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ORIGINAL ARTICLE

GFI1 as a novel prognostic and therapeutic factor for AML/MDS

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Genetic and epigenetic aberrations contribute to the initiation and progression of acute myeloid leukemia (AML). GFI1, a zinc-finger transcriptional repressor, exerts its function by recruiting histone deacetylases to target genes. We present data that low expression of *GFI1* is associated with an inferior prognosis of AML patients. To elucidate the mechanism behind this, we generated a humanized mouse strain with reduced *GFI1*expression (GFI1-KD). Here we show that AML development induced by onco-fusion proteins such as MLL-AF9 or NUP98-HOXD13 is accelerated in mice with low human *GFI1* expression. Leukemic cells from animals that express low levels of *GFI1* show increased H3K9 acetylation compared to leukemic cells from mice with normal human *GFI1* expression, resulting in the upregulation of genes involved in leukemogenesis. We investigated a new epigenetic therapy approach for this subgroup of AML patients. We could show that AML blasts from GFI1-KD mice and from AML patients with low GFI1 levels were more sensitive to treatment with histone acetyltransferase inhibitors than cells with normal *GFI1* expression levels. We suggest therefore that GFI1 has a dose-dependent role in AML progression and development. GFI1 levels are involved in epigenetic regulation, which could open new therapeutic approaches for AML patients.

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INTRODUCTION

Acute myeloid leukemia (AML) is a malignant disease of the bone marrow (BM) with accumulation of immature myeloid cells.[1,2] Despite a number of treatment options, including chemotherapy and allogeneic stem cell transplantation, prognosis of AML remains poor.[1,2] Myelodysplastic syndrome (MDS) is characterized by disturbed development of the myeloid-erythroid-megakaryocytic lineage.[3,4] Some MDS patients develop cytopenia of the myeloid compartment and may progress to AML.[3,4] The curative therapy for a limited number of patients is stem cell transplantation.[5] Initiation and progression of MDS and AML are driven, among other factors, by epigenetic alterations,[6,7] often induced by acquired mutations or altered levels of transcription factors[6,8–10] such as RUNX1, PU.1, BMI-1 and CEBPA.[6,10–14]

GFI1 is a transcriptional repressor regulating hematopoietic cell fates of the myeloid and lymphoid lineages.[15–31] Inherited mutations of *GFI1* have been reported in patients with severe congenital neutropenia.[32,33] In mice, *Gfi1* ablation affects quiescence and self-renewal of hematopoietic stem cells and the multilineage potential of early hematopoietic precursors, but also the differentiation of myeloid/lymphoid lineages at later stages.[32,33] Moreover, deletion of *Gfi1* in mice leads to an almost complete loss of mature neutrophils and an accumulation of immature myelomonocytic cells,[34,35] which can accelerate the development of a fatal myeloproliferative disease in the presence of an activated *Kras* gene.[31,36] The human *GFI1* gene is located on the p-arm of chromosome 1 (1p), and 1p deletions have been proposed as a potential prognostic marker for MDS.[37] Finally, a report of a small cohort of MDS patients suggested an association between reduced *GFI1* expression levels and an inferior prognosis.[38]

For this study, we generated mouse models that carry a human *GFI1* gene with different expression levels, that is, *GFI1* 'knock-in' and 'knock-down' animals. Using these models, we show that low *GFI1* expression accelerates the initiation and progression of AML in mice and renders AML cells more sensitive to histone acetyltransferase inhibitors (HATis) than to histone deacetylase inhibitors (HDACis) that are used in experimental therapies. Thus, *GFI1* expression levels not only predict disease outcome, but also represent a marker that can orient the choice of drugs in an epigenetic therapy.

MATERIALS AND METHODS

Study samples

Patient samples were obtained with informed consent before initiation of the treatment. Studies with mice were approved by local ethics committees (protocol number in Essen 11-4702). Data regarding

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the characteristics of patients from the different cohorts were published earlier.[39,40,36]

Mouse strains

NUP98-HOXD13 and *MLL-AF9* transgenic (*tg*) mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). *GFI1*-KI *GFI1*-KO, *Gfi1-GFP* mouse strains have been previously described.[20,36,25,41] Generation of the *GFI1*-KD/KD mouse was achieved by inserting a Neo cassette alongside the human *GFI1*-encoding cDNA into the murine *Gfi1* locus in an antisense direction, leading to an 80-90% reduction of normal *GFI1* expression.[36] Mice were housed in specific pathogen-free conditions at the animal facilities of University Hospital Essen. All experiments were conducted after approval by the local authorities (permission G1196/11). The percentage of blast cells was enumerated by technicians blinded for the genotype and values were confirmed by an experienced hematologist.

ChIP, ChIP-Seq and RNA-Seq analysis

For chromatin immunoprecipitation (ChIP), 1×10^7 cells were used as previously described,[42,43] using the polyclonal H3K9acetyl (ab4441; Abcam, Cambridge, UK) antibody. The CuffDiff R package was used to quantify changes in acetylation levels on gene promoters (Seattle, WA, USA). . For more details regarding RNA-Seq, please refer to Supplementary Methods.

CHIP-Seq and RNA-Seq data are available at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=qfcpeacwtronzqp&acc=GSE72671.

Gene expression arrays, analyses and mutational analysis

Gene expression arrays were performed as published[39] and are available at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=mpopwqokvhqnjkh&acc=GSE72489.

