CEBPA-dependent HK3 and KLF5 expression in primary AML and during AML differentiation

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The basic leucine zipper transcription factor CCAAT/enhancer binding protein alpha (CEBPA) codes for a critical regulator during neutrophil differentiation. Aberrant expression or function of this protein contributes to the development of acute myeloid leukemia (AML). In this study, we identified two novel unrelated CEBPA target genes, the glycolytic enzyme hexokinase 3 (HK3) and the krüppel-like factor 5 (KLF5) transcription factor, by comparing gene profiles in two cohorts of CEBPA wild-type and mutant AML patients. In addition, we found CEBPA-dependent activation of HK3 and KLF5 transcription during all-trans retinoic acid (ATRA) mediated neutrophil differentiation of acute promyelocytic leukemia (APL) cells. Moreover, we observed direct regulation of HK3 by CEBPA, whereas our data suggest an indirect regulation of KLF5 by this transcription factor. Altogether, our data provide an explanation for low HK3 and KLF5 expression in particular AML subtype and establish these genes as novel CEBPA targets during neutrophil differentiation.
explained by hypermethylation of its promoter as shown in primary AML and in several AML cell line models\(^1\). Still unclear is how KLF5 is transcriptionally regulated during granulocytic differentiation.

In this study we describe two novel CEBPA target genes, the glycolytic enzyme HK3 and the transcription factor KLF5, both of which are significantly downregulated in CEBPA-mutated AML patients.

**Results**

Low HK3 and KLF5 expression is associated with CEBPA-mutated AML. CEBPA is a master regulator of myeloid differentiation, whose expression peaks in the granulocyte-monocyte progenitor cell stage, and diminishes during terminal granulocyte differentiation\(^\text{3}\). Most importantly, in different AML subtypes CEBPA expression or function is inhibited by a variety of mechanisms\(^\text{3,5,6}\). In an attempt to test whether HK3 and KLF5, two genes with a recently discovered role in APL differentiation\(^\text{10,11,14}\), are novel CEBPA target genes, we compared their expression in a total of 90 CEBPA-mutated or CEBPA wild-type AML patients. HK3 and KLF5 were below the detection limit in 11 and 16 AML patient samples, respectively. We compared HK3 mRNA expression in CD34\(^+\) samples (\(n = 4\)) to 74 CEBPA-wt and 5 CEBPA-mutated AML samples. KLF5 expression in 4 CD34\(^+\) samples was compared to 70 CEBPA-wt and 4 CEBPA-mutated AML samples. A detailed analysis of HK3 and KLF5 mRNA levels in primary AML (FAB M0-M7) patient samples revealed a significantly lower expression of HK3 and KLF5 in CEBPA-mutated as compared to CEBPA wild-type AML samples (\(p < 0.01\)) (Figure 1a–b). As seen for other CEBPA target genes identified by screening AML patient cohorts, our results suggest that disrupting CEBPA function impairs HK3 and KLF5 expression\(^\text{15}\). Next, we confirmed that HK3 and KLF5 expression is significantly lower in CEBPA-mutated, but not CEBPA wild-type patients from analysis of a second AML patient cohort (\(n = 154\)). Since these cohorts contained patients with CEBPA single (SM) or double mutations (DM), we could address whether single or double mutations alter HK3 or KLF5 expression\(^\text{16}\). We found that HK3 or KLF5 expression was not significantly different from patient’s samples with either CEBPA SM or DM (Figure 1c–d). Taken together, our findings indicate that loss of HK3 or KLF5 expression correlated with CEBPA mutations, irrespective of single or double CEBPA mutations, whereas enhanced expression of HK3 or KLF5 correlated with wild-type CEBPA expression.

CEBPA-dependent induction of HK3 and KLF5 during neutrophil differentiation of APL cells. To experimentally test whether

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**Figure 1** HK3 and KLF5 expression is significantly downregulated in CEBPA-mutated AML patients. HK3 (a) and KLF5 (b) mRNA levels were measured by qPCR in total RNA extracted from primary AML (FAB M0-M7) blasts, CD34\(^+\) samples or granulocytes from healthy donors. Patient characteristics are summarized in Supplementary Table 1. HK3 (c) and KLF5 (d) levels in 154 patients from the Taskesen cohort with normal karyotype, expressing wild type CEBPA (WT), one allele mutated (SM) or two alleles mutated (DM). MWU: *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\), ****\(p < 0.0001\).
loss of CEBPA mRNA alters HK3 and KLF5 expression, we knocked down CEBPA in the two APL cell line models NB4 and HT93. This was accomplished using lentiviral vectors expressing two independent short hairpin (sh)RNAs targeting the CEBPA gene. Knockdown efficiency was evaluated by qPCR. CEBPA mRNA expression was normalized to the housekeeping gene HMBS and is shown as n-fold changes compared to untreated SHC002 control cells. HK3 (b) and KLF5 (c) mRNA expression in NB4 or HT93 APL cells was determined by qPCR and analyzed as in 2a. Impairment in granulocytic differentiation of CEBPA knockdown cells was shown by a reduction of the neutrophil marker granulocyte colony-stimulating factor receptor (GCSFR or CSF3R) (d) Data represent the mean ± s.d. of at least three independent experiments. (e), (f) CEBPA Knockdown efficiency at the protein level in NB4 and HT93 APL cells was confirmed by western blotting. GAPDH is shown as a loading control. MWU: *p < 0.05, **p < 0.01 and ***p < 0.001.

