Science
AAAS

Supplementary Materials for

MLL-Menin inhibition reverses preleukemic progenitor self-renewal induced by NPM1c mutations in mice

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This PDF file includes:

Materials and Methods
Figs. S1 to S13
Captions for Tables S1 to S6

Other Supplementary Materials for this manuscript include the following:

Table S1-S6

Materials and Methods

Animals

Dnmt3a$^{R878H/+}$ and *Npm1*$^{flox\text{-}cA/+}$ mice were generated as previously described (*11*, *12*). WT (C57Bl/6) mice were purchased from Taconic. CD45.1 recipient mice (B6.SJL - Model 4007) were purchased from Charles River and *MxCre* (B6.Cg-Tg(Mx1-cre)1Cgn/J, stock No: 003556) mice were purchased from The Jackson Laboratory. For PDX studies immunodeficient (NOG - EF NOG-sp/sp;ko/ko) mice (Taconic) were used as recipient mice without conditioning. *MxCre*, *Dnmt3a*$^{R878H/+}$ and *Npm1*$^{flox\text{-}cA/+}$ were crossed to generate *MxCreNpm1*$^{flox\text{-}cA/+}$, *MxCreNpm1*$^{flox\text{-}cA/+}$*Dnmt3a*$^{R878H/+}$ and *MxCreNpm1*$^{flox\text{-}cA/+}$*Dnmt3a*$^{R878H/+}$. Conditional alleles were activated in 6-8 week old mice *in vivo* by i.p. administration of pIpC. All animal experiments described in this study including the experiments with conditional knockout mice as well as mouse BM transplantation models were approved by, and adhered to guidelines of the institutional animal care and use (IACUC) committees of Memorial Sloan-Kettering Cancer Center and Dana-Farber Cancer Institute.

Transplantation Assays

CD45.1 recipient mice (Taconic) were lethally irradiated (900Gy) and transplanted with 50.000-100.000 sorted GMPs supplemented with 2x10^5 rescue BM cells by tail vein injections. Engraftment in the peripheral blood was monitored every 3-4 weeks. For secondary leukemia transplants mice were sub-lethally irradiated (450Gy) and transplanted with the indicated number of total BM or sorted cells.

Patient-derived xenografts

Samples for patient derived xenotransplantation (listed in Table S5) were obtained from PRoXe (www.proxe.org) depository(*26*). 1-10x10^5 cells were transplanted into unconditioned immunodeficient NOG mice (Taconic). The percentage of human $CD45^+$ cells in peripheral blood were analyzed by FACS. When median engraftment reached 1% of $hCD45^+$ cells in PB, the mice were randomized into 2 groups and treated with either with 0.1% VTP-50469 120mg/kg spiked-in or control chow for 28 days for short term treatment leukemia burden experiments. Engraftment in the peripheral blood was monitored every 3 weeks to assess leukemia burden. The mice were sacrificed at day 10 or 28 to assess leukemia burden in bone marrow and spleens. For survival study, mice were monitored for signs of leukemia and human CD45 engraftment in the blood.

Mutational Screening of MDS and sAML Samples

We studied 49 paired MDS/sAML cases by mutational screening of nine AML associated genes (*RUNX1, TP53, NPM1, NF1, FLT3, ASXL1, DNMT3A, IDH1 and IDH2*). Ficoll-enriched MDS/leukemic blast populations were used for DNA isolation followed by genomic analysis using GeneScan analysis, conventional Sanger sequencing and SNP array 6.0 profiling. Informed consent for biobanking and genomic analysis of MDS and sAML samples was given by all patients according to the Declaration of Helsinki. Approval was obtained from the ethical review board of the University of Ulm (ethical vote number 94/12 SFB 1074, Subproject B3).