Boundaries of *GFI1* expression

Two approaches were used to set the boundaries for *GFI1* expression levels in AML patients. In the first cohort of patients from Essen, we correlated expression levels with the outcome of patients and observed that boundaries defined as 0-5% (low), 6-60% (medium) and 61-100% (high, 100%) of *GFI1* expression predicted the outcome of patients. We revalidated these boundaries in a second patient cohort reported by Verhaak *et al.*[40]

RESULTS

Low expression levels of GFI1 influence AML prognosis

To test whether different doses of GFI1 play a role in AML pathogenesis we examined the association between different *GFI1* mRNA-expression levels and event-free survival in a cohort of AML patients from the University Hospital Essen (excluding AML-M3 patients, as they were treated differently). Lower levels of *GFI1* expression (see Materials and Methods) were associated with a significantly inferior outcome, while higher expression levels were associated with a better outcome (Supplementary Figure S1a). Low *GFI1* expression did not correlate with French-American-British (FAB) classification,[1,2] cytogenetic findings, age or sex (Supplementary Table S1).

We examined the association between *GFI1* expression levels and prognosis in another, independent AML cohort.[40] Low levels of *GFI1*expression were associated with inferior event-free survival and overall survival (Figures 1a and b). A correlation between low *GFI1* expression and age or sex was again not observed (Supplementary Table S2). Adverse cytogenetic findings, FAB M4E and M0, *CEBPa*-, *NRAS* mutations and elevated *EVI1* expression were more common among patients with low *GFI1* expression, whereas FAB M2, *NPM* and *FLT3* alterations were more common among patients with higher *GFI1* expression (Supplementary Table S2 and Figure 1c). Low *GFI1* expression was an independent prognostic factor for event-free survival in a multivariate Cox regressional analysis after adjustment for age, cytogenetic findings, *EVI1* expression, *NPM*, *CEBPa* and *NRAS* mutational status (Supplementary Table S3).

Finally, we examined the association between different gene expression signatures obtained from AML blasts and patient survival from an additional published study.[39] Here, clusters of patients were defined based on the correlation between gene expression signatures and specific disease entities (Valk *et al.*[39]and Figure 1d). *GFI1* expression levels were low in clusters 5 and 10 and associated with an inferior disease course (Figure 1e). However, *GFI1* expression was significantly higher in clusters 9, 12 and 13 (Figure 1d), and correlated with rather good prognosis (Figure 1e; for more details, see Valk *et al.*[39]), suggesting that low *GFI1*expression levels also negatively influence AML prognosis in these patients.

To explore why low *GFI1* expression is associated with inferior prognosis, we used the data sets of Valk *et al.*[39] and compared gene expression patterns obtained from blast cells with very low *GFI1* expression (5% lowest expression level) with blast cells with very high expression of *GFI1* (20% highest expression level, thus higher than 80%). Very low *GFI1* expression levels correlated with the gene expression signature found in leukemic stem cells (LSCs) and hematopoietic stem cells (Figure 1f and Supplementary Figure S1b), a pattern that is associated with poorer prognosis.[44]

Knock-down of GFI1 expression is associated with specific alteration of the hematopoietic system

To investigate how low levels of *GFI1* expression contribute to AML development, we used a previously described mouse strain,[36] in which the murine *Gfi1* gene is replaced by the human *GFI1* cDNA (denominated *GFI1*-KI mice, for 'knock-in'). By leaving the selectable neo marker gene in the genome, we generated another mouse strain (denominated *GFI1*-KD, for GFI1 'knock-down'), which expresses the human GFI1 protein at about 5-15% of the levels found in wild type (WT) or in *GFI1*-KI mice (Supplementary Figures S2a and b and Supplementary Table S4). Placement of the Neo cassette into a gene in the opposite direction of transcription has been described to lead to nonsense-mediated decay of the gene-specific mRNA (here, the GFI1 mRNA).[45] The antibody used detects both human GFI1 and murine Gfi1 (Supplementary Figure S2b). We used thymocytes, as Gfi1 is highly expressed in these cells and a quantification is more readily obtained. The *GFI1*-KI and *GFI1*-KD mouse strains represent experimental models with humanized *GFI1* genes and allow studying the function of GFI1 in a disease setting. In addition, the level of GFI1 expression in cells from *GFI1*-KD mice is within the same range (that is, 5-15% of WT levels) as observed in our AML patient cohort with inferior prognosis (that is, 5-15% of normal *GFI1* expression levels within the AML cohort). *GFI1*-KD mice exhibited an arrest of myeloid differentiation, a loss of neutrophil granulocytes, and an increase of granulocytic-monocytic progenitors (GMPs), which expanded *in vitro* faster than *GFI1*-KI GMPs (Supplementary Figures S2c–h). *GFI1*-KI mice carrying either one or two alleles of the human *GFI1* sequence did not show any abnormality or difference compared to *Gfi1*-WT animals, nor was the expression level of human *GFI1* different from the expression level of murine *Gfi1* (Supplementary Figure S2b and Khandanpour *et al.*[36]).

Reduction of GFI1 expression and loss of one Gfi1 allele accelerate MDS/AML progression in mice

LSCs can arise from GMPs in mice and humans.[46,47] We observed that GMPs from *GFI1*-KD mice generated more colonies in semisolid medium than GMPs from control animals, raising the possibility that reduction of *GFI1*expression may have an impact on MDS or AML stem cells. Therefore Lin^- cells (a fraction containing different hematopoietic progenitor and stem cells, including GMPs) from *GFI1*-KI and *GFI1*-KD mice were transduced with an *MLL-AF9*-encoding retrovirus (Figure 2a). The *MLL-AF9* translocation t(9;11)(q22;23) is found in a subset of AML patients and induces AML in mice.[48] Cells from *GFI1*-KD mice generated

