LSD1-mediated repression of *GFI1* super-enhancer plays an essential role in erythroleukemia

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Conflict of interest

The authors have no conflicts of interest to declare.

Abstract

Super-enhancers (SEs) consist of enhancer clusters with abundant binding of transcription factors (TFs) and cofactors. LSD1 is a histone modifier that eliminates SE activity. However, whether SE suppression by LSD1 is associated with leukemogenesis remains unknown. In erythro-megakaryocyte lineage leukemia cells, activation of the SE of *GFI1* (*GFI1*-SE) is related to induction of myeloid differentiation by LSD1 inhibitors NCD38 and NCD25 and to their anti-leukemia effect. Although functional TF-motifs were concentrated in an evolutionally conserved area, NCD38 barely induced additional TF recruitment. Instead, the transcription cofactors including LSD1, CoREST, HDAC1, and HDAC2 were evicted from *GFI1*-SE. Deletion of *GFI1*-SE impaired induction of myeloid differentiation by NCD38 and NCD25 in erythroleukemia cells. Gene set enrichment analysis revealed that the *GFI1*-SE deletion impaired NCD38-induced programs related to granulocyte differentiation and the CEBPA network, but restored NCD38-suppressed programs related to erythroid development, GATA1 targets, and acute myeloid leukemia (AML) clusters including FAB subtype M6 and AML with myelodysplastic syndrome-related chromosomal abnormalities. Ontologies of genes whose expression changes by NCD38 were cancelled due to the *GFI1*-SE deletion showed enrichment in

AML and neutropenia signatures. Collectively, our data suggest that sustainable repression of *GFI1*-SE by LSD1 is essential for sustenance of erythroleukemia cells.

Introduction

Networks of transcription factors (TFs) play a crucial role in hematopoiesis[1]. TFs function as key regulators for cell fate decisions such as stemness maintenance and terminal differentiation by coordination or cross-antagonism with other TFs. Dysregulation of differentiation is one of the causes of leukemia development[2]. This regulation partially occurs via enhancers near the gene loci of TF genes[3]. Recently, super-enhancers (SEs) have been identified as large clusters of stretched enhancers with abundant binding of TFs and cofactors to confer higher transcriptional activity than typical enhancers[4]. The precise regulation of SEs near the TF gene loci is essential to determine cell identity[4-6]. Moreover, SEs play pivotal roles not only in normal development but also in disease initiation and progression including oncogenesis[7-9]. Recent studies have reported that SEs in the proximity of oncogenes are often aberrantly activated in leukemia cells[10, 11]. However, little is known about whether silencing of SEs is involved in leukemia development or sustenance.

Lysine-specific demethylase 1 (LSD1, also known as KDM1A) is a histone demethylase that catalyzes the demethylation of mono- and di-methylated lysine 4 of histone H3 (H3K4me1/2)[12]. LSD1 is indirectly involved in the deacetylation of acetylated

lysine 27 of histone H3 (H3K27ac) via formation of a complex with RE1 silencing transcription factor corepressor (CoREST, also known as RCOR1) and histone deacetylase1 and 2 (HDAC1 and HDAC2, respectively)[13, 14]. Given that H3K4me1 and H3K27ac are surrogate markers of activated SEs[15, 16], LSD1 is presumed to function as an eraser of activated SEs. Deletion of *Lsd1* causes pancytopenia and increases the H3K27ac level on enhancers of *Lsd1* targets in murine hematopoietic cells[17]. *LSD1* is significantly overexpressed in a number of hematologic malignancies, including acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS)[18, 19]. Moreover, transgenic mice expressing a human *LSD1* isoform exhibit the development of T-cell lymphoblastic leukemia/lymphoma[20]. These reports clearly indicate that alterations in LSD1 expression lead to dysregulation of hematopoiesis and possible leukemia development.

To date, a number of LSD1 inhibitors have been developed, some of which are currently being investigated in clinical trials[21]. These inhibitors commonly induce myeloid differentiation to exert an anti-leukemia effect[18, 22-24]. However, the precise molecular mechanism is not fully understood. We previously developed the LSD1 inhibitors NCD38 and NCD25 and reported that these inhibitors are particularly effective in erythroleukemia and AML transformed from MDS or with MDS-related chromosomal abnormalities[23].

These inhibitors caused myeloid differentiation in such leukemia cells and activated several SEs near TF genes, including *GFI1*, which is a key regulator for neutrophil development[25] and a major partner in the hematopoietic TF network[1]. However, it is yet unclear how the SE localized 14 kb downstream of the *GFI1* gene body[23] (named *GFI1*-SE) is regulated and whether repression of *GFI1*-SE by LSD1 is critical to leukemogenesis in erythroleukemia cells. To clarify these points, we investigated the regulatory mechanism of *GFI1*-SE and the genetic programs influenced by *GFI1*-SE depletion.

Materials and methods

Cells and reagents

HEL, CMK11-5, UT7-EPO and K562 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin. Cells were maintained in our laboratory under condition without contamination with mycoplasma after validating the immunophenotype by fluorescence-activated cell sorting (FACS) analysis. NCD38 and NCD25 were synthesized as described previously[26], dissolved in dimethyl sulfoxide (DMSO), and used at 2 μM for all experiments.

Cell proliferation assay

After incubation of cells with 2 μM NCD38, 2 μM NCD25, or DMSO for 2 or 3 days, WST-8 reagent (Nacalai Tesque, Kyoto, Japan) was added. The data was obtained by a microplate reader (Tecan, Männedorf, Switzerland) according to the manufacturer's instructions.

Quantitative PCR

RNA and complementary RNA were synthesized as described in the supplementary

information. Quantitative PCR was performed using LightCycler480 System II (Roche, Basel, Switzerland) and a THUNDERBIRD SYBR qPCR mix (Toyobo, Osaka, Japan). Absolute quantification was calculated using recombinant DNA of targeted amplicons as a standard. Primer information is provided in Supplementary Table 1.

Western blotting

Total cell lysates were prepared as described in the supplementary information. We used anti-GFI1 [sc-8558 or sc-376949; Santa Cruz Biotechnology (SCB), Dallas, TX] and anti-ACTIN (sc-1616; SCB) as primary antibodies, and HRP-conjugated anti-goat (sc-2020; SCB) or anti-mouse (NA931v; GE Healthcare, Little Chalfont, UK) as secondary antibodies.

FACS

We used anti-CD11b-PE-Cy5 (ICRF44; eBioscience, San Diego, CA), and analyzed on FACS Calibur (BD Biosciences, San Jose, CA).

Chromatin immunoprecipitation (ChIP)

ChIP was performed as described previously[27]. For precipitation, anti-H3K27ac (39133; Active Motif, Carlsbad, CA), anti-GFI1 (sc-376949; SCB), anti-GFI1B (sc-28356X; SCB), anti-CEBPA (sc-61X; SCB), anti-TAL1 (ab155195; Abcam, Cambridge, UK), anti-GATA1 (ab11852; Abcam), anti-RUNX1 (ab23980; Abcam), anti- ERG (ab133264; Abcam), anti-LSD1 (ab17721; Abcam), anti-CoREST (ab32631; Abcam), anti-HDAC1 (ab7028; Abcam), anti-HDAC2 (ab7029; Abcam), or control rabbit IgG (sc-2027; SCB), and Dynabeads Protein G (Thermo Fisher Scientific, Waltham, MA) were used. After reverse crosslinking, ChIP DNA was purified with a MinElute PCR Purification kit (Qiagen, Hilden, Germany) and analyzed by quantitative PCR.

Reporter assays

A genomic region including *GFI1*-SE was amplified from HEL genomic DNA and cloned into the pGL3-promoter vector (Promega, Madison, WI). Deletion vectors and mutant vectors were constructed using PCR mutagenesis. Each vector and the Renilla luciferase reporter (phRL-TK, Promega) were cotransfected into HEL cells using Amaxa nucleofector technology (Lonza, Basel, Switzerland). After 48 hours, cells were treated with NCD38, NCD25, or DMSO. After a 24-hour treatment, luciferase activities were measured using the

Dual-Luciferase Reporter Assay System (Promega).

Genome editing

Genome editing was performed using CRISPR-Cas9 as described previously[23]. Briefly, two target-specific oligonucleotides were designed to cleave upstream and downstream sites of *GFI1*-SE using a bioinformatics tool[28, 29] (https://chopchop.rc.fas.harvard.edu/) and were inserted into the CRISPR-Cas9 genome editing vector (pX330-U6-Chimeric_BB-CBh-hSpCas9, plasmid ID: 42230; Addgene, Watertown, MA). Both vectors were transfected into HEL cells using Amaxa nucleofector technology. After limiting dilution and genotype screening, sublines with bi-allelic deletion of *GFI1*-SE (Δ*GFI1*-SE) were established. For control sublines, the non-inserted CRISPR-Cas9 genome editing vector was used.

Microarray experiments and analysis

Total RNA was extracted using an RNeasy Mini Kit (Qiagen). After quality check with an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA), 100 ng total RNA was labeled using a GeneChip WT Terminal Labeling Kit (Affymetrix, Santa Clara, CA) and hybridized to a

GeneChip Human Gene 2.0 ST Array (Affymetrix). Microarray data were analyzed using GeneSpring GX software (Agilent). All raw data were normalized and filtered based on the deviation of the values within a condition (SD < 0.1). Microarray data were submitted to the Gene Expression Omnibus and assigned to GSE128400. Gene set enrichment analysis (GSEA)[30, 31] was performed using GSEA v3.0 software (http://www.broadinstitute.org/gsea/index.jsp). The gene sets are listed in Supplementary Table 2. The gene ontology was analyzed using the Enrichr software[32, 33].

