GFI1 is required for *RUNX1/ETO* positive acute myeloid leukemia

Anna E. Marneth,[1#] Lacramioara Botezatu,[2#] Judith M. Hönes,[2,3#] Jimmy C.L. Israël,[1] Judith Schütte,[2] Lothar Vassen,[2] Robert F. Lams,[2] Saskia M. Bergevoet,[1] Laura Groothuis,[1] Amit Mandoli,[4] Joost H.A. Martens,[4] Gerwin Huls,[5] Joop H.Jansen,[1] Ulrich Dührsen,[2] Tobias Berg,[6] Tarik Möröy,[7] Christian Wichmann,[8] Mia-Chia Lo,[9] Dong-Er Zhang,[9] Bert A. van der Reijden[1] and Cyrus Khandanpour[2,10*]*

[1]Department of Laboratory Medicine, Laboratory of Hematology, Radboud Institute for Molecular Life Sciences, Radboud University Medical Centre, Nijmegen, The Netherlands; [2]Department of Hematology, West German Cancer Center, University Hospital Essen, University of Duisburg-Essen, Germany; [3]Department of Endocrinology, Diabetes and Metabolism, University Hospital Essen, University Duisburg-Essen, Germany; [4]Department of Molecular Biology, Faculty of Science, Radboud Institute for Molecular Life Sciences, Radboud University Nijmegen, The Netherlands; [5]Department of Hematology, University Medical Center Groningen, University of Groningen, The Netherlands; [6]Department of Medicine II - Hematology/Oncology, Goethe University, Frankfurt/Main, Germany; [7]Institut de recherches cliniques de Montréal (IRCM), Hematopoiesis and Cancer Research Unit, and Université de Montréal, Canada; [8]Department of Transfusion Medicine, Cell Therapeutics and Hemostaseology, Ludwig-Maximilian University Hospital, Munich, Germany; [9]Department of Pathology & Division of Biological Sciences, University of California San Diego, La Jolla, USA and [10]Department of Medicine A, Hematology, Oncology and Pneumology, University Hospital Münster, Germany;

AEM, LE and JMH contributed equally to this work

*[#]these authors contributed equally to this work; *co-corresponding authors.*

Correspondence: Cyrus.Khandanpour@uk-essen.de/ Cyrus.Khandanpour@ukmuenster.de/ Bert.vanderReijden@radboudumc.nl

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Supplement

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Anna E. Marneth[1#], Lacramioara Botezatu[2#], Judith M. Hönes[2,3#], Jimmy C.L. Israël[1], Judith Schütte[2], Lothar Vassen[2], Robert F. Lams[2], Saskia M. Bergevoet[1], Laura Groothuis[1], Amit Mandoli[3], Joost H.A. Martens[3], Gerwin Huls[5], Joop H.Jansen[1], Ulrich Dührsen[2], Tobias Berg[6], Tarik Möröy[7], Christian Wichmann[8], Mia-Chia Lo[9], Dong-Er Zhang[9] , Bert A. van der Reijden[1*] and Cyrus Khandanpour[2*]

