GFI1 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.[1] It functions as an aberrant transcription factor and contributes to AML development. We examined the role of the transcription factor Growth Factor Independence 1 (*GFI1*),[2] a RUNX1/ETO target gene, in the initiation and progression of this type of AML. We show here that GFI1 is required for the maintenance of RUNX1/ETO-induced leukemia and that loss/reduced expression of GFI1 impedes leukemia initiation and progression.

RUNX1/ETO impairs myeloid differentiation and increases expansion of the hematopoietic stem/progenitor pool.[3] 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.[4] Consequently, the RUNX1/ETO9a protein lacks the last two NHRs. The RUNX1/ETO9a transcript is expressed in most primary t(8;21) AML samples.[4] Forced expression of RUNX1/ETO9a in bone marrow (BM) cells causes a rapid development of leukemia in mice.[4] RUNX1/ETO induces a specific gene expression signature, impeding myeloid development and promoting leukemogenesis.[3] Interestingly, Ptasinska, Lin and many other colleagues showed that *GFI1* is among the target genes of RUNX1/ETO[3,5] (and many other publications). GFI1 is a transcriptional repressor and an oncogene in medulloblastoma and lymphoid tumors.[6] GFI1 binds NHR2,[7] a region of ETO essential for RUNX1/ETO9a-induced AML.[8] Since an interaction between RUNX1/ETO and GFI1 has been described,[7] and GFI1 represses its own transcription,[9] we investigated whether expression of *GFI1* is elevated in *RUNX1/ETO*$^+$ AML patients and whether GFI1 is important for RUNX1/ETO-induced leukemogenesis.

We first examined whether *GFI1* 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[10,11] showed that *GFI1* expression was elevated in *RUNX1/ETO*$^+$ AML samples compared to *RUNX1/ETO*$^-$ AML samples (P<0.001) and to normal BM or CD34$^+$ cells (Figure 1A). This finding was confirmed in independent cohorts (Figures 1B and 1C[10,11]). Interestingly, high *GFI1* expression also correlated with higher incidence of *NPM1* mutations, *FLT3*-ITD as well as *MLL*-rearrangements (Table 1). When we analyzed *GFI1* 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 *GFI1* expression has been implicated in lymphoid leukemia,[6] we investigated whether it is also important for *RUNX1/ETO*$^+$ leukemia induction and maintenance.

To understand whether loss of Gfi1 affects *RUNX1/ETO*$^+$ leukemia development, we studied the consequences of *Gfi1* ablation in murine models of RUNX1/ETO-induced AML. We retrovirally transduced Lineage negative (Lin$^-$) BM cells from *Gfi1* 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 Gfi1 reduced colony numbers by two-fold (Figure 1D), while the clonogenic capacity of non-transduced Gfi1 WT and KO cells was similar (data not shown). In a serial replating assay, the absence of Gfi1 inhibited the growth of transduced cells 6 to 10-fold compared to Gfi1 WT cells (Figure 1E). These data indicate that Gfi1 contributes to the cell growth and clonogenic capacity of *RUNX1/ETO9a*$^+$ cells or that loss of GFI1 abolished the transformative effect of RUNX1/ETO9a.

To verify the effects of Gfi1 loss on *RUNX1/ETO*-associated AML *in vivo*, we transplanted *Gfi1* WT mice with Gfi1 WT or KO Lin-BM cells expressing *RUNX1/ETO9a*. Following transplantation with *Gfi1 WT/RUNX1/ETO9* transduced cells, all mice developed AML within 250 days (Figure 1F). In contrast, only 3/10 mice transplanted with *Gfi1 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[12] with either a *Gfi1* 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-nitrosourea (ENU) triggers AML formation. During one year following poly(I:C) and ENU injections, 30% (7/22) of *RUNX1/ETO*$^+$*/Gfi1* WT mice developed *RUNX1/ETO*$^+$ myeloid leukemia, while the remaining mice developed lymphoma[12] (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*$^+$*/Gfi1 KO* mice developed leukemia or lymphoma (Figure 1G, upper panel). Thus, *Gfi1* is required for ENU/RUNX1/ETO-induced leukemia development.

