

The transcriptional corepressor CBFA2T3 inhibits all-trans retinoic acid-induced myeloid gene expression and differentiation in acute myeloid leukemia

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Running Title: *CBFA2T3 inhibits ATRA-mediated myeloid maturation.*

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ABSTRACT

CBFA2/RUNX1 partner transcriptional co-repressor 3 (CBFA2T3, also known as MTG16 or ETO2) is a myeloid translocation gene family protein that functions as a master transcriptional corepressor in hematopoiesis. Recently, it has been shown that CBFA2T3 maintains leukemia stem cell gene expression and promotes relapse in acute myeloid leukemia (AML). However, a role for CBFA2T3 in myeloid differentiation of AML has not been reported. Here, we show that CBFA2T3 represses retinoic acid receptor (RAR) target gene expression and inhibits all-trans retinoic acid (ATRA)-induced myeloid differentiation of AML cells. ChIP-Seq revealed that CBFA2T3 targets the RAR α /RXR α cistrome in U937 AML cells, predominantly at myeloid-specific enhancers associated with terminal differentiation. CRISPR/Cas9-mediated abrogation of CBFA2T3 resulted in spontaneous and ATRA-induced activation of myeloid-specific genes in a manner correlated with myeloid differentiation. Importantly, these effects were reversed by CBFA2T3 re-expression. Mechanistic studies showed that CBFA2T3 inhibits RAR target gene transcription by acting at an early step to regulate histone acetyltransferase recruitment, histone acetylation, and chromatin accessibility at RAR α target sites, independently of the downstream, RAR-mediated steps of transcription. Finally, we validated the inhibitory effect of CBFA2T3 on RAR in

multiple AML subtypes and patient samples. To our knowledge, this is the first study to show that CBFA2T3 down-regulation is both necessary and sufficient for enhancing ATRA-induced myeloid gene expression and differentiation of AML cells. Our findings suggest that CBFA2T3 can serve as a potential target for enhancing AML responsiveness to ATRA differentiation therapies.

Acute myeloid leukemia (AML) is a group of heterogeneous malignancies that arise from hematopoietic stem and progenitor cells (HSPCs) at various points in the hierarchy of myeloid development. A defining characteristic of AML is myeloid differentiation arrest, in which immature blasts suppress terminal maturation and thereby evade senescence and apoptosis. Under normal conditions, myeloid differentiation of HSPCs is facilitated by RAR α /RXR α -induced gene transcription, which occurs upon binding of RAR α to its ligand, all-trans retinoic acid (ATRA), the predominant endogenous metabolite of vitamin A. ATRA binding promotes the release of transcriptional co-repressors and the recruitment of transcriptional activators, resulting in activation of genes involved in terminal myeloid differentiation (1).

Deregulated retinoid signaling has been extensively studied in the context of t(15;17) AML (also known as acute promyelocytic leukemia, APL), which expresses a fusion protein between the promyelocytic leukemia protein (PML) and the retinoic acid receptor alpha (RAR α), known as

PML-RAR α . The PML-RAR α fusion protein is unresponsive to physiologic levels of ATRA and acts as a dominant inhibitor of retinoid signaling (2). A supraphysiological level of ATRA, however, is sufficient to activate PML-RAR α , which renders t(15;17) AML highly sensitive to ATRA differentiation therapy. Other subtypes of AML maintain resistance to both physiologic and therapeutic concentrations of ATRA, and the mechanisms they use to evade normal RAR signaling remain unclear. There are several reports that non-t(15;17) AMLs develop indirect means of repressing retinoid signaling and differentiation, such as loss-of-function mutations and transcriptional repression of key myeloid transcription factors, including CEBPA, RUNX1, and PU.1 (3,4). AML blasts can also up-regulate various cofactors and epigenetic enzymes that have been shown to directly or indirectly repress RAR α /RXR α transcriptional activation, including LSD1, MN1, and PRAME (5-8). These discoveries have led to the promising use of LSD1 and HDAC inhibitors, which can sensitize non-t(15;17) AML to ATRA-mediated differentiation and apoptosis (9-11).

CBFA2T3 (also known as ETO2, MTG16) is a hematopoietic corepressor that forms stoichiometric complexes with E-proteins (e.g. E2A, HEB) to facilitate exchange of coactivators including p300 and GCN5 histone acetyltransferases (HATs), with nuclear receptor corepressors (NCoR/SMRT) and HDACs (12,13). Underscoring its important role in hematopoietic transcriptional regulation, CBFA2T3 is frequently involved in leukemogenic translocations producing CBFA2T3 fusion proteins, including RUNX1-CBFA2T3 in therapy-related AML (14) and CBFA2T3-GLIS2 in pediatric AMLs (15). Wild-type CBFA2T3 plays important roles in both normal and malignant hematopoiesis, where it inhibits terminal erythro-megakaryocytic differentiation (16), maintains stemness of long-term hematopoietic stem cells (LT-HSCs) (17), and promotes the expansion of leukemia stem cells (LSCs) and AML relapse (18). Intriguingly, CBFA2T3-knockout in murine HSPCs promotes differentiation along the granulo-monocytic lineage at the expense of erythro-megakaryocytic development, phenocopying the effect of ATRA treatment (19,20).

We recently found that retinoic acid treatment rapidly downregulates CBFA2T3

expression in NB-4 t(15;17) APL cells (18). Based on these results, we hypothesized that there is an antagonistic crosstalk between CBFA2T3 and RAR-driven transcription. Here, we demonstrate that CBFA2T3 is causally involved in inhibiting ATRA-mediated myeloid gene expression and AML differentiation. We also show that CBFA2T3 function is linked to decreased chromatin accessibility at RAR α target genes via regulation of HAT recruitment and histone acetylation, independent of the downstream, RAR-involved steps of transcription. These results are supported by multiple complementary results at mechanistic, functional and biological levels. In particular, our loss-of-function and gain-of-function assays have demonstrated a causal role for CBFA2T3 in regulating ATRA/RAR signaling. Finally, we validated these results in multiple AML cell lines representing different AML subtypes, and in AML patient samples, suggesting that CBFA2T3 is a general inhibitor of ATRA/RAR-induced myeloid differentiation. Thus, CBFA2T3 may serve as a new therapeutic target to overcome ATRA resistance in AML.

RESULTS

CBFA2T3 targets the RAR/RXR cistrome.

Given the reported role of CBFA2T3 in inhibiting hematopoietic differentiation, along with the documented function of RAR in promoting myeloid differentiation, we hypothesized that CBFA2T3 inhibits RAR-dependent transcription. Because this predicts that CBFA2T3 should bind to RAR sites, we performed chromatin immunoprecipitation and sequencing (ChIP-Seq) assays and mapped the CBFA2T3 binding sites in U937 cells, an FAB M5 AML cell line minimally responsive to ATRA (21). CBFA2T3 ChIP-Seq was performed in both untreated (18) and CBFA2T3-over-expressed U937 cells to help identify the true binding sites of CBFA2T3. These binding sites were annotated to the nearest TSS (transcriptional start site) using HOMER (22). Next, to unbiasedly assess the function of the CBFA2T3-occupied genes, hypergeometric tests were performed to assess the enrichment of CBFA2T3-occupied genes across all known gene sets of the MSigDB database (Fig. S1). This analysis revealed highly significant enrichment of CBFA2T3 at (i) RAR α /PML-RAR α -bound genes, (ii) genes related to myeloid/leukocyte differentiation and activation,

and (iii) genes depleted in hematopoietic/leukemic stem cells (HSCs/LSCs) (Fig S1). Indeed, of the 22596 gene sets tested, the “MARTENS_BOUND_BY_PML_RARA_FUSION” gene set was found to be the most significantly enriched set for CBFA2T3 binding. Together, these results suggest that CBFA2T3 is capable of targeting the RAR α /PML-RAR α cistrome.

To further validate these results, we analyzed public ChIP-Seq datasets of both PML-RAR α /RXR α from PML-RAR α -transduced U937 cells (23), and endogenous RAR α from MV4-11 cells (an FAB M5 AML cell line) (24). Given that the above CBFA2T3 ChIP-Seq was performed in U937 cells not expressing PML-RAR α , we limited the following analyses to the shared binding sites between PML-RARA in U937 cells and RAR α in MV4-11 cells, thus excluding off-target peaks introduced by the PML moiety. Under these criteria, 21.1% and 23.5% of CBFA2T3 peaks contained RAR α sites in untreated and CBFA2T3-over-expressing U937 cells, respectively, and 63.6% of RAR α peaks were co-occupied by CBFA2T3 (Fig. 1A). Quantification of read densities from both experiments showed that ectopically-expressed CBFA2T3 specifically localized to the RAR cistrome in a distribution matching that of RAR α (Fig. 1B).