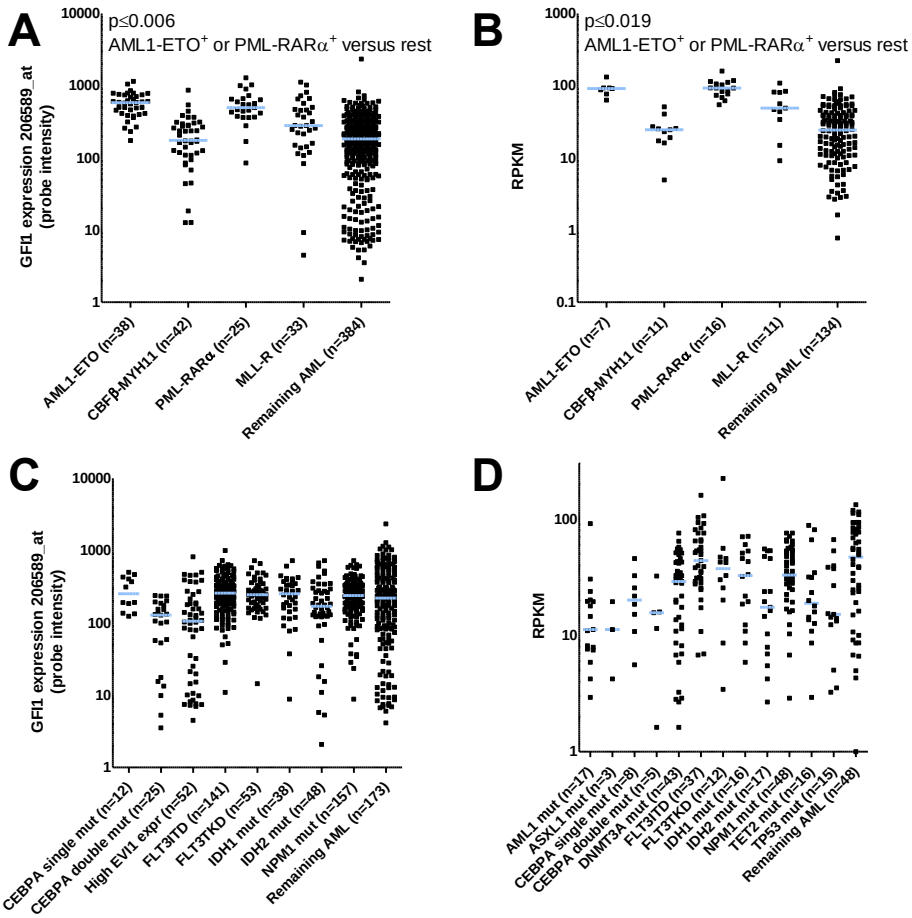

Figure S1. Further characterization of GFI1 expression in AML samples

(A) GFI1 expression in AML patients harboring recurrent chromosomal rearrangements [10]. Patients for which these data were not available, were excluded.

(B) GFI1 expression in AML patients harboring recurrent chromosomal rearrangements in a second cohort [11].

(C) Analysis of GFI1 expression in AML patients harboring molecular aberrations [10]. Patients for which molecular data were not available, were excluded. Expr = expression, mut = mutation.

(D) GFI1 expression in AML patients harboring molecular aberrations in a second cohort [11]. RPKM: reads per kilobase per million mapped reads.

Experimental procedures

Analysis of microarray and RNAseq data

The expression of ETO (205529_s_at) and GFI1 (206589_at) was studied in a publically available Affymetrix HG-U133 plus 2.0 microarray dataset containing 525 AML samples, 11 $CD34^+$ donor and 5 normal bone marrow (BM) control samples[10]. In addition, expression data from a publically available RNA-sequencing dataset containing 200 AML samples were analyzed[11]. The Mann-Whitney U test was performed to determine statically different median expression between sample groups (GraphPad Prism 5) and the chi-square test was performed to determine statistically different frequencies (IBM SPSS Statistics 22).

Patient samples

Bone marrow samples of AML patients were collected at time of diagnosis according to the protocol approved by the medical ethical committee (Commissie mensgebonden onderzoek) under registration number 2013/064. Patients consented to the use of their samples for research purposes.

RNA isolation, cDNA synthesis and qRT-PCR

RNA was isolated using the standard RNA-bee (Tel-Test Inc.) RNA isolation method. 1 µg of RNA was reverse-transcribed using M-MLV reverse transcriptase, First Strand buffer and DTT (Invitrogen), random hexamer primers (Roche) and dNTPs (GE Healthcare UK Limited). For quantitative real time polymerase chain reaction (qRT-PCR), cDNA was amplified by SYBR Green PCR Master Mix (Applied Biosystems)using a forward (F) and reverse (R) primer for GFI1 (F 5'-GAGCCTGGAGCAGCACAAAG, R 5'- GTGGATGACCTCTTGAAGCTCTTC, SYBR Green), PBGD (F 5'-GGCAATGCGGCTGCAA, R 5'-GGGTACCCACGCGAATCAC, R probe VIC-TAMRA 5'-CTCATCTTTGGGCTGTTTTCTTCC (Life Technologies)). qRT-PCR

was performed in the 7900 Real-Time PCR System (Applied Biosystems). cDNA was pre-incubated at 50ºC for 2 minutes and 95ºC for 10 minutes and amplified for 40 cycles. One cycle was composed of denaturation at 95ºC for 15 seconds and annealing/elongation at 60ºC for 60 seconds. Data was analyzed using 7900HT Sequence Detection Systems 2.4.1 (Applied Biosystems).

Cell culture

SKNO-1 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco/PAN Biotech GmbH), 1% penicillin/streptomycin (MP Biomedicals, LLC/Sigma) and 10 ng/ml granulocyte macrophage colony stimulating factor (GM-CSF, Immunotools) at a density of 0.2-1x10^6 cells/ml. Kasumi-1 cells were maintained in RPMI-1640 medium (Gibco) supplemented with 20% heat-inactivated FCS (Gibco/PAN Biotech GmbH) at a density of 0.3-1.2 x 10^6 cells/ml. 293T myc cells were cultured in DMEM GlutaMAX™ (Gibco) supplemented with 10% non-heat-inactivated FCS (PAN Biotech GmbH) and 1% penicillin/streptomycin (Sigma). All cells were maintained at 37ºC in 5% CO_2.

