# 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,[1] 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 Dührsen[1] and Cyrus Khandanpour[1,7]

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

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## 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

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## 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 proliferation[6] 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*$^{tg}$ 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*$^{tg}$ *NUP98/HOXD13*$^{tg}$ mice were injected intraperitoneally (i.p.), as shown previously.[6] For *Gfi1b*$^{fl/fl}$*MxCre*$^{tg}$*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*$^{wt/wt}$ mice not carrying the MxCre$^{tg}$ were injected with poly(I:C). Three weeks after transplantation of MLL-AF9-transduced lineage negative (Lin-) BM cells from *Gfi1b*$^{fl/fl}$*MxCre*$^{tg}$ 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.[23,27] 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 S1*).

### 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*$^{EGFP/wt}$),[13] 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$^{wt/wt}$ 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*$^{wt/wt}$ or *Gfi1b*$^{EGFP/wt}$ 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) administration[6] (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*.[33] 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

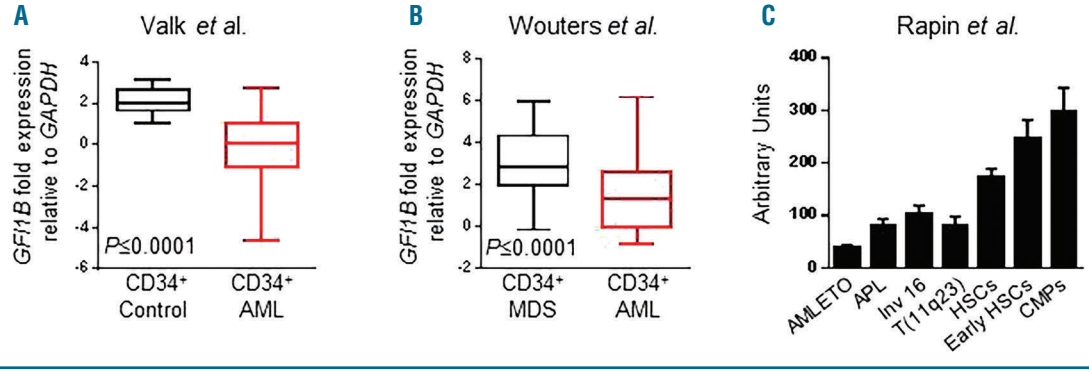