Finally, we visualized gene loci with high degrees of overlap between CBFA2T3 and RAR α (Fig. 1C). This revealed a number of myeloid-specific enhancers and promoters controlling the integrin CD11b (ITGAM), a marker of ATRA-mediated differentiation, PU.1 (SPI1), and CEBPA, master transcription factors required for terminal myeloid differentiation (25). Importantly, many of these regions have documented regulatory functions in myeloid differentiation, including the -14 kb SPI1 URE (upstream regulatory element), of which heterozygous deletion is sufficient to cause AML in mice (4), and the +34/+42 kb CEBPA enhancers, which drive neutrophil- and monocyte-specific CEBPA expression (26). These data were further integrated with H3K27ac ChIP-Seq, DNase HS-Seq, and RNA-Seq data from primary CD34+ HSCs and differentiated PBMCs, showing that these regulatory loci are conserved in multiple hematopoietic cell types and have higher transcriptional activity upon terminal myeloid differentiation (Fig. 1C). In summary, the results presented above support a functional role for

CBFA2T3 in regulating RAR α -dependent transcription.

CBFA2T3 abrogation de-represses myeloid-specific transcriptional program.

To further explore the functional involvement of CBFA2T3 in regulating RAR-mediated gene expression, we used CRISPR/Cas9-mediated gene editing to knock-out CBFA2T3. To increase editing frequency while facilitating screening of the edited clones, a dual gRNA strategy (27) was used to target exons 8 and 10, a region that encodes the NHR2 domain necessary for the corepressor function of MTG corepressors including CBFA2T3 (28-30). A U937 clone (U937 C) was identified to contain a single-nucleotide insertion in exon 8, resulting in a premature stop codon shortly downstream of the intron/exon junction (Fig. 2A). This led to the loss of detectable CBFA2T3 protein expression and significantly reduced CBFA2T3 mRNA expression due to nonsense-mediated decay (31) of CBFA2T3 transcripts (Figs. 2A, 2B and data not shown).

We assessed the global effect of CBFA2T3 loss on gene expression by performing whole-transcriptome Ampli-Seq assays in U937 WT and U937 C cells, treated with vehicle (DMSO) or ATRA (1 μ M) (Supplementary Table 2). Compared to U937 WT cells, U937 C cells expressed lower levels of CBFA2T3 expectedly, and higher levels of genes related to granulocyte-specific integrin signaling and activation, including *TYROBP*, *ITGAD*, and *CEACAM6* (32,33). In total, in the absence of ATRA, 75 genes were significantly upregulated whereas 54 genes were downregulated in U937 C cells.

Gene set enrichment analysis (GSEA) confirmed a genome-wide increase in the expression of CBFA2T3 target genes (Fig. 2C, top). The loss of CBFA2T3 also spontaneously increased the expression of integrin related genes, which are highly expressed in differentiated leukocytes (Fig. 2C, middle). Finally, and in agreement with the ChIP-Seq data, abrogation of CBFA2T3 also significantly upregulated ATRA-responsive genes (Fig. 2C, bottom). These results are consistent with an active role of CBFA2T3 in maintaining the normal repression of RAR target genes in U937 cells.

Next, we focused on a set of high-confidence ATRA-induced genes identified from U937 WT cells ($n=199$, FDR<.005), many of which

are bona fide ATRA-responsive genes, including *NCF1*, *CD38*, *CD11b/ITGAM* (Fig. 2D). Of these 199 genes, 82 (GROUP 1) were more highly expressed in U937 C cells compared to U937 WT cells, both with and without ATRA treatment (Fig. 2E). This shows that CBFA2T3 is required for maintaining the repression of these genes to below-basal levels.

Interestingly, a smaller subset of genes ($n=32$, GROUP 2) had a blunted response to ATRA in U937 C cells, prompting us to further characterize the functions of GROUP 1 and GROUP 2 genes. To this end, we compared gene sets enriched in each group, as well as in a control group of genes (OTHER, $n=81$) showing no differential response to ATRA between U937 WT and U937 C. This analysis revealed that GROUP 1 genes were enriched for genes related to myeloid/granulocytic differentiation and genes downregulated in normal and leukemic stem cells, which are known to express high levels of CBFA2T3 (18) (Fig. 2F). In contrast, GROUP 2 was enriched for genes that function as inhibitors of ATRA-mediated differentiation or antigens of LSCs, including *LYN*, *CD93*, and *CD36* (34-36). These GROUP 2 genes may not be directly regulated by CBFA2T3, but perhaps serve as a downstream, counter-regulatory response to promote resistance to myeloid differentiation.

Loss of CBFA2T3 sensitizes U937 cells to ATRA-induced myeloid differentiation.

We next sought to explore the biological effects of CBFA2T3 ablation on myeloid differentiation. Treatment of U937 C cells with 1 μ M ATRA decreased cell expansion in comparison to control U937 WT cells (Fig. 3A). Analyses of cell viability with MTT assays showed that U937 C cells had higher dose-dependent sensitivity to ATRA, with a nearly 3-fold reduction in ED₅₀ (Fig. 3B). Further analyses of cell morphology with Wright-Giemsa staining showed that, while U937 WT had minimal changes upon ATRA treatment as reported (21), U937 C displayed evidence of increased differentiation, as shown by decreased nuclear-to-cytoplasmic ratios, decreased basophilia, and loss of nucleoli in cytopsin cell preparations (Fig. 3C).

To test if the above effects were indeed due to the loss of CBFA2T3, we ectopically expressed CBFA2T3 in both U937 WT and U937 C cells. This not only increased CBFA2T3 protein expression in

U937 WT cells but also restored its expression in U937 C cells (Fig. 3D, top panel). Functionally, the re-expression of CBFA2T3 prevented the morphological changes seen in ATRA-treated U937 C cells (Fig. 3C) and reversed spontaneous and ATRA-induced activation of GROUP 1 genes (*ITGAM*, *CD68*, and *TYROBP*) in the U937 C cells (Fig. 3D). Ectopic CBFA2T3 also blunted the low-level ligand-dependent response of U937 WT cells (Fig. 3D). Together, these results support a causal role for CBFA2T3 in inhibiting ATRA responses of RAR target gene transcription.

CBFA2T3 function is linked to decreased chromatin accessibility independent of RAR α -involved steps of transcription.

Activation of transcription may occur through mechanisms dependent or independent of chromatin remodeling and accessibility. To provide a mechanistic insight into CBFA2T3 regulation of GROUP 1 genes, we focused on the CD11b gene (*ITGAM*) given its well-studied association with blast differentiation. We first assayed the effect of loss of CBFA2T3 expression on chromatin accessibility of the promoter and TSS regions of *ITGAM* by micrococcal nuclease (MNase) digestion assays (37). These regions include the -1 (UN1) and +1 (DN1) nucleosomes whose remodeling have been shown to be important for pre-initiation complex assembly and transcriptional activation (38) (Fig. 4B). We first noted that chromatin derived from U937 C cells was more sensitive to MNase digestion in comparison to U937 WT cells (Fig. 4A). This supports a possible global chromatin compaction effect of CBFA2T3. Next, MNase-qPCR assays were performed to determine site-specific accessibility at the -1/+1 nucleosomes (UN1/DN1) as well as the nucleosome-free region (NFR)/*ITGAM* TSS (Figs. 4B, 4C). In order to reveal gene-specific effects of CBFA2T3, we normalized the qPCR results to the house-keeping gene *RPL30*, whose expression was not affected by depletion of CBFA2T3 (data not shown), consistent with its lack of detectable binding by CBFA2T3 or RAR α (Fig. 4B). The MNase-qPCR results showed that at all assayed *ITGAM* TSS-proximal sites, the chromatin was more accessible in U937 C cells compared to U937 WT cells (Fig. 4C).

As a corepressor of E-proteins, CBFA2T3 has been shown to competitively prevent p300 and GCN5 recruitment (13,30,39). Analyses of ChIP-

Seq also showed that the E-protein HEB was present at the CBFA2T3-binding site at *ITGAM* NFR/TSS in multiple AML cells (GSM1122309 (40) and GSM585586 (41)). We thus asked if CBFA2T3 inhibits chromatin accessibility by inhibiting HAT recruitment and histone acetylation. ChIP-qPCR assays were performed to measure p300 and GCN5 recruitment and H3K27 and H3K9 acetylation, which are catalyzed by p300 and GCN5, respectively, to mark active, accessible chromatin. As shown in Fig. 4D, the loss of CBFA2T3 expression in U937 C cells strongly increased p300 and GCN5 recruitment to *ITGAM* NFR and DN1 with minimal effects on their levels at the *RPL30* site. Acetyl-H3K27 and acetyl-H3K9 were also increased at NFR, DN1 and, to a lesser extent, at UN1 in U937 C cells compared to U937 WT cells (Fig. 4D).

Since these results were obtained in the absence of exogenous RAR ligands, we hypothesized that CBFA2T3 regulated an early chromatin step prior to RAR α recruitment. This predicted that, by making chromatin accessible to RAR α , the loss of CBFA2T3 should render transcription susceptible to enhanced regulation by RAR α under both DMSO and ATRA conditions. To test this, we ectopically expressed RAR α in U937 WT and U937 C cells. Consistent with the prediction, ectopic RAR α in U937 C cells dramatically increased expression of CD11b upon ATRA treatment (Fig. 4E). Empty vector-transduced U937 WT cells did not respond to ATRA (Fig. 4E), consistent with the idea that the chromatin was inaccessible to RAR α binding. However, by increasing RAR α expression, ectopic RAR α in WT cells manifested a ligand-dependent gene transcription in magnitude comparable to that seen in the empty-vector-transduced U937 C cells (Fig. 4E). This shows that sufficiently high levels of RAR α (which mirrors PML-RAR α expression) may partially overcome the inaccessible state of CBFA2T3-bound RAR cistromic sites to augment transcriptional activation upon ATRA treatment. In U937 C cells, by increasing chromatin accessibility, loss of CBFA2T3 expression allows endogenous RAR α to bind and induce target gene activation in response to ATRA.

