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Destabilization of AETFC through C/EBPα-mediated repression of LYL1 contributes to t(8;21) leukemic cell differentiation

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The AML1-ETO fusion protein is produced by the t(8;21) translocation, which is the most common chromosomal abnormality in acute myeloid leukemia (AML). Although AML1-ETO alone is insufficient to cause leukemia, it is necessary for maintaining leukemia and therefore represents a therapeutic target. This notion has been supported by several lines of evidence: (i) transient suppression of AML1-ETO by small interfering RNA (siRNA) increases susceptibility of the leukemic cells to differentiation and delays leukemogenesis *in*

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vivo (1, 2); (ii) in a mouse model harboring fully developed leukemia, switching off AML1-ETO leads to leukemia regression (3); (iii) in an AML1-ETO9a (AE9a)-driven leukemic mouse model, myeloid differentiation of leukemic cells triggered by panobinostat (an HDAC inhibitor) was attributed to AE9a degradation (4); and (iv) mechanistic studies revealed that depletion of AML1-ETO in leukemic cells leads to a genome-wide epigenetic reprogramming and changes in transcription factor binding, resulting in myeloid differentiation and loss of leukemia maintenance (5).

We previously found that, in leukemic cells, AML1-ETO is stabilized and functions through the AML1-ETO-containing transcription factor complex (AETFC), which contains multiple transcription (co)factors that include AML1-ETO, CBFβ, E proteins HEB and E2A, hematopoietic bHLH transcription factor LYL1, LIM domain protein LMO2 and its binding partner LDB1 (6). These AETFC components mutually stabilize each other and cooperatively bind and regulate target genes, and AETFC integrity and proper conformation are essential for leukemogenesis (6). Thus, destabilization of AETFC provides a strategy to target AML1-ETO. Notably, it has been generally proposed that the stability of a protein complex can be reflected by its sensitivity to overexpression versus depletion of individual components (7). First, many complexes can be destabilized by overexpression of individual components that, in a dosage-dependent manner, make promiscuous interactions that change the topology of the complex and thereby destabilize it. This mechanism, known as “dosage sensitivity”, is widely applicable to the regulation of protein functions in organisms ranging from yeast to human (8), including the interplay among the key transcription factors in hematopoiesis and leukemogenesis (9). Second, other complexes show a lack of sensitivity (termed “robustness”) to component overexpression, likely because they possess strong multivalent interactions that cannot be altered by dosage increase, but can be perturbed by depletion, of individual components (10).

In this study, we investigated a means to destabilize AETFC, as well as the underlying mechanism. Following the principle described above, we first examined whether overexpression of AETFC components could affect the stability of the complex. In addition, several known interacting partners of AETFC components, including C/EBPα, TAL1 and ID1, were also analyzed. We transduced Kasumi-1 cells with retroviruses expressing HEB, E2A, E2-2, LDB1, LYL1, LMO2, C/EBPα, TAL1 or ID1 (Supplementary Figure S1a), and determined the protein levels of each AETFC component by immunoblot. The results showed that overexpression of the AETFC components failed to destabilize the complex (Figure 1a). Thus, this result, in combination with our previous observation that knockdown of AETFC components in Kasumi-1 cells leads to degradation of the complex (6), reflects the “robustness” of AETFC. This result is also consistent with the extremely strong biochemical stability of AETFC that we previously established (6).

Unexpectedly, overexpression of C/EBPα dramatically decreased the protein levels of all AETFC components (Figure 1a) and led to an accompanying inhibition of Kasumi-1 cell growth (Supplementary Figure S1b). To verify the loss-of-function of AETFC, we performed RNA-seq of the cells. Gene set enrichment analysis (GSEA) revealed that previously identified (6) effects of AETFC-loss on both the up- and downregulated target genes tend to be mimicked by C/EBPα overexpression; this was confirmed by RT-qPCR

analysis of representative genes (Figure 1b). GSEA also revealed that the genes associated with myeloid differentiation are enriched, whereas those associated with hematopoietic stem cells are depleted, in the C/EBPα-activated genes (Supplementary Figure S2), consistent with the function of C/EBPα in myeloid differentiation (11). We next employed the AE9a-driven leukemic mouse model to investigate whether C/EBPα overexpression could affect leukemogenesis. We observed that C/EBPα overexpression induces myeloid differentiation of the mouse leukemia cells and delays leukemogenesis *in vivo*, as indicated by an increased frequency of $CD11b^+$ cells and a significantly extended survival time of the mice (Figure 1c). Thus, these results suggest that AETFC destabilization can be achieved by overexpression of C/EBPα, which is associated with cell differentiation and delayed leukemogenesis; however, the mechanism of how C/EBPα destabilizes AETFC is unclear.