CEBPA binding to the HK3 or KLF5 promoters activates transcription. Our findings prompted us to investigate if HK3 and KLF5 are direct transcriptional targets of CEBPA. To this end, we first analyzed the genomic regions surrounding exon 1 of these genes for putative CEBPA binding sites using Matinspector 8.0. We identified two putative CEBPA binding sites in both the HK3 and the KLF5 genomic regions analyzed (Figure 3a and c). Chromatin immunoprecipitation (ChIP) revealed binding of CEBPA at positions +821/+2963 and −385/−1576 relative to the transcriptional

Figure 2 | Genetic inhibition of CEBPA impairs HK3 and KLF5 upregulation during neutrophil differentiation of APL cells. (a) NB4 or HT93 APL cell lines were stably transduced with pLKO.1 lentiviral vectors expressing non-targeting or two independent CEBPA-targeting shRNAs. APL control and CEBPA knockdown cells were differentiated with 1 μM ATRA for 4 days. Knockdown efficiency in NB4 (top panels) or HT93 (bottom panels) APL cells was validated by qPCR. CEBPA mRNA expression was normalized to the housekeeping gene HMBS and is shown as n-fold changes compared to untreated SHC002 control cells. HK3 (b) and KLF5 (c) mRNA expression in NB4 or HT93 APL cells was determined by qPCR and analyzed as in 2a. Impairment in granulocytic differentiation of CEBPA knockdown cells was shown by a reduction of the neutrophil marker granulocyte colony-stimulating factor receptor (GCSFR or CSF3R) (d) Data represent the mean ± s.d. of at least three independent experiments. (e), (f) CEBPA Knockdown efficiency at the protein level in NB4 and HT93 APL cells was confirmed by western blotting. GAPDH is shown as a loading control. MWU: *p < 0.05, **p < 0.01 and ***p < 0.001.
start site on the HK3 and KLF5 genomic regions, respectively (Figures 3b and d). We next asked whether CEBPA activates HK3 and KLF5 transcription from these sites using promoter reporter assays. CEBPA co-expression with the different luciferase promoter reporters resulted in a significant, dose-dependent activation of HK3 promoter A (up to 37-fold) and KLF5 (up to 11-fold) transcription, respectively (Figures 3e and g). The HK3 promoter B containing the CEBPA binding site was only induced 1.5-fold (Supplementary Figure S2a). To confirm the specific CEBPA-induction of the promoter activity we mutated the CEBPA binding sites on the HK3 promoter A and the KLF5 promoter. Indeed, inactivating the CEBPA binding site in the HK3 promoter significantly inhibited transcription (Figure 3f). Surprisingly, however, introducing single or double CEBPA binding sites mutations in the KLF5 promoter did not attenuate transcriptional activity of the reporter construct (Supplementary Figure S2b).

**Ectopic expression of CEBPA significantly induces HK3 and KLF5 transcription.** To confirm CEBPA-dependent transcriptional regulation of HK3 and KLF5, we overexpressed CEBPA in HT93 APL cells by transiently transfecting a CEBPA expression plasmid. Ectopic expression of CEBPA in HT93 cells resulted in a significant 5.5- and 12.3-fold induction of HK3 and KLF5 gene expression, respectively (Figures 4a and b). CEBPA transfection efficiency was assessed by qPCR and Western blotting (Figure 4d and e). In addition, induction of the direct CEBPA target CEBPE was measured as a positive control of CEBPA activity (Figure 4c). Next, we used K562 leukemic cell lines expressing 4-OHT inducible full-length CEBPA (CEBPA-p42-estrogen receptor (ER)), a truncated isoform (CEBPA-p30-ER), or a p42 isoform with a mutation preventing interaction with E2F and neutrophil differentiation (CEBPA-BRM2-ER). After 24 h of 4-OHT treatment, we observed a significant 2.5-fold transcriptional activation of KLF5 (p < 0.05) upon CEBPA-p42-ER activation only, but not in ER control, nor in CEBPA-p30-ER, or mutated CEBPA-BRM2-ER activated K562 cells (Figure 4f), suggesting that induction of KLF5 is a specific function of the full length CEBPA p42 isoform. Interestingly, HK3 mRNA was not detectable in K562 cells indicating that different HK isoforms may be active in these cells. Induction of CEBPE was measured as a positive control (Figure 4g). Altogether, our findings demonstrate that CEBPA is a crucial positive regulator of HK3 and KLF5 during granulocytic differentiation.