Notably, in the absence of ATRA, ectopic RAR α strongly reduced the high basal-level transcription seen in U937 C cells (Fig. 4E, DMSO, “VECTOR” vs. “RAR α ”). This shows that ectopic

RAR α can function both as a potent repressor and as a potent activator, in the absence of CBFA2T3, under DMSO and ATRA conditions, respectively, thus supporting the idea that CBFA2T3 is not directly involved in modulating RAR α 's transcriptional activities. This was further indicated by co-immunoprecipitation assays showing that CBFA2T3 was incapable of binding to RAR α in 293T cells overexpressing both proteins (Fig. 4F). In contrast, CBFA2T3 strongly bound to the HEB E-protein expectedly (13).

Taken together, these results support the idea that CBFA2T3 and RAR α function at separate, sequential steps in ATRA/RAR α -dependent gene transcription. CBFA2T3 inhibits chromatin accessibility to limit RAR α recruitment thereby preventing ATRA-induced, RAR α -mediated gene activation. Mechanistically, CBFA2T3 precludes p300 and GCN5 HAT recruitment and histone acetylation, consistent with its demonstrated corepressor function for E-proteins (13,30,39).

CBFA2T3 generally inhibits ATRA-mediated effects in various AML cell types.

To extend these findings to other AML cell types, we first examined HL-60 cells, an M2 AML that is relatively less mature than U937 cells (42). Depletion of CBFA2T3 using two independent shRNAs (Fig. 5A) inhibited cell growth in a manner synergistic with ATRA treatment (Fig. 5B). Although CBFA2T3 knockdown alone can inhibit HL-60 cell proliferation, it was insufficient to prevent long-term cell-expansion or induce maximal differentiation without concomitant ATRA treatment (Fig. 5C). Assessing the effects of loss- and gain-of-CBFA2T3 on basal and ATRA-induced expression of *ITGAM* showed that CBFA2T3 knockdown resulted in high-level, constitutive expression of CD11b in HL-60 cells (Fig. 5D). In overexpression assays, whereas ATRA can modestly induce *ITGAM* expression in a time-dependent manner, these effects were almost completely blocked by ectopic expression of CBFA2T3 (Fig. 5E). Finally, we provided evidence that depletion of CBFA2T3 promotes ATRA responses in multiple other AML cell lines, including SKNO-1, a model of M2, t(8;21) $^+$ AML, and THP-1, a model of M5 AML (Fig. 6A).

CBFA2T3 antagonizes RAR signaling in primary AML patients.

Finally, we sought to document the antagonistic effects between CBFA2T3 and RAR α in clinical AML samples. To this end, we examined two of the largest AML microarray studies, the GSE14468 AML patient cohort ($n=526$) (43) and the multicenter MILE cohort (GSE13159, $n=1066$) (44), both of which include patients spanning multiple cytogenetic categories, FAB morphology categories, and prognoses. As a surrogate for RAR α transcriptional activity, we computed a “gene set score” for each patient, representing the mean expression of all genes belonging to a particular gene set, z-scored across patients to prevent skewing towards highly-expressed genes (see Methods). Using this metric, we observed highly significant, negative correlations between CBFA2T3 expression and the “BROWN_MYELOID_CELL_DEVELOPMENT_UP” gene set (163 genes), as well as the “MARTENS_TRETINOIN_RESPONSE_UP” set (856 genes) (Fig. 6B).

Next, representing the “CHYLA_CBFA2T3_TARGETS_UP” gene set ($n=373$) (containing CBFA2T3 target genes upregulated in CBFA2T3-knockout murine HSPCs) as a third, fill variable revealed that patients with low CBFA2T3 levels and high RAR α target gene expression also expressed higher levels of CBFA2T3 target genes. This shows decreased functionality of CBFA2T3 (and not merely decreased CBFA2T3 expression) in these low-CBFA2T3 and high RAR α -activity patients. These data suggest a functional antagonism between CBFA2T3 and RAR/myeloid differentiation pathways, thus extending CBFA2T3/RAR α antagonism to diverse AML patient populations.

DISCUSSION

Together, these results demonstrate the novel finding that CBFA2T3 is a general inhibitor of the RAR-dependent myeloid differentiation program in AML. Our ChIP-Seq analyses reveal a strong, genome-wide overlap between CBFA2T3 and the RAR α cistrome in U937 cells, and our functional studies, encompassing both loss-of-function and gain-of-function assays, demonstrate a direct, repressive effect of CBFA2T3 at these genomic loci. Mechanistically, multiple lines of evidence support the idea that CBFA2T3 mediates its anti-ATRA/RAR α effects by acting at an early step to regulate HAT recruitment, histone acetylation and chromatin accessibility at RAR α

target sites. This restricts RAR α recruitment, thus inhibiting ATRA-dependent, RAR α -mediated target gene transcription. An RAR α -independent role for CBFA2T3 in antagonizing HAT recruitment and histone acetylation is well supported by its previously demonstrated corepressor function for E-proteins (13,39), which are part of the multi-protein pioneer transcription factor complex important for lineage specification in hematopoiesis (12,45). Thus, optimal binding of RAR α to its myeloid-specific target genes may require the assistance from these pioneer factors and their associated chromatin remodeling enzymes to increase chromatin accessibility. CBFA2T3 inhibits this step to preclude ATRA-induced, RAR α -mediated transcription. Adding further support to this idea, we found that ATRA-sensitive AML cells, including NB-4 and MV-4-11, and U937 cells overexpressing PML-RAR α , all had detectable levels of apo-RAR α binding at myeloid-specific genes such as *ITGAM*, whereas no such binding of endogenous apo-RAR α can be detected in ATRA-resistant AML cells such as wild type U937 cells (data not shown). Our Ampli-Seq studies showed that depletion of CBFA2T3 does not affect RAR α expression (data not shown). Most AML cells, including those belonging to minimally differentiated AML (M0/M1), also express a functional RAR α (46). Thus, it is possible that a pre-occupied, poised RAR α (or PML-RAR α) on chromatin sites is a major determinant of the ATRA sensitivity of AML cells. Finally, given that CBFA2T3 also binds to HDACs, a role for CBFA2T3 in early chromatin-dependent steps of RAR α target gene transcription also sheds new light on the synergism between HDAC inhibitors and ATRA in promoting AML differentiation (47).

We identified 82 genes (GROUP 1) that were more highly expressed in U937 C upon ATRA treatment. Most of these genes play roles in functionally mature granulocytes, and the enrichment analysis returned many neutrophilic gene ontologies related to phagocytosis, superoxide production, and chemotaxis. Notably, however, many GROUP 1 genes are specifically expressed in the monocytic lineage, such as *CD68*, *EMR1*, and *C2*, among others (48-50). These results align with the fact that while RAR α signaling has most commonly been associated with granulocytic differentiation, there is significant crosstalk with monocytic-macrophage differentiation, especially

in combination with vitamin D treatment (51). This bi-lineage gene expression may also represent a “promiscuous” phase of multi-lineage gene expression, which has been shown to immediately precede loss of multipotency and lineage commitment in hematopoietic progenitor cells (52).

Although not directly addressed in this study, a number of observations support the notion that ATRA sensitivity may be linked to changes in CBFA2T3 expression. For example, we previously found that NB-4 cells, which are highly susceptible to ATRA-mediated differentiation, rapidly terminate active CBFA2T3 transcription upon ATRA treatment (18). Additionally, CBFA2T3 levels are increased in relapsed AML, a phenomenon that is driven mostly by M4 and M5 subtypes of AML. M4/M5 blasts exhibit a high degree of myelomonocytic differentiation and relatively low CBFA2T3 expression, which may present a selective pressure, favoring the expansion of high-CBFA2T3 subclones that have a greater capacity to resist RAR α signaling and terminal differentiation.

Interestingly, GCN5 functions as an important driver of this post-remission upregulation of CBFA2T3 (18). Recently, GCN5 was also shown to promote ATRA resistance in multiple AML subtypes (53). Therefore, the degree to which this depends on CBFA2T3 up-regulation is an important direction of future studies. Although HDAC inhibitors have been shown to promote ATRA sensitivity in vitro, their off-target toxicities have limited their clinical efficacy (54). CBFA2T3 may therefore serve as a new target to allow specific induction of the RAR-driven myeloid differentiation pathway. Conceivably, this may be achieved by combining different approaches, for example, by inhibiting GCN5 to down-regulate CBFA2T3 expression, by preventing CBFA2T3 interactions with transcription factors such as E-proteins (39), or by preventing CBFA2T3 oligomerization (15). These methods offer the hope of extending the success of ATRA differentiation therapy for t(15;17) AML to a majority of AML patients.

