor (MEP) and granulocytes (Mac1<sup>+</sup>Gr-1<sup>+</sup>). Expression was measured by RT-qPCR and fold change calculated relative to the housekeeping gene snoRNA202. **(B)** Percentage of Hoxa9/Meis1 cells in indicated subpopulations ( $n=5$ ). **(C)** Survival curves for cohorts of mice transplanted with 1000 cells of indicated Hoxa9/Meis1 subpopulations or bulk cells ( $n=5/arm$ ).



**Figure S4. Absence of miR-155 impairs colony formation *in vitro*.** Related to Figure 4. **(A)** Growth in liquid culture of miR-155<sup>+/+</sup> and miR-155<sup>-/-</sup> bm cells transduced with Hoxa9/Meis1. The proliferation rate represents the cell counts relative to the seeding cell number of  $n=4$  biological replicates/arm. Statistical significance was calculated using the Student's t-test (two-tailed). **(B)** Colony counts of Hoxa9/Meis1<sup>155+/+</sup> and Hoxa9/Meis1<sup>155-/-</sup> cells shown as mean per 1000 plated cells for  $n=6$  biological replicates. **(C)** Expression of miR-155 in independently generated cell lines overexpressing Hoxa9/Meis1/155 sponge relative to cells transduced with Hoxa9/Meis1/scr ( $n=6$ /arm).



**Figure S5. MiR-155 expression is elevated in all samples in the LAML-TCGA dataset. Related to Figure 5. (A)** Correlation of miR-155 and *HOXA9* levels ( $n=38$ ) and **(B)** correlation of miR-155 and *MEIS1* levels ( $n=38$ ) in CN-AML NPM1 mutated subgroup of the LAML-TCGA dataset. **(C)** MiR-155 expression in following sorted subpopulations of CN-AML patient samples; CD34<sup>+</sup>CD38<sup>-</sup> ( $n=15$ ), CD34<sup>+</sup> ( $n=17$ ), CD34<sup>-</sup> ( $n=17$ ) and myeloid enriched cells (ME,  $n=17$ ).

## **Supplemental Tables**

**Table S1. Differentially expressed genes of Hoxa9/miR-155 and Hoxa9/Meis1 cell lines.**

**Table S2. Overlap of miR-155 targets and overlap between Hoxa9/miR-155 or Hoxa9/Meis1 with a conditional Hoxa9 cell line.**

**Table S3. Gene ontology pathway analysis of differentially expressed genes in Hoxa9/miR-155.**

**Table S4. Differentially expressed genes of Hoxa9/Meis1 155KO and Hoxa9/Meis1 155WT cells.**

**Table S5. Chromosome 16 Meis1 peaks.**

**Table S6. Histone marks on chr16 in Hoxa9/ctrl cells.**

**Table S7. Histone marks on chr16 in Hoxa9/Meis1 cells.**

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