Bioinformatics tools

Our previous ChIP-sequencing data for H3K27ac (accession number: DRA003526) was visualized with the Integrative Genomics Viewer (IGV) software[34, 35] version 2.4 (Broad Institute, Cambridge, MA; https://www.broadinstitute.org/igv). Genomic regions conserved between humans and mice were identified using the ECR browser[36] (http://ecrbrowser.dcode.org/). The genomic sequence spanning the *GFI1*-SE locus was obtained from the UCSC genome browser[37] (http://genome.ucsc.edu/). DNA sequence alignments were performed using the T-coffee server[38] (http://tcoffee.vital-it.ch/apps/tcoffee/do:regular). TF-motifs were analyzed using the

JASPAR database[39] (http://jaspar.genereg.net/).

Statistical analysis

ANOVA followed by Dunnett's test was used for multiple comparison analysis of luciferase reporter activities. All tests were two-sided and a p-value < 0.05 was considered significant.

Results

Relevance of *GFI1*-SE activation to myeloid differentiation and anti-leukemia effect in erythro-megakaryocyte lineage leukemia cells

To investigate the role of *GFI1*-SE in the regulation of GFI1 expression and myeloid differentiation by LSD inhibitors, we compared the changes in H3K27ac levels in the *GFI1*-SE region, *GFI1* transcript levels, a myeloid marker CD11b, an erythroid marker CD235a, cytology, and cell growth following exposure to NCD38 or NCD25 in four erythro-megakaryocyte lineage leukemia cells. The H3K27ac level was elevated up to 25-fold in HEL cells and up to 3-fold in CMK11-5 cells, but not in UT7-EPO cells and K562 cells (Fig. 1a). LSD1 expression was comparable across all cell types and was not changed by exposure to NCD38 and NCD25 (Supplementary Figure 1). In response to the activation of *GFI1*-SE and regardless of the basal H3K27ac values, the *GFI1* transcript level increased in HEL and CMK11-5 cells but not in UT7-EPO and K562 cells (Fig. 1b). Induction of CD11b and neutrophil-like cytology including nuclear segmentation, as well as attenuation of CD235a were also observed only in HEL and CMK11-5 cells (Fig. 1c, Supplementary Figure 2&3). Cell growth was suppressed in HEL and CMK11-5 cells, but barely suppressed in UT7-EPO and K562 cells after 48-hour treatment (Fig. 1d). These data

suggest that the activation of *GFI1*-SE by NCD38 and NCD25 appears to correlate with the induction of myeloid differentiation and subsequent anti-leukemia effect in erythro-megakaryocyte leukemia cells. Although minimal efficacy was observed in UT7-EPO cells after 72-hour treatment, we observed no changes in *GFI1*-SE, *GFI1* expression, and CD11b level, except for attenuation of CD235a (Supplementary Figure 4a-c), suggesting that only this delayed effect is induced in a GFI1-independent manner. We also found *GFI1* upregulation with *GFI1*-SE activation in MDS-L cells transformed from MDS cells[40] but not in THP-1 cells with MLL-AF9 (Supplementary Figure 5), although both were moderately suppressed by NCD38 and NCD25 as previously reported[23]. This data suggests that *GFI1*-SE repression might be essential in MDS overt AML where the border with erythroleukemia has been partially altered[41].

GFI1-SE activation by the coordination of several TFs

Our first aim was to understand which TFs play a significant role in *GFI1*-SE activation by NCD38 or NCD25. Homology search between humans and mice uncovered a highly conserved area (CA) with more than 70% homology in *GFI1*-SE (Fig. 2a). The CA was most strongly activated by NCD38 according to the reconstruction of our previous

ChIP-sequencing data (DRA003526). In murine erythroleukemia cells, treatment with NCD38 or NCD25 elevated the H3K27ac level in the CA, upregulated the level of the *Gfi1* transcript, and induced the granulocyte marker Gr-1 (Supplementary Figure 6). The CA included five CpG sites (CG1-5) (Supplementary Figure 7); CG2-5 were almost fully open across the cell lines we used in the steady state, suggesting that DNA methylation status is not involved in CA activation, while CG1 was open only in CMK11-5 cells, which might relate to the basal H3K27ac level in *GFI1*-SE (Supplementary Figure 8).

Next, to ascertain the impact of the CA on enhancer activity, we cloned the most H3K27ac-enriched area of *GFI1*-SE including the CA into a luciferase vector (wild-type; WT) and generated two types of deletion mutants: a *GFI1*-SE mutant lacking the CA (ΔCA) or the non-CA portion (Δnon-CA). A reporter assay performed after transfection to HEL cells and exposure to NCD38 or NCD25 for 24 hours showed that Δnon-CA induced a high reporter activity that was comparable to the WT, while ΔCA failed to induce reporter activity (Fig. 2b). These data indicate that CA is required to activate *GFI1*-SE.

Within the CA, we identified binding motifs for TFs including GFI1/GFI1B, CEBPA, TAL1, RUNX1, and GATA1 (Supplementary Figure 7). Thus, we generated *GFI1*-SE mutants in which only the binding motifs of each TF were mutated and performed

reporter assays (Fig. 2b). We found that all single TF-motif mutants except the construct with a mutation in the CEBPA upstream-motif (CEBPA #1) exhibited significantly reduced levels of reporter activity compared with the WT after exposure to NCD38 [0.50, 0.61, 0.46, 0.56, and 0.50 of the WT level in constructs with mutations in GFI1/GFI1B-motifs, a CEBPA downstream-motif (#2), a TAL1-motif, a RUNX1-motif, or a GATA1-motif, respectively] and to NCD25 (0.53, 0.66, 0.51, 0.51, and 0.57 of the WT level in the same order as NCD38 exposure, respectively). The GFI1/GFI1B/GATA1/RUNX1/TAL1/CEBPA multiple-motifs mutant (5TFs-mt) achieved the same level of reporter activity reduction as the ΔCA mutant after exposure to NCD38 (0.28 and 0.30 of the WT level in ΔCA and 5TFs-mt, respectively) and NCD25 (0.25 and 0.30 of the WT level in ΔCA and 5TFs-mt, respectively). These data suggest that *GFI1*-SE activation by NCD38 and NCD25 is not controlled by any one TF but rather appears to be regulated by the coordination of various TFs.

Sustainable occupation of TFs and release of the LSD1 complex by NCD38 in *GFI1*-SE

Based on reporter assay results, we hypothesized that the recruitment of several TFs would be required to activate *GFI1*-SE. However, ChIP analysis showed that exposure to NCD38 barely changed the occupancy level of TFs, including GFI1, CEBPA, TAL1, RUNX1, GATA1, and ERG, in *GFI1*-SE (Fig. 3a). Although GFI1B was recruited by more than 2-fold, it is a key transcriptional repressor to silence myeloid-specific genes and consequently switch cell fate towards the erythroid lineage[42]. In addition, the GFI1B-motif mutant did not completely eliminate the reporter activity of the CA. We therefore considered it to be unlikely that GFI1B alone dominantly regulates *GFI1*-SE. In contrast, we and other groups demonstrated previously that LSD1 forms a complex with CoREST, HDAC1, and HDAC2 to silence gene expression and that NCD38 can dissociate LSD1 from GFI1B and RUNX1[27, 43]. ChIP data clearly showed that all members of the LSD1 complex were significantly detached from *GFI1*-SE by NCD38 (Fig. 3b). These findings suggest that *GFI1*-SE can be sufficiently activated by pre-bound TFs after the LSD1 complex has detached from *GFI1*-SE following exposure to NCD38.

Attenuation of NCD38 or NCD25-induced myeloid differentiation by complete loss of *GFI1*-SE in erythroleukemia cells

We next examined whether *GFI1*-SE is essential for LSD1 inhibitor-mediated myeloid differentiation in erythroleukemia cells. Using the CRISPR-Cas9 genome editing system, sublines with complete loss of *GFI1*-SE (Δ*GFI1*-SE) were established from HEL cells (Supplementary Figure 9). ChIP analysis after reinduction of wild-type *GFI1*-SE or each single TF-motif mutant to Δ*GFI1*-SE cells showed that direct recruitment of TFs was reduced in all mutants, indicating that *GFI1*-SE is important as an aggregation and action point of multiple TFs (Supplementary Figure 10). The *GFI1* transcript level following exposure to NCD38 or NCD25 was reduced by approximately 80% in Δ*GFI1*-SE cells (Fig. 4a). This transcriptional reduction caused a decrease in the level of GFI1 protein (Fig. 4b). Finally, induction of CD11b by NCD38 or NCD25 was attenuated in Δ*GFI1*-SE cells (Fig. 4c). We also established sublines with deletion of the CA from murine erythroleukemia cells (ΔCA-MEL, Supplementary Figure 11a). In these sublines, induction of the *Gfi1* transcript and Gr-1 by NCD38 or NCD25 was suppressed (Supplementary Figure 11b-d). These data indicate that *GFI1*-SE is essential for the upregulation of GFI1 and subsequent myeloid differentiation following exposure to NCD38 or NCD25 in erythroleukemia cells.