EXPERIMENTAL PROCEDURES

Cell culture, chemicals, plasmids, primers, and antibodies. AML cells were maintained in RPMI 1640 with 10% fetal bovine serum. Kasumi-1, U937, SKNO-1, and THP-1 cells have been previously described (18), and the HL-60 cell line

was a gift from Herman Sintim (55). Lentiviral particles were produced in HEK293T cells as previously described (39). All-trans retinoic acid (ATRA) and validated shRNAs targeting CBFA2T3 were obtained from Millipore Sigma (St. Louis, MO, USA). For CBFA2T3 over-expression experiments, cDNA of the full-length, NM_005187 isoform was ordered from Dharmacon (Clone ID: 5227349, Lafayette, CO, USA) and cloned into the pCDH-MSCV-EF1 α -Puro lentivector (System Biosciences, Palo Alto, CA). For RAR α over-expression experiments, FLAG-RAR α (pcDNA-FLAG-RAR α , Addgene plasmid # 35555) was cloned into the pCDH-MSCV-EF1 α -Puro lentivector. All vectors were Sanger sequenced to verify correct inserts, and stably-transduced clones were selected for 48 hours following infection with 2 μ g/ μ L puromycin. CBFA2T3 protein was probed with an in-house antibody raised against the NHR1 domain. β -actin (A2228, Millipore Sigma) or TBP (AB28175, Abcam, Cambridge, MA) were used as loading controls. All shRNA, gRNA, and qPCR primer sequences are listed in Supplementary Table 1.

CRISPR-Cas9 deletion of CBFA2T3. gRNA sequences targeting exon 8 and 10 of CBFA2T3 (NM_005187) were obtained from Gene Script (Piscataway, NJ, USA), cloned into the dual-cassette pKLV2.2-h7SKgRNA5-hU6gRNA5-PGKpuro-W (56) lentivector (Addgene #72666), and transduced into U937 cells stably expressing Cas9 from the pKLV2-EF1 α Bsd2ACas9-W lentivector (Addgene #67978). Following puromycin selection, cells were diluted to 48 cells/10 mL in RPMI 1640 diluted 1:2 with 45 μ m-filtered media collected from the bulk U937 population and seeded in 100 μ L wells. Wells receiving a single cell were marked the following day and grown up to clonal populations. Clones were assessed for CBFA2T3 protein expression, and knock-out clones were subject to PCR amplification of the targeted regions for sequencing. Control U937 WT cells were obtained in an identical process but initially transduced with the empty lentivector lacking gRNAs. FLAG-tagged HEB plasmid has been previously described (30).

Gene expression, pathway, and patient analyses. Reverse transcription-quantitative PCR (RT-qPCR) was performed as previously described (39). 18S

ribosomal RNA was used for normalization control. For all RT-qPCR results in figures, experiments were performed in triplicate, and bars represent mean values while error bars represent standard deviation. Whole-genome AmpliSeq analysis was performed on duplicate samples using Ion AmpliSeq Kit for Chef DL8 and Ion 540 Kit-Chef (Thermo Fisher Scientific, Waltham, MA, USA), and edgeR (57) was used to assess differential expression. Gene set enrichment analyses (58) was performed using fgsea (59) against MSigDB 30 C2 V5.1 gene sets. P-values were empirically derived using 100 000 permutations. AML patient microarray studies, GSE14468 (43) and GSE13159 (44), were downloaded from GEO using GEOquery (60) and analyzed in R. To calculate “gene set scores” for each patient, we averaged the z-score (relative to all patients) of every gene in the gene set, i.e.,

$$G.S.(P) = \frac{\sum_{i=1}^n (P_i - \bar{P})/\sigma}{n},$$

where σ represents standard deviation, P_i represents the raw expression value of gene i in an individual patient, \bar{P} represents the average expression of gene i across all patients, and n represents the number of genes in a given gene set. Ontological enrichment of genes was performed with the clusterProfiler package (61).

Chromatin immunoprecipitation sequencing (ChIP-Seq) and analysis. ChIP-Seq of over-expressed CBFA2T3 in U937 cells was performed as previously described, and ChIP-Seq data from endogenous CBFA2T3 is available at GSE126953 (18). Additional ChIP data for PML-RAR α /RAR α in U937 and MV4-11 cells were downloaded from SRA studies SRP001549 (23) and SRP103029 (24), respectively. Raw FASTQ files were aligned to hg19 with STAR (62) and peak calling, peak overlaps, motif enrichment, and tag count distributions were assessed using HOMER (22). CBFA2T3 peak ontology enrichment was performed with the chipenrich package (63). CBFA2T3 antibody (SC-9741) was obtained from (Santa Cruz Biotechnology, Dallas, TX, USA). Sequencing was performed at the Genome Technology Access Center at Washington University in St. Louis, MO. Peaks were visualized in IGV, and additional H3K27ac, DNase, and RNA-Seq signal tracks were imported directly from the DeepBlue epigenomic data server (64).

Chromatin accessibility and ChIP-qPCR assays. MNase digestion and qPCR assays were performed as previously described (37). Briefly, we crosslinked chromatin in U937 cells with 4% paraformaldehyde and lysed the cells using the SimpleChip Enzymatic Chromatin IP Kit (Cell Signaling Technology, Danvers, MA, USA). Nuclei was further lysed in hypotonic lysis buffer (20 mM HEPES (pH 7.5), 0.25 M sucrose, 3 mM MgCl₂, 0.2% NP-40, 3 mM 2-mercaptoethanol), and extracted chromatin was subject to MNase digestion for 20 min at 37° C with varying concentrations. Following reverse cross-linking, DNA was extracted by Phenol:Chloroform:Isoamyl Alcohol 25:24:1. qPCR was used to quantify specific sequences (UN1/NFR/DN1) and the RPL30 exon 3 locus was used as a normalization control. ChIP-qPCR was performed using the SimpleChip Enzymatic Chromatin IP Kit as previously described (18) GCN5 and p300 antibodies were from Santa Cruz (SC-20698 and SC-584, respectively) H3K9ac and H3K27ac antibodies were from Abcam (ab32129 and ab4729, respectively).

Assessment of ATRA sensitivity. Dose-response sensitivity to ATRA was assessed by seeding cells in duplicate 100 μ L wells at an initial concentration of 60 000 cells/mL, with increasing concentrations of ATRA (0, .0008, .004, .02, .1, .5, 2.5 μ M). Cell viability was assessed with the MTT assay 5 days later according to manufacturer’s instructions (Promega, Madison, WI, USA). Log-logistic models were fit with the drc package (65). To assess cell morphology, cells were washed with PBS and diluted to a concentration of 5E5 cells/mL, and cytopsins were prepared using a Thermo Shandon Cytospin 4 at 500 rpm for 5 minutes. Slides were fixed in methanol for 1 minute and stained with Wright-Giemsa (WS16, Millipore Sigma) according to manufacturer’s instructions. Slides were imaged on a Leica DM6 B bright-field microscope. For cell expansion assays, cells were seeded in duplicate at 10E4 cells/mL in 200 μ L of RPMI 1640 with 1 μ M ATRA and counted with a hemocytometer. Fresh media with ATRA was added every other day to maintain a constant volume.

Coimmunoprecipitation assay. 293T cells were transfected using TurboFect Transfection Reagent (Thermo Fisher Scientific). Cells were lysed in lysis

buffer (20 mM HEPES, pH 7.9, 1 mM EDTA, 20% glycerol, and protease inhibitors) containing 400 mM NaCl and 0.5% NP-40. Cell extracts were immunoprecipitated with CBFA2T3 antibody (SC-9741). Following washing with lysis buffer, bound proteins were subjected to Western blot analysis.

Input lanes show 1.5 % of the total. Anti-FLAG M2 antibody was from Sigma. HEB antibody was from Santa Cruz (SC-357).

Data Availability. The ChIP-Seq results from CBFA2T3-transduced U937 cells have been deposited to NIH GEO under GSE126953.

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Author contributions

NS, CG, and JZ performed experiments, collected the data, and analyzed the results. JZ and NS developed the original idea. NS wrote the first draft. NS and JZ wrote and finalized the manuscript with assistance from CG. All authors have read and approved the final submission.

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Conflict of Interests

The authors have no competing interests to declare.

Abbreviations

CBFA2T3, core-binding factor, runt domain, alpha subunit 2, translocated to, 3, also known as MTG16 or ETO2; AML, acute myeloid leukemia; PML, promyelocytic leukemia; ATRA, all-trans retinoic acid; RAR, retinoic acid receptor; HSPCs, hematopoietic stem and progenitor cells; LSCs, leukemia stem cells; TSS, transcriptional start site, ITGAM, integrin subunit alpha M, also known as CD11b; HAT, histone acetyltransferase; GCN5, general control non-depressible; p300, E1A binding protein p300.

Figure Legends

Figure 1. CBFA2T3 is highly enriched at genomic loci occupied by RAR α . A. Peak overlap between CBFA2T3 peaks (endogenous and exogenously expressed) and RAR α cistrome in U937 cells. Significance of overlap was assessed with hypergeometric tests. B. Read coverage from α -CBFA2T3 ChIP-Seq in U937 cells within the RAR α cistrome. Background regions were randomly selected with HOMER to match GC-content of RAR α cistromic sites. C. IGV tracks depicting CBFA2T3 and RAR α binding in U937 and MV4-11 cells. α -H3K27ac ChIP-Seq, DNase-Seq and RNA-Seq data from human CD34+ HSPCs (immature) and PBMCs (differentiated) were visualized to demarcate active enhancer regions related to myeloid differentiation. Tracks were normalized by [1,000,000 / (totalReadCount)], and all tracks corresponding to the same ChIP or cell type (HSPCs vs. PBMCs) were set to the same scaling factor.

