



EUROPEAN
HEMATOLOGY
ASSOCIATION



Ferrata Storti
Foundation

Gfi1b: a key player in the genesis and maintenance of acute myeloid leukemia and myelodysplastic syndrome

Aniththa Thivakaran,¹ Lacramioara Botezatu,¹ Judith M. Hönes,^{1,2} Judith Schütte,¹ Lothar Vassen,¹ Yahya S. Al-Matary,¹ Pradeep Patnana,¹ Amos Zeller,³ Michael Heuser,³ Felicitas Thol,³ Razif Gabdoulline,³ Nadine Olberding,¹ Daria Frank,¹ Marina Suslo,¹ Renata Köster,¹ Klaus Lennartz,⁴ Andre Görgens,^{5,6} Bernd Giebel,⁵ Bertram Opalka,¹ Ulrich Dührsen¹ and Cyrus Khandanpour^{1,7}

¹Department of Haematology, West German Cancer Center, University Hospital Essen, University of Duisburg-Essen, Essen, Germany; ²Department of Endocrinology, Diabetes and Metabolism, University Hospital Essen, University of Duisburg-Essen, Essen, Germany; ³Department of Haematology, Haemostaseology, Oncology, and Stem Cell Transplantation, Medical University of Hannover, Germany; ⁴Institute for Cell Biology, University Hospital Essen, University of Duisburg-Essen, Essen, Germany; ⁵Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Essen, Germany; ⁶Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden and ⁷Department of Medicine A, Hematology, Oncology and Pneumology, University Hospital Münster, Germany

Haematologica 2018
Volume 103(4):614-625

ABSTRACT

Differentiation of hematopoietic stem cells is regulated by a concert of different transcription factors. Disturbed transcription factor function can be the basis of (pre)malignancies such as myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML). Growth factor independence 1b (*Gfi1b*) is a repressing transcription factor regulating quiescence of hematopoietic stem cells and differentiation of erythrocytes and platelets. Here, we show that low expression of *Gfi1b* in blast cells is associated with an inferior prognosis of MDS and AML patients. Using different models of human MDS or AML, we demonstrate that AML development was accelerated with heterozygous loss of *Gfi1b*, and latency was further decreased when *Gfi1b* was conditionally deleted. Loss of *Gfi1b* significantly increased the number of leukemic stem cells with upregulation of genes involved in leukemia development. On a molecular level, we found that loss of *Gfi1b* led to epigenetic changes, increased levels of reactive oxygen species, as well as alteration in the p38/Akt/FoXO pathways. These results demonstrate that *Gfi1b* functions as an oncosuppressor in MDS and AML development.

Correspondence:

cyrus.khandanpour@uk-essen.de

Received: June 29, 2017.

Accepted: January 5, 2018.

Pre-published: January 11, 2018.

doi:10.3324/haematol.2017.167288

Check the online version for the most updated information on this article, online supplements, and information on authorship & disclosures: www.haematologica.org/content/103/4/614

©2018 Ferrata Storti Foundation

Material published in *Haematologica* is covered by copyright. All rights are reserved to the Ferrata Storti Foundation. Use of published material is allowed under the following terms and conditions:

<https://creativecommons.org/licenses/by-nc/4.0/legalcode>.

Copies of published material are allowed for personal or internal use. Sharing published material for non-commercial purposes is subject to the following conditions:

<https://creativecommons.org/licenses/by-nc/4.0/legalcode>,

sect. 3. Reproducing and sharing published material for commercial purposes is not allowed without permission in writing from the publisher.



Introduction

Myelodysplastic syndrome (MDS) is characterized by disturbed function of the myeloid compartment of the bone marrow (BM),¹ leading in some cases to acute myeloid leukemia (AML).² AML is characterized by an accumulation of immature myeloid blasts in the BM.² Hematopoietic development, among other functions, is regulated by transcription factors (TFs).³ Functional dysregulation of several TFs^{4,5} can induce malignant transformation. The hematopoietic^{TF} Growth factor independence 1b (*Gfi1b*) regulates dormancy and proliferation⁶ of hematopoietic stem cells (HSCs), the development of erythroid and megakaryocytic cells,⁷⁻¹⁰ as well as B and T cells.¹¹⁻¹³ Constitutive deletion of *Gfi1b* in mice is embryonically lethal at day E15 due to bleeding and anemia.⁹ Conditional loss of *Gfi1b* leads to a significant expansion of functional HSCs in the BM and peripheral blood.⁶ In human primary hematopoietic progenitors, forced expression of *GFI1B* results in expansion of immature erythroblasts and repression of myeloid differentiation.¹⁴ *Gfi1b* exerts its function by recruiting histone modifying enzymes, such as CoREST, G9a, LSD1 or HDACs, to induce deacetylation of H3K9, demethylation of H3K4 and/or methylation of H3K9.¹⁵⁻¹⁸ We report that a reduced level or absence of *GFI1B* negatively influences the prognosis of MDS/AML patients. Moreover, we present evidence that loss/reduced expression of *Gfi1b* promotes AML development in different murine models of human AML.

Furthermore, reduced expression of *Gfi1b* in murine models of human leukemia leads to a higher number of leukemic stem cells (LSCs). On a molecular level, aberrant regulation of the ROS/p38/Akt/FoXO pathway as a consequence of reduced *Gfi1b* level might contribute to these phenotypic changes.

Methods

Study samples

Characteristics of different patient cohorts have been described previously.¹⁹⁻²⁵

Boundaries of *GFI1B* expression

To set boundaries for *GFI1B* expression levels in AML and MDS patients, we correlated expression levels with the survival outcome of patients.

Mice

Gfi1b^{fl/fl} and *Gfi1b^{EGFP/WT}*, *MxCre*, *NUP98/HOXD13* and *Kras* mice have been described previously.^{6,26-28} Mice were housed in specific pathogen-free conditions in the animal facility of University Hospital Essen. All mouse experiments were performed with the approval of the local ethics committee for animal use (authorization n. G1196/11).

Poly(I:C) treatment

MxCre^{fl} mice harboring the poly(I:C) inducible Cre recombinase gene under the control of the *Mx1* promoter were crossed to *Gfi1b^{fl/fl}* mice. To conditionally delete the *Gfi1b* alleles in the *NUP98/HOXD13* MDS mouse model, *Gfi1b^{fl/fl}MxCre^{fl}* *NUP98/HOXD13^{fl}* mice were injected intraperitoneally (i.p.), as shown previously.⁶ For *Gfi1b^{fl/fl}MxCre^{fl}Kras^{+/fl}* mice, two poly(I:C) injections were sufficient to activate the *Kras* oncogene and delete the *Gfi1b* alleles. As a control, *Gfi1b^{fl/fl}* or *Gfi1b^{EGFP/WT}* mice not carrying the *MxCre^{fl}* were injected with poly(I:C). Three weeks after transplantation of MLL-AF9-transduced lineage negative (Lin-) BM cells from *Gfi1b^{fl/fl}MxCre^{fl}* or *Gfi1b^{fl/fl}MxCre^{wt}* mice, primary recipient mice were injected with poly(I:C) 4 times every second day.

Isolation, retroviral transduction, and transplantation of murine hematopoietic progenitor cells

Mouse leukemia was induced by transplanting Lin- BM cells that were retrovirally transduced with the *MLL-AF9* oncofusion gene as well as the GFP-encoding gene, as previously described.^{4,27} For the limiting dilution assay, different numbers of leukemic cells were retransplanted into sublethally irradiated (3 Gy) secondary recipient mice (3-4 mice/group). The frequency of functional LSCs was determined using ELDA software.²⁹

ChIP and ChIP-Seq analyses

Chromatin Immunoprecipitation (ChIP) and ChIP-Seq analyses were performed as previously described.^{4,27} Data are available from: <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE88934>

Online Supplementary Appendix

Details on the experimental procedures and figures are available in the *Online Supplementary Appendix*.

Results

Low level of *GFI1B* are indicative of an inferior prognosis of MDS and AML patients

To obtain a first insight into the role of *GFI1B* in AML prognosis, we analyzed two well-annotated published

data sets.^{19-21,25} In these sets, CD34⁺ leukemic cells and CD34⁺ control HSCs were used. CD34⁺ leukemic cells represent a fraction in which LSCs are enriched, whereas CD34⁺ cells from healthy donors represent a fraction of cells in which HSCs are enriched.^{21,30} *GFI1B* showed lower expression in CD34⁺ AML blasts compared to CD34⁺ control HSCs (Figure 1A). MDS can progress to AML, and therefore, we wanted to elucidate how *GFI1B* expression changes during the progression of MDS to AML. Again, *GFI1B* showed a lower expression in AML blasts compared to *GFI1B* expression in CD34⁺ cells from the BM of patients with MDS (Figure 1B).

We also analyzed an independent data set, which provided whole genome expression data for LSCs in different types of AML as well as different human hematopoietic progenitor cells.^{20,25} *GFI1B* showed a lower expression in human LSCs of different AML subtypes compared to its expression in normal human myeloid progenitors (GMPs) or HSCs (Figure 1C).²⁰ GMPs and HSCs are two fractions from which LSCs arise in mice and humans.³¹

We analyzed whether *GFI1B* level might also be informative regarding the prognosis of MDS and AML patients. Based on available expression data of *GFI1B* and the associated survival data, we could distinguish two distinct populations with regard to *GFI1B* expression (Figure 2A). A low level of *GFI1B* (see Methods section and *Online Supplementary Appendix* for details) in leukemic blast cells was associated with inferior outcome with regard to overall survival (OS) of all AML patients (Figure 2B) as well as OS and event-free survival (EFS) in the group of patients with no overt cytogenetic aberrations (Figure 2C and D). We also performed a multivariate analysis, including additional factors such as age, sex and cytogenetic status, as well as mutational status of certain genes. There was a tendency for a very low *GFI1B* level to be an independent prognostic marker ($P=0.12$), but this did not reach a level of significance (*data not shown*). Low *GFI1B* expression might be associated with an inferior prognosis, but other confounding factors contribute to this association. Finally, we examined whether low *GFI1B* expression (the lowest 5% compared with the highest 20% of expression levels) was associated with a certain gene expression signature to obtain a first insight into how *GFI1B* might influence prognosis. We performed Signaling Pathway Enrichment using Experimental Datasets (SPEED) analysis (see *Online Supplementary Appendix*) on two separate studies,^{21,22} for which expression data of the full length *GFI1B* and associated clinical data were available. Low level of *GFI1B* expression was associated with a reactive oxygen species (ROS)-mediated signature pathway as well as activation of mitogen-activated protein kinase (MAPK), JAK, TGFB and TLR signaling pathways (Figure 2E).