GFI1-SE is required for the lineage switch from erythroid to myeloid and for the disruption of AML programs upon NCD38 treatment

To investigate the cellular machinery controlled by *GFI1*-SE upon NCD38 treatment in erythroleukemia cells, we conducted gene expression profiling of *GFI1*-SE-non-depleted (control) or Δ*GFI1*-SE HEL cells after treatment with DMSO or NCD38 for 24 hours (Fig. 5a). Subsequently, gene set enrichment analysis (GSEA) was performed to compare DMSO-treated control cells with NCD38-treated control cells to extract gene sets that are influenced by NCD38 treatment, as well as to compare NCD38-treated control cells with NCD38-treated Δ*GFI1*-SE cells to extract gene sets that are influenced by the *GFI1*-SE deletion upon NCD38 treatment. The LSD1 signature that we identified in our previous report[23] was induced by NCD38, whereas this induction was suppressed by the *GFI1*-SE deletion (Supplementary Figure 12), indicating that the gene induction by NCD38 is at least partially mediated by *GFI1*-SE. In terms of differentiation programs, gene sets regulating granulocyte differentiation and the CEBPA network[44, 45] were induced by NCD38 in control cells and suppressed by the *GFI1*-SE deletion upon NCD38 treatment (Fig. 5b). Sets of genes differentially expressed during erythroid development and GATA1 target genes[46, 47] were suppressed by NCD38 but were restored by the *GFI1*-SE deletion (Fig. 5c).

These data indicate that *GFI1*-SE is important in the NCD38-mediated switch from an erythroid to granulocytic lineage by positively regulating the CEBPA-directed machinery and negatively regulating the GATA1-directed machinery. Moreover, we performed GSEA using an officially available gene expression dataset of AML[48] (Fig. 5d and Supplementary Figure 13). Programs characterizing AML cluster 7 and cluster 8 were downregulated by NCD38, whereas this downregulation was significantly impaired by the *GFI1*-SE deletion. According to the original paper containing the dataset[48], the former cluster included all cases registered as FAB subtype M6 and the latter cluster included many cases with MDS-related chromosomal abnormalities including complex karyotypes, deletion of 7q, and monosomy 7. These data suggest that *GFI1*-SE may be important in the exertion of anti-leukemia effects by NCD38 against programs for erythroleukemia or AML with myelodysplasia-related changes.

Finally, we identified 225 genes that were induced more than two-fold by NCD38 when comparing DMSO-treated and NCD38-treated control HEL cells. Among these, only in 17 genes, the NCD38-mediated inductions were attenuated by less than 50% due to the *GFI1*-SE deletion, according to our comparison between DMSO-treated and NCD38-treated Δ*GFI1*-SE HEL cells (Supplementary Table 3). Similarly, we found 57

genes whose expression levels were suppressed by less than 50% by NCD38. Among these, only in 5 genes, the NCD38-mediated suppressions were recovered more than two-fold due to the *GFI1*-SE deletion. According to our previous data set (GSE68348)[23], most of these 22 genes were strongly influenced in CMK11-5 cells following exposure to NCD38. Gene ontology analysis using the Human Phenotype Ontology database revealed that these 22 genes influenced by the *GFI1*-SE deletion upon NCD38 treatment were enriched in signatures related to AML and neutropenia (Fig. 5e). These data suggested that this subset of genes is regulated in a *GFI1*-SE-dependent manner upon NCD38 treatment and may be involved in myeloid differentiation and AML-related programs.

Discussion

In this study, we demonstrated a relationship between myeloid differentiation and activation of *GFI1*-SE by two LSD1 inhibitors, NCD38 and NCD25, in erythro-megakaryocyte lineage leukemia cells. The effect of the LSD1 inhibitors on *GFI1*-SE activity was dependent on the conserved area between humans and mice. Multiple motifs of TFs including RUNX1, GATA1, CEBPA, TAL1, GFI1, ERG, and GFI1B were localized in this CA and mutation of each motif partially impaired the enhancer ability of *GFI1*-SE. The deletion of *GFI1*-SE attenuated the induction of GFI1 and CD11b by NCD38 and NCD25 in erythroleukemia cells. GSEA revealed that NCD38 induced a lineage switch from erythroid to myeloid by activating a granulocytic differentiation program and suppressing an erythroid development program in erythroleukemia cells. However, the *GFI1*-SE deletion clearly impaired this NCD38-induced lineage switch.

The TF network is an essential system for determining cell fate [1]. SEs of TF genes are reported to contain a number of TF-motifs and play an important role as an action point for TFs in controlling the overall TF network[3, 49]. GFI1 is a key regulator for neutrophil development and is reported to be negatively correlated to some TFs including GFI1B, which is a key regulator for erythroid development[1, 25, 42]. Therefore, *GFI1*-SE functions as

a key conductor in the TF network by aggregating the regulation by TFs and controlling GFI1 expression. Silencing of *GFI1*-SE is likely important in directing differentiation to the erythroid lineage.

Our ChIP results revealed that additional recruitment of TFs is not necessary for *GFI1*-SE activation by NCD38. Instead, histone-repressive modifiers including LSD1, CoREST, HDAC1, and HDAC2 were evicted from *GFI1*-SE by NCD38. These modifiers are known to form a complex and associate with several TFs[14, 43, 50, 51]. Our previous comprehensive proteome analysis found that LSD1 interacts with GFI1B and RUNX1, and that NCD38 selectively dissociates LSD1 from GFI1B without disruption of the LSD1-CoREST-HDAC1/2 complex in erythroleukemia cells[27]. This finding has been further supported by another report using a different LSD1 inhibitor[52]. Overall, our data suggest that *GFI1*-SE remains primed by the cooperative binding of TFs but can be sufficiently obscured by recruitment of the LSD1-CoREST-HDAC1/2 complex via TFs such as GFI1B in erythroleukemia cells. Therefore, cross-antagonism between GFI1 and GFI1B may occur by recruitment of the LSD1-CoREST-HDAC1/2 complex rather than by increased binding of opposing TFs. Moreover, a recent study reported that another LSD1 inhibitor was associated with increased chromatin accessibility in an MLL-AF9 mice

leukemia model[53]. Although this study demonstrated that this increased accessibility is mediated by a key TF, PU.1, our data suggest that dissociation of the LSD1-CoREST-HDAC1/2 complex from enhancers may also increase chromatin accessibility at least when using NCD38.

Our results demonstrated that *GFI1*-SE activation affects the anti-leukemia effect of NCD38 and NCD25 in erythro-megakaryocyte lineage leukemia cells. According to our GSEA results, the gene sets characterizing AML clusters including FAB subtype M6 or AML harboring MDS-related chromosomal abnormalities were impaired by NCD38, whereas this impairment was cancelled by the *GFI1*-SE deletion. Gene ontology analysis showed that genes whose expression change by NCD38 was canceled by the *GFI1*-SE deletion were enriched in signatures related to neutropenia and AML. Recent reports have accumulated evidence of a relationship between aberrant SE activation and leukemogenesis[11, 54-56]. Pharmacological suppression of aberrant SE activation has been considered as an option for leukemia treatment. In contrast, a direct association between aberrant repression of SEs and leukemogenesis has been demonstrated in only a few reports[57, 58]. Our data provide a novel finding that sustainable repression of *GFI1*-SE by LSD1 is essential for the sustenance of erythroleukemia cells. Furthermore, as this

repression can be easily reversed by the LSD1 inhibitors NCD38 and NCD25, LSD1-mediated repression of SEs is a promising target for erythroleukemia. However, it remains unclear what causes the differences in the responses of similar erythro-megakaryocyte lineage leukemia cells to NCD38 and NCD25. Although DNA methylation status in the CA was not significantly changed across these cells, a recent paper has demonstrated that a combination of LSD1 inhibitors and a DNA hypomethylating agent, 5-azacytidine, yielded improved efficacy against AML[59]. DNA methylation status surrounding the entire gene body of GFI1 might be involved. In addition, the delayed attenuation of CD235a and minimal growth suppression in UT7-EPO might be caused by activation of other SEs such as *ERG*-SE, as we previously demonstrated that *ERG*-SE activation by NCD38 contributed to the attenuation of CD235a but not CD11b[27]. Further clarification of the epigenetics anomaly network centered on SEs is required to develop more effective epigenetics-targeted therapies in erythroleukemia cells.

LSD1 inhibitors have been tested in several clinical trials targeting AML[21]. Although LSD1 inhibitors experimentally exert an anti-leukemia effect against multiple AML subtypes in mouse models and human cell lines, AML cells frequently exhibit resistance to LSD1 inhibitors[23, 24, 60]. Our data also suggest that the activation status of

GFI1-SE or subsequent *GFI1* upregulation might act as a surrogate marker to predict an anti-leukemia effect, at least in erythroleukemia and megakaryoblastic leukemia cells. Determining whether such SE reactivation correlates with the anti-leukemia effect of LSD1 inhibitors in future clinical studies will help to select patients suitable for LSD1 inhibitor administration. Furthermore, our GSEA results showed that a CEBPA network was induced by NCD38 and negatively regulated by the *GFI1*-SE deletion, while a gene set of GATA1 targets was suppressed by NCD38 and recovered by the *GFI1*-SE deletion, suggesting that the lineage switch from erythroid to myeloid by NCD38 is at least partially executed by machineries directed by CEBPA and GATA1 in erythroleukemia cells. In contrast, a gene set characterizing the AML cluster with CEBPA mutations was prone to enrichment in NCD38-treated cells and was not influenced by the *GFI1*-SE deletion. These data raise the possibility that CEBPA mutations may be an adverse marker of the anti-leukemia effect of NCD38. This possibility is also supported by a previous report in which deletion of *Cebpa* was described to generate resistance of leukemia cells to LSD1 inhibition, despite the use of a different LSD1 inhibitor and a different leukemia mouse model[53]. Further clinical studies are necessary to clarify what type of gene alterations influence the efficacy of LSD1 inhibitors.