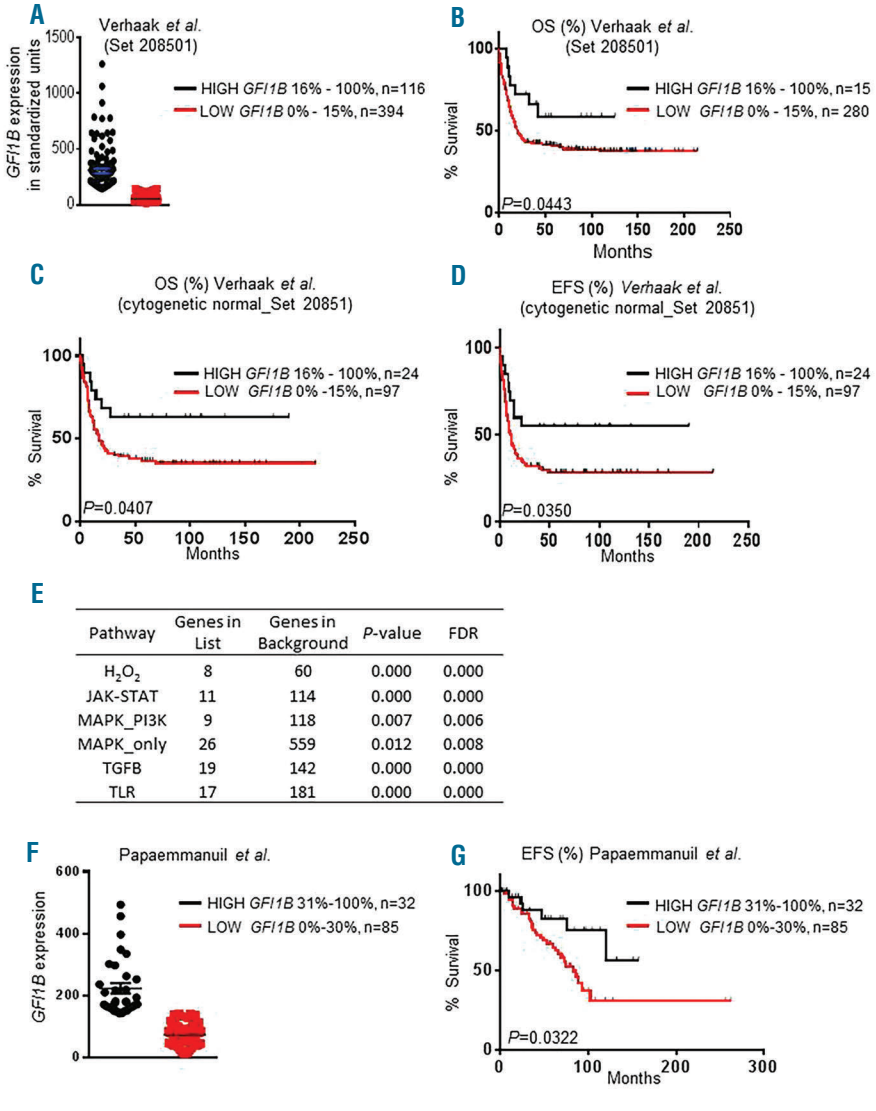

**Figure 1. Correlation between different *GFI1B* expression levels and myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML) prognosis.** (A) Expression of *GFI1B* in CD34$^+$ AML cells (n=269) compared to CD34$^+$ control cells (n=8) based on the patient cohort published by Valk *et al.*;[21] $P$≤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] $P$≤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 $Gfi1b^{wt/wt}MxCre^{tg}$ mice, and after poly(I:C) administration, we observed mice for the emergence of disease (Figure 4A).

While $Gfi1b^{wt/wt}MxCre^{tg}Kras^{+/fl}$ 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 $Gfi1b^{wt/wt}MxCre^{tg}$ 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 $Gfi1b^{wt/wt}MxCre^{tg}$ 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 $Gfi1b^{wt/wt}MxCre^{tg}$ (*Gfi1b*-expressing) cells (Figure 4F). However, there were no major qualitative differences concerning cytological findings and or blood parameters




| Pathway | Genes in List | Genes in Background | *P*-value | FDR |
|---|---|---|---|---|
| $H_2O_2$ | 8 | 60 | 0.000 | 0.000 |
| JAK-STAT | 11 | 114 | 0.000 | 0.000 |
| MAPK_PI3K | 9 | 118 | 0.007 | 0.006 |
| MAPK_only | 26 | 559 | 0.012 | 0.008 |
| TGFB | 19 | 142 | 0.000 | 0.000 |
| TLR | 17 | 181 | 0.000 | 0.000 |

**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; $H_2O_2$), 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.

