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**GF1 is required for RUNX1/ETO positive acute myeloid leukemia**

RUNX1/ETO (Acute Myeloid Leukemia 1-Eight Twenty One) is an onco-fusion protein produced as a consequence of the t(8;21)(q22;q22) translocation. It functions as an aberrant transcription factor and contributes to AML development. We examined the role of the transcription factor Growth Factor Independence 1 (GF1), a RUNX1/ETO target gene, in the initiation and progression of this type of AML. We show here that GF1 is required for the maintenance of RUNX1/ETO-induced leukemia and that loss/reduced expression of GF1 impedes leukemia initiation and progression.

RUNX1/ETO impairs myeloid differentiation and increases expansion of the hematopoietic stem/progenitor pool. Two main RUNX1/ETO transcripts have been described. The transcript encoding the longest protein isoform contains almost the entire ETO protein including four nerve homology regions (NHRs). Alternative splicing incorporates ETO exon 9a and prematurely truncates the fusion protein. Consequently, the RUNX1/ETO9a protein lacks the last two NHRs. The RUNX1/ETO9a transcript is expressed in most primary (8;21) AML samples. Forced expression of RUNX1/ETO9a in bone marrow (BM) cells causes a rapid development of leukemia in mice. RUNX1/ETO induces a specific gene expression signature, impeding myeloid development and promoting leukemogenesis. Interestingly, Ptasiniska, Lin and many other colleagues showed that GF1 is among the target genes of RUNX1/ETO13 (and many other publications). GF1 is a transcriptional repressor and an onco-gene in medulloblastoma and lymphoid tumors. GF1 binds NHR2, a region of ETO essential for RUNX1/ETO9a-induced AML. Since an interaction between RUNX1/ETO and GF1 has been described, and GF1 represses its own transcription, we investigated whether expression of GF1 is elevated in RUNX1/ETO-AML patients and whether GF1 is important for RUNX1/ETO-induced leukemogenesis.

We first examined whether GF1 gene expression was increased in RUNX1/ETO AML samples. Analysis of a publicly available cohort of over 500 AML patients and their expression data sets showed that GF1 expression was elevated in RUNX1/ETO AML samples compared to RUNX1/ETO AML samples (<0.001) and to normal BM or CD34+ cells (Figure 1A). This finding was confirmed in independent cohorts (Figures 1B and 1C). Interestingly, high GF1 expression also correlated with higher incidence of NPM1 mutations, FLT3-ITD as well as MLL rearrangements (Table 1). When we analyzed GF1 expression in the context of other types of AML, we observed that only low-intermediate risk AML patients with PML-RARα mutation have comparable expression levels to RUNX1/ETO positive AML in two independent cohorts, while all the other types have lower expression levels (Online Supplementary Figure S1A-D). As high GF1 expression has been implicated in lymphoid leukemia, we investigated whether it is also important for RUNX1/ETO leukemia induction and maintenance.

To understand whether loss of GF1 affects RUNX1/ETO leukemia development, we studied the consequences of GF1 ablation in murine models of RUNX1/ETO-induced AML. We retrovirally transduced Lineage negative (Lin) BM cells from GF1 wildtype (WT) or knockout (KO) mice with RUNX1/ETO9a IRES GFP (shortly RUNX1/ETO9a) and tested their clonogenic capacity in a colony forming cell (CFC) assay. The absence of GF1 reduced colony numbers by two-fold (Figure 1D), while the clonogenic capacity of non-transduced GF1 WT and KO cells was similar (data not shown). In a serial replating assay, the absence of GF1 inhibited the growth of transduced cells 6 to 10-fold compared to GF1 WT cells (Figure 1E). These data indicate that GF1 contributes to the cell growth and clonogenic capacity of RUNX1/ETO9a cells or that loss of GF1 abolished the transformative effect of RUNX1/ETO9a.

To verify the effects of GF1 loss on RUNX1/ETO-associated AML in vivo, we transplanted GF1 WT mice with GF1 WT or KO Lin-BM cells expressing RUNX1/ETO9a. Following transplantation with GF1 WT/RUNX1/ETO9a transduced cells, all mice developed AML within 250 days (Figure 1F). In contrast, only 3/10 mice transplanted with GF1 KO/RUNX1/ETO9a transduced cells died of leukemia (Figure 1F).