While C/EBPα has been shown to physically interact with AML1-ETO (12), this interaction is relatively weak compared with the interactions among other factors (e.g., the interactions among AETFC components and the interactions of TAL1 and ID1 with E proteins), and thus is insufficient to mediate a “dosage sensitivity” effect that destabilizes AETFC (8). We therefore examined whether C/EBPα overexpression can affect AETFC in other ways. Using RNA-seq and RT-qPCR, we found that C/EBPα overexpression leads to a significant decrease of *LYL1* mRNA, but not other AETFC component mRNAs (Figure 2a). Our previous characterization of one-to-one interactions within AETFC revealed a central position of LYL1 (i.e., LYL1 interacts strongly with E proteins and LMO2 and weakly with AML1-ETO and LDB1) (6). We thus speculated that loss of LYL1 could disrupt AETFC. To confirm this, we analyzed the integrity of AETFC in the presence and absence of LYL1 by co-immunoprecipitation (co-IP) assay, and we found that, without LYL1, LMO2 and LDB1 cannot be integrated into a complex with AML1-ETO and E proteins (Figure 2b, i). Thus, LYL1 appears to act as a linker for the AML1-ETO-E proteins (AE-E) and the LMO2-LDB1 parts of AETFC. This mechanism was held valid for the endogenous AETFC, as knockdown of LYL1 in Kasumi-1 cells led to reduced amounts of LMO2 and LDB1 that bind to AML1-ETO (Supplementary Figure S3). An analysis of Kasumi-1 nuclear extract indicated that knockdown of LYL1 led to a dramatic degradation of LMO2 and LDB1, as well as decreased HEB and E2A (Figure 2b, ii); AML1-ETO was lagged behind in this degradation process likely due to a different degradation mechanism for AML1-ETO relative to other AETFC components. In contrast, knockdown of TAL1, a homologue of LYL1, did not show such an effect (Figure 2b, ii). Conversely, overexpression of LYL1 in the C/EBPα-overexpressed Kasumi-1 cells rescued AETFC stability, and the extent of restoration of different AETFC components correlates with the interaction strength and spatial distance between these components and LYL1 (Figure 2b, iii). Taken together, these results suggest that downregulation of LYL1 by C/EBPα contributes to the AETFC destabilization.

To investigate whether LYL1 is directly regulated by C/EBPα and to gain a genome-wide view of C/EBPα binding, we performed a ChIP-seq analysis of the overexpressed C/EBPα in Kasumi-1 cells. The results showed that C/EBPα directly binds to an approximate −1 kb region of the *LYL1* locus (Figure 2c, i), which was confirmed by ChIP-qPCR (Figure 2c, ii). Recently published ChIP-seq data also indicated that this region is physiologically bound by endogenous C/EBPα in myeloid cell lines (Supplementary Figure S4). Interestingly, this −1 kb region is distinct from the previously reported promoter region (within 542 bp upstream

of *LYL1* transcription start site) bound by ETS and GATA factors (13). C/EBPα is known mostly as a transcriptional activator and, according to our RNA-seq data, overexpressed C/EBPα in Kasumi-1 cells activates more genes relative to repressed genes (Supplementary Figure S5a). However, it has also been established that C/EBPα can repress genes (14) (e.g., *MYC*, *MYB* and *GATA2*); and we observed direct binding and downregulation of these genes by C/EBPα in Kasumi-1 cells (Supplementary Figure S5b and c). To validate that C/EBPα directly represses *LYL1* transcription, we performed a luciferase reporter assay and observed that the transcriptional activity of the *LYL1* promoter is decreased upon C/EBPα overexpression in a dosage-dependent manner (Figure 2c, iii). Furthermore, GSEA revealed a strong correlation between the genes upregulated by C/EBPα and those derepressed by LYL1 knockdown (Supplementary Figure S6a), suggesting that repression of LYL1 to some degree recapitulates the effect of C/EBPα overexpression.