To study the consequences of reduced *GFI1* expression in human leukemia cells, we used two *GFI1* shRNA constructs[13] to silence GFI1 in *RUNX1/ETO*$^+$ SKNO-1 and Kasumi-1 cells. In a proliferation competition experiment, cells transduced with either of the two shRNAs against GFI1 were rapidly overgrown by non-transduced cells, while this was not the case for control-transduced cells (Figure 1H, I). To study whether *GFI1* could be a downstream target of RUNX1/ETO, ChIP-seq experiments were performed. *GFI1* promoter occupancy by RUNX1/ETO was analyzed in three *RUNX1/ETO*$^+$ primary AML samples and the *RUNX1/ETO*$^+$ cell line Kasumi-1.[14] A strong RUNX1/ETO signal within intron 1 of the *GFI1* gene was found in all RUNX1/ETO ChIP-seq profiles (Figure 2A and data not shown), confirming that *GFI1* 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 *GFI1* 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. GFI1 ChIP-seq revealed that RUNX1/ETO and GFI1 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 GFI1, we compared their genome-wide occupancies. We found that the RUNX1/ETO, RUNX1 and GFI1 binding profiles were highly comparable, since clustering was based on peak width and inten-

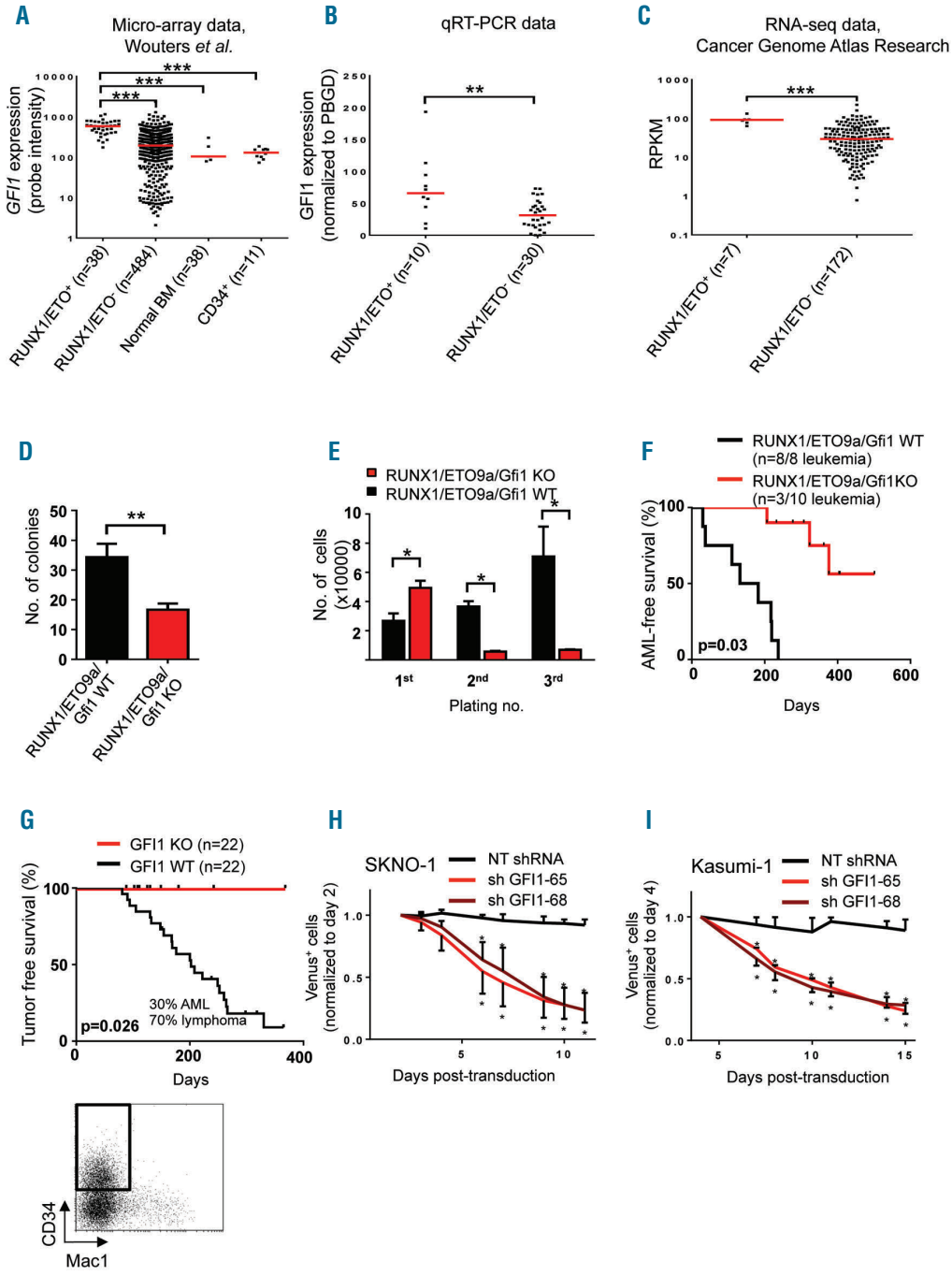