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**Figure 3 | CEBPA binds to and activates the HK3 and the KLF5 promoters.** Schematic representation of a 6 kb human HK3 (a) and a 5 kb human KLF5 (c) genomic regions retrieved from an online database. MatInspector software predicted two putative CEBPA binding sites (squares) in the DNA sequences analyzed. In vivo binding of CEBPA to these CEBPA consensus sites in the HK3 (b) or KLF5 (d) genomic regions was shown by chromatin immunoprecipitation (ChIP) in NB4 APL cells. As a negative control for the different pull downs, absence of GAPDH amplification is shown. *unspecific band, primer dimer. Two HK3 (e–f) and one KLF5 genomic region (g) containing the CEBPA binding sites were PCR amplified from genomic DNA of NB4 cells using proof reading Pfu DNA polymerase and cloned into the pGLO.10-basic vector. H1299 cells were transiently transfected with 40 ng of either HK3 promoter reporter construct A (e), construct A with mutated CEBPA binding site (f, wild-type GAAAGAC, mutated GGTCGAC) or the KLF5 promoter reporter construct (g), together with pcDNA3.1 empty vector or increasing concentrations (40–80–120 ng) (e,g) or 80 ng of CEBPA expression vector (f). The promoter activity is shown as relative light units (RLU) relative to pcDNA3.1 control transfected cells. Results are the means ± s.d. of at least triplicate transfections. MWU: **p < 0.01, ***p < 0.001.
Discussion

Reduced CEBPA expression or loss of function mutations are commonly observed in AML suggesting that the deregulation of CEBPA function is a major event in the development of AML. Identifying new CEBPA target genes and associated CEBPA-dependent pathways in myeloid differentiation may provide novel insights into the differentiation block as well as into ancillary cellular functions found in AML. Interestingly, CEBPA has been described as a pivotal regulator of various metabolic processes in different cell types. Nevertheless, whether CEBPA regulates metabolic activity in myeloid cells is of yet unclear. Our findings now link CEBPA to glycolysis via direct positive regulation of the glycolytic enzyme HK3.

In general, AML is defined by a block in differentiation and an increased proliferation of immature myeloid progenitors. Additionally, loss of CEBPA-mediated cell-cycle arrest is crucial for the development of AML. Since KLF5 regulates genes involved in cell cycle regulation and apoptosis, it may represent a relevant downstream effector of CEBPA-induced cell cycle arrest. In the myeloid lineage, KLF5 induces differentiation and functions as a tumor suppressor and its low expression in AML is partially explained by epigenetic silencing. Importantly, low KLF5 expression is associated with poor overall survival. In this study, we suggest an additional mechanism for low KLF5 levels in AML that is loss of positive regulation of KLF5 due to impaired CEBPA function. Since mutating the CEBPA binding sites in the KLF5 promoter constructs did not change the responsiveness of these reporters to CEBPA expression, CEBPA may indirectly activate KLF5 transcription. The KLF5 promoter lacks a TATA box, but contains a GC-rich region that is activated by the transcription factor Sp1. Moreover, CEBPA can functionally interact with Sp1, e.g. in regulating the CD11c integrin gene. We propose, that CEBPA induces KLF5 transcription, similarly to CD11c regulation, via Sp1.

Figure 4 | Ectopic expression of CEBPA activates HK3 and KLF5 transcription. (a–e) HT93 cells were transiently transfected with pcDNA3.1 empty control or a CEBPA expression vector. HK3 (a) and KLF5 (b) mRNA expression was quantified by qPCR. Data were normalized to HMBS and are shown as n-fold regulation as compared to control transfected cells. Induction of CEBPE mRNA, a direct target gene of CEBPA, was measured as a positive control for CEBPA activity (c). Results are the means ± s.d. of at least triplicate transfections. CEBPA transfection efficiency was measured by qPCR (d) and western blotting (e). GAPDH is shown as a loading control. (f–g) Different CEBPA-ER fusion constructs were induced by treating the respective K562 cell lines with 5 μM Tamoxifen for 24 h. HK3 (f) or KLF5 (g) mRNA expression was quantified by qPCR as in 2a. Expression of the CEBPA target CEBPE was measured as positive control for CEBPA activation in wildtype CEBPA p42 expressing K562 cells. MWU: *p < 0.05, **p < 0.01.
In conclusion, we identified HK3 and KLF5 as novel CEBPA-regulated genes in AML and during APL differentiation underlying their tumor suppressor function in AML as well as their role in granulopoiesis.