Figure 2. CBFA2T3 deletion enhances transcriptional response to ATRA in U937 cells. A. CBFA2T3 deletion strategy depicting targeted Sanger sequencing of clone C, and western blot demonstrating loss of CBFA2T3 protein in U937 C cells. B. Volcano plot depicting genes with significantly altered expression (red) between U937 C and U937 WT. Differential expression testing was performed with QL F-tests in edgeR. Ampli-Seq was performed in duplicate for each biological sample. C. Gene set enrichment analysis (GSEA) shows significant enrichment of CBFA2T3 target genes, integrin pathway genes, and ATRA-response genes among the genes up-regulated in U937 C. P values were empirically derived with permutation testing. D. Heatmap of GROUP 1 and GROUP 2 genes among four sample groups (U937 WT/C +/- ATRA), scaled across samples (rows). Among the genes that were highly/significantly upregulated in U937 WT upon ATRA treatment ($FDR < .005$, $n=199$), GROUP 1 genes were defined as those having higher expression in U937 C upon ATRA treatment ($U937\text{C}/WT > 1.2$, $n=82$) and GROUP 2 as those with lower expression ($U937\text{C}/WT < .8$, $n=36$). E. Normalized expression (TPM) of GROUP 1 and GROUP 2 genes shows that GROUP 1 genes are more highly expressed in U937 C under basal conditions (DMSO). Significance assessed with the Mann-Whitney U test. F. Enrichment of gene sets within GROUP 1 and GROUP 2 gene categories assessed with the hypergeometric distribution and p values adjusted using the Benjamini-Hochberg method. Ratio represents the fraction of GROUP 1/2 genes also belonging to a given gene set. Significant results ($FDR < .05$) are labeled with Q value.

Figure 3. U937 C cells exhibit increased sensitivity to ATRA-mediated differentiation. A. U937 WT and U937 C cells were treated with 1 μM ATRA over 5 days and cell counts were recorded after trypan-blue staining ($n=2/\text{group}$). Generalized linear models were fit to assess cell count as a function of treatment and timepoint, and Wald tests were used to determine the significance of ATRA treatment as a predictive variable. B. The MTT assay was used to assess viable cell counts ($n=2$) after five days of ATRA treatment over a range of concentrations. A four-parameter log-logistic model was used to fit curves and estimate ED₅₀ concentrations. P-value represents the significance of cell line (WT/C) as model variable obtained from the Wald test. C. Wright-Giemsa stains of U937 cells transduced with pCDH-MSCV-CBFA2T3 or an empty vector control and treated for 5 days with 1 μM ATRA. Slides were imaged at 100X magnification. D. Upper left, western blot analysis showing CBFA2T3 protein expression in empty vector and pCDH-MSCV-CBFA2T3 transduced U937 WT and U937 C cells, using TBP as a loading control. Upper right and Bottom, RT-qPCR was performed to measure CBFA2T3, ITGAM, CD68, and TYROBP gene expression after a 48 hours treatment with 1 μM ATRA in U937 WT/C cells +/- pCDH-MSCV-CBFA2T3 ($n=3$). In the western blot analysis, ectopic CBFA2T3 was expressed at lower levels in U937 C cells than its levels in U937 WT cells. This may conceivably be due to possible processing of CBFA2T3 transcripts by CAS9/sgRNA2 in the U937 C cells. In RT-qPCR assays, the primers used to detect CBFA2T3 expression were complementary to a 5' region of the CBFA2T3 transcript, which may detect both unprocessed and processed CBFA2T3 transcripts, explaining why the result did not show reduced expression of the ectopic CBFA2T3 transcripts in the U937 C cells. Student's t-test was used to assess significance ("*" = $<.05$, "****" = $<.01$, "*****" = $<.001$, "*****" = $<.0001$).

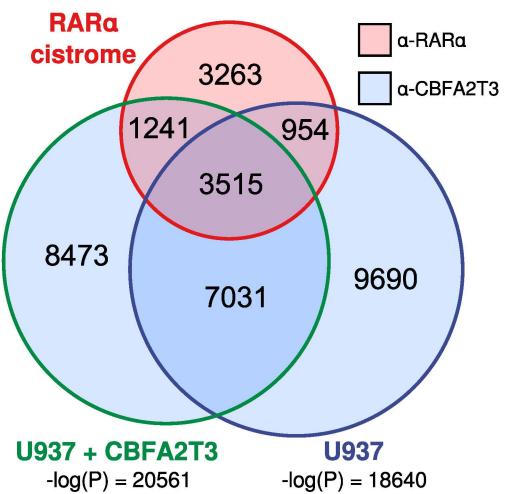
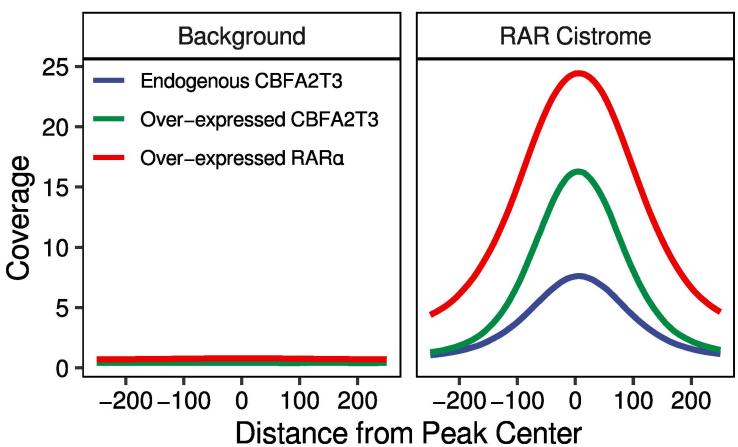
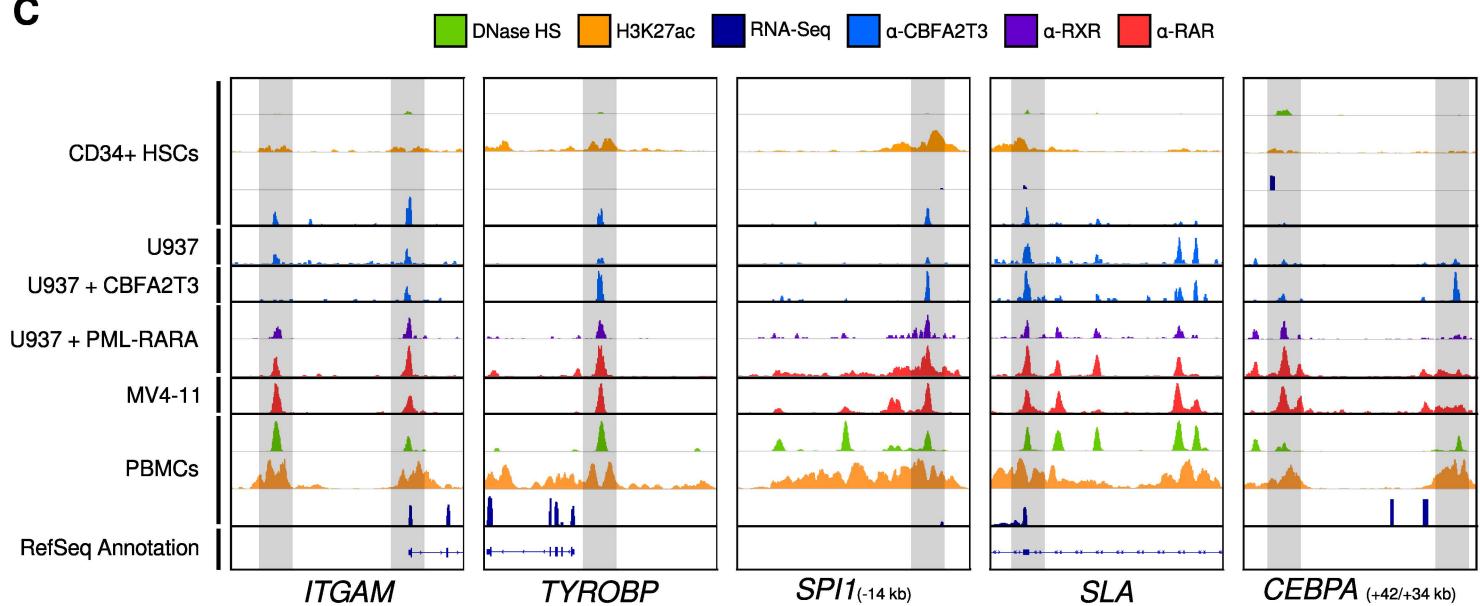
Figure 4. Loss of CBFA2T3 promotes increased accessibility at the CD11b (*ITGAM*) locus.