In conclusion, activation of *GFI1*-SE by NCD38 is induced by dissociation of the LSD1-CoREST-HDAC1/2 complex from *GFI1*-SE and leads to the induction of programs related to the lineage switch from erythroid to myeloid, as well as impairment of programs related to erythroleukemia and AML with MDS-related chromosomal abnormalities. Sustainable repression of *GFI1*-SE by LSD1 is essential for the sustenance of erythroleukemia cells and therefore may be a surrogate adverse marker to predict the anti-leukemia effect of LSD1 inhibitors.

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Author contributions

G.T. performed all experiments, analyzed data, and wrote the paper. M.K. designed and conducted all experiments, analyzed data, provided funding, and wrote the paper. R.Y. and M.H. assisted with several experiments. T.S. provided LSD1 inhibitors. K.K., A.T-K. and A.A supervised the experiments and edited the paper.

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Figure Legends

Fig. 1. Correlation of *GFI1*-SE re-activation with myeloid differentiation and anti-leukemia effects in erythroleukemia and megakaryocytic leukemia cells

(a) ChIP-qPCR analysis of H3K27ac in *GFI1*-SE, (b) quantitative PCR analysis of the *GFI1* mRNA level, (c) FACS analysis of CD11b, and (d) a cell proliferation assay, on HEL, CMK11-5, UT7-EPO and K562 cells after 48-hour treatment with 2 μM NCD38, 2 μM NCD25, or DMSO. The Y-axes in (a), (b), and (d) indicate the % input of H3K27ac, the % GAPDH, and the fold change of viable cells measured by MTS assay, respectively. The mean fluorescence intensity (MFI) is presented in (c). FACS experiments were performed independently twice. Other experiments were performed independently three times. The means (± SD) are shown in (a), (b), and (d) and representative data are shown in (c). Mean values are described on the top of each bar in (a) and (b). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

Fig. 2. Role of TFs in *GFI1*-SE activation by NCD38 and NCD25 in erythroleukemia cells

(a) Homology search of *GFI1*-SE between human and mouse. The top portion shows the

reconstructed ChIP-sequencing data of H3K27ac around the *GFI1* locus and *GFI1*-SE using the DRA003526 data set and referencing human genome assembly hg19. The bottom portion shows a comparison of the human genome area presented in the top portion to the murine genome assembly mm10. The conserved area (CA), with homology of more than 70% over 100 base-pairs in *GFI1*-SE, is indicated by a red box. (b) Transcriptional activity analysis of *GFI1*-SE by a reporter assay. The left panel presents schematic diagrams of site-directed deletion or mutagenesis in the *GFI1*-SE region. The center and right panels show reporter activity of HEL transfectants with each indicated vector after a 48-hour treatment with 2 μM NCD38 and 2 μM NCD25, respectively, in comparison to DMSO. Each data set is presented as the relative ratio to the luciferase activity of an empty-vector transfectant treated with DMSO, after normalization of firefly luciferase activity to Renilla luciferase activity. Experiments were performed independently three times and the means (± SD) are shown. ΔCA: mutant lacking CA; Δnon-CA: mutant lacking the non-CA portion; GFI1/GFI1B mt: mutant of two GFI1/GFI1B-motifs; CEBPA #1 or #2 mt: mutant of a mutated CEBPA-motif #1 or #2, respectively; TAL1 mt: mutant of a TAL1-motif; RUNX1 mt: mutant of a RUNX1-motif; GATA1 mt: mutant of a GATA1-motif; 5TFs-mt: mutant of GFI1/GFI1B/CEBPA/TAL1/RUNX1/GATA1 multiple-motifs. The location of all

TF-motifs in *GFI1*-SE is indicated in Supplementary Figure 8. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ (ANOVA followed by Dunnett's test).

Fig. 3. Alteration in recruitment of TFs and the LSD1 complex on *GFI1*-SE after exposure to NCD38

(a) ChIP-qPCR analysis for GFI1, GFI1B, CEBPA, TAL1, RUNX1, GATA1, and ERG. (b) ChIP-qPCR analysis for LSD1, CoREST, HDAC1, and HDAC2. Data are presented as the fold change of enrichment calculated by dividing the % input of each TF on *GFI1*-SE in the NCD38 treatment cells by that in the DMSO treatment cells. Experiments were performed independently three times and the means (± SD) are shown.

Fig. 4. Attenuation of myeloid differentiation by NCD38 and NCD25 in Δ*GFI1*-SE erythroleukemia cells

(a) Changes in *GFI1* mRNA levels after 48-hour treatment with 2 μM NCD38 or NCD25. The fold change was calculated in each subline by dividing the *GFI1* mRNA level in NCD38 or NCD25 by that in DMSO after normalization to each *GAPDH* transcript. Experiments were performed independently three times and the means (± SD) are shown.

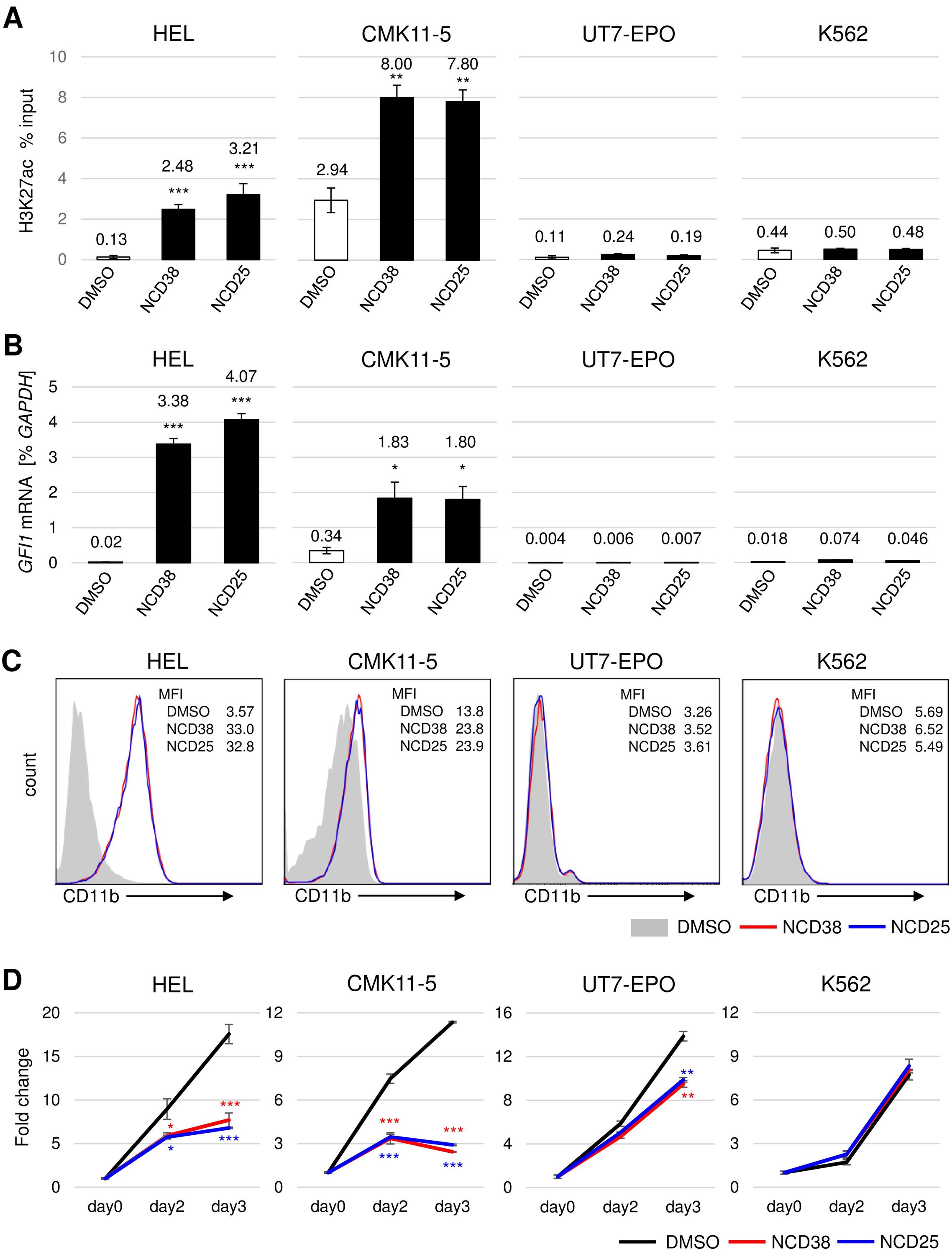
(b) Western blot analysis for the protein expression level of *GFI1* after 48-hour treatment with 2 μM NCD38, 2 μM NCD25, or DMSO. Actin is used as a control. (c) FACS analysis of CD11b. The histogram shows CD11b expression on the cell surface of each subline after 48-hour treatment with 2 μM NCD38, 2 μM NCD25, or DMSO. The filled histograms indicate DMSO-treated cells. The mean fluorescence intensity (MFI) is shown. The experiments in (b) and (c) were performed independently twice and representative data are shown.