Since retroviral-mediated oncofusion protein expression can lead to non-physiologically high expression levels, we used a second system, in which expression of RUNX1/ETO is induced endogenously. Conditional RUNX1/ETO knock-in mice with either a GF1 WT or KO background were injected with poly(I:C) to activate RUNX1/ETO expression. Subsequent treatment with the DNA-damaging agent N-ethyl-N-nitosourea (ENU) triggers AML formation. During one year following poly(I:C) and ENU injections, 30% of RUNX1/ETO/GF1 WT mice developed RUNX1/ETO myeloid leukemia, while the remaining mice developed lymphoma (Figure 1G, upper panel). The myeloid leukemia appearing in these animals was characterized by expression of CD34 (Figure 1G, lower panel) and a lack of CD4 or CD8 surface markers (data not shown). In contrast, none of the RUNX1/ETO/GF1 KO mice developed leukemia or lymphoma (Figure 1G, upper panel). Thus, GF1 is required for ENU/RUNX1/ETO-induced leukemia development.

To study the consequences of reduced GF1 expression in human leukemia cells, we used two GF1 shRNA constructs to silence GF1 in RUNX1/ETO SKNO-1 and Kasumi-1 cells. In a proliferation competition experiment, cells transduced with either of the two shRNAs against GF1 were rapidly overgrown by non-transduced cells, while this was not the case for control-transduced cells (Figure 1H, I). To study whether GF1 could be a downstream target of RUNX1/ETO, ChIP-seq experiments were performed. GF1 promoter occupancy by RUNX1/ETO was analyzed in three RUNX1/ETO primary AML samples and the RUNX1/ETO cell line Kasumi-1. A strong RUNX1/ETO signal within intron 1 of the GF1 gene was found in all RUNX1/ETO ChIP-seq profiles (Figure 2A and data not shown), confirming that GF1 is indeed one of its direct targets. In addition, these RUNX1/ETO profiles were similar (Figure 2B and data not shown) to RUNX1 ChIP-seq profiles in Kasumi-1 cells, which were generated using an antibody that does not recognize RUNX1/ETO. Furthermore, the GF1 gene contained several RUNX1 putative binding motifs, one of which was located in the promoter and transcription start site (TSS) associated with the the RUNX1/ETO peak. GF1 ChIP-seq revealed that RUNX1/ETO and GF1 peaks partially overlapped (Figure 2A), which could mean that the proteins interact and influence each other’s function.

To gain more insight into the interplay between RUNX1/ETO and GF1, we compared their genome-wide occupancies. We found that the RUNX1/ETO, RUNX1 and GF1 binding profiles were highly comparable, since clustering was based on peak width and inten-
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Figure 1. GFI1 is highly expressed in RUNX1/ETO+ AML and required for growth of RUNX1/ETO+ cells. (A) Micro-array data of GFI1 expression in RUNX1/ETO+ and remaining AML samples, normal bone marrow (BM) and CD34+ cells from an AML patient cohort published by Wouters et al. Samples without information on large chromosomal aberrations were excluded from this graph. The red lines indicate the median (***P<0.001). (B) qRT-PCR data of GFI1 expression in RUNX1/ETO+ AML versus other AML samples. GFI1 expression was normalized to PBGD expression. The red lines indicate the median (**P<0.01). (C) RNA-seq data of GFI1 expression in AML patients harboring recurrent chromosomal rearrangements from an AML patient cohort published by Cancer Genome Atlas Research. RPKM: reads per kilobase per million mapped reads. The red lines indicate the median (**P<0.001). (D) Colony forming cell (CFC)-assay using RUNX1/ETO9a-transduced murine Lineage (Lin−) Gfi1 WT or KO bone marrow (BM) cells (mean±SD, n=3 for each genotype, **P<0.003). (E) Serial replating assay with RUNX1/ETO9a-transduced murine Lin− cells derived from Gfi1 WT or Gfi1 KO mice in liquid culture. After 6-10 days the number of cells was counted and 1000 cells/well were serially replated two more times (mean±SD, n=3). The experiment was performed at least three times in triplicates. One representative experiment is shown for each serial plating assay (**P<0.01). (F) Kaplan-Meier curve of AML-free survival for mice transplanted with Lin− cells from Gfi1 WT or KO mice transduced with RUNX1/ETO9a (n=8/8 leukemia, n=3/10 leukemia). (G) Upper panel: Kaplan-Meier curve showing tumor-free survival of conditional RUNX1/ETO knock-in mice that were treated with poly(I:C) to activate RUNX1/ETO and with ENU to induce DNA damage and as a consequence, tumors. Lower panel: Representative FACS plot of ENU-induced AML in RUNX1/ETO knock-in mice showing the frequency of CD34+ cells. (H) Knockdown (KD) of GFI1 in the RUNX1/ETO+ cell line SKNO-1 using two established GFI1 shRNAs (shGFI1-65 and shGFI1-68) in a growth competition experiment over time. The normalized percentage of GFI1 or non-targeting (NT) shRNA transduced cells (Venus+) is shown (mean±SD, three cultures, *P<0.05). (I) Similar to D, in RUNX1/ETO+ Kasumi-1 cells two established GFI1 shRNAs (shGFI1-65 and shGFI1-68) constructs were used in a growth competition experiment over time. The normalized percentage of GFI1 or non-targeting (NT) shRNA transduced cells (Venus+) is shown (mean±SD, three cultures, *P<0.05).
sity instead of differential binding (Figure 2B, left panel) at almost all of the ~40,000 binding sites identified. Genomic annotation revealed that co-occupancy was not specific only for TSS/promoter areas, but also for intergenic and intronic regions (10,379-14,241 sites each) (Figure 2B, middle panel). Only a minority of binding sites (~2,000) could be identified with either RUNX1 (1,597 peaks) or RUNX1/ETO (441) binding, but these displayed equal GFI1 occupancy, suggesting no preferential colocalization of GFI1 and WT or mutant RUNX1 (Figure 2B, right panel). To investigate whether GFI1 regulates RUNX1/ETO DNA binding in vivo we determined RUNX1/ETO DNA binding using ChIP-seq in spleen cells of leukemic mice transplanted with Gfi1 WT or Gfi1 KO/RUNX1/ETO9a-transduced cells. We found that RUNX1/ETO binds at many sites, such as the Sfpi1 locus (Figure 2C). Interestingly, a change in RUNX1/ETO occupancy at the Gfi1 promoter region was observed in Gfi1 KO cells (Figure 2C), suggesting that an altered binding of RUNX1/ETO could deregulate Gfi1 expression. Using a cutoff of 3-fold, 83 regions showed increased RUNX1/ETO occupancy (Figure 2D), whereas 210 showed decreased occupancy. Decreased RUNX1/ETO occupancy regions were associated with genes of the Hippo, Rap1, Cancer and TGFβ pathways. These results suggest that Gfi1 is regulating RUNX1/ETO binding and activation at genes involved in leukemic transformation.