We also examined whether *GFI1B* expression level influences survival and disease progression from MDS to AML using a separate set of data.^{25,27} Again, we could distinguish two different populations with regard to *GFI1B* expression (low and high) (Figure 2F): low expression of *GFI1B* correlated with poor EFS (Figure 2G).

Anguita *et al.*³² observed a positive correlation between the expression of a mutated form of *GFI1B*, which acts in a dominant-negative manner, and the expression of *MLLT3* and a negative correlation with regard to SPI1. In addition, Chowdhury *et al.* described a negative correlation between *GFI1B* expression and *MEIS1*.³³ In our patient cohorts, we also found an inverse correlation

between *GFI1B* expression level and SPI1 expression as well as *MEIS1* and a positive correlation with *MLLT3* (Online Supplementary Figure S4).

Reduced expression level or loss of *Gfi1b* promotes progression of MDS to AML in a murine MDS model

To investigate a connection between *Gfi1b* level and AML, we used different mouse strains and models of human leukemia. We used one strain in which both *Gfi1b* alleles can be conditionally deleted in the hematopoietic system upon poly(I:C) administration, resembling absence of *Gfi1b* expression (*Gfi1b^{fl/fl}MxCre^{tg}*).⁶ In a second mouse model, one coding allele of *Gfi1b* is replaced by enhanced green fluorescence protein (*EGFP*) cDNA (*Gfi1b^{EGFP/wt}*),¹⁵ which leads to a lower expression level of *Gfi1b* (see below). Finally, wild-type mice were used to model normal/high *Gfi1b* expression. To study whether reduced *Gfi1b* expression accelerates MDS to AML progression, we crossed the above-mentioned mouse strains with *NUP98/HOXD13^{tg}* mice, which represent a model for human MDS/AML.³⁴

We first used the *Gfi1b:EGFP* knock-in reporter mouse strain and crossed these mice with *NUP98/HOXD13^{tg}* mice (Figure 3A). Loss of one allele of *Gfi1b* shortened the latency period of AML development (Figure 3B). In BM cells derived from heterozygous leukemic mice, the expression of *Gfi1b* mRNA and *Gfi1b* protein levels were reduced to approximately 50% compared to BM cells from *Gfi1b^{w^t/w^t}* leukemic mice (Online Supplementary Figure S2A and B). Furthermore, we found that the *EGFP* expression level and hence *Gfi1b* expression level was significantly lower in the myeloid blasts when the disease onset was within the first 250 days compared to *Gfi1b* expression in blasts from mice that developed overt leukemia more than 250 days after birth (Figure 3C). The leukemic cells from *Gfi1b^{w^t/w^t}* or *Gfi1b^{EGFP/w^t}* animals showed no significant differences with regard to surface marker expression, spleen size, white blood cell and platelet counts, or cytological appearance, but showed significant differences with regard to hemoglobin and red blood cells (Figure 3D, Online Supplementary Figure S2C-F and *data not shown*), which might be due to a potential dose-dependent role of *Gfi1b* in erythropoiesis.^{6,9}

We next examined how complete absence of *Gfi1b* influences MDS to AML progression. We used the *Gfi1b* conditional knockout mouse model (*Gfi1b^{fl/fl}MxCre^{tg}*), whereby the expression of *Gfi1b* can be conditionally abrogated in the hematopoietic system upon poly(I:C) administration⁶ (Figure 3E). The absence of *Gfi1b* resulted in a substantially earlier onset of AML with a median survival time of approximately 50 days ($P < 0.0001$) (Figure 3F). Cre-mediated excision was verified to be efficient in leukemic *Gfi1b^{fl/fl}MxCre^{tg}NUP98/HOXD13^{tg}* mice after poly(I:C) administration with non-excised *Gfi1b* alleles below detection levels (Figure 3G), and this was associated with practically no expression of *Gfi1b* mRNA and protein (Online Supplementary Figure S2A and B).⁶ Leukemic cells from *Gfi1b^{fl/fl}MxCre^{tg}* and *Gfi1b^{fl/fl}MxCre^{tg}* animals showed no significant differences in spleen size, white blood cells or cytological appearance but significant differences in hemoglobin, red blood cells and platelet counts (Figure 3H and Online Supplementary Figure S2G-J and *data not shown*), which might be due to a dose-dependent role of *Gfi1b* in erythropoiesis.^{6,9} The absence of *Gfi1b* led to a reduced frequency of myeloid cells (Figure 3I, left, middle, and Online Supplementary Figure S3A-C). CD117 (c-Kit) was uniformly higher expressed on all *Gfi1b*-deficient blast cells (derived from *Gfi1b^{fl/fl}MxCre^{tg}* mice compared to *Gfi1b* expressing blasts (*Gfi1b^{fl/fl}MxCre^{wt}*) (Figure 3I, right). Finally, there was no difference with regard to apoptosis in *NUP98/HOXD13^{tg}* mice (Online Supplementary Figure S3D). In our murine model of MDS/AML development, we did not observe a positive correlation between *Gfi1b* and *Mllt3* expression nor a negative correlation between *Gfi1b* and *Spi1* expression, which might be disease context-dependent and thus not reproducible in all types of AML (Online Supplementary Figure S3A and B). We also analyzed the expression level of *Meis1*, since Chowdhury *et al.* observed a negative correlation between *GFI1B* and *MEIS1*.³⁵ We were able to confirm this finding for this model of AML (Online Supplementary Figure S4C).

Loss of *Gfi1b* promotes the progression of myeloproliferative disorder in a conditional *Kras* mouse model

To validate the results above in a second model, we used mice conditionally expressing a mutated form of

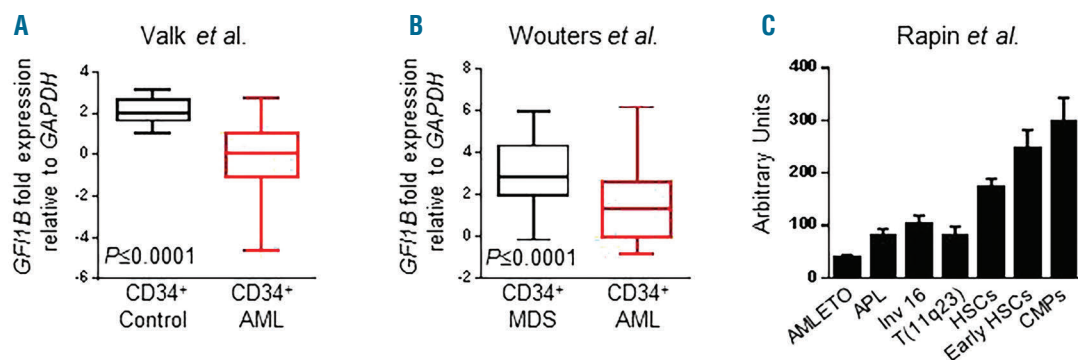


Figure 1. Correlation between different *GFI1B* expression levels and myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML) prognosis. (A) Expression of *GFI1B* in CD34⁺ AML cells (n=269) compared to CD34⁺ control cells (n=8) based on the patient cohort published by Valk *et al.*;²² $P \leq 0.0001$. (B) Expression of *GFI1B* in CD34⁺ MDS cells (n=23) compared to CD34⁺ AML cells (n=501) based on the patient cohort published by Wouters *et al.*;¹⁹ $P \leq 0.0001$. (C) Expression of *GFI1B* in leukemic stem cells (LSCs)²⁰ of different AML subtypes compared to normal hematopoietic stem cells (HSCs) or common myeloid progenitor cells (CMPs) in published gene expression arrays.³¹

Kras. RAS mutations are found in 5-10% of AML patients.² These mice harbor a transcriptional stop codon flanked by loxP sites upstream of a mutated Kras allele and, after removal of the stop codon, develop myeloproliferative disorder.³⁵ We crossed these mice with *Gfi1b^{fl/fl}MxCre^{tg}* or *Gfi1b^{wi/wi}MxCre^{tg}* mice, and after poly(I:C) administration, we observed mice for the emergence of disease (Figure 4A).