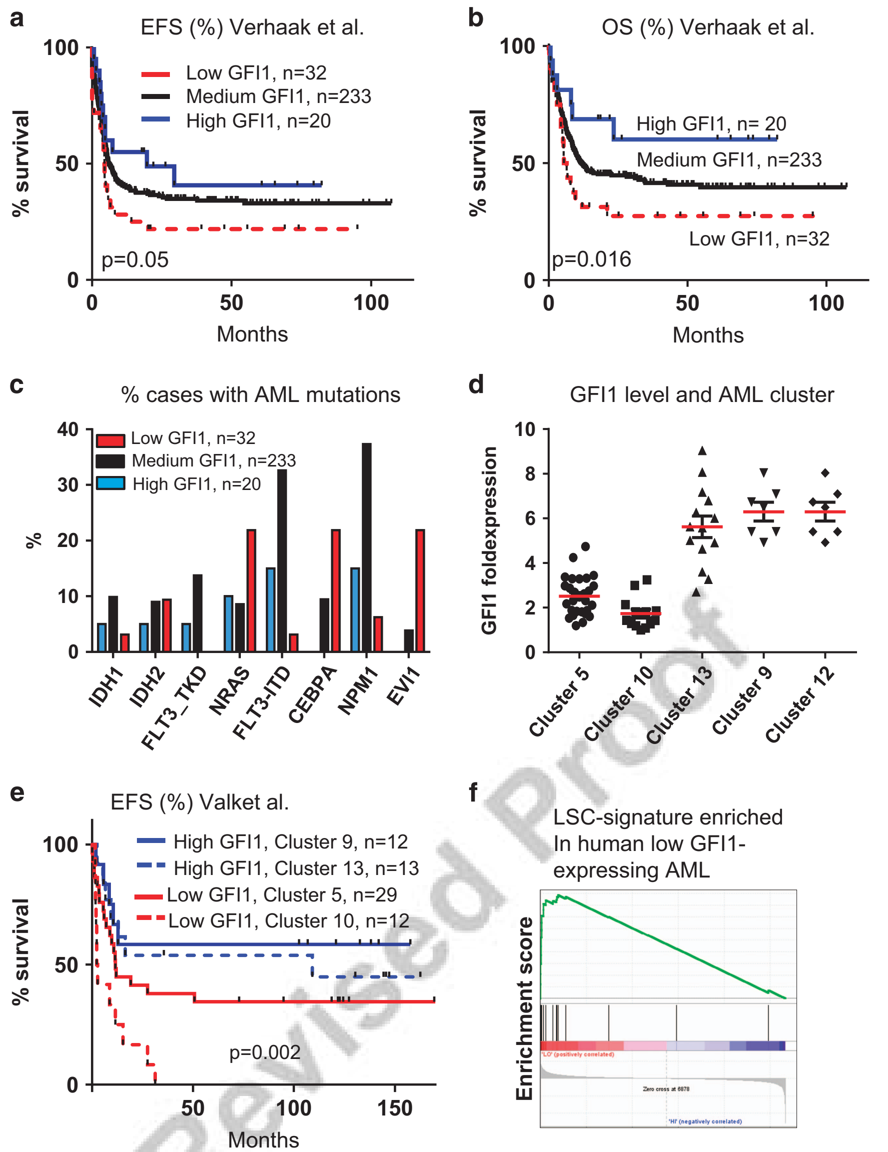

Figure 1. Correlation between *GFI1* expression level differences and AML prognosis as well as establishment of a humanized mouse model to study the role of different GFI1 levels. (**a**) Event-free survival (EFS) of AML patient cohort from the publication of Verhaak *et al.*[40] with regard to *GFI1* expression ($P=0.05$). (**b**) Overall survival (OS) of the AML patient cohort from the publication of Verhaak *et al.*[40] with regard to *GFI1* expression ($P=0.016$). (**c**) Frequency of mutations of certain known AML drivers in AML cells with low, medium and high *GFI1* expression. (**d**) Relative expression levels of *GFI1* in different clusters based on the patient cohort published by Valk *et al.*[39] (**e**) Event-free survival of AML patient cohorts from the publication of Valk *et al.*[39] with regard to *GFI1* expression ($P=0.002$). (**f**) Gene set enrichment analysis (GSEA) of low *GFI1*-expressing human leukemic cells with resemblance to gene expression signature in leukemic stem cells (LSCs). Normalized enrichment score (NES) = 2.3; $P=2.38\times10^{4}$.

increased numbers of colonies in semisolid medium as well as liquid culture compared to *GFI1*-KI animals (Supplementary Figures S3a–d). Mice that received *MLL-AF9*-transduced *GFI1*-KD cells succumbed much faster to leukemia than mice transplanted with *MLL-AF9*-transduced *GFI1*-KI cells (Figure 2b). The leukemia emerging in all animals showed no major qualitative differences with respect to cell surface marker, cytological findings or blood parameters (Figure 2c and Supplementary Figures S3e–g). Reduced *GFI1*expression levels were maintained in leukemic cells from mice transplanted with *MLL-AF9*-transduced *GFI1*-KD cells (Supplementary Figure S3h). However, higher levels of blast cells were observed in the BM and blood of leukemic mice transplanted with *MLL-AF9*-transduced cells from *GFI1*-KD mice, yet no differences were detectable in spleen (Figure 2d and Supplementary Figures S3i–m and S4).

Retroviral overexpression of *MLL-AF9* can deliver other results than transgenic overexpression of *MLL-AF9*.[49] We validated our results by crossing *MLL-AF9-tg* mice[49] with *GFI1*-KD and *GFI1*-KI animals. Presence of one *GFI1*-KD allele alone accelerated leukemia development significantly ($P=0.0014$) compared with *MLL-AF9-tg* animals that carry one *GFI1*-KI allele (Figure 2e). We never observed *MLL-AF9-tg* mice with two *GFI1*-KD alleles, suggesting that this combination is potentially lethal. The different AML mouse cohorts did not differ with respect to expression of surface proteins or microscopic appearance of blast cells (Figures 2f and g).