We also investigated the motif composition of GFI1, RUNX1 and RUNX1/ETO common binding sites. This revealed enrichment of the RUNX1/ETO and GFI1 binding sequences (Figure 2E), suggesting that co-occupancy could be the result of each protein binding independently to the DNA. Further research is needed to elucidate the exact mechanism causing high Gfi1 expression in RUNX1/ETO+ AML patients and the functional interplay between GFI1 and RUNX1/ETO in induction and maintenance of AML. The human and mouse Gfi1 promoters share regions with very high sequence similarity, including the region around the TSS. RUNX1/ETO9a occupancy was also found in this region in two independent samples from murine leukemic RUNX1/ETO9a/Gfi1 WT cells (Figure 2F and data not shown).

Our findings reveal that expression of Gfi1 is higher in RUNX1/ETO+ AML samples compared to other AML types and that absence of Gfi1 delays the growth of RUNX1/ETO9a+ cells both in vitro and in vivo. The fact that loss of Gfi1 negatively influences leukemia development might seem contradictory to our previous reports indicating that reduced levels of Gfi1 (10-20% of physiological level) accelerate AML development.11 We propose that GFI1 might have context-dependent roles in leukemogenesis and, in the case of RUNX1/ETO, it might be required for sustained growth. In addition, AML with t(8;21)(q22;q22) translocation may be different from other types of leukemia since the RUNX1/ETO fusion protein directly binds to GFI1.

Thus, our results demonstrate an important role for GFI1/Gfi1 in the onset and maintenance of RUNX1/ETO+ AML.

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**Table 1. Molecular, cytological and cytogenetic characteristics of AML with regard to GFI1 expression.**

<table>
<thead>
<tr>
<th>Chromosomal rearrangements</th>
<th>RUNX1/ETO+ AML</th>
<th>PML-RARα+ AML</th>
<th>Samples with highest 25% GFI1 expression in remaining AML samples</th>
<th>Samples with lowest 75% GFI1 expression in remaining AML samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBFβ-MYH11</td>
<td>0/7</td>
<td>0.0</td>
<td>0/16 0.0</td>
<td>2/45 4.4</td>
</tr>
<tr>
<td>MLL-Rearrangements</td>
<td>0/7</td>
<td>0.0</td>
<td>0/16 0.0</td>
<td>8/45 17.8</td>
</tr>
</tbody>
</table>