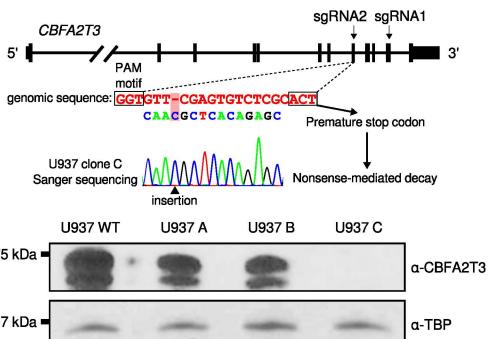
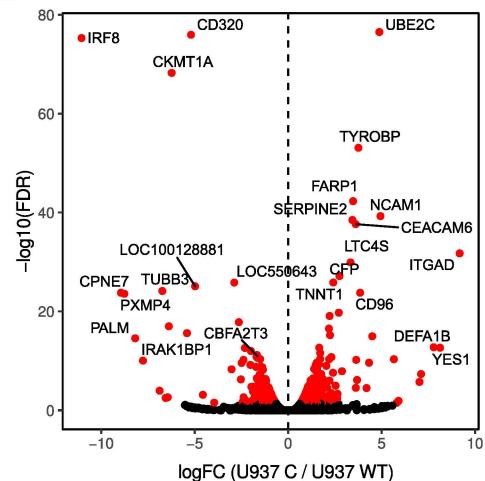
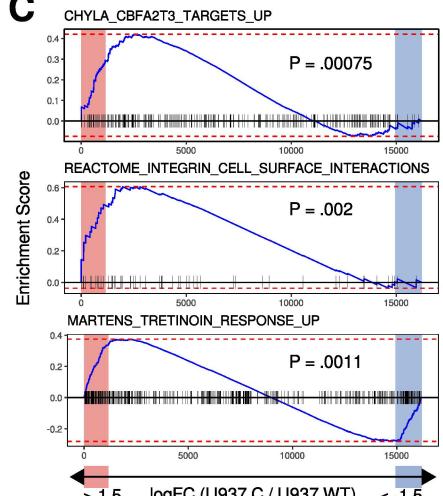
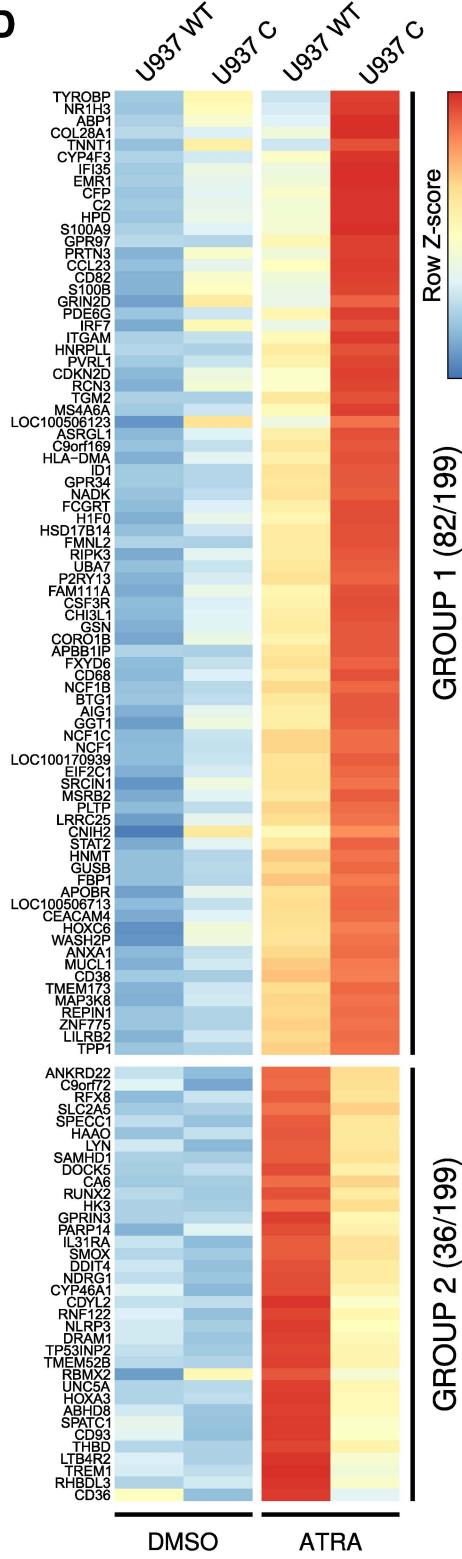
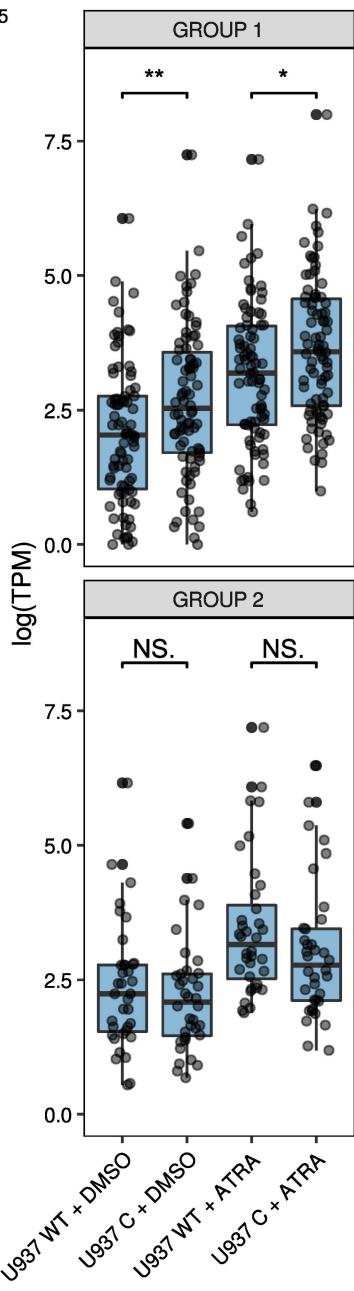
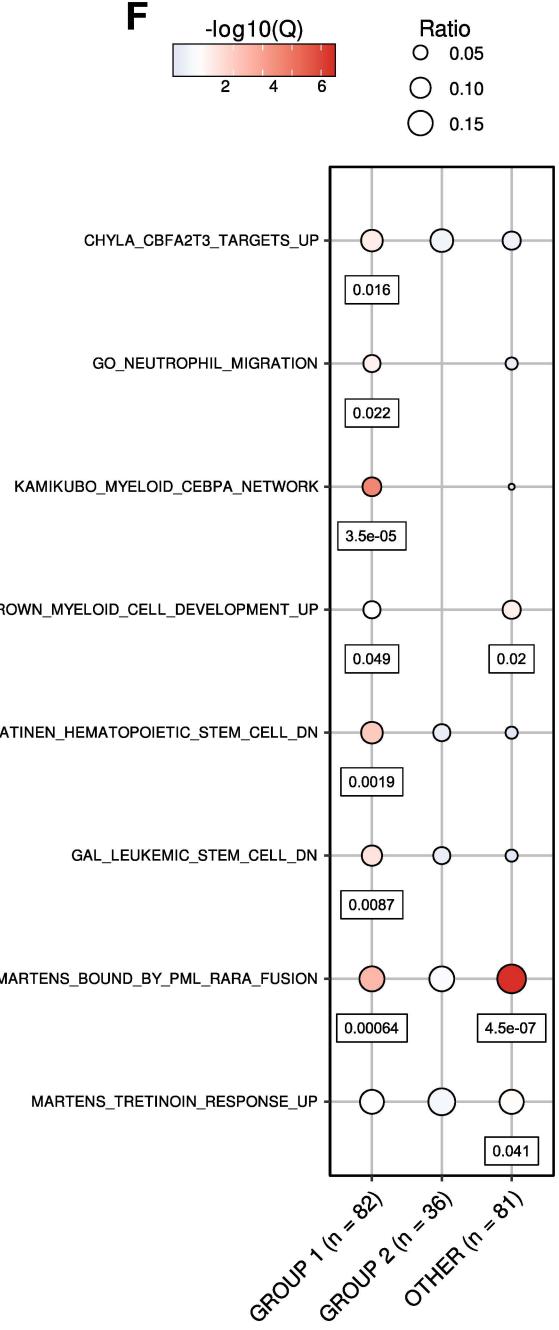
A. Chromatin isolated from U937 WT or U937 C was subject to digestion with increasing concentrations of MNase, and nucleosomal-length DNA distribution was visualized with gel electrophoresis. B. IGV tracks (from Figure 1) showing the specific sites analyzed in our MNase- and ChIP-qPCR experiments. CBFA2T3 and RAR α bind strongly to this region, and higher CBFA2T3 binding (indicated in CD34+ HSCs, which express high levels of CBFA2T3) is associated with lower DNase and H3K27ac signals. The RPL30 site (right) shows a lack of CBFA2T3/RAR α binding. The cell-type and track annotations apply to both *ITGAM* and *RPL30* gene loci. C. Relative (digested/undigested) nucleosomal protection of *ITGAM* sites was quantified via qPCR with specific primers (UN1,NFR,DN1) to ensure a predominantly mononucleosomal distribution. Experiment was performed in technical triplicates and normalized to primers targeting a *RPL30* nucleosomal site. D. ChIP-qPCR shows increased association of p300 and GCN5 at the NFR and DN1 sites, along with corresponding increases in H3K27 and H3K9 acetylation (H3K27ac/H3K9ac), in U937 C cells under basal conditions. Experiment was performed in technical triplicates and a non-specific IgG antibody was used as a negative control for IP efficiency. E. U937 WT and U937 C were transduced with pCDH-MSCV-FLAG-RARA or an empty vector control and treated with 1 μ M ATRA for 24 hours. RT-qPCR was performed to measure *ITGAM* expression. F. 293T cells were co-transduced with CBFA2T3, FLAG-HEB, and FLAG-RAR α , and cell lysates were immunoprecipitated with the α -CBFA2T3 antibody. FLAG-RAR α was detected by anti-FLAG M2 antibody. FLAG-HEB was detected by an anti-HEB antibody.