Fig. 5. Involvement of *GFI1*-SE in the lineage switch from erythroid to myeloid programs and in impairment of AML programs upon NCD38 treatment

(a) Scatter plot of the expression profiling. Genes that are changed by more than 2-fold are plotted in log10 raw intensity values and representative gene names including *GFI1* are indicated. (b) GSEA of gene sets involved in regulation of granulocyte differentiation and the CEBPA network. (c) GSEA analysis of gene sets involved in differentially expressed genes during erythroid development and GATA1 targets. (d) GSEA analysis of gene sets of AML cluster 7 and 8 from datasets previously reported by Valk et al[48]. In (a) to (d), the left panels and right panels present results from comparisons between DMSO treatment and

NCD38 treatment in control HEL cells and between control HEL cells and Δ*GFI1*-SE HEL cells upon NCD38 treatment, respectively. The normalized enrichment score (NES) and the false discovery rate (FDR) q-value in GSEA are presented at the bottom of each panel. (e) Gene ontology analysis of genes influenced by *GFI1*-SE upon NCD38 treatment. The left pie charts show genes whose expression was significantly changed by NCD38 in white pies. Among these, genes whose NCD38-mediated inductions were attenuated by less than 50% or whose NCD38-mediated suppressions were recovered more than two-fold due to the *GFI1*-SE deletion are presented in black pies. The list of these genes and the fold changes are presented in Supplementary Table 3. The right panel presents the gene ontology result obtained from these 22 genes with Human Phenotype Ontology provided by the Enrichr software. FC denotes fold change.

Fig. 1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HEL
CMK11-5
UT7-EPO
K562
H3K27ac % input
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2.48 ***
3.21 ***
2.94
8.00 **
7.80 **
0.11
0.24
0.19
0.44
0.50
0.48
DMSO
NCD38
NCD25
B
GFI1 mRNA [% GAPDH]
0.02
3.38 ***
4.07 ***
0.34
1.83 *
1.80 *
0.004
0.006
0.007
0.018
0.074
0.046
C
count
CD11b
MFI
DMSO 3.57
NCD38 33.0
NCD25 32.8
DMSO 13.8
NCD38 23.8
NCD25 23.9
DMSO 3.26
NCD38 3.52
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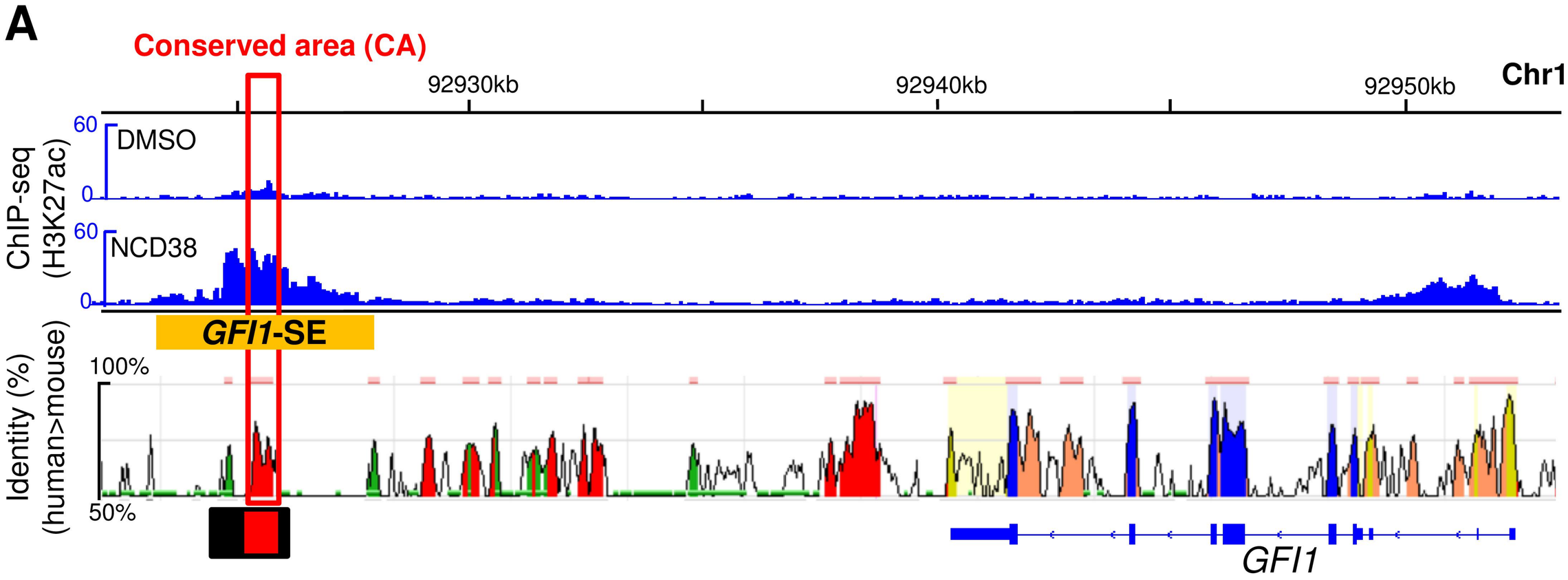
D
Fold change
day0
day2
day3

Fig. 2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92930kb
92940kb
92950kb
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ChIP-seq (H3K27ac)
60
0
DMSO
NCD38
GFI1-SE
Identity (%) (human>mouse)
100%
50%
GFI1

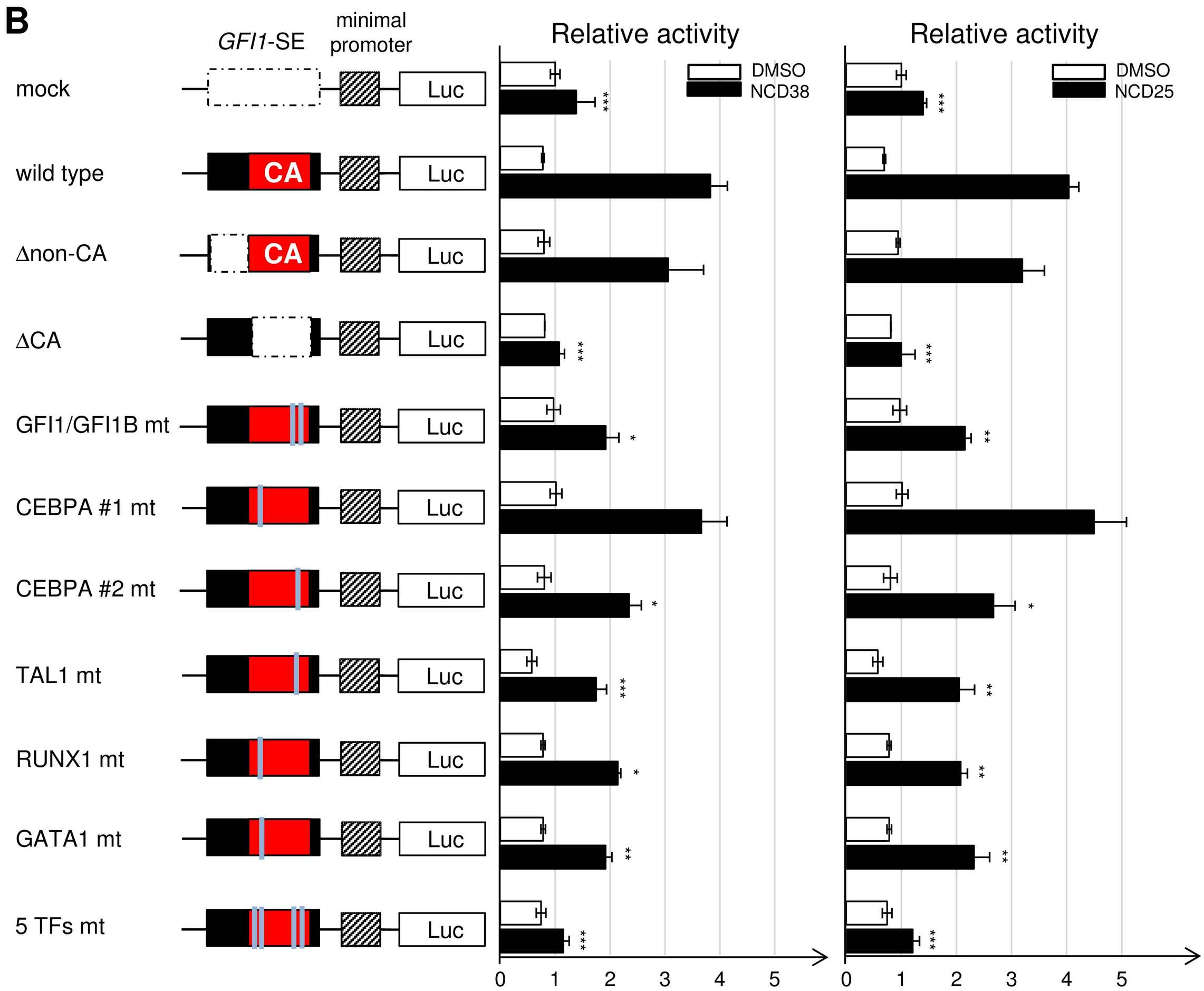
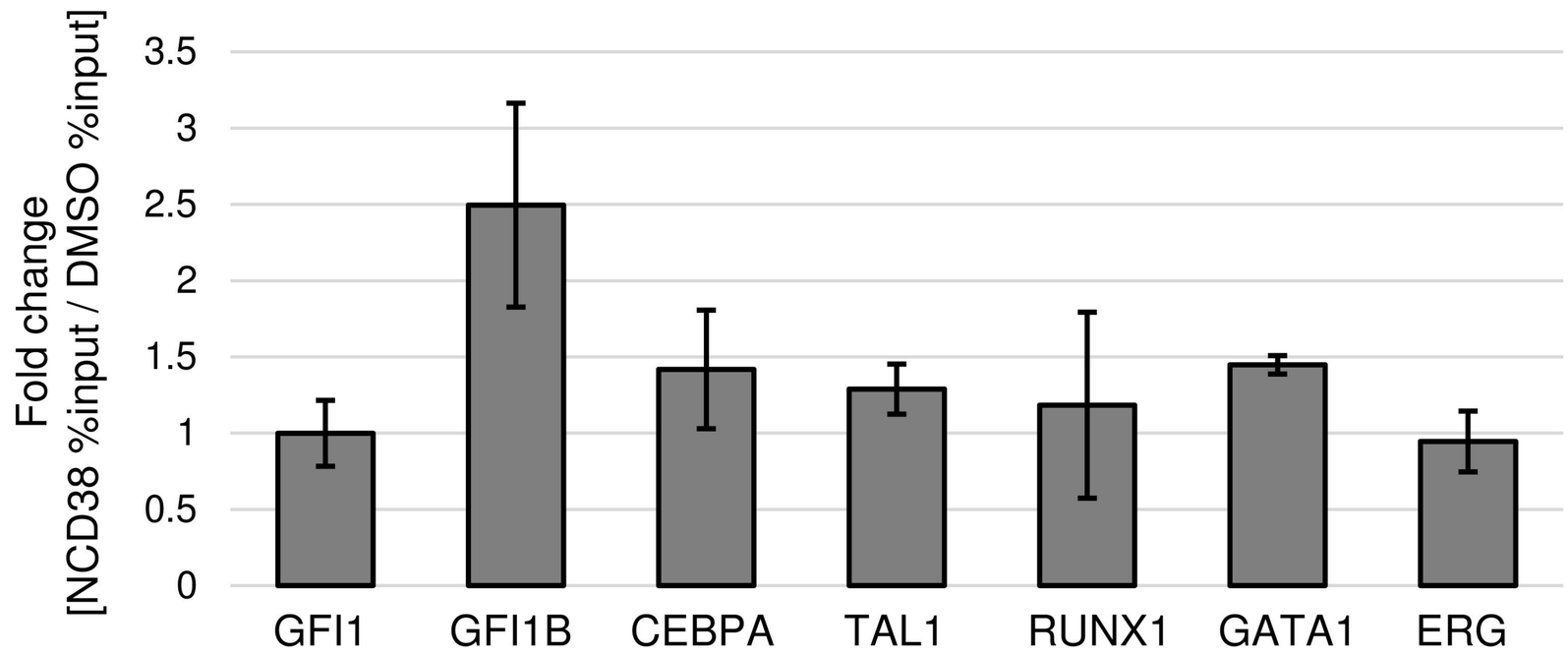