We next investigated whether LYL1 repression contributes to leukemic cell differentiation. We simultaneously overexpressed LYL1 and C/EBPα in Kasumi-1 cells and assessed their differentiation. The results showed that the LYL1 overexpression reduces the number of C/EBPα-induced $CD11b^+$ cells (Supplementary Figure S6b). Furthermore, we observed that knockdown of LYL1 enhances the ability of Kasumi-1 cells to differentiate upon induction by Vitamin D3, which otherwise shows very subtle effect on the cells (Supplementary Figure S6c). These results suggest that the LYL1 depletion can release the differentiation blockage in leukemic cells, although it is insufficient to induce a complete differentiation as does C/EBPα (Supplementary Figure S6c). This insufficiency is likely because C/EBPα activates many genes required for myeloid differentiation, while LYL1 depletion and AETFC destabilization can release the repression of some genes but cannot fully activate them.

In summary, our study first demonstrated an AETFC “robustness” in leukemic cells, which confer on the complex a resistance to a destabilization strategy based on overexpression of AETFC components. However, we found that overexpression of C/EBPα can destabilize AETFC by direct repression of the core component LYL1 at the transcriptional level, and that the depletion of LYL1 causes AETFC disruption that increases susceptibility of the leukemic cells to differentiation (Figure 2d). The important role of C/EBPα in t(8;21) leukemia development and treatment has been established (11) and recently re-emphasized by several interesting studies showing that depletion of AML1-ETO activates a C/EBPα-dominated transcriptional network (15) and that C/EBPα overrides the repressive activity of AML1-ETO (16). Our studies provide a new mechanism by which C/EBPα can destabilize AETFC, suggesting restoration of C/EBPα as a strategy for leukemia therapy, and further identifying LYL1 as a new therapeutic target in t(8;21) leukemia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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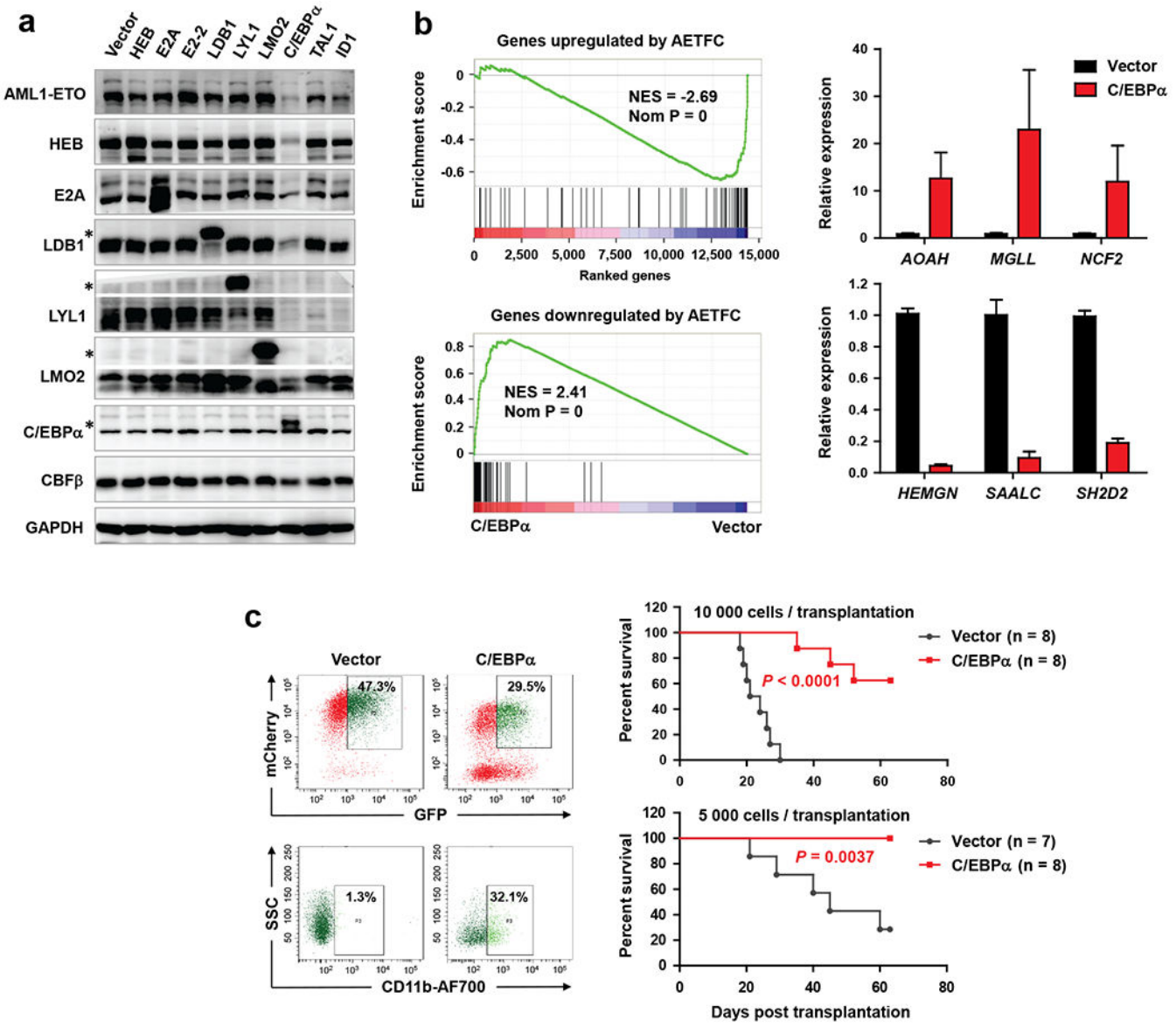