To test the role of *GFI1* expression levels in another model, we used the *NUP98-HOXD13-tg* mice (Figure 3a)[50] that are an established MDS/AML disease model recapitulating the t(2;11)(q31;p15) translocation in human AML. They show features of

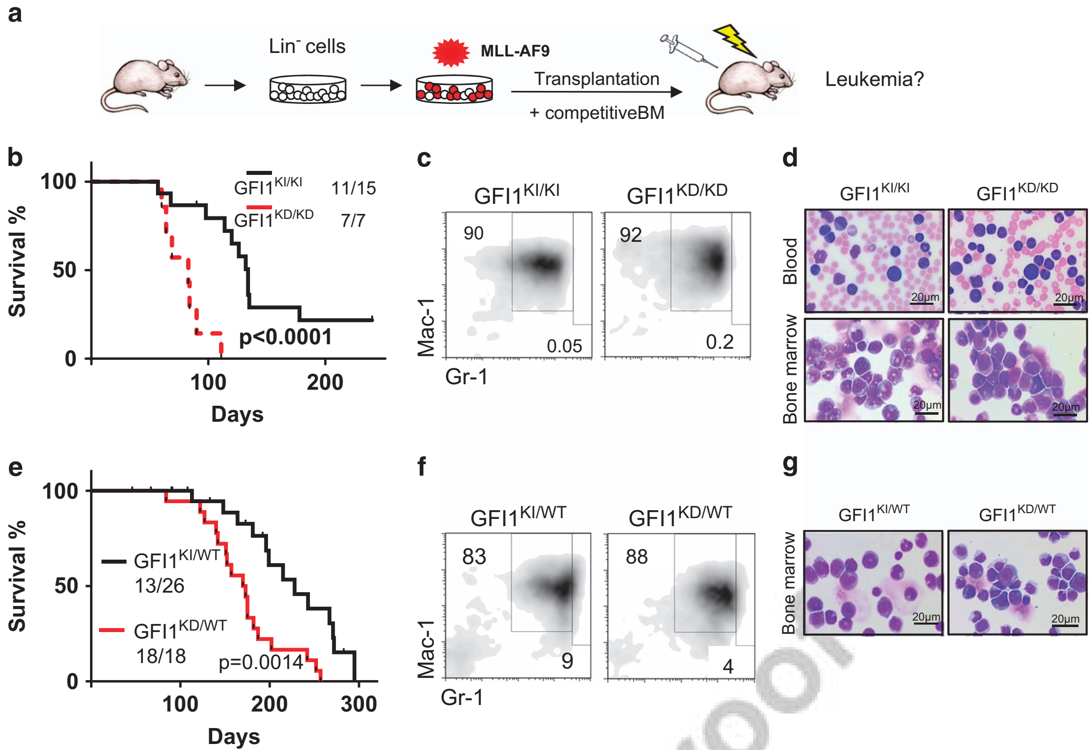

Figure 2. Low level of GFI1 accelerates AML development. (**a**) Schematic representation of isolating and transduction of Lineage-negative cells with MLL-AF9-expressing retrovirus. The transduced cells were transplanted in lethally irradiated mice alongside 100 000 WT, non-malignant BM cells. (**b**) Survival of the mice transplanted with *GFI1*-KD and *GFI1*-KI ($P < 0.0001$). The numbers indicate how many mice of the total cohort died of AML. (**c**) Cell surface staining of the BM of the indicated mice cohorts for Mac1 and Gr1. (**d**) Wright-Giemsa staining of BM cytospins and blood smears (bar represents 20 μm). (**e**) Survival of *GFI1*-KD/WT and *GFI1*-KI/WT mice transgenically expressing *MLL-AF9*. Number indicates how many mice of the total cohort died of AML ($P = 0.0014$). (**f**) Cell surface staining of the BM of the indicated mice cohorts. (**g**) Wright-Giemsa staining of BM cytospins (bar represents 20 μm).

human MDS such as dysplasia of different lineages and increased apoptosis, and a fraction of mice develop full-blown leukemia.[50] *GFI1*-KD increased the incidence and shortened the latency period of AML development in *NUP98-HOXD13* mice significantly ($P = 0.0001$) compared to *NUP98-HOXD13* animals with normal *GFI1/Gfi1* levels (*GFI1*-KI or *Gfi1*-WT mice) (Figure 3b and Supplementary Figure 5a). Leukemic cells from both populations showed no qualitative differences with regard to surface marker expression, white blood cells, platelet, hemoglobin counts, spleen size or cytologic appearance (Figures 3c and d, and Supplementary Figures S5b–f). Thus, reduction of *GFI1* expression in leukemic cells promotes AML progression in two different experimental models simulating human AML or MDS to AML progression.