While *Gfi1b^{wi/wi}MxCre^{tg}Kras^{wi/fl}* mice developed a lethal myeloproliferative disorder with a median survival of approximately 25 days, loss of *Gfi1b* significantly shortened the latency period of the disease to a median survival of approximately seven days (Figure 4B). There was no difference with regard to cytological appearance, number of myeloid cells or level of apoptosis (Figure 4C and D and *Online Supplementary Figure S5A-D*). We also did not observe any significant difference with regard to white blood counts, platelet counts or spleen size but a significant difference in hemoglobin and red blood cells between *Gfi1b^{fl/fl}MxCre^{tg}* and *Gfi1b^{wi/wi}MxCre^{tg}* animals (*Online Supplementary Figure S5E-H*), which might be due to the role of *Gfi1b* in erythropoiesis.^{6,9}

Loss of *Gfi1b* promotes the progression of AML initiated by retroviral *MLL-AF9* expression

The Mixed Lineage Leukemia (*MLL*) gene is a common target for chromosomal translocations.² *MLL-AF9* is a fusion protein frequently occurring in a subset of AML patients,² and its expression in hematopoietic progenitors has been linked to the induction of AML in mice.³⁶ As a third AML mouse model, we thus used mice that developed AML through the induction of *MLL-AF9* expression, the product of the t(9;11)(q22;p23) translocation. Lin- BM cells derived from *Gfi1b^{wi/wi}MxCre^{tg}* or *Gfi1b^{fl/fl}MxCre^{tg}* mice were transduced with a retrovirus expressing *MLL-AF9* and transplanted into lethally irradiated C57BL/6J mice. For Cre-mediated excision of *Gfi1b* in the transplanted cells, mice were injected with poly(I:C) three weeks after transplantation (Figure 4E). Poly(I:C)-injected mice with *MLL-AF9*-transduced *Gfi1b^{fl/fl}MxCre^{tg}* (*Gfi1b*-deficient) cells succumbed faster to leukemia than mice injected with poly(I:C) and transplanted with *MLL-AF9*-transduced *Gfi1b^{wi/wi}MxCre^{tg}* (*Gfi1b*-expressing) cells (Figure 4F). However, there were no major qualitative differences concerning cytological findings and or blood parameters

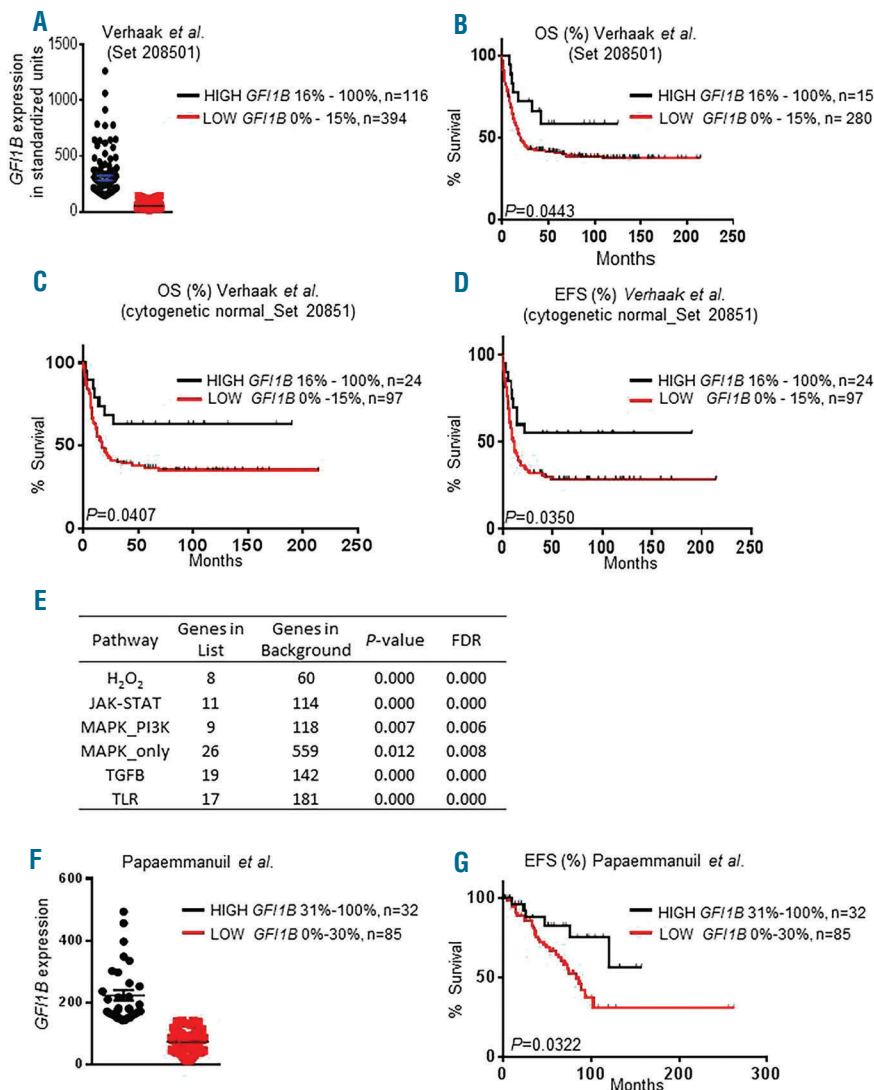


Figure 2. Different *Gfi1B* levels are indicative of prognosis of myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML) patients. (A) High expression of *Gfi1B* in human AML cells (n=116) compared to lower expression of *Gfi1B* (0-15%) in AML cells (n=394) based on the patient cohort published by Verhaak et al.²² (B) Overall survival (OS) from patients described in Verhaak et al.²² with regard to *Gfi1B* expression (P=0.0443). (C) Overall survival (OS) from patients described in Verhaak et al.²² (restricted to cytogenetically normal patients) with regard to *Gfi1B* expression; P=0.0407. (D) Same as in (C) but with regard to event-free survival (EFS); P=0.0350. (E) Analysis of signaling pathways with low *Gfi1B* expression (the lowest 5% compared with the highest 20% of expression levels) in a bigger dataset from Verhaak et al.²² Analysis was performed by Signaling Pathway Enrichment using Experimental Datasets (SPEED) analysis. Pathways such as reactive oxygen species (ROS; H₂O₂), MAPK, JAK, TGFB and TLR are highly significant. (F) High expression of *Gfi1B* (31-100%) in human MDS patients (n=32) compared to lower expression of *Gfi1B* (0-30%) in MDS patients (n=85) based on the patient cohort published by Papaemmanuil et al.²³ (G) EFS of patients described in Papaemmanuil et al.²³ with regard to *Gfi1B* expression; P=0.032.