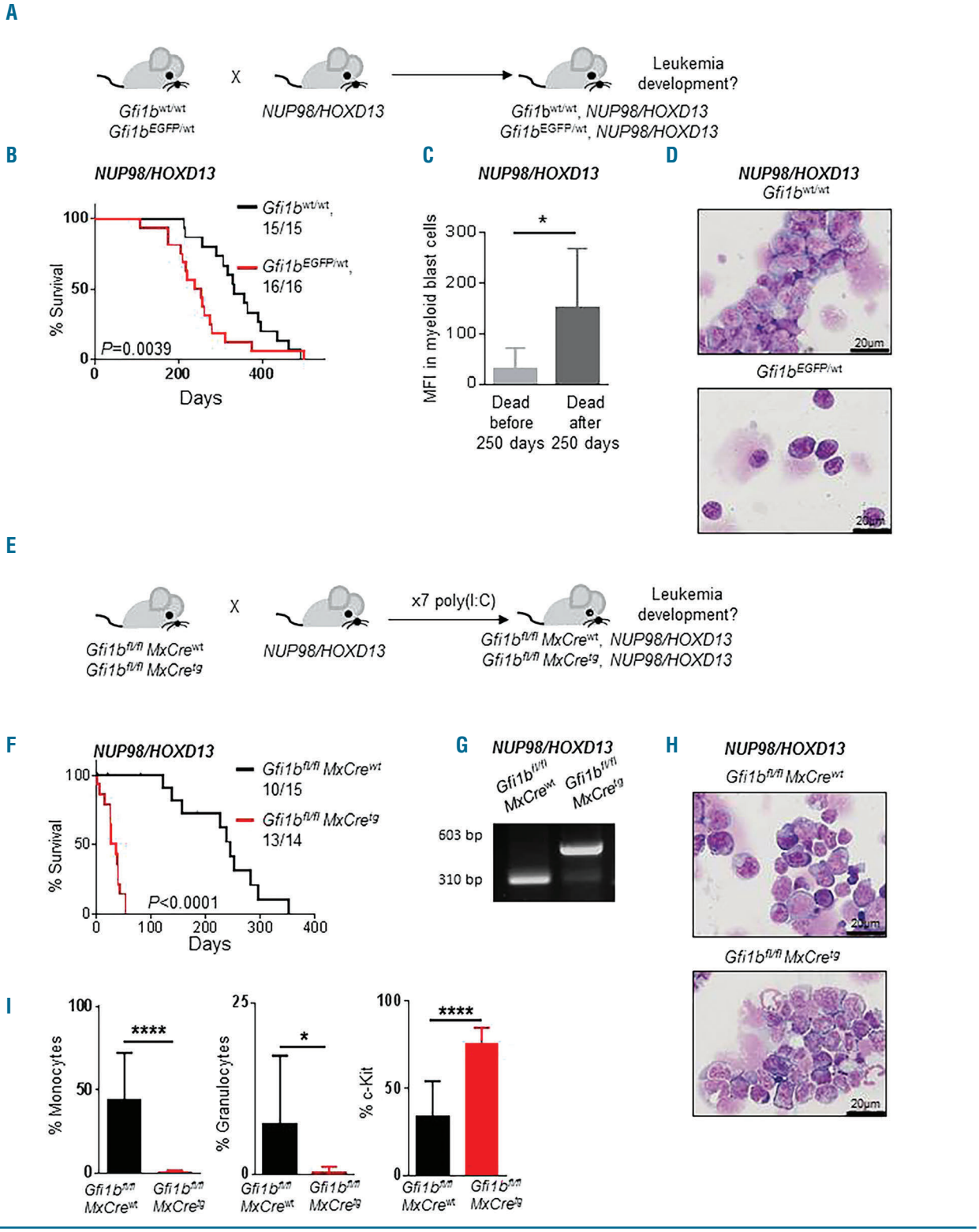



**Figure 3. Low level or absence of *Gfi1b* accelerates the progression of myelodysplastic syndrome (MDS) to acute myeloid leukemia (AML) in the *NUP98/HOXD13* MDS mouse model.** (A) Crossing of the *Gfi1b*$^{wt/wt}$ and *Gfi1b*$^{EGFP/wt}$ mouse strains with the *NUP98/HOXD13* mouse model. (B) Survival 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) cytospins 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*$^{KO/KO}$) 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 cytospins 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*$^{wt}$ 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 functional 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-