To test whether deletion of one Gfi1 allele can also promote an earlier onset of AML, we used *Gfi1-GFP* mice in which one murine *Gfi1* allele was replaced by an *EGFP* cDNA, enabling monitoring *Gfi1* promoter activity *in vivo*.[41] These animals are heterozygous for *Gfi1* and show reduced *Gfi1* expression (Supplementary Table S4). We crossed these mice to *NUP98-HOXD13-tg* animals. Loss of one *Gfi1* allele increased the incidence and shortened the latency of AML development compared to *Gfi1*-WT *NUP98-HOXD13-tg* animals (Figure 3e). In BM cells derived from heterozygous mice the expression of *Gfi1* mRNA was reduced to 50% of WT levels (Supplementary Figure S5g). Similarly, expression of Gfi1 protein was reduced to about 50% of WT levels in thymocytes derived from heterozygous mice (Supplementary Figure S5h). The leukemic cells from *Gfi1* WT/WT or *Gfi1EGFP*/WT animals showed no obvious differences with regard to surface marker expression, white blood cell, platelet, hemoglobin count, spleen size or cytologic appearance (Figures 3f and g, and Supplementary Figures S5i–m). *EGFP* expression levels (and hence *Gfi1* expression levels) in the blast cells from *Gfi1-EGFP*/WT mice were significantly lower when the disease onset was before 300 days after birth compared to *EGFP* expression in the blast cells from mice in which the disease appeared later than 300 days after birth (Supplementary Figure S5n), suggesting that a leukemia with lower *Gfi1* expression levels in blast cells emerges earlier than a leukemia with higher *Gfi1* expression levels.

We next investigated whether deletion of one *GFI1* allele might also play a role in human MDS/AML development. Since *GFI1* is located on chromosome 1p22 and chromosome 1 deletions have been associated with initiation and progression of MDS and AML,[37] we examined the minimal deleted region of five MDS patients with chromosome 1 deletions. The minimal common deleted region encompassed the *GFI1* locus, and one patient showed a deletion only comprising the *GFI1* locus and part of the neighboring genes (Figure 3h).

Reduced GFI1 expression correlates with altered gene expression and histone acetylation patterns

We next tested whether *GFI1*-KD mice were suitable for modeling the influence of low *GFI1* expression on AML pathogenesis in human patients. We performed a Gene Set Enrichment Analysis using mRNA expression data from leukemic cells derived from *NUP98-HOXD13-GFI1*-KD and -*GFI1*-KI mice. Leukemic cells from animals with low *GFI1* expression showed enrichment of genes belonging to 'cluster 5', which was defined by Valk *et al.*[39] as an AML subgroup with low *GFI1* expression and poor prognosis (Figure 3i). Hence, our murine model with low *GFI1* expression