Virus production

For production of retrovirus, 1 x 10^7 293T myc cells were plated onto 140 mm dishes. When confluency reached 70%, cells were transiently transfected with 2.25 µg pCL-Eco retroviral packaging plasmid (Imgenex) and 20 µg MiGR1 RUNX1/ETO9a IRES-GFP plasmid[4] using the calcium-phosphate transfection method. Medium was refreshed 16 hours after transfection and viral supernatant was harvested 40 and 70 hours after transfection.

Isolation and expansion of murine hematopoietic progenitor cells

BM cells were isolated from the femurs, tibia and humeri of 6-8 week old male or female mice. Lineage$^+$ (Lin$^+$) cells were depleted using the Lineage Cell Depletion Kit (Miltenyi Biotec) according to manufacturer's instructions. For *in vitro* experiments, Lin$^-$ cells were cultured in stem cell medium (SCM): Iscove's Modified Dulbecco's Medium (IMDM, Gibco) supplemented with 20% FCS (PAN Biotech GmbH), 1% penicillin/streptomycin (Sigma), 20 ng/ml recombinant mouse Stem Cell Factor (Miltenyi Biotec), 10 ng/ml recombinant mouse Interleukin-3 (Miltenyi Biotec) and 10 ng/ml recombinant human Interleukin-6 (Miltenyi Biotec). When Lin$^-$ cells were used for *in vivo* experiments, the cells were cultured in Marrow Max Bone Marrow Medium (Gibco) supplemented with cytokines as described for SCM. Cells were cultured at a density of 1x10^6 cells/ml.

Retroviral transduction of murine hematopoietic progenitor cells

Murine Lin$^-$ cells were transduced three days after isolation on two consecutive days. Viral supernatant was centrifuged twice onto retronectin (Takara Bio Inc.) coated plates (20 µg/ml) at ≥4600 rpm for ≥90 minutes at 4^{o}C. Supernatant was aspirated and Lin$^-$ cells (1x10^6 cells/ml) were spun onto the retrovirus coated plates at 1200 rpm, 4^{o}C, for 10 minutes. Subsequently, 2 µg/ml polybrene (Millipore) was added to the cells.

***In vitro* liquid culture and Colony Forming Cell (CFC) Assay of murine hematopoietic progenitor cells**

To study cell expansion in liquid culture, 3'000-5'000 *RUNX1/ETO9a* transduced cells were sorted using BD FACS DIVA Cell Sorter (BD Biosciences) and cultured in triplicate in 150 µl SCM medium on non-tissue culture treated 96 well plates. Every two days 50 µl fresh medium was added and the cell number was assessed 5-7 days later. For CFC Assay, approximately 1'000 sorted GFP$^+$ cells were seeded in semi-solid medium (Stem Cells, M3434). Colony

formation and cell number was analyzed 7-10 days later. The student's t test was performed to determine statically different values (GraphPad Prism 5).

ChIP-seq

Chromatin was harvested as previously described[14]. ChIPs were performed using the following specific antibodies Rabbit against RUNX1 (directed against the C-terminal region that is absent from RUNX1/ETO, ab23980, Abcam), Rabbit against RUNX1/ETO (C15310197, Diagenode), Goat against GFI1 (8558X, Santa Cruz) and analyzed by ChIP-sequencing (26148230). Relative occupancy was calculated as fold over background, for which the second exon of the Myoglobin gene or the promoter of the H2B gene was used. Illumina high-throughput sequencing End repair was performed using the precipitated DNA of ±6 million cells (3–4 pooled biological replicas) using Klenow and T4 PNK. A 3'-protruding A base was generated using Taq polymerase, and adapters were ligated. The DNA was loaded on gel and a band corresponding to ±300 bp (ChIP fragment+adapters) was excised. The DNA was isolated, amplified by PCR and used for cluster generation on the Genome analyzer (Illumina, San Diego, CA, USA) and HiSeq 2000 (Illumina). The 50-bp tags were mapped to the human genome HG19 using the eland program allowing 1 mismatch or Burrows-Wheeler Aligner. For each base pair in the genome, the number of overlapping sequence reads was determined and averaged over a 10-bp window and visualized in the UCSC genome browser (http://genome.ucsc.edu). For processing and manipulation of SAM/BAM files, SAM tools were used. All ChIP-seq data can be downloaded from Gene Expression Omnibus accession numbers GSE23730 and GSE103255. The token for the private access is: ubolusgifhofvcr

Chromatin immunoprecipitation (ChIP) of murine RUNX1/ETO9a-HA.