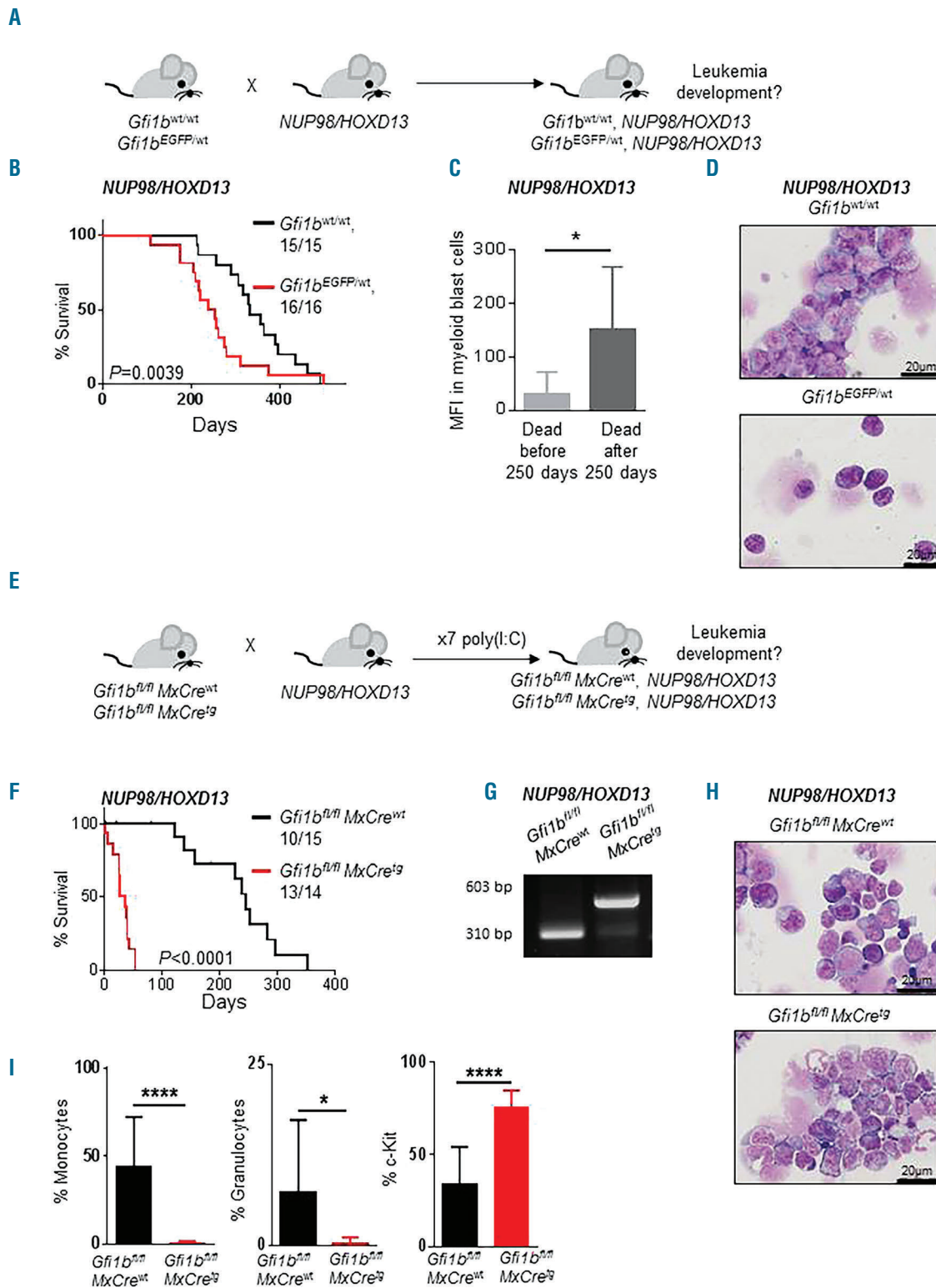


Figure 3. Low level or absence of *Gfi1b* accelerates the progression of myelodysplastic syndrome (MDS) to acute myeloid leukemia (AML) in the *NUP98/HOXD13* MDS mouse model. (A) Crossing of the *Gfi1b*^{wt/wt} and *Gfi1b*^{EGFP/wt} mouse strains with the *NUP98/HOXD13* mouse model. (B) Survival of *Gfi1b*^{wt/wt} and *Gfi1b*^{EGFP/wt}*NUP98/HOXD13* transgenic mice; *P*=0.0039. Number of mice succumbing to AML is indicated. (C) Mean fluorescence intensity (MFI) of the GFP expression level (and hence *Gfi1b* promoter activity) in *Gfi1b*^{EGFP/wt} mice that died of AML before 250 days (*n*=7) or after 250 days (*n*=5); *P*=0.0272. (D) Wright-Giemsa staining of bone marrow (BM) cytopsin from representative *Gfi1b*^{EGFP/wt} and *Gfi1b*^{wt/wt} *NUP98/HOXD13* leukemic mice (bar=20 μm). (E) Crossing of the *Gfi1b*^{fl/fl}*MxCre*^{wt} and *Gfi1b*^{fl/fl}*MxCre*^{tg} mouse strains with the *NUP98/HOXD13* mouse model. After Cre-mediated deletion of the *Gfi1b* gene upon poly(I:C) administration, the mice were monitored for signs of leukemia. (F) Survival of *Gfi1b*^{fl/fl}*MxCre*^{wt} (*Gfi1b*^{wt/wt}) and *Gfi1b*^{fl/fl}*MxCre*^{tg} (*Gfi1b*^{fl/fl}) mice transgenically expressing *NUP98/HOXD13* after poly(I:C) administration; *P*<0.0001. Number of mice succumbing to AML is indicated. (G) Polymerase chain reaction genotyping of DNA from BM cells of poly(I:C)-injected *Gfi1b*^{fl/fl}*MxCre*^{wt} and *Gfi1b*^{fl/fl}*MxCre*^{tg} *NUP98/HOXD13* leukemic mice. (H) Wright-Giemsa staining of BM cytopsin from representative poly(I:C)-injected *Gfi1b*^{fl/fl}*MxCre*^{wt} and *Gfi1b*^{fl/fl}*MxCre*^{tg}*NUP98/HOXD13* leukemic mice (bar=20 μm). (I) The frequency of monocytes (Mac-1hiGr-1int) (left panel, *****P*<0.0001), granulocytes (Mac-1hiGr-1hi) (middle panel, **P*=0.0206) and CD117⁺ (c-Kit) cells (right panel, *****P*<0.0001) in the BM of mice described in (F) (*n*=15 for *Gfi1b*^{fl/fl}*MxCre*^{wt} mice; *n*=13 for *Gfi1b*^{fl/fl}*MxCre*^{tg}*NUP98/HOXD13* mice).

(Figure 4G and data not shown). We did not observe a significant change in the number of overall myeloid cells or apoptosis level in the different settings (Figure 4H and Online Supplementary Figure S6A-D).

Loss of *Gfi1b* increases the number of LSCs

Loss of *Gfi1b* leads to an expansion in the number of functional HSCs;⁶ therefore we investigated whether the same applies to LSCs. We performed a limiting dilution assay by transplanting *MLL-AF9* leukemic BM cells derived from poly(I:C)-treated *Gfi1b^{fl/fl}MxCre^{tg}* or *Gfi1b^{fl/fl}MxCre^{tg}* leukemic mice into sublethally irradiated congenic mice (Figure 5A). *Gfi1b*-deficient *MLL-AF9* BM cells had a LSC frequency of 1:3500 compared with an LSC frequency of 1:63000 cells in *Gfi1b*-expressing leukemic cells (Figure 5B). The increased number of func-

tional LSCs in *Gfi1b*-deficient leukemic cells could explain why loss of *Gfi1b* accelerated disease progression, as it has already been shown that a higher number of LSCs is associated with a poor prognosis of leukemia patients.³⁷ However, this hypothesis needs to be confirmed in independent experiments.

Loss of *Gfi1b* induces gene expression changes supporting AML development

To further study the molecular function of *Gfi1b* in AML, we performed whole genome gene expression analysis using *Gfi1b*-expressing and *Gfi1b*-deficient *NUP98/HOXD13^{tg}* leukemic mice (Figure 6A). This model was used since the difference between *Gfi1b*-deficient and *Gfi1b*-expressing leukemic cells was most striking in the *NUP98/HOXD13^{tg}* mouse model. Using gene set enrich-

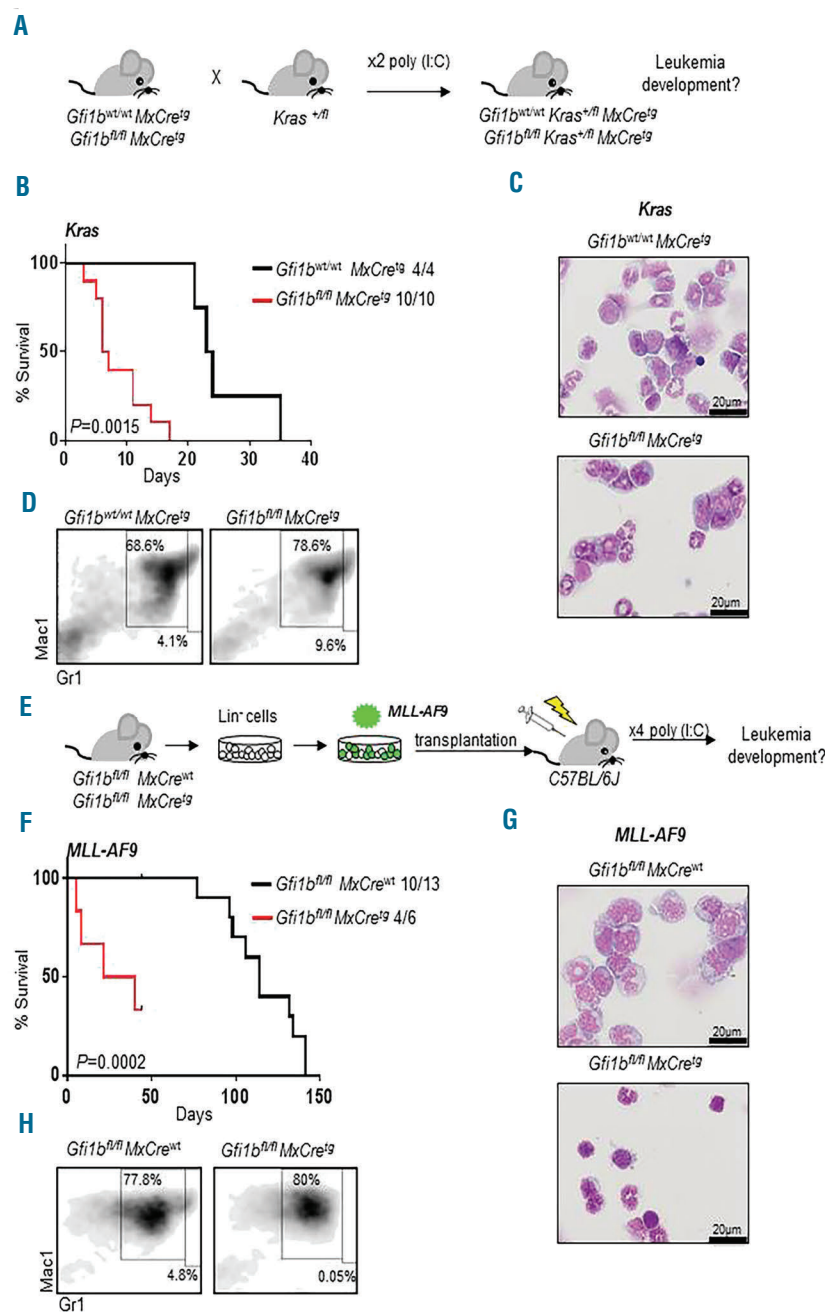


Figure 4. Absence of *Gfi1b* accelerates the progression of myeloproliferative disorder and acute myeloid leukemia (AML). (A) Crossing of the *Gfi1b^{wt/wt}MxCre^{tg}* and *Gfi1b^{fl/fl}MxCre^{tg}* mouse strains with the *Kras^{+/fl}* mouse model. (B) Survival of *Gfi1b^{wt/wt}MxCre^{tg}* and *Gfi1b^{fl/fl}MxCre^{tg}* mice transgenically expressing *Kras* after poly (I:C) administration; **** $P < 0.0015$. Numbers indicate the number of mice succumbing to AML. (C) Wright-Giemsa staining of bone marrow (BM) cytopsin from representative *Gfi1b^{wt/wt}MxCre^{tg}* and *Gfi1b^{fl/fl}MxCre^{tg}* leukemic mice transgenically expressing *Kras* after poly (I:C) administration (bar=20 μ m). (D) Flow cytometric analysis of the BM from the leukemic mice shown in (B) with regard to Gr-1 and Mac-1 expression. (E) Isolation, transduction and transplantation of lineage-negative (Lin⁻) cells from *Gfi1b^{fl/fl}MxCre^{wt}* and *Gfi1b^{fl/fl}MxCre^{tg}* mice with *MLL-AF9*-expressing retrovirus. After Cre-mediated deletion of the *Gfi1b* gene upon poly(I:C) administration, the mice were monitored for signs of leukemia. (F) Survival of the mice transplanted with *Gfi1b^{fl/fl}MxCre^{wt}* and *Gfi1b^{fl/fl}MxCre^{tg}* *MLL-AF9* transduced cells; *** $P = 0.0002$. Number of mice succumbing to AML is indicated. (G) Wright-Giemsa staining of BM cytopsin from the leukemic mice described in (F) (bar=20 μ m). (H) Flow cytometric analysis of the BM from the leukemic mice described in (F) with regard to Gr-1 and Mac-1 expression.