B
GFI1-SE
minimal promoter
Relative activity
DMSO
NCD38
NCD25
mock
wild type
Δnon-CA
ΔCA
GFI1/GFI1B mt
CEBPA #1 mt
CEBPA #2 mt
TAL1 mt
RUNX1 mt
GATA1 mt
5 TFs mt
CA
Luc
0
1
2
3
4
5

Fig. 3

A


Fold change
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3
2.5
2
1.5
1
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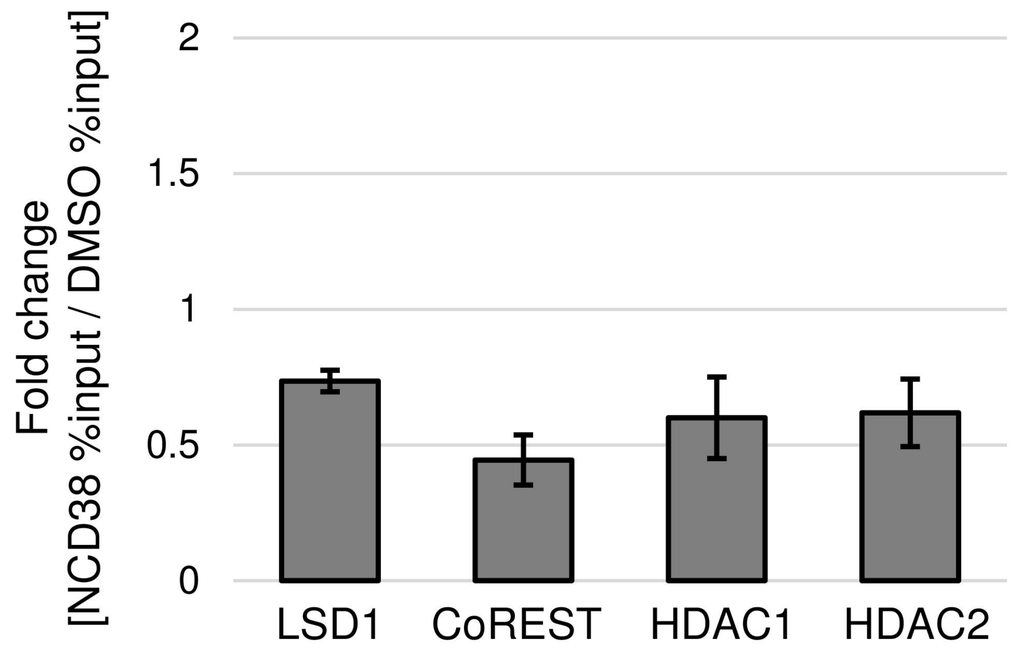
GFI1
GFI1B
CEBPA
TAL1
RUNX1
GATA1
ERG

B


Fold change
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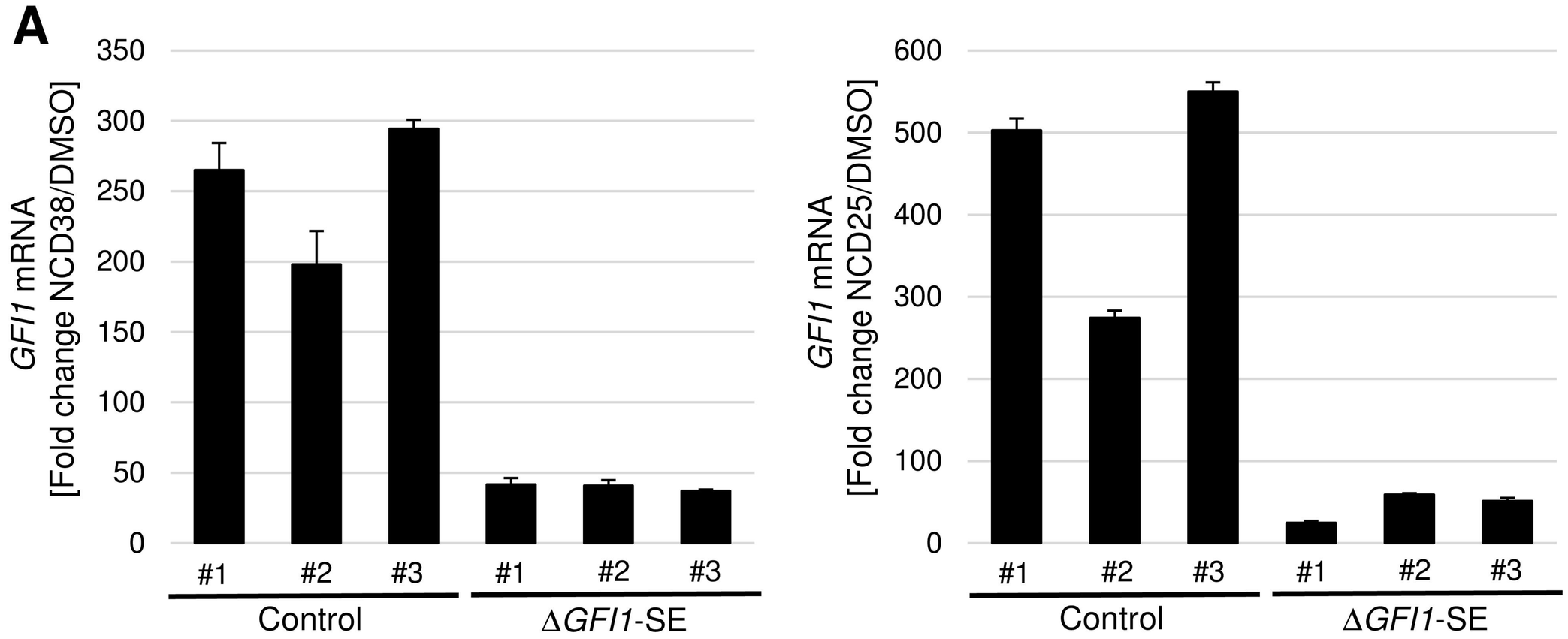
1.5
1
0.5
0
LSD1
CoREST
HDAC1
HDAC2