Figure 5. CBFA2T3 inhibits ATRA response in more immature HL-60 AML. A. CBFA2T3 mRNA expression in HL-60 cells 72 hours after transduction with SCR (scrambled shRNA control) or CBFA2T3-specific shRNAs (#164, or #165). Whole cell lysates for immunoblotting were similarly collected 72 hours after transduction. B. Transduced HL-60 cells were grown with 1 μ M ATRA until reaching confluence, and counts were recorded after trypan-blue staining (n=2/group). Wald test used to assess treatment effect on cell expansion. C. Wright-Giemsa stains of HL-60 cells transduced with shCBFA2T3#164 or SCR and treated for 5 days with 1 μ M ATRA. Slides were imaged at 100X magnification. D. RT-qPCR was performed to measure *ITGAM* gene expression after a 48 hours treatment with 1 μ M ATRA (n=3). Student's t-test was used to assess significance ("*" = <.05, "**" = <.01, "***" = <.001). E. Stably-transduced HL-60 cells were treated with DMSO or ATRA for 2 and 4 days (fresh ATRA/media added daily) and *ITGAM* expression was measured (n=3) via RT-qPCR.

Figure 6. CBFA2T3 suppresses RAR α function in multiple AML cell lines and across AML patients.

A. One week following transduction, SKNO.1, U937, and THP-1 cells were grown with 1 μ M ATRA until reaching confluence, and counts were recorded after trypan-blue staining (n=2/group). Wald test was used to assess treatment effect on cell expansion. B. Pearson correlation among AML patients between CBFA2T3 expression and "gene set scores" (see Methods). High CBFA2T3 levels are associated with low expression of "BROWN_MYELOID_DEVELOPMENT_UP" (genes increasing with myeloid differentiation) and "MARTENS_TRETINOIN_RESPONSE_UP" (genes up-regulated in ATRA-treated NB-4 cells) in GSE14468 (n=526) and GSE13159 (n=1066). Fill of each point represents patient's "CHYLA_CBFA2T3_TARGETS_UP" score (genes that are increased in CBFA2T3 $^{+/-}$ murine HSPCs).