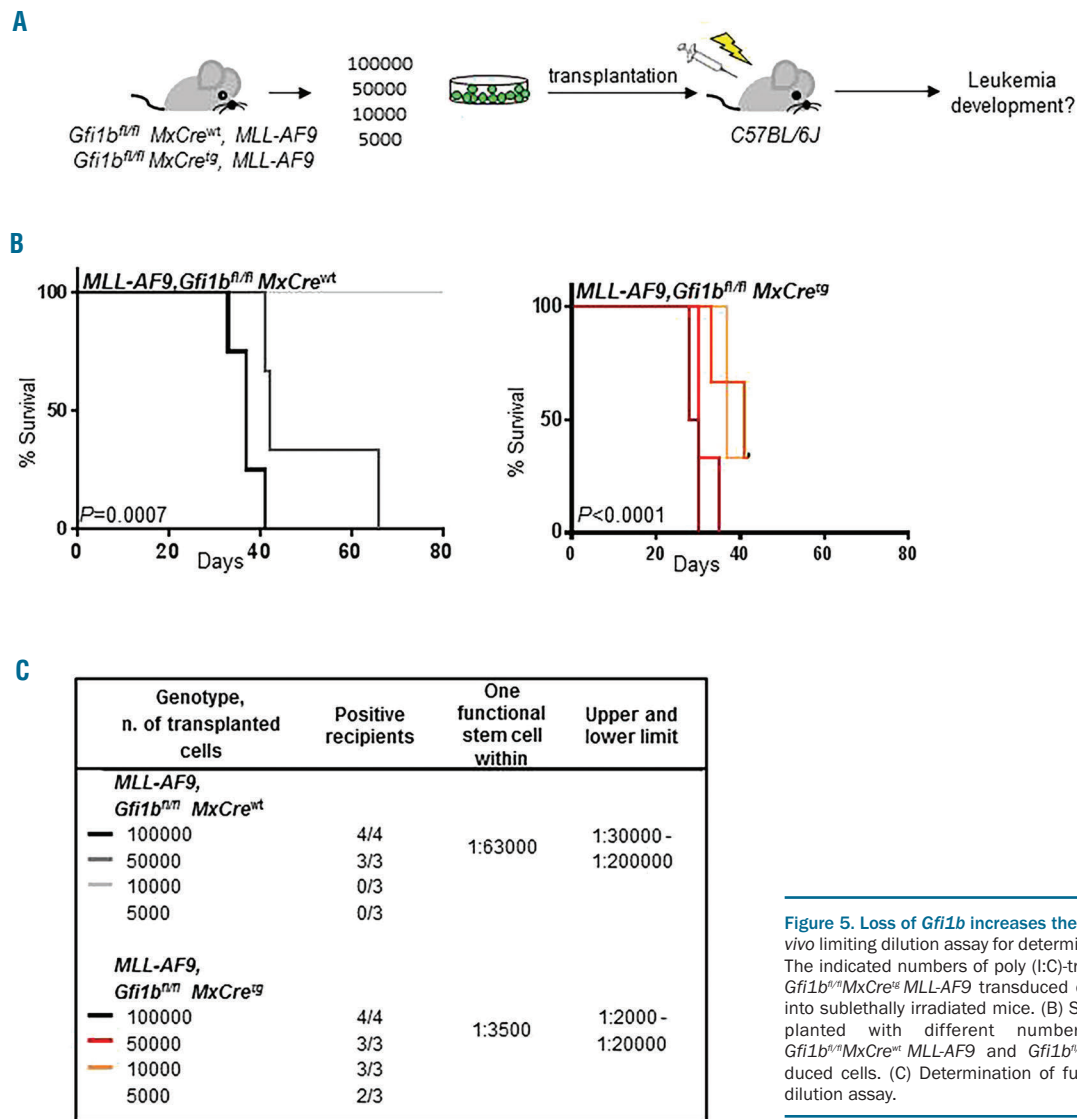


Figure 5. Loss of *Gfi1b* increases the stemness of LSCs. (A) *In vivo* limiting dilution assay for determination of functional LSCs. The indicated numbers of poly(I:C)-treated *Gfi1b*^{fl/fl}*MxCre*^{wt} and *Gfi1b*^{fl/fl}*MxCre*^{tg} MLL-AF9 transduced cells were retransplanted into sublethally irradiated mice. (B) Survival of the mice transplanted with different numbers of poly(I:C)-treated *Gfi1b*^{fl/fl}*MxCre*^{wt} MLL-AF9 and *Gfi1b*^{fl/fl}*MxCre*^{tg} MLL-AF9 transduced cells. (C) Determination of functional LSCs by limiting dilution assay.

ment analysis (GSEA), loss of *Gfi1b* was associated with a signature showing enrichment of genes involved in AML development as well as regulation of stemness (Figure 6B). This is of interest since we observed an increase in the number of LSCs upon deletion of *Gfi1b*.

Gfi1b recruits different histone-modifying enzymes, among them HDACs,¹⁶ to its target genes. This in turn leads to deacetylation of H3K9, which leads to epigenetic silencing of the particular *Gfi1b* target genes.¹⁶ We, therefore, analyzed the genome-wide H3K9 acetylation level of leukemic blasts from *Gfi1b*-expressing and *Gfi1b*-deficient *NUP98/HOXD13*^{tg} leukemic mice. Loss of *Gfi1b* leads to a genome-wide increase in H3K9 acetylation level (Figure 6C). In a subsequent step, we analyzed those genes, which showed an elevated level of H3K9 acetylation in *Gfi1b*-deficient leukemic cells compared to the H3K9 acetylation level of the same genes found in *Gfi1b*-expressing leukemic cells. Using GSEA, we found a significant enrichment of gene sets associated with the regulation of cell growth and MAPK signaling (Figure 6D). We also performed a Kyoto encyclopedia of genes and

genomes (KEGG) pathway analysis of those genes, which exhibited differentially H3K9 acetylated promoter areas in *Gfi1b*-expressing and *Gfi1b*-deficient leukemic cells. We found a number of processes involved in erythroid regulation (Online Supplementary Figure S7A and B), which is a main function of *Gfi1b* and hence underscores the validity of our results.^{6,9,16} Finally, we analyzed the differentially acetylated genes in *Gfi1b*-deficient and *Gfi1b*-expressing leukemic cells and compared these gene sets based on the gene sets provided by the Molecular Signatures Database (MSigDB). Using this approach, we repeatedly found signatures associated with p38 (Figure 6E).

We observed increased H3K9 acetylation of the promoter area of genes involved in stem cell function in *Gfi1b*-deficient leukemic cells, and these epigenetic changes correlated with the gene-expression changes described above (Figure 6B). As described, gene expression arrays revealed an enrichment of a stem cell/leukemic stem cell gene signature in *Gfi1b*-deficient leukemic cells. To validate these results, we selected 14 different genes