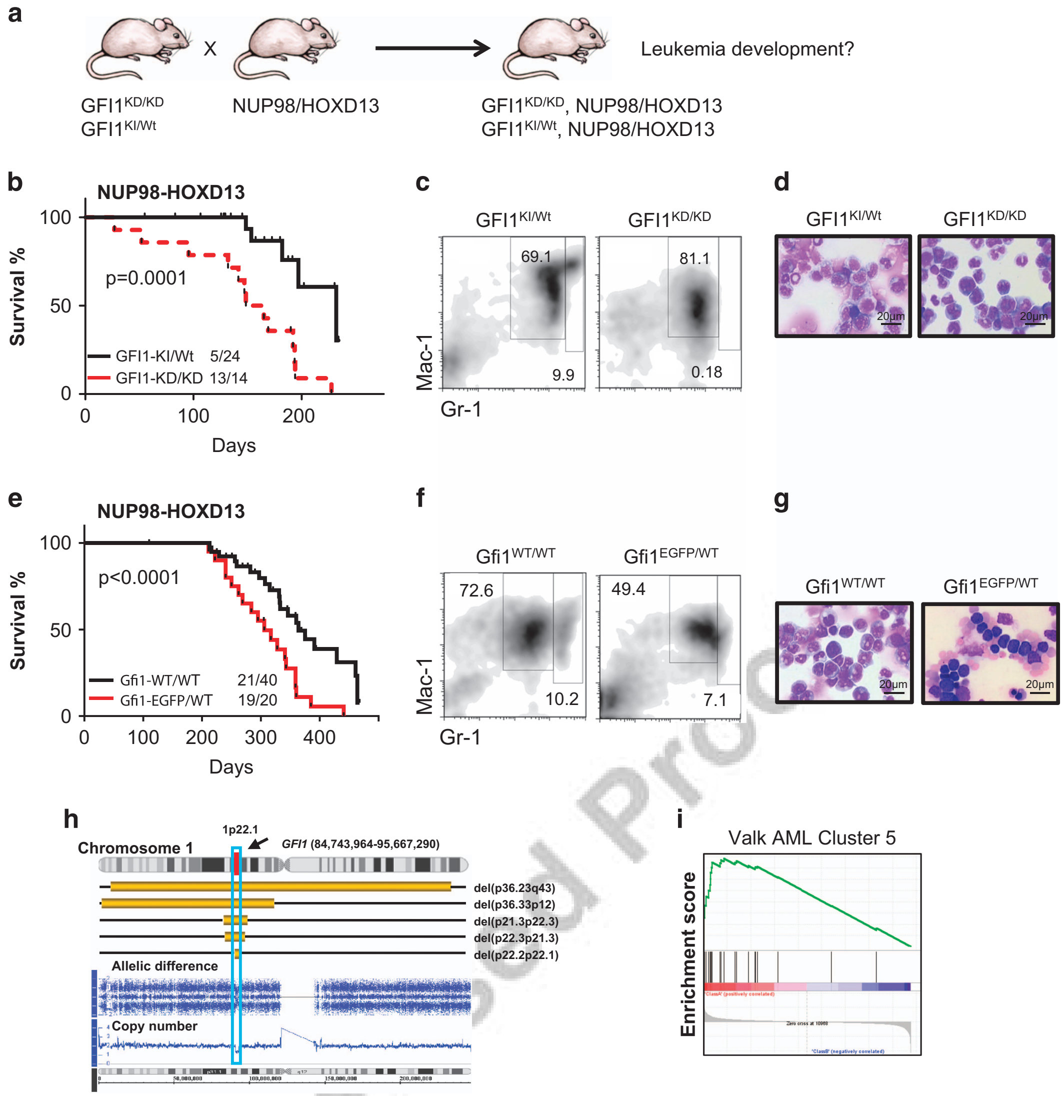

Figure 3. Low level of GFI1 accelerates AML development in a murine MDS model. (**a**) Schematic representation of the crossing of the indicated mouse strains with *NUP98-HOXD13* mice. (**b**) Survival of the mice with the indicated genotype ($P = 0.0001$). The numbers indicate how many mice of the total cohort died of AML. (**c**) Cell surface staining of the BM of the indicated mouse cohorts for Mac1 and Gr1. (**d**) Wright-Giemsa staining of BM cytospins (bar represents 20 µm). (**e**) Survival of *Gfi1-EGFP*/WT and *Gfi1*-WT/WT mice transgenically expressing *NUP98-HOXD13* ($P < 0.0001$). The numbers indicate how many mice of the total cohort died of AML. (**f**) Cell surface staining of the BM of the indicated mouse cohorts for Mac1 and Gr1. (**g**) Wright-Giemsa staining of BM cytospins (bar represents 20 µm). (**h**) SNP array of different human MDS samples with chromosome 1 deletion with regard to the different deleted regions. (**i**) Gene set enrichment analysis (GSEA) of *GFI1*-KD *NUP98-HOXD13* leukemic cells with resemblance to cluster 5 of the Valk *et al.* publication. Normalized enrichment score (NES) = 2; $P = 0.008$.

appears to well recapitulate the situation in the subset of human AML with a more aggressive disease course.

To elucidate the effect of reduced *GFI1* expression in leukemic cells we focused on the *NUP98-HOXD13-tg* mouse model, as it shows a number of features typical for human AML and, in addition, NUP98-HOXD13 is expressed as a transgene, which induces AML over a longer period of time and subsequent to a precondition resembling MDS. This is closer to the human situation than the induction of AML by retroviral transduction of the *MLL-AF9* onco-fusion protein. We studied one key function of GFI1, which is the de-acetylation of histone H3 at lysine 9 (H3K9)[51] and observed increased acetylation at this residue in a subset of GFI1 target gene promoters in leukemic cells from *NUP98-HOXD13-GFI1*-KD mice compared with cells from *NUP98-HOXD13-GFI1*-KI mice (Figures 4a and b). Functional analysis of differentially acetylated genes showed an implication in Gene Ontology Biological functions related to chromatin organization, modification and transcription regulation (Supplementary Figure S6a) as well as Kyoto Encyclopedia of Genes and Genomes pathways associated with cancer (Supplementary Figure 6b). Increased acetylation levels correlated positively with increased mRNA expression levels between NUP98-HOXD13 Gfi1-KD and Gfi1-KI AML samples at Gfi1 target genes (Supplementary Figure S6c). Among the 1177 GFI1 target genes with increased levels of acetylation and quantifiable mRNA levels, 302 showed a significant change in mRNA expression and 95% of these showed an increase in expression levels (Figure 4c). Further analysis of these genes showed that they were enriched in pathways regulating cancer development, including leukemia and cell signaling (Supplementary Figures S6d and e). These results suggest that reduced GFI1 levels lower the efficiency of de-acetylation, leading to the altered expression patterns of target genes involved in cancer and in particular leukemia.