Figure 1. Destabilization of AETFC by overexpression of C/EBPα and its role in cell differentiation and leukemogenesis.

(**a**) Immunoblot analysis of AETFC components in Kasumi-1 cells upon overexpression of indicated proteins. Note that overexpression of C/EBPα, but not the AETFC components, leads to a decrease of AETFC components. Overexpression of TAL1 or ID1 only decreases LYL1, suggesting different mechanism(s) relative to C/EBPα. Asterisks denote the larger sizes of exogenous tagged proteins relative to the endogenous ones. (**b**) RNA-seq and GSEA (*left*) and RT-qPCR (*right*) analyses of Kasumi-1 cells expressing C/EBPα, showing that overexpression of C/EBPα impairs the function of AETFC in regulation of both up- and downregulated genes. In the *right* panel, data are presented as mean ± standard deviation (SD) of three independent experiments with triplicates each time. (**c**) Myeloid differentiation of the AML1-ETO9a-expressing mouse leukemic cells (*left*) and delayed leukemogenesis *in vivo* (*right*) caused by overexpression of C/EBPα. In the *right* panel, shown are Kaplan-

Meier survival curves of indicated numbers of mice transplanted with 10 000 or 5 000 leukemic cells; *P* values are calculated by the log rank test.

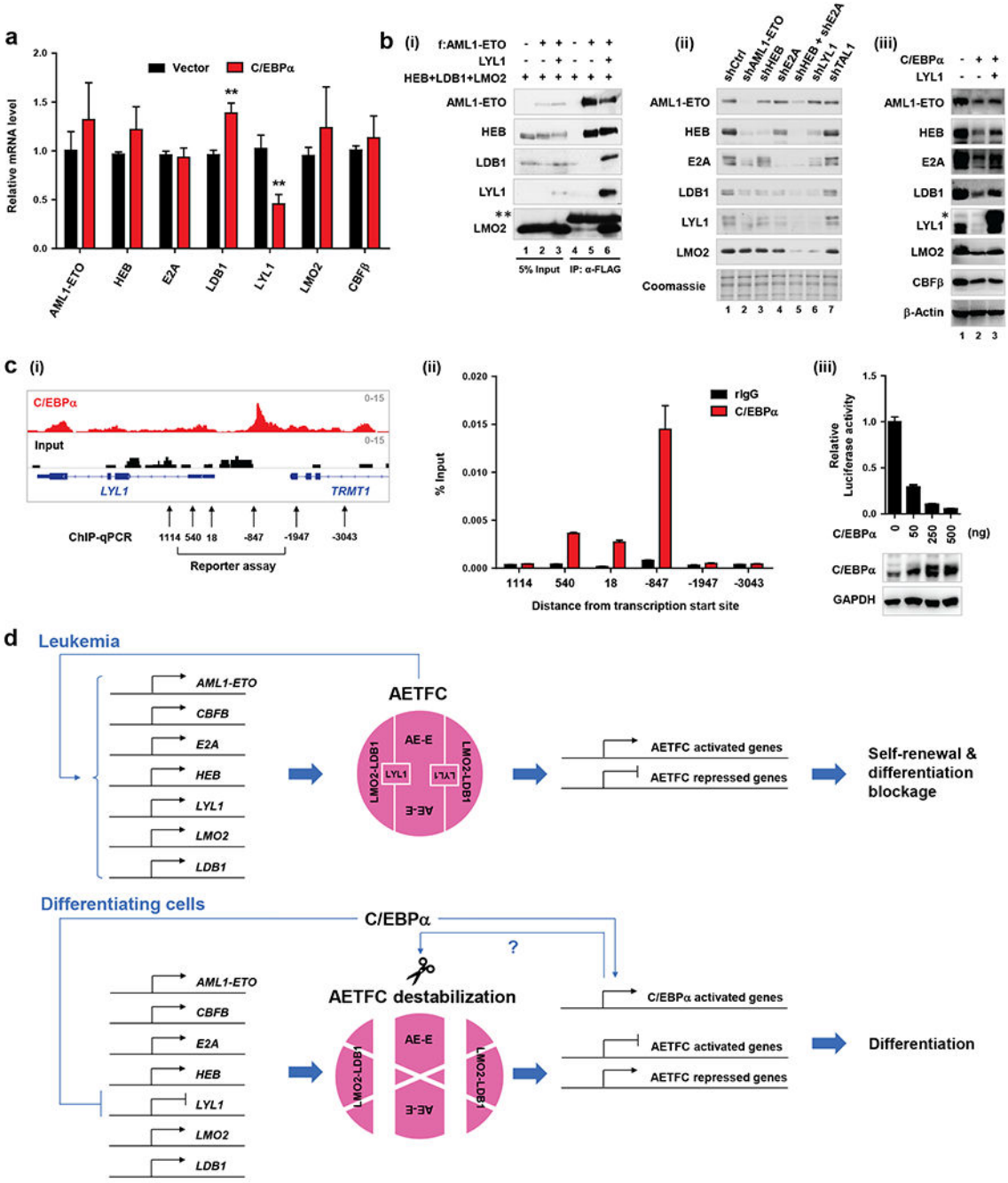

Figure 2. Direct repression of the core AETFC component LYL1 by C/EBPα, leading to disruption of AETFC.

(**a**) RT-qPCR analysis of mRNA levels of AETFC components in Kasumi-1 cells upon C/EBPα overexpression. Note that only *LYL1* mRNA is decreased. Data are presented as mean ± SD of three independent experiments with triplicates each time; $^{**}P < 0.01$; two-tailed t test. (**b**) The role of LYL1 in AETFC stabilization. (**i**) Co-IP analysis of AETFC integrity in 293T cells co-transfected with FLAG-tagged (f:) AML1-ETO and indicated components, showing that LYL1 is required for interaction between the AML1-ETO–HEB