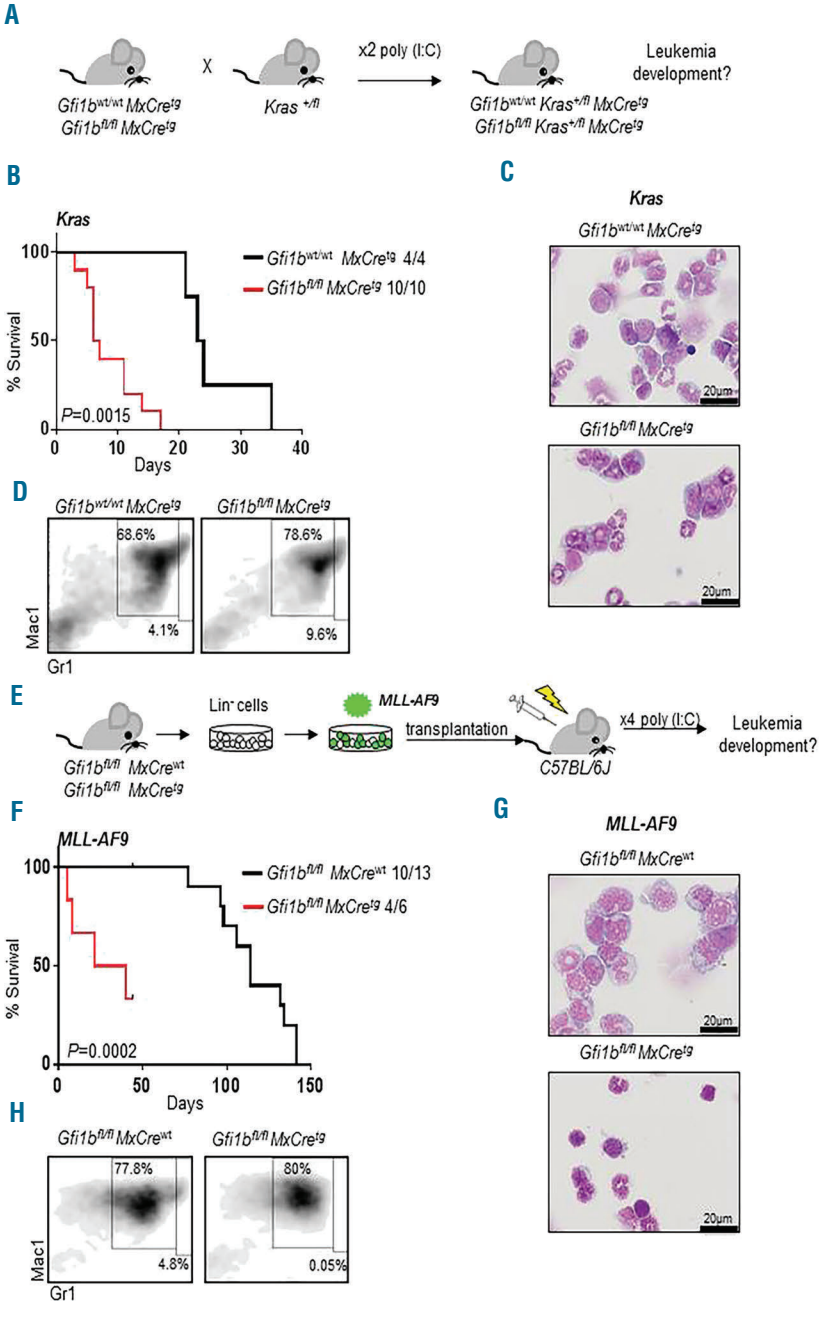
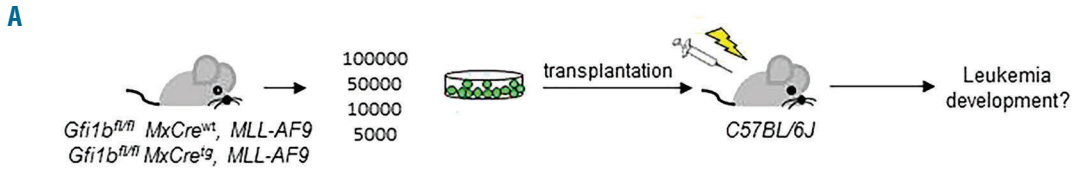
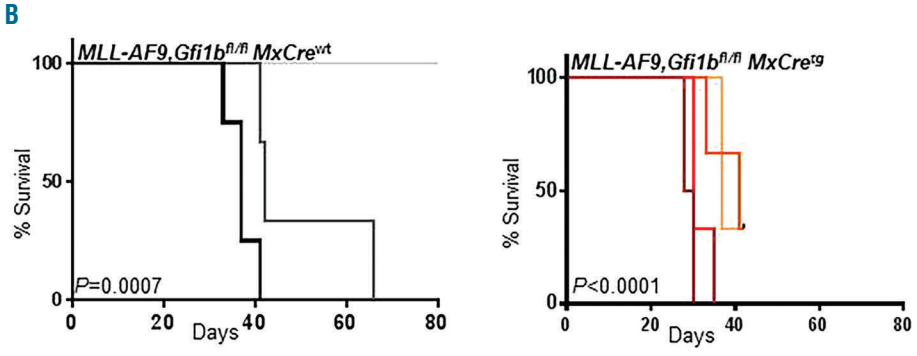