Fig. 4

A


GFI1 mRNA
[Fold change NCD38/DMSO]
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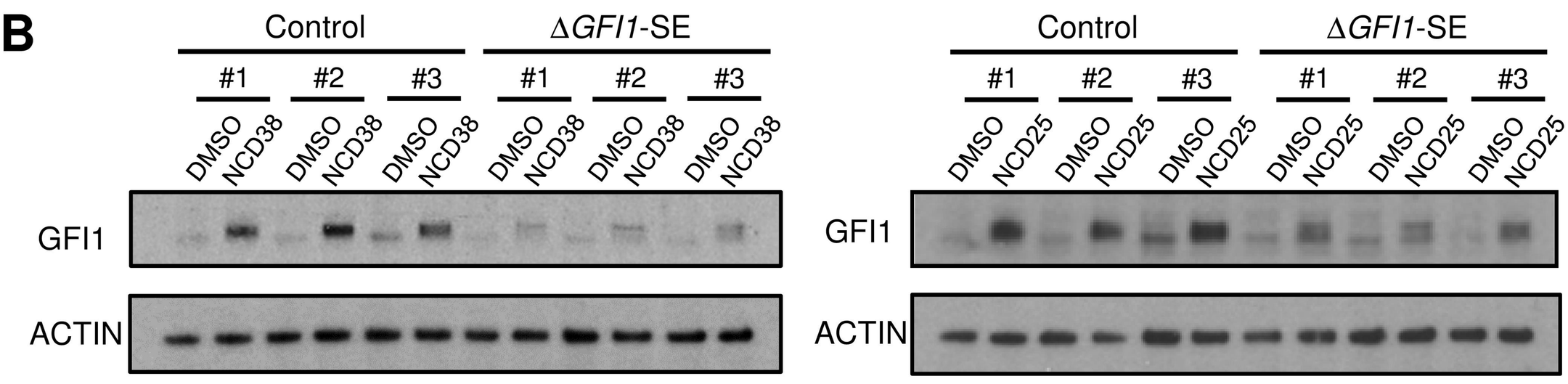
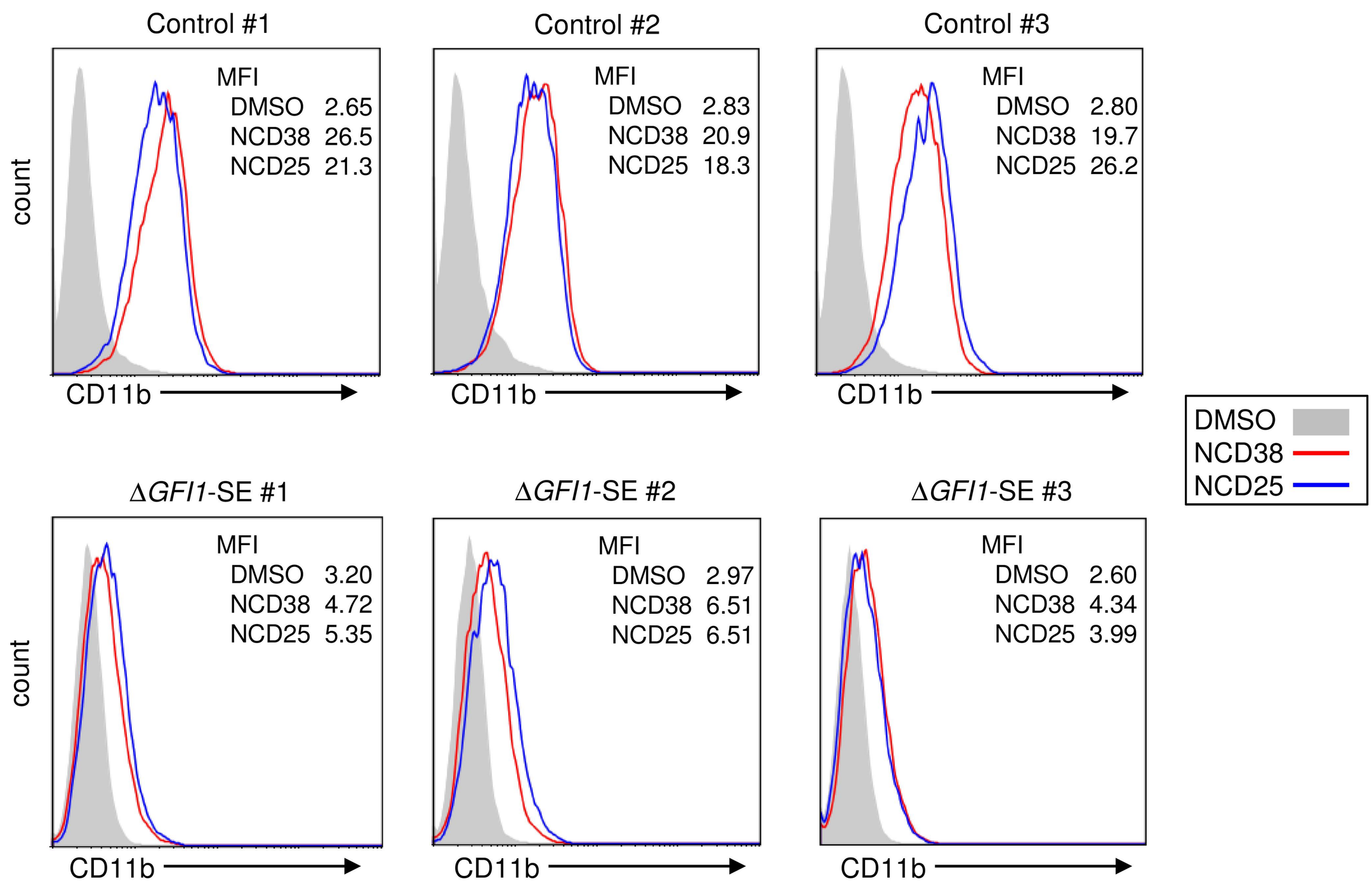
300
250
200
150
100
50
0
#1 #2 #3 #1 #2 #3
Control
ΔGFI1-SE
GFI1 mRNA
[Fold change NCD25/DMSO]
600
500
400
300
200
100
0
#1 #2 #3 #1 #2 #3
Control
ΔGFI1-SE

B


Control
ΔGFI1-SE
#1 #2 #3 #1 #2 #3
DMSO NCD38 DMSO NCD38 DMSO NCD38 DMSO NCD38 DMSO NCD38 DMSO NCD38
GFI1
ACTIN
Control
ΔGFI1-SE
#1 #2 #3 #1 #2 #3
DMSO NCD25 DMSO NCD25 DMSO NCD25 DMSO NCD25 DMSO NCD25 DMSO NCD25
GFI1
ACTIN

C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DMSO 2.65
NCD38 26.5
NCD25 21.3
count
CD11b
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DMSO 2.83
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CD11b
Control #3
MFI
DMSO 2.80
NCD38 19.7
NCD25 26.2
CD11b
DMSO
NCD38
NCD25
ΔGFI1-SE #1
MFI
DMSO 3.20
NCD38 4.72
NCD25 5.35
count
CD11b
ΔGFI1-SE #2
MFI
DMSO 2.97
NCD38 6.51
NCD25 6.51
CD11b
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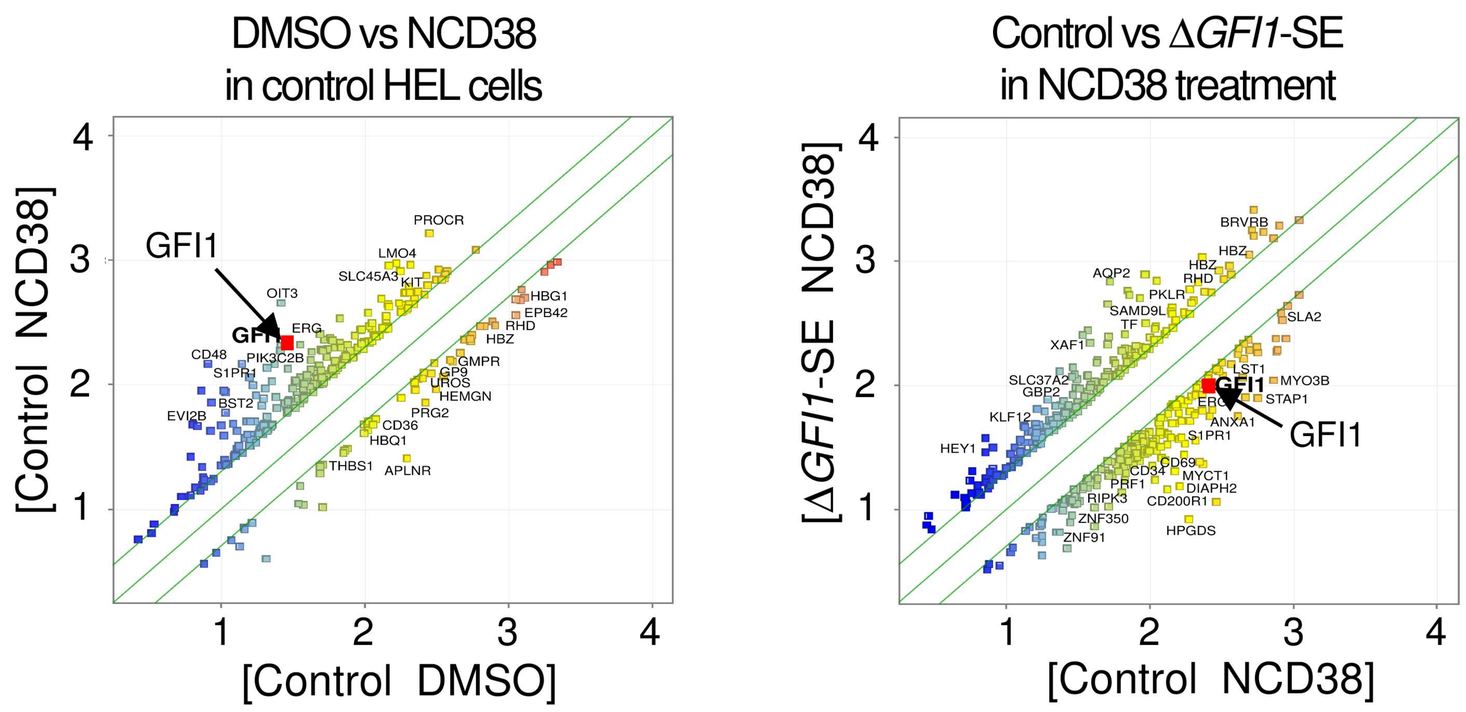
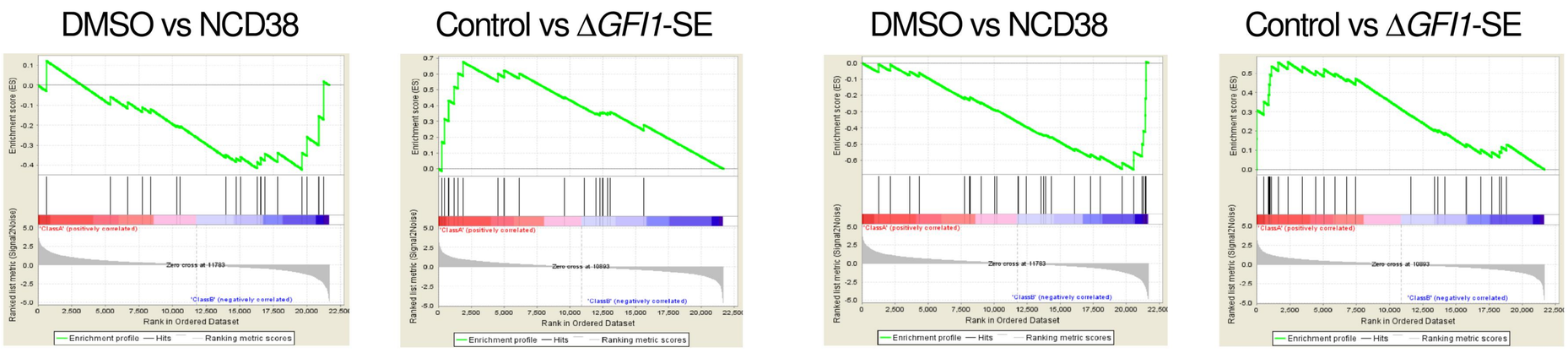
NCD38 4.34
NCD25 3.99
CD11b