Isolation of bone marrow and peripheral blood cells

Mice were sacrificed, and tibiae, femurs, coxae and vertebral columns were crushed in IMDM medium (Sigma-Aldrich) supplemented with 2% FBS. For cell sorting, lineage depletion was performed using EasySep™ Mouse Hematopoietic Progenitor Cell Isolation Kit (STEMCELL

Technologies). For analysis of PB, four to five drops of blood were collected from the vena facialis into an EDTA-coated tube. Blood cells were analyzed using a hematology system (Hemavet 950; Drew Scientific). For FACS analysis, erythrocytes were lysed with 1 ml of BD Pharm Lyse buffer, washed with PBS and stained in PBS with 2%FBS.

FACS Analysis and Sorting

Blood and BM cells were stained for 30min on ice using FACS antibodies: CD4 (Biolegend, clone GK1.5), CD8 (Biolegend, clone 53-6.7), CD11b (Biolegend, clone M1/70), Gr-1 (Biolegend, clone RB6-8C5), B220 (Biolegend, clone RA3-6B2), TER119 (Biolegend,TER119), CD16/32 (Biolegend, clone 93), cKit (Biolegend, clone 2B8), Sca-1 (Invitrogen, clone D7), CD150 (Biolegend, clone TC15-12F12.1), CD48 (Biolegend, clone HM48-1), CD34 (eBioscience, clone RAM34), human CD45 (Biolegend, clone H130), mouse CD45 (Biolegend, clone 30-F11), mouse CD45.2 (Biolegend, clone 104). Cells were analyzed on an LSR Fortessa flow cytometer (BD). Data were analyzed with FlowJo software (Tree Star). For FACS sorting, FACSAria cell sorters were used (BD).

Colony-formation Assays

Colony-forming unit (CFU) assays were performed by plating 500 LSK or 5000 GMPs into 3ml of Methocult GF M3434 (STEMCELL Technologies). For relating assays cells were resuspended in warm IMDM on day 7, counted and the same number of input cells used in the primary CFU was plated in secondary CFUs. Colonies were quantified on day 7.

Histology, Cytospins and microscopic imaging

Histology samples from Formalin fixed spleen, liver and bones were paraffin embedded and sectioned by ServiceBio (ServiceBio, Boston, MA). Imaging was done using the REVOLVE 4 microscope (ECHO).

Cell Culture

Human cell line OCI-AML3 carrying *NPM1* Type A mutation was cultured in RPMI supplemented with 10% FBS at 37ºC in a humidified incubator (*27*, *28*). *Npm1*$^{flox\text{-}cA/+}$*Dnmt3a*$^{R878H/+}$ mutant mouse cell line SIIIL12 was derived from primary mouse stem and progenitor cells that were serially replated in colony forming assay M3434 (Stemcell Technologies) and then transferred into myeloid medium (IMDM with 15% FBS, supplemented with mIL6, mSCF and mIL3 (Stemcell technologies). Cells were cultured at 37°C and 5% CO_2.

Virus production and transduction

Viral supernatants were generated by co-transfection of HEK293-T cells with lentiviral (sgRNA expression plasmid improved-scaffold-pU6-sgRNA-EF1Alpha-puro-T2A-RFP (ipUSEPR)) or retroviral (MIG-Cre, MSCV-IRES-puro, MSCV-*Meis1*-IRES-puro, MSCV-*HoxA9/Meis1*-IRES-hygro) expression vectors with packaging and envelope vectors (Human Lenti: PAX2 and VSV-G; Mouse retro: psiEco). Human Menin sgRNA sequences (1.CACCTGCTGCGATTCTACGA; 2.GAACGTTGGTAGGGATGACG**;** 3. GAGGCTGTTCCATATGACAT) and previously published MLL1/2 sgRNAs (*14*) were cloned into ipUSEPR**.** Cells were spin infected with an MOI of 4 and 0.8ug/ml polybrene at 2000rpm at 37°C for 1.5h and the supernatant was replaced with fresh medium immediately after spin infection. Cells were sorted for GFP (MIG) or RFP (ipUSERP) or selected using puromycin

(Sigma) or hygromycin (Calbiochem). MIG-Cre transduced LSK and GMPs cells were cultured in Stemspan medium (STEMCELL technologies) supplemented with 1% l-glutamine, 1% penicillin/streptomycin, 50 ng/ml mFlt3, 50 ng/ml mouse thrombopoietin (mTPO), and 50 ng/ml mouse stem cell factor (mSCF)(STEMCELL technologies).