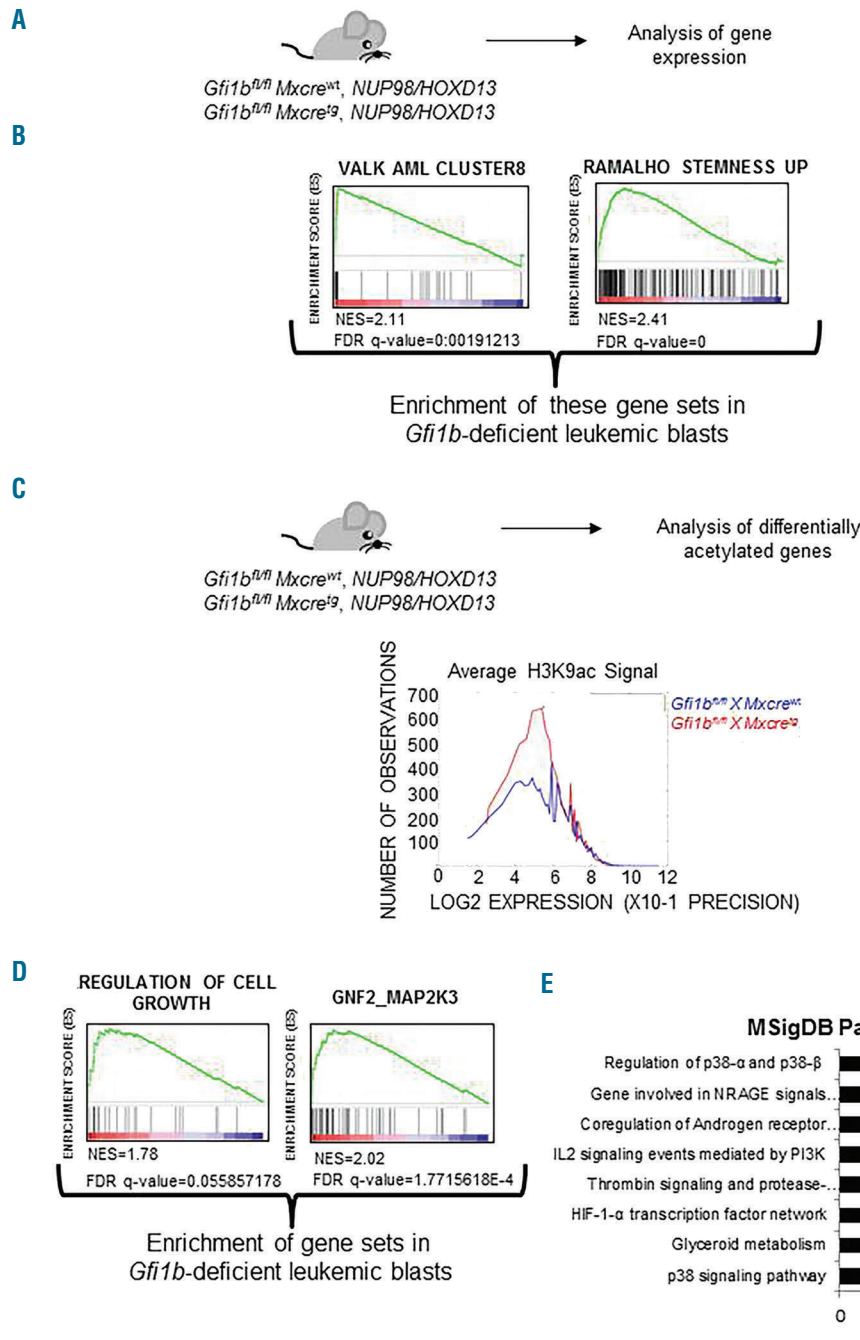


Figure 6. Loss of *Gfi1b* induces gene expression changes supporting acute myeloid leukemia (AML) development. (A) Microarray analysis was performed with *Gfi1b*^{fl/fl}*MxCre*^{wt}*NUP98/HOXD13* and *Gfi1b*^{fl/fl}*MxCre*^{tg}*NUP98/HOXD13* leukemic cells. (B) Based on the results of the micro-array analysis, a Gene set enrichment analysis (GSEA) of leukemic cells from *Gfi1b*^{fl/fl}*MxCre*^{wt}*NUP98/HOXD13* and *Gfi1b*^{fl/fl}*MxCre*^{tg}*NUP98/HOXD13* mice was performed. As a result, *Gfi1b* deficient leukemic cells showed an enrichment of the gene set of VALK AML cluster 8 with a normalized enrichment score (NES) of 2.1 and false discovery rate (FDR) of q-val=0.00191213. *Gfi1b* deficient cells showed also an enrichment for RAMALHO STEMNESS with an NES=2.41 and an FDR q-val=0. (C) ChIP and ChIP-Seq analysis was performed with *Gfi1b*^{fl/fl}*MxCre*^{wt}*NUP98/HOXD13* and *Gfi1b*^{fl/fl}*MxCre*^{tg}*NUP98/HOXD13* leukemic cells. ChIP-Seq analysis for differences in the frequency of H3K9 acetylation of *Gfi1b*-deficient (*Gfi1b*^{fl/fl}*MxCre*^{tg}) leukemic blasts from *NUP98/HOXD13* mice compared to leukemic cells with normal *Gfi1b* expression (*Gfi1b*^{fl/fl}*MxCre*^{wt}). (D) GSEA of genes with an elevated acetylation level in *Gfi1b* deficient mice are associated with regulation of cell growth NES=1.78 and FDR q-val=0.055857178 and GNF2_MAP2K3 NES=2.02 and FDR q-val=1.7715618E-4. (E) Upon analyzing the differentially acetylated genes and using the MSigDB Pathway approach, we found significant enrichment of different pathways, among them the p38 pathway. P-value was used to rank the enrichment.

(*Abcg2*, *Gata3*, *Igta2*, *Thy1*, *Cd24a*, *Pecam1*, *Prom1*, *Plaur*, *Klf4*, *Mycn*, *Ptch1*, *Pecam1*, *Sav1*, and *Notch1*), which were differentially expressed by more than 2-fold between *Gfi1b*-expressing and *Gfi1b*-deficient leukemic mice in the gene expression arrays. We selected these genes based on their diverse role in regulating stem cell function. We then examined these genes and confirmed that these genes were also differentially expressed using RT-PCR (Online Supplementary Figure S8A).

Loss of *Gfi1b* leads to increased ROS levels and decreased levels of activated p38

To obtain further insight into the molecular mechanism behind our observation we compared the whole genome

gene expression pattern in murine *Gfi1b*-expressing and *Gfi1b*-deficient leukemic cells based on an AltAnalyze approach (see Online Supplementary Appendix). Using the same algorithm, we compared the gene expression pattern found in AML blasts with low *GFI1B* expression and high *GFI1B* expression (data obtained from published studies from Valk *et al.*²¹ and Verhaak *et al.*²²). For the analysis of the human dataset, we analyzed the 10% of patients with the lowest and the 20% of patients with the highest *GFI1B* expression level in order to have enough observations from which to draw any conclusions. Then we compared which pathways were similarly deregulated in the human and murine leukemia sets. ROS and MAPK signaling were among the pathways differentially expressed between both murine and human

Gfi1b/*GFI1B*-deficient/low and *Gfi1b*/*GFI1B* high-expressing leukemic cells (Figure 7A). As ROS plays an important role in the pathogenesis of AML,³⁸ we examined the level of ROS. For non-malignant HSCs, it was shown that HSCs with low ROS had a high self-renewal capacity.³⁹ In contrast, HSCs with elevated ROS were mostly located in the vascular niche, had a reduced self-renewal capacity, and were more restricted with regard to their differentiation potential.³⁹ Based on this and our previous report that loss of *Gfi1b* leads to higher level of ROS in HSCs,⁶ we examined whether ROS level might differ between *Gfi1b*-deficient and *Gfi1b* expressing LSCs. Due to the difficulty of defining a distinct LSC population in each set of AML samples, we used CD117 (c-Kit) expression as a surrogate marker to define a population which is enriched for LSCs. CD117 expression has been used to identify a fraction that is enriched for LSCs.⁴⁰ We identified two distinct populations in the CD117⁺ blast cells that differ with regard to their ROS expression (a population with low ROS expression and a population with high ROS expression). Loss of *Gfi1b* led to an increased level of ROS (defined as the mean fluorescent intensity, MFI) in both ROS-low and ROS-high populations (Figure 7B-D and *Online Supplementary Figure S8B*).

Altered activity of the AKT pathway in *Gfi1b*-deficient AML

Elevated levels of ROS promote AML development, but ROS also activates various redox-sensitive signaling transduction cascades,⁴¹ including the MAPK pathway, which limits the stemness of the affected cells, at least in a non-malignant setting.⁴² In the presence of ROS, the MAPK pathway component p38 is activated, which subsequently results in an exhaustion of the HSC population.⁴³ It has also been shown that activation of p38 limits oncogenic transformation.⁴⁴ Despite a higher level of ROS, in our models, *Gfi1b*-deficient leukemic cells have escaped p38 activation, indicated by a decreased level of phosphorylated p38 compared to *Gfi1b*^{fl/fl}*MxCre*^{wt}*NUP98/HOXD13*³⁸ (Figure 7E). The fact that *Gfi1b* might directly or indirectly regulate p38 is also supported by the analysis of differentially acetylated genes in *Gfi1b*-deficient and *Gfi1b*-expressing leukemic cells. Because decreased p38 levels are associated with higher oncogenic potential,⁴⁴ this could partially explain the higher number of functional LSCs we observed in the *Gfi1b*-deficient leukemic cells. Activation of p38 leads to an increased level of Akt^{Ser473}.⁴⁵ Akt^{Ser473} activity inversely correlates with the number of LSCs in AML.⁴⁶ We thus examined the connection between loss of *Gfi1b* and Akt^{Ser473} and found that the level of phosphorylated Akt^{Ser473} is reduced in *Gfi1b*-deficient leukemia (Figure 7F). Akt represses the function of FoXO3, and since FoXO3 acts as an oncogene in AML,⁴⁶ we determined the FoXO3 protein level. *Gfi1b*-deficient leukemic cells showed an increased expression of FoXO3 protein in the nucleotide (NER) and cytoplasmatic (CER) cell fraction compared to the expression level of FoXO3 in *Gfi1b*-expressing leukemic cells (Figure 7G). To obtain an insight into whether this increased level of FoXO3 also has any functional consequences, we re-examined the whole genome expression datasets in *Gfi1b*-expressing and *Gfi1b*-deficient leukemic cells and found an enrichment of FoXO3 binding sites among the promoter areas of those genes, which were differentially expressed between cells with

absence of *Gfi1b* expression and cells with intact expression of *Gfi1b* (*Online Supplementary Figure S9*), showing that altered level of FoXO3 might be one additional explanation for our observations (Figure 7H).

Discussion

In the datasets analyzed by us, *GFI1B* was expressed at a lower level in LSCs compared to the control. Low *GFI1B* was also indicative of an inferior prognosis for MDS and AML patients, with the caveat that these statements are based on retrospective studies. Larger prospective studies would be required to make such a claim on a solid basis. We previously reported that low *GFI1* expression level in AML blasts was associated with poor outcome and here we report that low *GFI1B* expression levels were associated with poor survival. This might appear surprising since *GFI1* and *GFI1B* repress each other, but in our cohorts we observed that low *GFI1B* expression level can also be associated with low *GFI1* expression (*data not shown*), therefore, the reciprocal regulation between *GFI1* and *GFI1B* might be different in leukemic cells. We postulate that *GFI1B* plays a dose-dependent role in human/murine AML pathogenesis. Anguita *et al.* showed that a mutated dominant-negative form of *GFI1B* contributes to AML development. These reports show how altering the function of *GFI1B* can influence normal and malignant development. Recent studies have highlighted the role of different isoforms of *GFI1B* in the course of erythroid and megakaryocytic development.^{6,47-49} It remains to be elucidated whether altering the expression of these isoforms might also contribute to AML development.