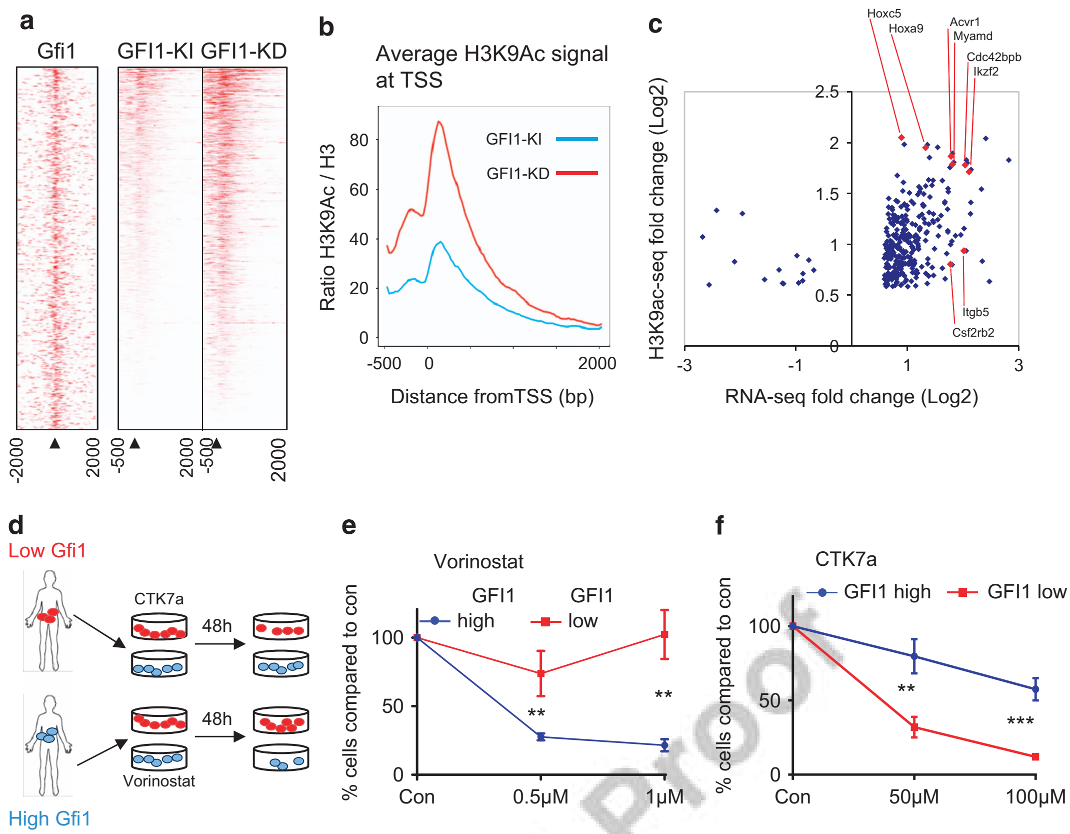

Figure 4. A low level of GFI1 is associated with certain epigenetic and gene expressional changes and opens new possibilities for therapeutic approach. (**a**) Heatmap representing enrichment of GFI1 relative to an IgG control at GFI1 target genes. The first row represents a window of 4 kb centered on the transcription start site (TSS) of the genes. The second and third rows show heatmaps representing enrichment of H3K9ac relative to its H3 control in leukemic cells from *NUP98-HOXD13*, *GFI1*-KI and *GFI1*-KD mice. Each row represents a 2.5-kb window, extending −0.5 and +2 kb from the gene TSS. (**b**) Histogram showing the average distribution of H3K9ac relative to the TSS across selected genes in leukemic cells from *GFI1*-KD and *GFI1*-KI mice. (**c**) Scatter plot comparing the log2-fold increase in H3K9 acetylation (ChIP-Seq) between *Nup98-HoxD13-GFI1*-KD and *-GFI1*-KI leukemic samples (*y* axis) versus the log2-fold increase in mRNA expression (RNA-Seq) between *NUP98-HOXD13-GFI1*-KD and *GFI1*-KI leukemic cells, for genes with greater than 1.5-fold change in both experiments and a *P*-value <0.25. (**d**) Scheme of isolating leukemic cells from the indicated patients with either low or high *GFI1* expression in their blast cells on treating them with either Vorinostat or CTK7a. $n=3$ for each treatment condition. (**e**) Treatment of the leukemic cells from the indicated patients with Vorinostat (**$P<0.009$). $n=3$ for each treatment condition. (**f**) Treatment of the leukemic cells from the indicated patients with CTK7a (**$P=0.0035$; ***$P=0.00046$). $n=3$ for each treatment condition.

Epigenetic therapy as a novel approach to treat low *GFI1*-expressing leukemic cells

HDACi have been used in a subset of patients with myeloid malignancies when other more aggressive therapeutic options are not suitable due to poor health of the patients.[52,53] These approaches have not generally been successful. We hypothesized that in low *GFI1*-expressing cells H3K9 deacetylation might be less efficient than in cells with normal *GFI1* expression, and that HDACi treatment of patients with low *GFI1* expression levels in blasts would be counter-productive (Supplementary Figure S7). HATi treatment of low *GFI1*-expressing patients might be a more promising approach since it could revert the increased acetylation of H3K9 and thus counteract the effect of reduced expression of *GFI1*.

GFI1-KI or *GFI1*-KD mice were transduced retrovirally with *MLL-AF9* and then treated with either Vorinostat (HDACi)[52] or CTK7a (a HATi).[54] We used low concentrations of Vorinostat or CTK7a to recapitulate the attainable levels *in vivo*.[53,54] *GFI1*-KI cells responded to treatment with Vorinostat by growth reduction; however, *GFI1*-KD cells were more resistant to this treatment even at higher doses (Supplementary Figures S8a and b). Upon exposure of low *GFI1*-expressing cells to CTK7a, already lower concentrations of CTK7a impeded the growth of *GFI*-KD cells expressing *MLL-AF9* (Supplementary Figure S8c). Thus, *GFI1* expression levels might determine whether leukemic cells respond better to HDACi or HATi. To test this further in human AML, we used Kasumi1 and K562 cells,[55] which express different GFI1 levels (Supplementary Figure S8d). High *GFI1*-expressing Kasumi1 cells were responsive to Vorinostat, whereas low *GFI1*-expressing K562 cells were significantly more sensitive to CTK7a (Supplementary Figures S8e and f).

Next, we used published data sets regarding cellular response to drug treatment and resulting gene expression patterns of a number of established AML cell lines, including MonoMac6, HL-60, Kasumi1, THP1, P31FUJ (high *GFI1* expression), CESS, ML2 and GDM1 (low *GFI1* expression).[55] Cell lines with high *GFI1* expression had a significantly lower IC50 than cell lines with lower *GFI1* expression levels (Supplementary Figure S8g), confirming our results with Kasumi1 and K562 cells. Finally, we subjected primary *NUP98-HOXD13*-expressing leukemic cells from *GFI1*-KI and *GFI1*-KD mice (Supplementary Figures S8i–k) or human samples with low and high *GFI1* expression (Supplementary Figure S8h) to treatment with CTK7a and Vorinostat (Figures 4d–f and Supplementary Table S5) and observed that low *GFI1*-expressing murine or human primary AML cells were more resistant to treatment with Vorinostat than with CTK7a (Figures 4e and f, and Supplementary Figures S8j and k).We also examined the effect of HATis on non-malignant hematopoietic progenitor cells, by treating GFI1-KI and GFI1-KD Lin^{-} cells. HATis had a significantly reduced effect on these cells compared to the malignant cells (Supplementary Figure S9).