CRISPR/Cas9 mediated Knock-outs

CRISPR mediated knock-out of *MLL1* and *MLL2* as well as *Cas9*-expressing OCI-AML3 were generated as previously described (*14*). Other sgRNAs were designed using the Zhang laboratory's CRISPR design tool (*29*). All guides were cloned into an improved scaffold pU6-sgRNA-EF1Alpha-puro-T2A-RFP (ipUSEPR) vector (*30*). *Menin, MLL1* and *MLL2* KOs were validated by western blot of RFP^+ sorted cells on day 5 post transduction and by next generation sequencing of amplicons generated from genomic target loci of edited cells. *Npm1c/Dnmt3a* mouse cells were electroporated with Cas9/sgRNA RNP complexes using the Neon Transfection system 10µl tip kit (Invitrogen™). $1x10^5$ cells/replicate were washed twice with PBS and resuspended in 10µl Buffer T. 7.5pmol of recombinant Cas9 (TrueCut™ Cas9 Protein v2, Invitrogen™) and 7.5pmol of sgRNA (TrueGuide, Invitrogen™) were incubated for 5min at RT to form complexes and then added to the cells. Guide RNAs used were *sgNegative* (A35519), *sgMeis1-1* (GGGGUUCCUCCUGAACGAG), *sgMeis1-2* (CRISPR475067_CR), *sgRpa3* (CRISPR113015_CR). Electroporation was performed according to manufacturer's instructions using 1 pulse of 1350V for 35ms. Electroporated cells were dispensed into pre-warmed antibiotic-free myeloid medium.

Immunoblotting

Western blots were performed as previously described (*31*) using the following antibodies: anti-Menin1 (A300-105A, Bethyl), anti-ßActin (AC026, ABclonal), MLL1n (A300-086, Bethyl), MLL2 (D6X2E, Cell signaling).

RNA isolation and Quantitative real-time PCR

RNA was isolated using the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. For sorted HSCs and small cell numbers (<100,000), cells were lysed in 50–100 µl of RNA extraction buffer, and RNA was isolated with the PicoPure RNA isolation kit (ThermoFisher). RNA was reverse transcribed with the SuperScript VILO cDNA synthesis kit (Invitrogen, Carlsbad, CA). For qRT-PCR, the ViiA 7 Real-Time PCR system (Applied Biosystems Grand Island, NY) was used with 384-well plates. Taqman gene expression assays (Invitrogen, Carlsbad, CA) were used for real-time quantitative PCR. *GAPDH* was used as the housekeeping gene for normalization. Relative gene expression was calculated by the comparative ΔΔ cycle threshold method. Mouse probes used: *Gapdh* (Mm99999915_g1), *Hoxa9* (Mm00439364_m1); Human probes used: *GAPDH* (Hs02786624_g1), *HOXA5* (Hs00430330_m1), *HOXB5* (Hs00357820_m1), *MEIS1* (Hs01017441_m1), *PBX3* (Hs00608415_m1). Mouse ChIP-qPCR primers used were: *b-Actin* (F: GACACCCAACCCCGTGACG, R: GCGGCCATCACATCCCAG), *Meis1* (F: TCACCACGTTGACAACCTCG , R: GCTTTCTGCCACTCCAGCTG), *Hoxa9* (F: GGAATAGGAGGAAAAAACAGAAGAGG, R: TGTATGAACCGCTCTGGTATCCTT).

RNA sequencing

RNA quality for RNA sequencing was checked on the Agilent TapeStation (Agilent, Santa Clara, CA). Poly-A tail selection and library preparation were performed using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (New England Biolabs, Ipswich, MA). Sequencing was done using the Illumina Next Gen Sequencing NextSeq platform (Illumina, San Diego, CA) with 20-30 million 75bp, single-end reads.