Fig. 5

A

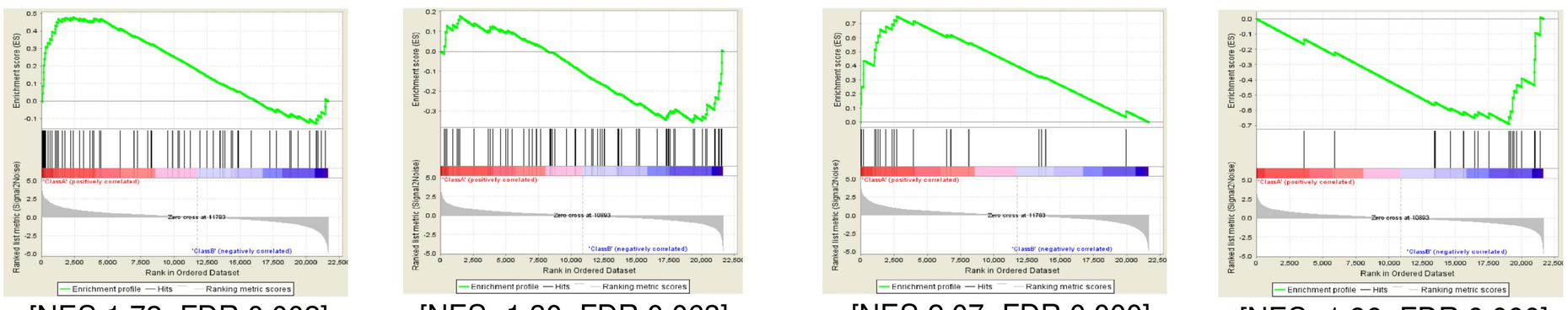


B



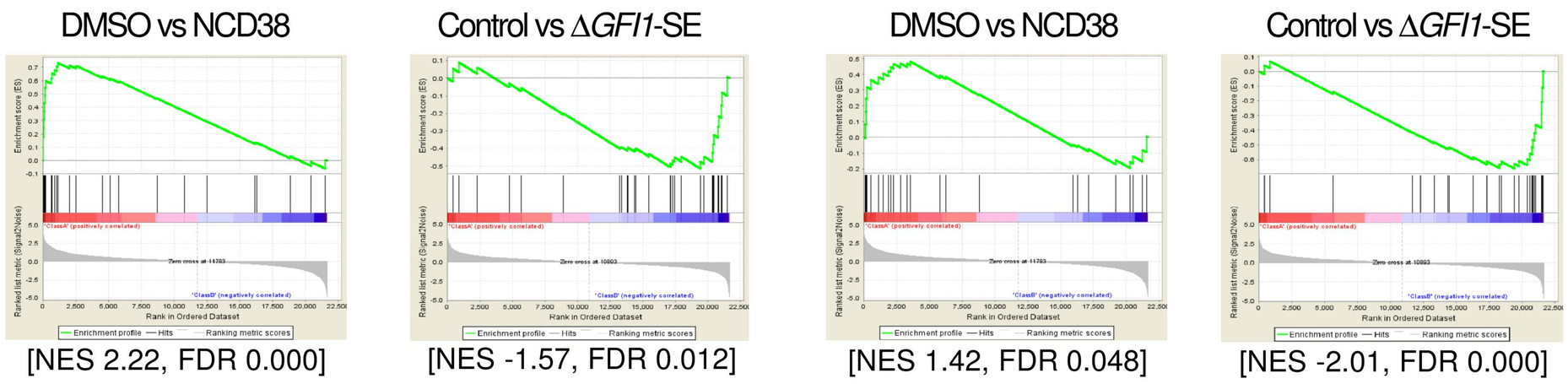
[NES -1.20, FDR 0.095] [NES 1.90, FDR 0.000] [NES -1.98, FDR 0.000] [NES 1.79, FDR 0.002]

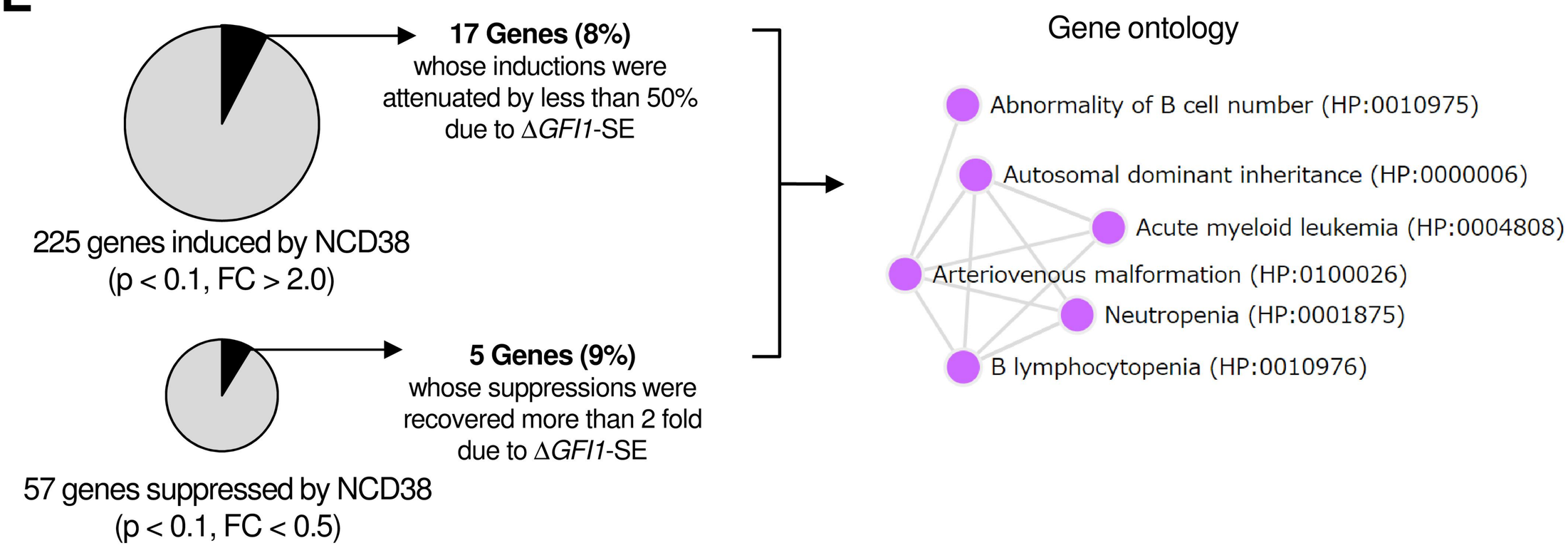
C



[NES 1.72, FDR 0.002] [NES -1.30, FDR 0.063] [NES 2.07, FDR 0.000] [NES -1.96, FDR 0.000]

D



[NES 2.22, FDR 0.000] [NES -1.57, FDR 0.012] [NES 1.42, FDR 0.048] [NES -2.01, FDR 0.000]

E