Figure 1. **GFI1 is highly expressed in *RUNX1/ETO*⁺ AML and required for growth of *RUNX1/ETO*⁺ cells.** (A) Micro-array data of *GFI1* expression in *RUNX1/ETO*⁺ and remaining AML samples, normal bone marrow (BM) and CD34⁺ cells from an AML patient cohort published by Wouters *et al.*[10] Samples without 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.[11] RPKM: reads per kilobase per million mapped reads. The red lines indicate the median (****P*<0.0001). (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=10 for Gfi1 KO and n=8 for *Gfi1* WT, *P*=0.003). (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 in one of the Gfi1 wild type RUNX1/ETO positive sarcomas. (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).

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

	RUNX1/ETO⁺ AML		*PML-RARα*⁺ AML		Samples with highest 25% *GFI1* expression in remaining AML samples		Samples with lowest 75% *GFI1* expression in remaining remaining AML samples	
Chromosomal rearrangements	Freq	%	Freq	%	Freq	%	Freq	%
CBFβ-MYH11	0/7	0.0	0/16	0.0	2/45	4.4	9/111	8.1
MLL-Rearrangements	0/7	0.0	0/16	0.0	8/45	17.8*	3/111	2.7
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
DNMT3a 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	22/25	88.0	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 ($P<0.05$).

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 TSSs/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 TGFB 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/RUNX1 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.