Chromatin Immunoprecipitation

DMSO or VTP-50469 treated cells were crosslinked with 1% methanol-free formaldehyde (ThermoFisher) for 7-10 min at room temperature, followed by quenching with 100 mM Tris pH8.0 and 25 mM Glycine, and the cells were lysed in SDS buffer. Cytoplasm was lysed using 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 5 mM EDTA, 1% SDS for 10 min and nuclei were precipitated by centrifugation at 10,000×*g*. Nuclei were resuspended in 66 mM Tris-HCl pH 8.0, 100 mM NaCl, 5 mM EDTA, 1.7% Triton X-100, 0.5% SDS and sheared using E100S (Covaris) to chromatin fragments of 200-400 base-pair DNA size. 1-5 µg of chromatin were used per immunoprecipitation with anti-MEN1 (A300-105A, Bethyl), MLL1n (A300-086, Bethyl) or H3K4me3 (ab8580, Abcam) antibodies and protein-A magnetic beads (Dynal). Immunoprecipitated DNA fragments were eluted and de-crosslinked in 100 mM $NaHCO_3$, 100 mM NaCl, 1% SDS, and quantified by TAPE (Agilent) and Qubit (ThermoFisher). 1-10 ng of DNA was used in preparation of Illumina-compatible libraries using ThruPlex DNA kit (Rubicon Genomics) and subjected to sequencing using Illumina Next Gen Sequencing NextSeq platform (Illumina, San Diego, CA) to obtain 1-5×10^7 unique sequencing paired-end tags.

ChIP-seq and RNA-seq data analyses

Raw Illumina sequencing output was converted to FASTQ format using bcl2fastq (v2.17). Reads (37- or 75-mers) were trimmed for quality using trimmomatic (v0.36; minimum trimmed length 34bp), aligned to the human or mouse genome (GRCh37/hg19 or mm10) and associated Trinity CTAT gene GTF reference (GRCh37_v19_CTAT_lib_Feb092018 or Mouse_M16_CTAT_lib_Feb092018, respectively, with External RNA Controls Consortium [ERCC] spike-in sequence references appended, as appropriate) using STAR (v2.6.1b), sorted and duplicates marked/removed with picard pipeline tools (v2.9.4) (*32*). Final "deduped" .BAM files were indexed using SAMtools (v1.2). For Chip-seq, total signal was assessed around transcription start site (TSS) regions using the sitepro tool from the CEAS (Cis-regulatory Element Annotation System) package (using 4kb windows off-centered around TSS regions [1kb upstream, 3kb into gene bodies] with 200bp tiling and input-subtracted signal summed across each TSS window), based on signal from WIG files generated using IGVtools (v2.3.98) (*33*). For each ChIP target a floor of detectable signal was determined from average signal across all TSS windows; windows with signal below threshold were set to zero. ChIP-seq data visualizations were produced using IGVtools (TDF signal pileups) and ngs.plot (pileup heatmaps). For RNA-seq, raw counts calculated with HTSeq (htseq-count, v0.6.1pl) (*34*). Duplicate reads were marked with picard tools (v2.9.4) and removed before further processing. Differential RNA-seq expression was calculated using the BioConductor DESeq2 package (v1.22.1), using raw unnormalized per-gene counts. Scatterplots of RNAseq data were generated using the basepair online analysis tool (basepairtech.com). Gene set enrichment analysis was performed using and GSEA v3.0 software. All gene sets used are summarized in table S3.

Statistical analysis

Statistical tests were used as specified in figure legends. Generation of plots and statistical analyses were performed using Prism version 8 (GraphPad). Errors bars represent standard deviation, unless otherwise indicated. We used One-way ANOVA or Student's t-test (unpaired, two-tailed) to assess significance between treatment and control groups. Log-rank (Mantel-Cox) test was used to assess significance in survival experiments to calculate p values. $P<0.05$ was considered significant. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.