**Figure 4. Absence of Gfi1b accelerates the progression of myeloproliferative disorder and acute myeloid leukemia (AML).** (A) Crossing of the *Gfi1b*$^{wt/wt}$*MxCre*$^{tg}$ and *Gfi1b*$^{fl/fl}$*MxCre*$^{tg}$ mouse strains with the *Kras*$^{+/fl}$ mouse model. (B) Survival of *Gfi1b*$^{wt}$*/wtMxCre*$^{tg}$ 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) cytospins from representative *Gfi1b*$^{wt/wt}$*MxCre*$^{tg}$ and *Gfi1b*$^{fl/fl}$*MxCre*$^{wt}$ leukemic mice transgenically expressing Kras after poly(I:C) administration (bar=20 µm). (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 cytospins from the leukemic mice described in (F) (bar=20 µm). (H) Flow cytometric analysis of the BM from the leukemic mice described in (F) with regard to Gr-1 and Mac-1 expression.




C

| Genotype, n. of transplanted cells | Positive recipients | One functional stem cell within | Upper and lower limit |
|---|---|---|---|
| *MLL-AF9, Gfi1b$^{fl/fl}$ MxCre$^{wt}$* | | | |
| 100000 | 4/4 | 1:63000 | 1:30000 - 1:200000 |
| 50000 | 3/3 | | |
| 10000 | 0/3 | | |
| 5000 | 0/3 | | |
| *MLL-AF9, Gfi1b$^{fl/fl}$ MxCre$^{tg}$* | | | |
| 100000 | 4/4 | 1:3500 | 1:2000 - 1:20000 |
| 50000 | 3/3 | | |
| 10000 | 3/3 | | |
| 5000 | 2/3 | | |