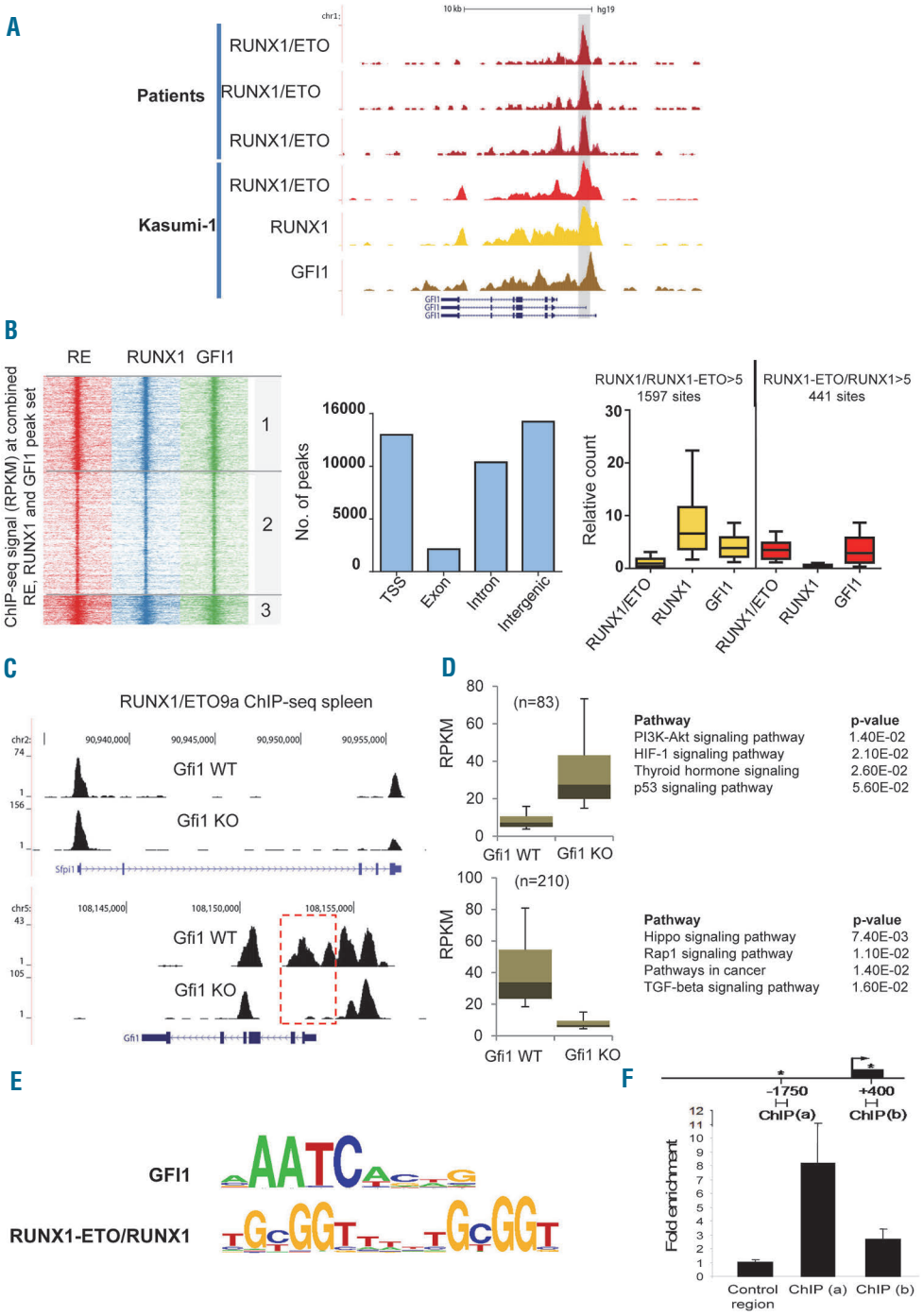

Figure 2. RUNX1/ETO, RUNX1 and GFI1 occupy the *GFI1* locus and highly similar regions genome-wide. (A) ChIP-seq results on the *GFI1* locus (hg19 chr1:92,938,000-92,953,033). Conservation with the mouse genome is indicated. Profiles 1-3: GFI1, RUNX1/ETO and RUNX1 binding to the *GFI1* locus in primary *RUNX1/ETO*$^+$ AML samples.[14] 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 (left) at regions increased in RUNX1/ETO binding (top) or decreased in RUNX1/ETO binding (bottom) in Gfi1 KO leukemic cells. Altered binding of RUNX1/ETO to mouse *Gfi1* promoter in *Gfi1* KO leukemic cells from spleen: 83 regions showed increased RUNX1/ETO occupancy (top), while 210 showed decreased occupancy (bottom). KEGG pathway enrichment of genes associated with regions of altered RUNX1/ETO9a binding. RPKM: 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.

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