DISCUSSION

Our findings indicate that reduced *GFI1* expression levels represent a marker for an adverse AML prognosis and the most beneficial epigenetic therapy. Data from clinical studies indicated that reduced expression of *GFI1* in AML blasts correlates with inferior prognosis of AML patients. The analysis of gene expression arrays from leukemic cells with low *GFI1* expression revealed enrichment of gene signatures that are found in LSCs as well as in hematopoietic stem cells and that are associated with poor prognosis.[44] There is an association between low *GFI1* expression, increased incidence of *EVI1* expression and adverse cytogenetic findings. However, these two findings do not completely explain the inferior prognosis conferred by low *GFI1* expression, since low *GFI1* expression is an adverse prognostic factor even after adjusting for cytogenetics and *EVI1* expression. Moreover, Cluster 5 of the Valk *et al.*[39] study also features low *GFI1* expression, an inferior prognosis but not increased expression of *EVI1*. We hypothesize that the epigenetic changes associated with low GFI1 doses and the resulting gene expression changes in the context of a LSC signature contribute to a number of different pathways, conferring inferior prognosis. The hypothesis that reduced levels of GFI1 promote leukemia development is also supported by the finding that low *GFI1* expression levels are associated with an inferior prognosis in MDS patients[38] and accelerate progression of CML from a chronic to an accelerated phase.[56] In addition, a *GFI1* gene expression signature was associated with pediatric AML relapse.[57] Finally, downregulation of *Gfi1* expression in the process of leukemia development seems not to be restricted to myeloid malignancies. Upon development of T-acute lymphoid leukemia in a murine acute lymphoid leukemia-model, *Gfi1* expression is downregulated upon transition of normal lymphoid cells to premalignant and full-blown leukemic cells.[58]

Myeloid differentiation block and accelerated AML development in GFI1 knock-down mice

To study the effect of *GFI1* downregulation on leukemogenesis, we generated humanized *GFI1* ‘knock-down’ mice (*GFI1*-KD). The phenotypes of *GFI1*-KD mice, such as monocytosis and absence of neutrophils, are likely a direct consequence of reduced GFI1 expression and not due to presence of the Neo cassette, as several other, independently generated Gfi1-deficient mouse models also typically show monocytosis and absence of neutrophils when the Neo cassette is removed,[35] or in ‘knock-in’ mice expressing a P2A loss-of-function mutant of Gfi1 without presence of a Neo cassette.[59] In a conditional Gfi1 mouse that also lacks a Neo cassette,[25] loss of both alleles of Gfi1 also leads to monocytosis and absence of neutrophils. Finally, Zarebski *et al.*[28] showed that Lin^{-} BM cells transduced with a retroviral vector expressing the dominant-negative Gfi1N382S form cause also monocytosis and neutropenia, confirming that inhibition or ablation of Gfi1 directly causes monocytosis and neutropenia without the presence of a Neo cassette.

Q4

We avoided using *GFI1*-deficient mice, as *GFI1* expression is still detectable in human AML samples and is not absent. In addition, among all AML patients characterized so far, no known loss of function or complete loss of both alleles of *GFI1* has been detected (Khandanpour, Maciejewsiki *et al.*, unpublished), suggesting that complete loss of *GFI1* might not be beneficial for development of human AML.

Our finding that knock-down of GFI1 was associated with a block of differentiation of the myeloid compartment and increased numbers of GMPs further supports our hypothesis. A block of myeloid differentiation is a hallmark of AML and, in addition, both in humans and in mice, LSCs can originate from the GMP fraction.[36,46] A higher number of GMPs, alongside a block of differentiation, increases the probability that additional events lead to a full-blown AML. Low expression of *GFI1* increased the incidence and shortened the latency of AML development in three different models of human AML and MDS, which is evidence for a critical role of *GFI1* expression levels in AML development.

GFI1 expression levels affect histone acetylation and predict response to epigenetic therapies

GFI1 exerts its repression by recruiting HDACs to its target genes, leading to deacetylation of H3K9 and decreased expression of target genes.[15–17,32,33] Here we show that lower GFI1 levels lead to higher histone H3K9 acetylation at the GFI1 target gene compared to cells with normal WT *GFI1* expression levels. We propose, therefore, that low levels of GFI1 are no longer able to repress genes critical for AML development, suggesting that GFI1 may act as an oncosuppressor at higher expression levels in myeloid cells (Supplementary Figure S5). Although we cannot rule out that these effects are due to reduced activity of HDACs, it is conceivable that reduced GFI1 expression leads to reduced recruitment of HDACs to GFI1 target genes and consequently to the observed reduced de-acetylation.

Epigenetic therapy such as treatment with HDCAi has become more prevalent for patients who could not be treated by conventional therapy, but results have been disappointing.[52,53,60] Low *GFI1* expression levels correlated with a resistance to treatment with HDACi, most likely because low levels of GFI1 impair efficient deacetylation of GFI1 target genes. It is likely that cancer-promoting genes are among those targets. A further inhibition of deacetylation by HDACi would therefore not reverse this mechanism. As a result, the roughly 10-15% of AML patients who have low expression of *GFI1* in their blasts might be non-responsive to HDACi and would thereby mask the benefits that an HDACi therapy would have on other AML/MDS patients.

We postulate instead that treatment with HATis could potentially reverse the increased acetylation and also expression of GFI1 target genes. Our data with the HATi CTK7a support this idea and indicate that blocking HATs might be a new strategy to overcome this resistance in low *GFI1*-expressing patients. This concept needs to be confirmed in *in-vivo* murine models and possibly in future interventional studies, as no major study has yet used Vorinostat or CTK7a as a single agent for therapy of MDS patients. Nevertheless, our study supports the hypothesis that GFI1 acts in myeloid malignancies in a dose-dependent manner, and that GFI1 expression levels are a candidate biomarker for prognosis and can potentially help in the decision for a specific epigenetic therapy approach for a subset of AML/MDS patients.

CONFLICT OF INTEREST

Cyrus Khandanpour received travel reimbursement for attending scientific conferences from Amgen and Chugai. Jaroslaw Maciejewski received speaker honoraria from Celgene and Alexion. The remaining authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

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