A**B****C****Fig. 1**

A**B****C****D****E****F****Fig. 2**

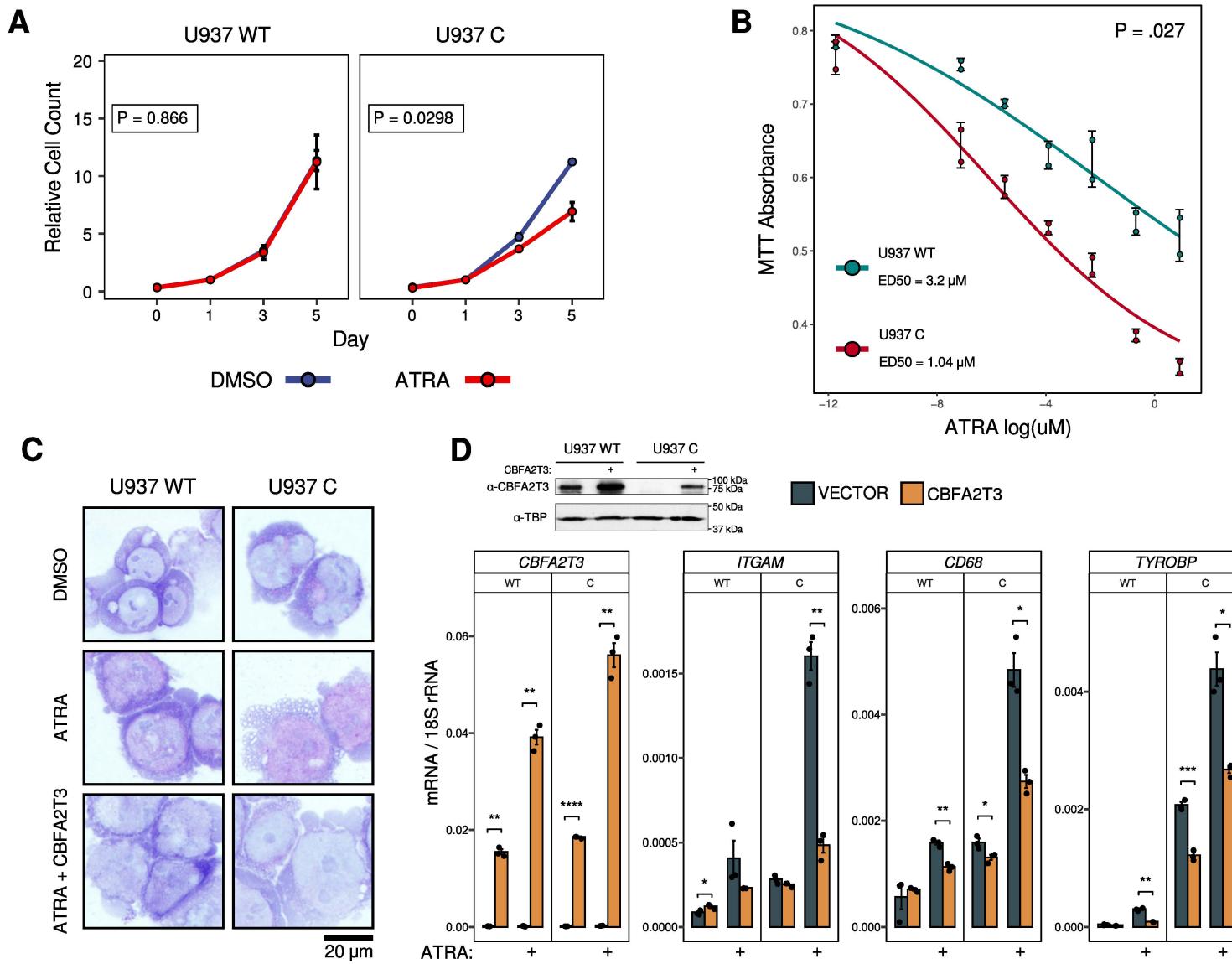


Fig. 3

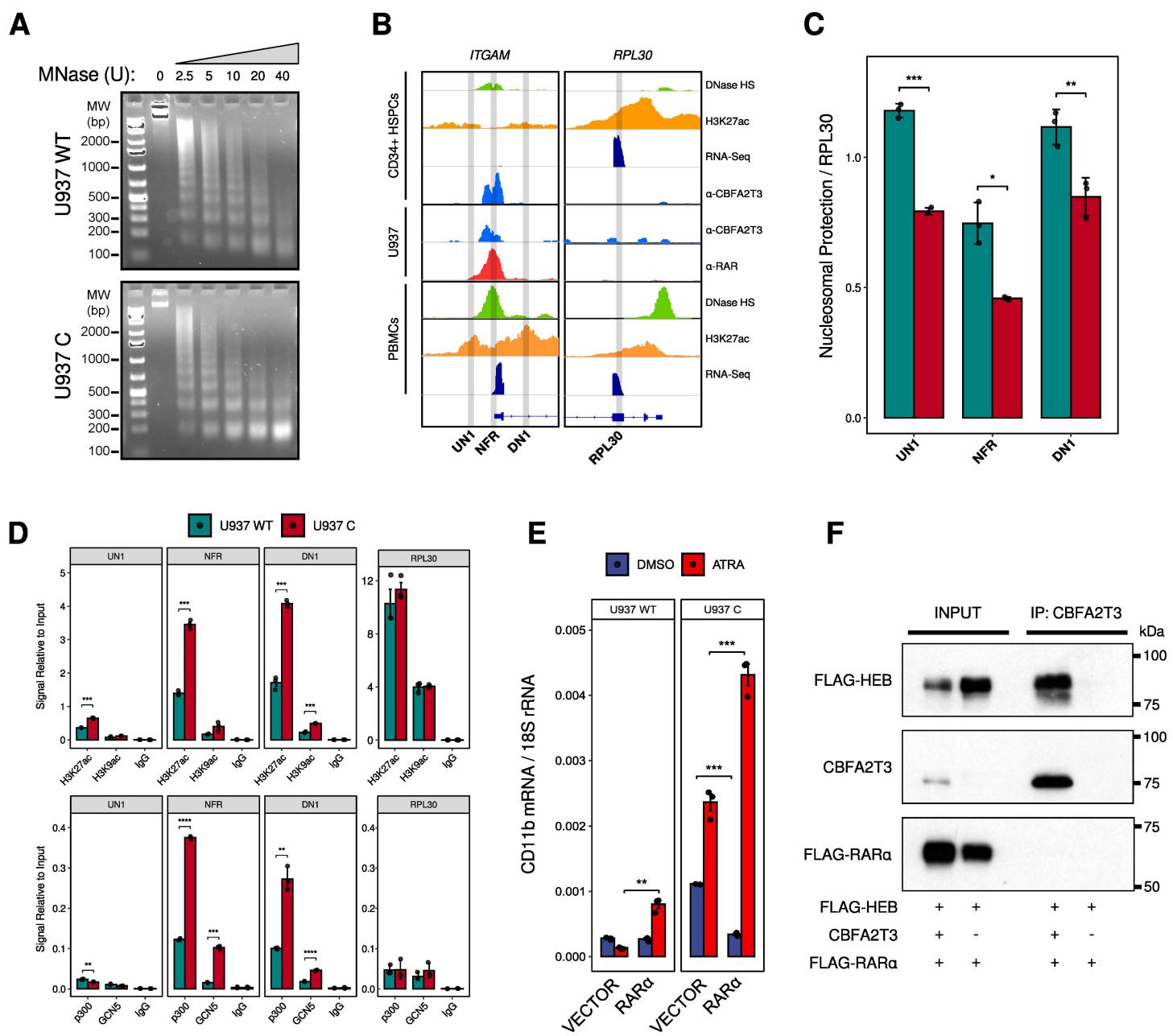


Fig. 4

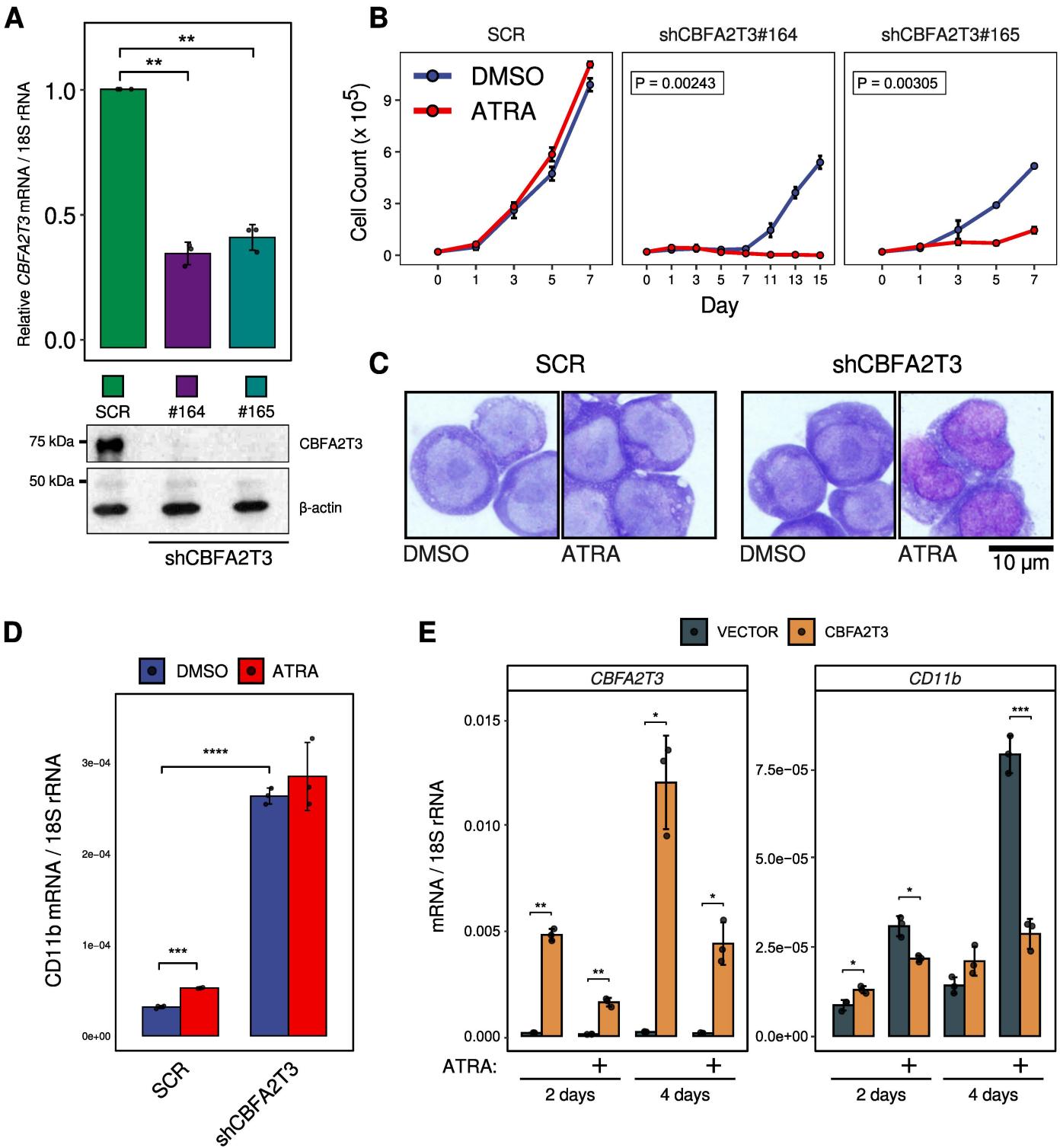
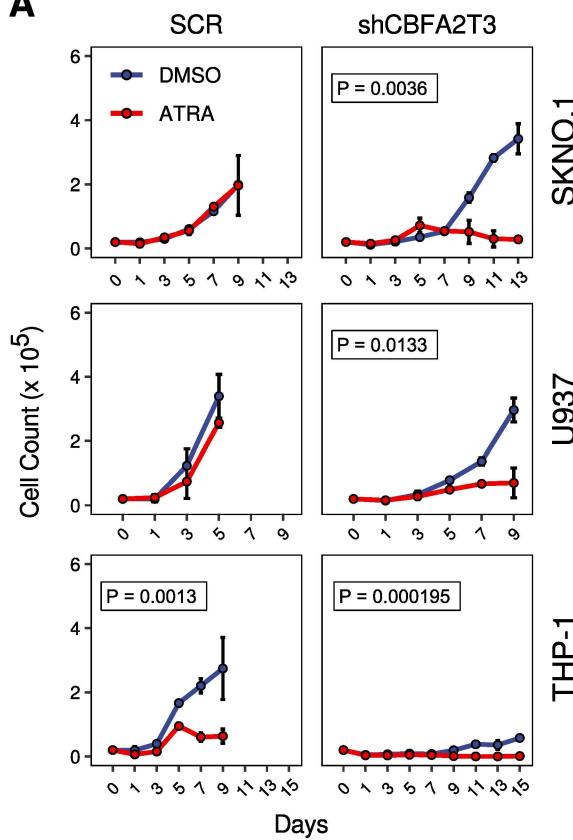
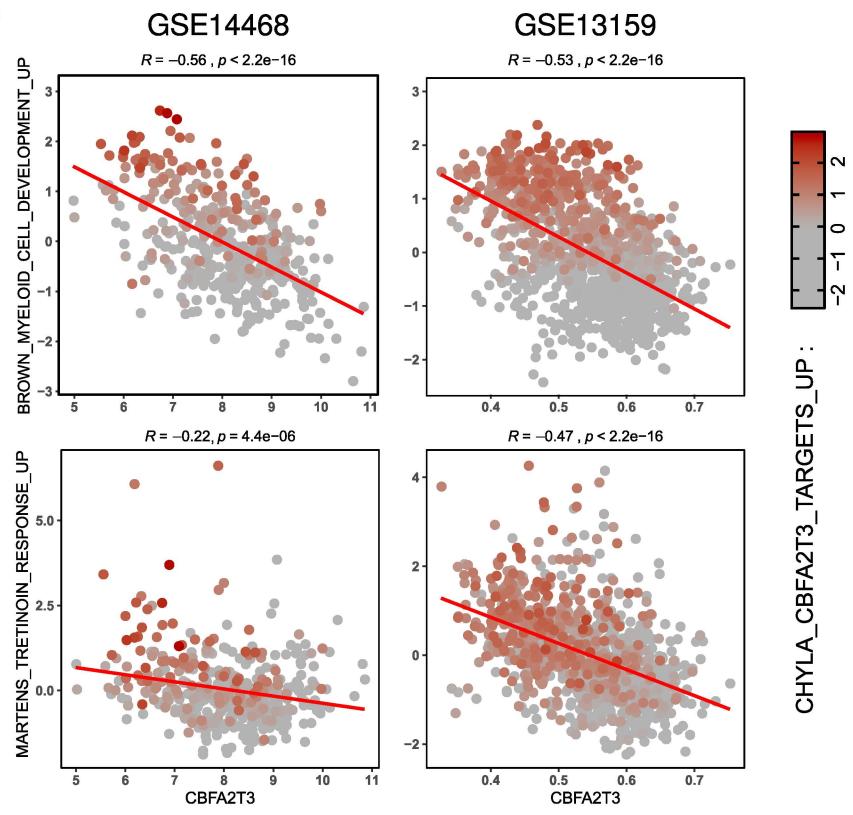


Fig. 5

A**B****Fig. 6**

The transcriptional corepressor CBFA2T3 inhibits all-trans retinoic acid–induced myeloid gene expression and differentiation in acute myeloid leukemia
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