Formaldehyde cross-linked RUNX1/ETO9a expressing leukemic splenocytes derived from *Gfi1* WT or *Gfi1* KO mice were sonicated to generate chromatin fragments with sizes ranging from 2 kb to 500 bp. Chromatin immunoprecipitation (ChIP) was performed using rabbit-anti-HA antibody (Santa Cruz) and rabbit-IgG (Sigma-Aldrich) as previously described (Li et al.). Briefly, 2 mg of sheared chromatin and 10 µg of antibody were used in each ChIP reaction. To confirm the binding of RUNX1/ETO9a to mouse Gfi1 promoter, the precipitated DNA was amplified by PCR with primers flanking the two RUNX1 consensus sites, respectively, performing 40 cycles of 95°C - 60°C - 72°C for 30 seconds at each temperature. For each qPCR reaction, 0.25 µM of each primer and 1/100 of ChIP DNA of input genomic DNA were used. To analyze the fold enrichment of Gfi1 promoter regions by anti-HA ChIP, the Ct values of qPCR with input genomic DNA were first used to normalize the enrichment level of each DNA region by anti-HA antibody (ΔCt). Then the enrichment level of a control region by anti-HA antibody was set to 1 and the differences between the Gfi1 promoter regions and the control region were calculated ($\Delta\Delta$Ct). The sequences of primers used are: [for the distal RUNX1/ETO9a consensus site, ChIP (a) in Figure 2F] F 5'-CAAGGATACCCCCTTCCTGT-3', R 5'-CTTGCTTTCGGGAGAGACTG-3'; [for the proximal RUNX1/ETO9a consensus site, ChIP (b) in Figure 2F] F 5'-GGGGACAGGTTTTACCACTG-3', R 5'-TTGGACCCTCGGATACTCTG-3'.

Peak calling, tag counting and peak distribution analyses

Peak calling algorithm MACS1.3.3 (Zhang et al., 2008) was used to detect the binding sites at a p-value cut off for peak detection of 10^{-6}. Tags within a given region were counted and adjusted to represent the number of tags within a 1 kb region. Subsequently the percentage of these tags as a measure of the total number of sequenced tags of the sample was calculated and displayed as a heat map. To determine genomic locations of binding sites, the peak file was analyzed using a script that annotates binding sites according to all RefSeq

genes. With this script every binding site is annotated either as promoter (-500 bp to the Transcription Start Site), exon, intron or intergenic (everything else).

Mice

All animalexperiments were approved by the ethical committee of the University HospitalEssen, Germany and Institut de Recherches Cliniques de Montréal (IRCM), Canada. *Gfi1* KO[15] and mice with a conditional RUNX1/ETO knockin allele[12] were generated as previously described. Mx-Cre^{+} mice were purchased form Jackson Laboratory (Bar Harbor, ME, USA).All mice were bred and housed in single ventilated cages and specific pathogen-free conditions in the animal facility of University Hospital Essen or of the Institut de Recherches Cliniques de Montréal (IRCM). All animal experiments were approved by the ethics committee and local authorities in Germany under document number G1196/11.

Inducible *RUNX1/ETO* knock-in mice

Mice harboring the poly(I:C) inducible Cre recombinase gene (Mx1 promoter) and an *RUNX1/ETO* knockin allele, in which a transcriptional stop cassette 5' to the RUNX1/ETO fusion site was bracketed by loxP sites, were crossed with *Gfi1* WT or *Gfi1* KO mice. Poly(I:C) was injected intraperitoneally (IP) three times over a six-day period into 3-9 week old mice at a dose of 500 µg per injection. N-ethyl-N-nitrosourea (ENU) was administered IP once a at a dose of 100mg/kg after finishing the poly(I:C) injection. Mice were censored if they had BM failure, indicated by a hemoglobin level lower than 8 dg/dl, platelets below 100/fl and/or less than 2/fl leukocytes.

Transplantation experiments

For primary transplantations,5-7.5x10^5 RUNX1/ETO9a transduced cells (GFP^+, 5 days after Lin^- cell isolation) from *Gfi1* KO or*Gfi1* WT mice were transplanted into lethally irradiated (10 Gy) *Gfi1* WT mice together with 1,5 x 10^5 fresh competitive BM cells from *Gfi1* WT mice.

All transplanted mice were monitored every other day and when moribund, the mice were sacrificed and analyzed.

Analysis of transplanted mice

Expression of surface molecules on BM and spleen cells of moribund mice was analyzed by staining the cells with the following fluorochrome-conjugated antibodies: CD11b, Gr-1, B220, Ter119, CD8a, CD4 and c-Kit (Biolegend). Surface marker expression was detected using BD FACS Scan flow cytometer (Becton Dickinson). The student's t test was performed to determine statically different values and the Log-rank (Mantel-Cox) test was used to compare survival curves (GraphPad Prism 5).