**Molecular aberrations**

| RUNX1 mutation        | 0/7 0.0 0/16 0.0 | 1/45 2.2* 16/111 14.4 |
| CEBPα mutation        | 0/7 0.0 0/16 0.0 | 1/45 2.2 12/111 10.8  |
| DNMT3α mutation       | 0/7 0.0 0/16 0.0 | 13/45 28.9 30/111 27.0 |
| FLT3-ITD              | 1/7 14.3 5/16 31.3 | 14/45 31.1* 17/111 15.3 |
| FLT3-TKD              | 0/7 0.0 0/16 0.0 | 6/45 13.3 6/111 5.4    |
| IDH1 mutation         | 0/7 0.0 0/16 0.0 | 7/45 15.6 9/111 8.1    |
| IDH2 mutation         | 0/7 0.0 0/16 0.0 | 5/45 11.1 12/111 10.8  |
| NPM1 mutation         | 0/7 0.0 0/16 0.0 | 20/45 44.4* 28/111 25.2 |
| FLT3-ITD and NPM1 mutated | 0/7 0.0 0/16 0.0 | 9/45 20.0* 7/111 6.3 |
| TET2 mutation         | 1/7 14.3 0/16 0.0 | 2/45 4.4 13/111 11.7  |
| TP53 mutation         | 0/7 0.0 0/16 0.0 | 2/45 4.4 13/111 11.7  |

**FAB classification**

| M0             | 0/38 0.0 0/25 0.0 | 4/110 3.6 14/333 4.2 |
| M1             | 2/38 5.3 0/25 0.0 | 24/110 21.8 71/333 21.3 |
| M2             | 32/38 84.2 2/25 8.0 | 25/110 22.7 71/333 21.3 |
| M3             | 0/38 0.0 2/25 8.0 | 0/110 0.0 1/333 0.3  |
| M4             | 4/38 10.5 1/25 4.0 | 24/110 21.8 63/333 18.9 |
| M5             | 0/38 0.0 0/25 0.0 | 30/110 27.3 86/333 25.8 |

Freq: frequency *indicates statistical significance (P<0.05).
Figure 2. RUNX1/ETO, RUNX1 and GFI1 occupy the GFI1 locus and highly similar regions genome-wide. (A) ChIP-seq results on the GFI1 locus (hg19 chr1:92,938,000-92,953,033). Conservation with the mouse genome is indicated. Profiles 1-3: GFI1, RUNX1/ETO and RUNX1 binding to the GFI1 locus in primary RUNX1/ETO+ AML samples. Profiles 4-6: RUNX1/ETO binding to the GFI1 locus in the RUNX1/ETO+ cell line Kasumi-1. (B) ChIP-seq on Kasumi-1 cells showing that RUNX1/ETO, RUNX1 and GFI1 occupy the same genomic regions. Left panel: RUNX1/ETO, RUNX1 and GFI1 occupancy on all RUNX1/ETO, RUNX1 and GFI1 peaks. When clustering analysis was performed, peaks were clustered based on peak width and intensity, rather than on regions that were bound by either transcription factor versus multiple transcription factors. Middle panel: genomic distribution of regions occupied by RUNX1/ETO, RUNX1 and GFI1. RE: RUNX1/ETO. Right panel: to study whether GFI1 preferentially interacted with RUNX1/ETO over RUNX1, GFI1 occupancy was studied on largely RUNX1-specific (left) or largely RUNX1/ETO-specific (right) binding sites. (C) RUNX1/ETO9a binding to mouse Sfpi1 and Gfi1 loci in spleen cells of leukemic mice transplanted with Gfi1 WT or Gfi1 KO/RUNX1/ETO9a-transduced cells. The red box indicates altered RUNX1/ETO9a occupancy in Gfi1 KO cells. (D) Occupancy of RUNX1/ETO9a at regions increased in RUNX1/ETO binding (top) or decreased in RUNX1/ETO binding (bottom) in Gfi1 KO leukemic cells from spleen: 83 regions showed increased RUNX1/ETO9a binding, RPMK: reads per kilobase per million mapped reads. (E) Shown are the consensus sequences for GFI1 and RUNX1/ETO found in our ChIP-seq profiles. (F) Binding profile of HA-tagged RUNX1/ETO9a on mouse Gfi1 promoter region obtained with anti-HA antibody. The positions of the predicted transcription initiation site (arrow) and the two RUNX1 consensus sites (asterisks) are indicated. The numbers indicate the positions relative to the transcription initiation site (+1bp). ChIP (a) and ChIP (b) are the regions analyzed by PCR following ChIP assays.
Anna E. Marneth, a,b Lacramioara Botetzatu, a,b Judith M. Hönes, a,b,c,d Jimmy C.L. Israel, d Judith Schütte, e Lothar Vassen, f Robert F. Laufs, a Saskaia M. Bergeveit, a Laura Groothuis, a Amir Mandal, j Joost H.A. Martens, a Gerrwin Huls, a Joop H. Jansen, h Ulrich Dühren, a Tobias Berg, g TARik Möröy, f Christian Wichmann, a Mia-Chia Lo, a Dong-Er Zhang, a Bert A. van der Reijden* and Cyrus Khandanpour2,10*.

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

The team contributed equally to this work.

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

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


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