Loss of *Gfi1b* in our murine models increased the number of LSCs on a functional level. These data are in line with our previous reports that loss of *Gfi1b* leads to an expansion of functional HSCs.⁶ On a molecular level, loss of *Gfi1b* resulted in an increased level of H3K9ac among its target genes, which is in line with other reports regarding the epigenetic function of *Gfi1b*.^{11,16} Among these target genes are a number of genes involved in the regulation of leukemogenesis and stem cell regulation, indicating that the absence of *Gfi1b* leads to a gene expression signature that directly or indirectly contributes to an increased number of LSCs.

Both murine and human data also indicated a possible connection between *Gfi1b* and ROS/p38/Akt signaling. P38 and Akt^{Ser473} activation limit oncogenic and stemness potential.^{43,44,46} Conceivably, lower expression of these proteins would increase the oncogenic potential. P38 and Akt were down-regulated in *Gfi1b*-deficient leukemic cells *in vivo*. In line with this, Saleque *et al.* demonstrated that *Gfi1b* is involved in the regulation of p38 and that reduced *Gfi1b* levels are associated with lower p38 signaling.⁵⁰ In addition to the ROS/p38/Akt/FoXO3 signaling cascade, other pathways were altered. It remains to be elucidated which role these pathways might play in the pathogenesis of human and murine AML. In addition, how *Gfi1b*/*GFI1B* regulates ROS, p38, Akt and FoXO3 levels remains to be analyzed.

In summary, epigenetic changes and alteration of the ROS/p38/Akt/FoXO3 signaling cascade might facilitate the progression of normal hematopoietic cells to LSCs. In the future, testing will be needed to determine whether alter-

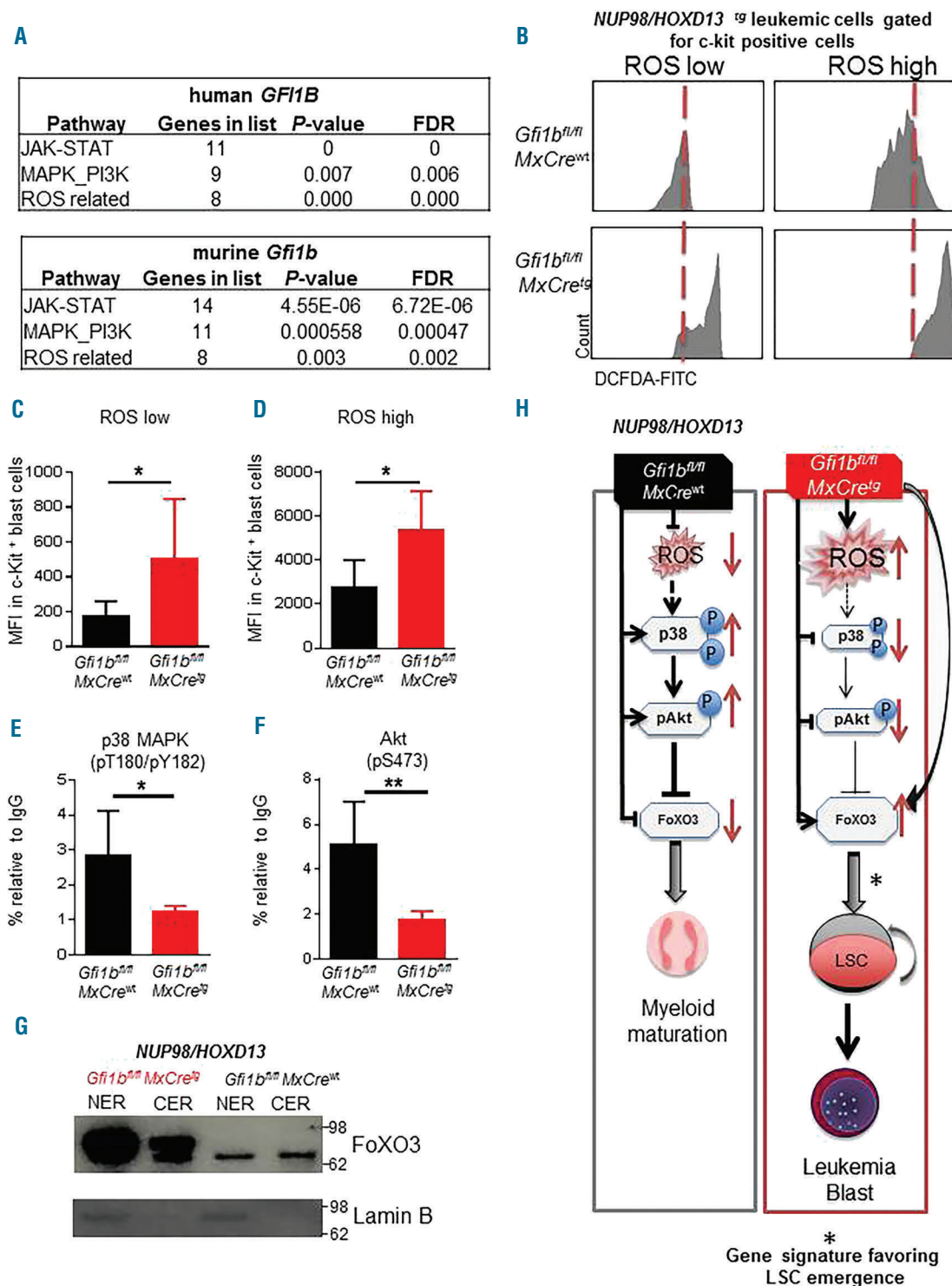


Figure 7. Loss of *Gfi1b* deregulates acute myeloid leukemia (AML) signaling pathway. (A) Analysis to see which pathways were enriched in *GFI1B* low-expressing blast cells in two independent sets of myelodysplastic syndrome (MDS) or AML patients compared to the expression pattern found in *GFI1B* high-expressing MDS/AML blast cells. The same approach was repeated for *Gfi1b*-expressing and *Gfi1b*-non-expressing leukemic cells from our mice experiments. As an overlap, enrichment was observed in pathways of JAK-STAT-, MAPK- and ROS-related signaling. (B) Representative flow cytometric analysis of bone marrow (BM) from *Gfi1b*^{fl/fl}*MxCre*^{tg}*NUP98/HOXD13*^{tg} mice compared to *Gfi1b*^{fl/fl}*MxCre*^{wt}*NUP98/HOXD13*^{tg} mice showing the gating strategy for determining ROS low and ROS high levels. (C) Mean fluorescence intensity (MFI) for ROS in the ROS-low population of c-Kit⁺ blast cells derived from *Gfi1b*^{fl/fl}*MxCre*^{wt}*NUP98/HOXD13*^{tg} (n=6) and *Gfi1b*^{fl/fl}*MxCre*^{tg}*NUP98-HOXD13*^{tg} (n=5); *P=0.0488. (D) Mean fluorescence intensity (MFI) for ROS in the ROS-high population of c-Kit⁺ blast cells derived from *Gfi1b*^{fl/fl}*MxCre*^{wt}*NUP98-HOXD13*^{tg} (n=6) and *Gfi1b*^{fl/fl}*MxCre*^{tg}*NUP98/HOXD13*^{tg} (n=5); *P=0.0191. (E) Flow cytometric analysis of p38 MAPK (pT180/pY182) in CD117⁺ blast cells derived from *Gfi1b*^{fl/fl}*MxCre*^{wt}*NUP98/HOXD13*^{tg} (n=5) and *Gfi1b*^{fl/fl}*MxCre*^{tg}*NUP98/HOXD13*^{tg} (n=6); *P=0.0144. (F) Flow cytometric analysis of Akt (pS473) in c-Kit⁺ blast cells derived from *Gfi1b*^{fl/fl}*MxCre*^{wt}*NUP98/HOXD13*^{tg} (n=4) and *Gfi1b*^{fl/fl}*MxCre*^{tg}*NUP98/HOXD13*^{tg} (n=5); **P=0.0040. (G) FoXO3 protein level was detected in nuclear extraction (NER)- and cytoplasmic extraction (CER)-derived BM cells from *Gfi1b*^{fl/fl}*MxCre*^{tg}*NUP98/HOXD13*^{tg} and *Gfi1b*^{fl/fl}*MxCre*^{wt}*NUP98/HOXD13*^{tg}. (H) Working model hypothesis: normal levels of *Gfi1b* lead to reduced ROS levels, resulting in normal maturation and differentiation of progenitor cells. Loss of *Gfi1b* in leukemic cells is associated with higher ROS levels, which have been shown to promote AML development. However, through a still undefined mechanism, this results in lower levels of p38 MAPK and pAkt and higher levels of unphosphorylated FoXO3, which might explain the increased number of functional leukemic stem cells in the *Gfi1b*-deficient AML population. LSC: leukemic stem cells.

ation of the ROS pathway could be a targeted therapeutic approach to treat AML patients with low GFI1B expression.

Acknowledgments

We thank the animal facility of University Hospital Essen.

Funding

This work was supported by the Deutsche Forschungsgemeinschaft and the IFORES program of University Hospital Essen.