**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

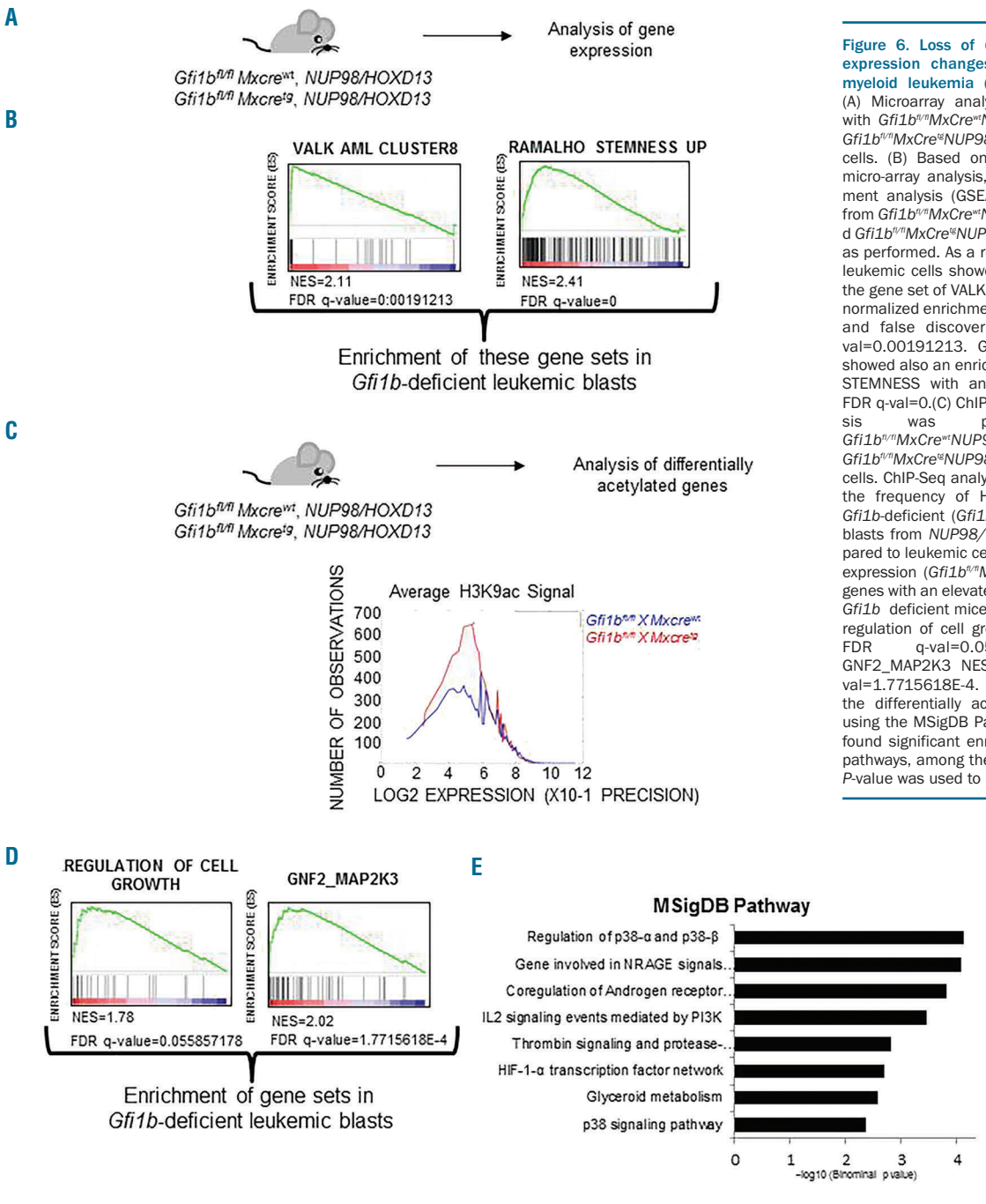



**Figure 6. Loss of *Gfi1b* induces gene expression changes supporting acute myeloid leukemia (AML) development.** (A) Microarray analysis was performed with *Gfi1b*$^{fl/fl}$*MxCre*$^{wt}$*NUP98/HOXD13* and *Gfi1b*$^{fl/fl}$*MxCre*$^{tg}$*NUP98/HOXD13* leukemic cells. (B) Based on the results of the micro-array analysis, a Gene set enrichment analysis (GSEA) of leukemic cells from *Gfi1b*$^{fl/fl}$*MxCre*$^{wt}$*NUP98/HOXD13*$^{tg}$ and *Gfi1b*$^{fl/fl}$*MxCre*$^{tg}$*NUP98/HOXD13*$^{tg}$ 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*$^{tg}$ 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, Itga2, 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*$^{tg}$ (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/FoXO signaling cascade might facilitate the progression of normal hematopoietic cells to LSCs. In the future, testing will be needed to determine whether alter-

A

| human *GFI1B* | | | |
|---|---|---|---|
| Pathway | Genes in list | *P*-value | FDR |
| JAK-STAT | 11 | 0 | 0 |
| MAPK_PI3K | 9 | 0.007 | 0.006 |
| ROS related | 8 | 0.000 | 0.000 |

| murine *Gfi1b* | | | |
|---|---|---|---|
| Pathway | Genes in list | *P*-value | FDR |
| JAK-STAT | 14 | 4.55E-06 | 6.72E-06 |
| MAPK_PI3K | 11 | 0.000558 | 0.00047 |
| ROS related | 8 | 0.003 | 0.002 |