and the LMO2-LDB1 parts of AETFC. Double asterisk denotes immunoglobulin signal. (**ii**) Immunoblot analysis of AETFC in Kasumi-1 cell nuclear extract upon knockdown of indicated components, showing that knockdown of LYL1 leads to AETFC degradation. Nuclear extract was used in this assay to exclude any cytoplasmic AETFC components. Also shown are knockdowns of E proteins and TAL1 as positive and negative controls, respectively. (**iii**) Rescue of AETFC stability by overexpression of LYL1 in the C/EBPα-overexpressing Kasumi-1 cells. Note that the stronger LYL1-interacting AETFC component shows a better restoration extent, suggesting a possible stepwise restoration of the complex. Asterisk denotes the exogenous tagged LYL1. (**c**) C/EBPα directly represses the transcription of the *LYL1* gene. (**i**) ChIP-seq analysis of overexpressed C/EBPα in Kasumi-1 cells, showing its binding to the *LYL1* locus. Arrows with numbers and bracket denote the regions selected for ChIP-qPCR and promoter reporter assays. (**ii**) ChIP-qPCR validation of C/EBPα binding to the indicated regions in the *LYL1* locus. An anti-C/EBPα antibody was used in this ChIP experiment, and a rabbit immunoglobulin G (rIgG) was used as a negative control. (**iii**) Luciferase reporter assay showing repression of the *LYL1* promoter by C/EBPα. A dosage-dependent effect of C/EBPα was revealed by transfection of different amounts of C/EBPα plasmid and immunoblot analysis of protein levels. (**d**) A working model. In leukemic cells, the robustness of AETFC is maintained by both the strong multivalent interactions within AETFC and a positive feedback loop in the transcriptional network (*upper*). Overexpression of C/EBPα specifically and directly represses LYL1, and thereby breaks the connection between the AML1-ETO–E (AE-E) and the LMO2-LDB1 parts of AETFC, leading to AETFC destabilization (*lower*). Potentially also involving other C/EBPα-activated genes (denoted by a question mark), these molecular events trigger degradation of AETFC/AML1-ETO and differentiation of leukemic cells.