References

- Tefferi A, Vardiman JW. Myelodysplastic syndromes. *N Engl J Med.* 2009; 361(19):1872-1885.
- Dohner H, Estey E, Grimwade D, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood.* 2017;129(4):424-447.
- Calero-Nieto FJ, Ng FS, Wilson NK, et al. Key regulators control distinct transcriptional programmes in blood progenitor and mast cells. *EMBO J.* 2014;33(11):1212-1226.
- Hones JM, Botezatu L, Helness A, et al. GFI1 as a novel prognostic and therapeutic factor for AML/MDS. *Leukemia.* 2016; 30(6):1237-1245.
- Gentner B, Pochert N, Rouhi A, et al. MicroRNA-223 dose levels fine tune proliferation and differentiation in human cord blood progenitors and acute myeloid leukemia. *Exp Hematol.* 2015;43(10):858-868.
- Khandanpour C, Sharif-Askari E, Vassen L, et al. Evidence that growth factor independence 1b regulates dormancy and peripheral blood mobilization of hematopoietic stem cells. *Blood.* 2010;116(24):5149-5161.
- Laurent B, Randrianarison-Huetz V, Marechal V, Mayeux P, Dusanter-Fourt I, Dumenil D. High-mobility group protein HMGB2 regulates human erythroid differentiation through trans-activation of GFI1B transcription. *Blood.* 2010;115(3):687-695.
- Foudi A, Kramer DJ, Qin J, et al. Distinct, strict requirements for Gfi-1b in adult bone marrow red cell and platelet generation. *J Exp Med.* 2014;211(5):909-927.
- Saleque S, Cameron S, Orkin SH. The zinc-finger proto-oncogene Gfi-1b is essential for development of the erythroid and megakaryocytic lineages. *Genes Dev.* 2002; 16(3):301-306.
- Vassen L, Beauchemin H, Lemsaddek W, Krongold J, Trudel M, Moroy T. Growth factor independence 1b (gfi1b) is important for the maturation of erythroid cells and the regulation of embryonic globin expression. *PLoS One.* 2014;9(5):e96636.
- van der Meer LT, Jansen JH, van der Reijden BA. Gfi1 and Gfi1b: key regulators of hematopoiesis. *Leukemia.* 2010; 24(11):1834-1843.
- Schulz D, Vassen L, Chow KT, et al. Gfi1b negatively regulates Rag expression directly and via the repression of FoxO1. *J Exp Med.* 2012;209(1):187-199.
- Vassen L, Okayama T, Moroy T. Gfi1b:green fluorescent protein knock-in mice reveal a dynamic expression pattern of Gfi1b during hematopoiesis that is largely complementary to Gfi1. *Blood.* 2007;109(6):2356-2364.
- Osawa M, Yamaguchi T, Nakamura Y, et al. Erythroid expansion mediated by the Gfi-1B zinc finger protein: role in normal hematopoiesis. *Blood.* 2002;100(8):2769-2777.
- Vassen L, Fiolka K, Moroy T. Gfi1b alters histone methylation at target gene promoters and sites of gamma-satellite containing heterochromatin. *EMBO J.* 2006; 25(11):2409-2419.
- Saleque S, Kim J, Rooke HM, Orkin SH. Epigenetic regulation of hematopoietic differentiation by Gfi-1 and Gfi-1b is mediated by the cofactors CoREST and LSD1. *Mol Cell.* 2007;27(4):562-572.
- McGhee L, Bryan J, Elliott L, et al. Gfi-1 attaches to the nuclear matrix, associates with ETO (MTG8) and histone deacetylase proteins, and represses transcription using a TSA-sensitive mechanism. *J Cell Biochem.* 2003;89(5):1005-1018.
- Duan Z, Zarebski A, Montoya-Durango D, Grimes HL, Horwitz M. Gfi1 coordinates epigenetic repression of p21Cip/WAF1 by recruitment of histone lysine methyltransferase G9a and histone deacetylase 1. *Mol Cell Biol.* 2005;25(23):10338-10351.
- Wouters BJ, Lowenberg B, Erpelinck-Verschueren CA, van Putten WL, Valk PJ, Delwel R. Double CEBPA mutations, but not single CEBPA mutations, define a subgroup of acute myeloid leukemia with a distinctive gene expression profile that is uniquely associated with a favorable outcome. *Blood.* 2009;113(13):3088-3091.
- Rapin N, Bagger FO, Jendholm J, et al. Comparing cancer vs normal gene expression profiles identifies new disease entities and common transcriptional programs in AML patients. *Blood.* 2014;123(6):894-904.
- Valk PJ, Verhaak RG, Beijin MA, et al. Prognostically useful gene-expression profiles in acute myeloid leukemia. *N Engl J Med.* 2004;350(16):1617-1628.
- Verhaak RG, Wouters BJ, Erpelinck CA, et al. Prediction of molecular subtypes in acute myeloid leukemia based on gene expression profiling. *Haematologica.* 2009;94(1):131-134.
- Papaemmanuil E, Gerstung M, Malcovati L, et al. Clinical and biological implications of driver mutations in myelodysplastic syndromes. *Blood.* 2013;122(22):3616-3627.
- Gerstung M, Pellagatti A, Malcovati L, et al. Combining gene mutation with gene expression data improves outcome prediction in myelodysplastic syndromes. *Nat Commun.* 2015;6:5901.
- Bagger FO, Sasivarevic D, Sohi SH, et al. BloodSpot: a database of gene expression profiles and transcriptional programs for healthy and malignant haematopoiesis. *Nucleic Acids Res.* 2016;44(D1):D917-924.
- Al-Matary YS, Botezatu L, Opalka B, et al. Acute myeloid leukemia cells polarize macrophages towards a leukemia supporting state in a Growth factor independence 1 dependent manner. *Haematologica.* 2016; 101(10):1216-1227.
- Botezatu L, Michel LC, Helness A, et al. Epigenetic therapy as a novel approach for GFI136N-associated murine/human AML. *Exp Hematol.* 2016;44(8):713-726 e714.
- Khandanpour C, Krongold J, Schuette J, et al. The human GFI136N variant induces epigenetic changes at the Hoxa9 locus and accelerates K-RAS driven myeloproliferative disorder in mice. *Blood.* 2012; 120(19):4006-4017.
- Hu Y, Smyth GK. ELDA: Extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. *J Immunol Methods.* 2009; 347(1-2):70-78.
- Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med.* 1997;3(7):730-737.
- Goardon N, Marchi E, Atzberger A, et al. Coexistence of LMPP-like and GMP-like Leukemia Stem Cells in Acute Myeloid Leukemia. *Cancer Cell.* 2011;19(1):138-152.
- Anguita E, Gupta R, Olariu V, et al. A somatic mutation of GFI1B identified in leukemia alters cell fate via a SPI1 (PU.1) centered genetic regulatory network. *Dev Biol.* 2016;411(2):277-286.
- Chowdhury AH, Ramroop JR, Upadhyay G, Sengupta A, Andrzejczyk A, Saleque S. Differential transcriptional regulation of meis1 by Gfi1b and its co-factors LSD1 and CoREST. *PLoS One.* 2013;8(1):e53666.
- Lin YW, Slape C, Zhang Z, Aplan PD. NUP98-HOXD13 transgenic mice develop a highly penetrant, severe myelodysplastic syndrome that progresses to acute leukemia. *Blood.* 2005;106(1):287-295.
- Chan IT, Kutok JL, Williams IR, et al. Conditional expression of oncogenic K-ras from its endogenous promoter induces a myeloproliferative disease. *J Clin Invest.* 2004;113(4):528-538.
- Krivtsov AV, Twomey D, Feng Z, et al. Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9. *Nature.* 2006;442(7104):818-822.
- Meyer LH, Eckhoff SM, Queudeville M, et al. Early relapse in all is identified by time to leukemia in NOD/SCID mice and is characterized by a gene signature involving survival pathways. *Cancer Cell.* 2011; 19(2):206-217.
- Hole PS, Darley RL, Tonks A. Do reactive oxygen species play a role in myeloid leukemias? *Blood.* 2011;117(22):5816-5826.
- Jang YY, Sharkis SJ. A low level of reactive oxygen species selects for primitive hematopoietic stem cells that may reside in the low-oxygenic niche. *Blood.* 2007;110(8):3056-3063.
- Wang Y, Krivtsov AV, Sinha AU, et al. The

- Wnt/beta-catenin pathway is required for the development of leukemia stem cells in AML. *Science*. 2010;327(5973):1650-1653.
41. Hole PS, Zabkiewicz J, Munje C, et al. Overproduction of NOX-derived ROS in AML promotes proliferation and is associated with defective oxidative stress signaling. *Blood*. 2013;122(19):3322-3330.
 42. Essers MA, Offner S, Blanco-Bose WE, et al. IFNalpha activates dormant haematopoietic stem cells in vivo. *Nature*. 2009;458(7240):904-908.
 43. Ito K, Hirao A, Arai F, et al. Reactive oxygen species act through p38 MAPK to limit the lifespan of hematopoietic stem cells. *Nat Med*. 2006;12(4):446-451.
 44. Bulavin DV, Fornace AJ Jr. p38 MAP kinase's emerging role as a tumor suppressor. *Adv Cancer Res*. 2004;92:95-118.
 45. Park S, Chapuis N, Tamburini J, et al. Role of the PI3K/AKT and mTOR signaling pathways in acute myeloid leukemia. *Haematologica*. 2010;95(5):819-828.
 46. Sykes SM, Lane SW, Bullinger L, et al. AKT/FOXO signaling enforces reversible differentiation blockade in myeloid leukemias. *Cell*. 2011;146(5):697-708.
 47. Polfus LM, Khajuria RK, Schick UM, et al. Whole-Exome Sequencing Identifies Loci Associated with Blood Cell Traits and Reveals a Role for Alternative GFI1B Splice Variants in Human Hematopoiesis. *Am J Hum Genet*. 2016;99(3):785.
 48. Schulze H, Schlagenhaut A, Manukjan G, et al. Recessive grey platelet-like syndrome with unaffected erythropoiesis in the absence of the splice isoform GFI1B-p37. *Haematologica*. 2017;102(9):e375-e378.
 49. Vassen L, Khandanpour C, Ebeling P, et al. Growth factor independent 1b (Gfi1b) and a new splice variant of Gfi1b are highly expressed in patients with acute and chronic leukemia. *Int J Hematol*. 2009;89(4):422-430.
 50. Sengupta A, Upadhyay G, Sen S, Saleque S. Reciprocal regulation of alternative lineages by Rgs18 and its transcriptional repressor Gfi1b. *J Cell Sci*. 2016;129(1):145-154.