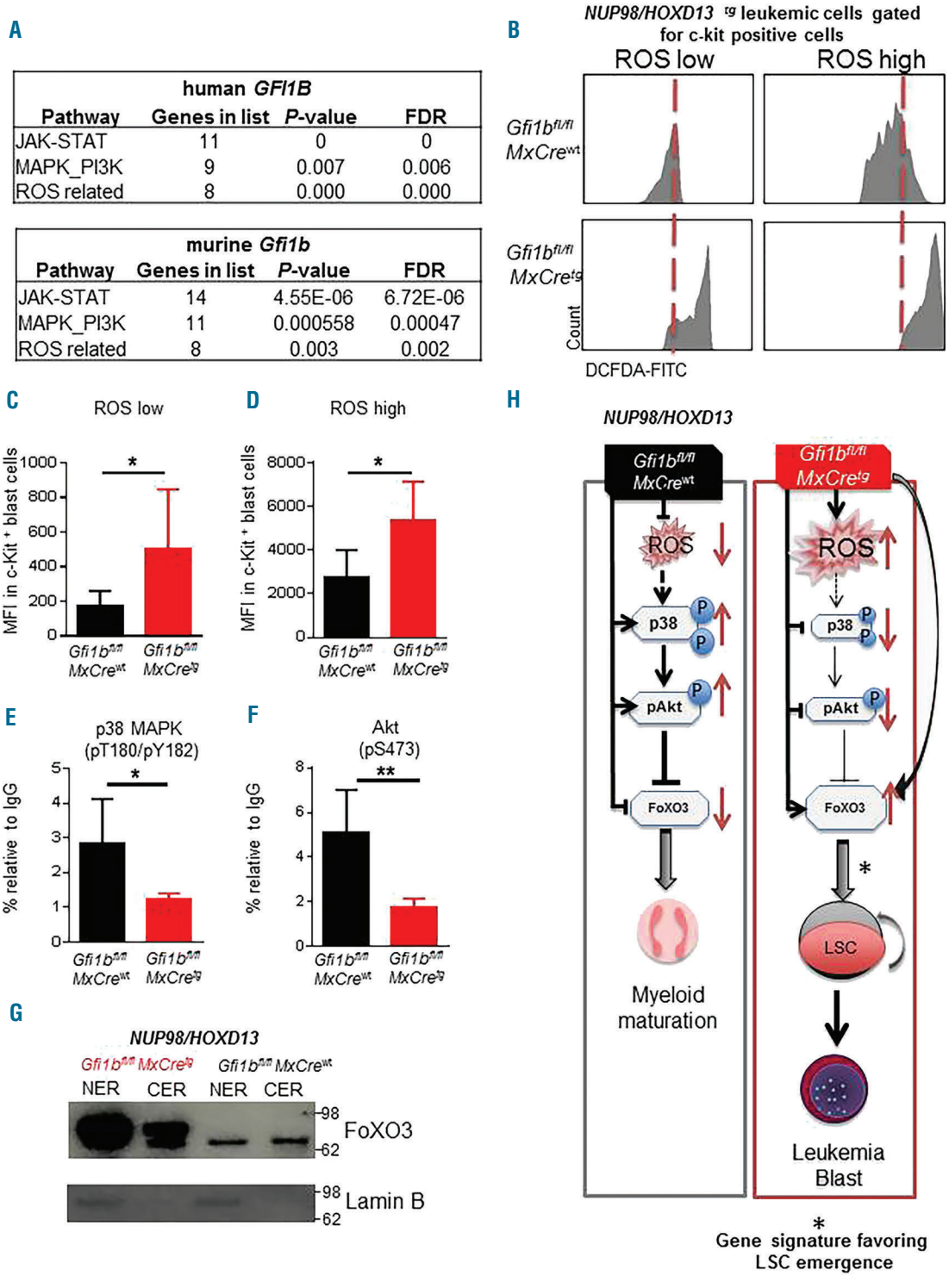



**Figure 7. Loss of *Gfi1b* deregulates acute myeloid leukemia (AML) signaling pathway.** (A) Analysis to see which pathways were enriched in *GFI1B* low-expressing blast cells in two independent sets of myelodysplastic syndrome (MDS) or AML patients compared to the expression pattern found in *GFI1B* 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.

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