**Methods**

**Patient samples, cell lines and cell culture conditions.** Fresh leukemic blast cells from untreated AML patients at diagnosis obtained at the Inselspital Bern (Switzerland) were used as described according to the French-American-British (FAB) classification and cytogenetic analysis. All leukemic samples displayed a blast count of >90% after separation of mononuclear cells using a Ficol gradient (Lymphoprep™, Axon Lab AG, Switzerland) as described previously11. PBMC and granulocytes obtained from normal, healthy donors were isolated using a Ficol gradient.

The human acute promyelocytic leukemia (APL) cells line NB4 and HT93 were maintained in RPMI-1640 and Sigma-Aldrich) with 10% fetal calf serum (FCS) (Biochrom, AG) 50 U/mL penicillin and 50 µg/ml streptomycin (Sigma-Aldrich) in a 5% CO2-95% air humidified atmosphere at 37°C.

For differentiation experiments, NB4 cells were seeded at a density of 0.2 × 10⁶/ml and treated with 1 µM all-trans retinoic acid (ATRA, dissolved in DMSO, Sigma Aldrich) as indicated. Successful neutrophil differentiation was assessed by light microscopy using May-Grünwald-Giemsa (Merck, Darmstadt, Germany) stained cells and by surface expression of the differentiation marker CD11b (ImmunoTools)

Briefly, 5 × 10⁶ cells were collected, washed, and incubated with monoclonal mouse phycocyanin (PE)-labeled anti-human-CD11b for 20 min at 4°C. Fluorescence intensities were measured by a FACS Calibur Flow cytometer (Becton Dickinson, Basel, Switzerland) and analyzed using FlowJo software.

K562-CEPA-p42-ER, K562-CEPA-p30-ER, K562-CEPA-BRM2-ER and K562-CEPA-ER were kindly provided by Prof. G. Behre and were maintained in RPMI-1640 supplemented with 10% fetal calf serum (FCS), 50 U/mL penicillin and 50 µg/ml streptomycin in a 5% CO2-95% air humidified atmosphere at 37°C. K562 CEPA-ER cells were differentiated as described9 by addition of 5 µM 4-OHT (Sigma-Aldrich).

**Chromatin immunoprecipitation (ChIP).** ChIP was performed using the ChIP-IT Express Chromatin Immunoprecipitation Kit (ChIP-IT Express, Active Motif, Rixensart, Belgium) according to the manufacturer’s recommendations. For immunoprecipitation, an anti-CEBPα antibody (sc-61X Santa Cruz, CA, US) was used. Antibodies against acetyl-histone H3 (Stratagene, La Jolla, CA, US) and IgG (Pepablot, Upstate, Millipore) served as positive and negative controls, respectively.

PCR was performed using the following primers: HK3 promoter A, F: 5‘-GGGCTTCTGGGAGGTTCTCAA, R: AGGTGTCTTCTTCAAGGGCCAGG-3’. HK3 promoter B, F: TCCAGCTCTGATGCTCTCCA, R: GTTCGAGTGTCGACAAGACAG-3’, R: 5’-TGGAGGATGAGTGGTGATGAC-3’, K562 promoter B, F: 5’-GGGCCATGATGGGTGCTCTG-3’, R: 5’-GTTGGAAGACGCGGAGAGAAG-3’. As a negative control for the different pulldowns, absence of GAPDH amplification is shown9.

**Human HK3 and KLF5 promoter reporter assays and mutagenesis.** Two HK3 promoter regions and one KLF5 promoter region containing the CEBPA binding sites were PCR amplified from genomic DNA of NB4 APL cells using proof reading Pfu DNA Polymerase (Stratagene) and cloned into the pGL4.10-HK3 AACCAGAAGGCCC; KLF5 promoter A, F: 5’-TGCCTTCAGAGAATGGCTGATGAC -3’. Amplification is switch sufficient for induction of granulocytic development from bipotential myeloid progenitors. Mol. Cell. Biol. 18, 4301–4314 (1998).

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Author contributions

E.A.F. and M.H. performed the experimental research, analyzed the data and drafted the article. G.B. provided essential CEBPA reagents and revised the article. M.F.F. and B.E.T. instigated the experimental design and revised the drafted article. M.P.T. designed the project, wrote the paper and gave final approval of the submitted manuscript.

Additional information

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