Inactivation of the p53-KLF4-CEBPA axis in acute myeloid leukemia.

Research Article

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Abbreviations: AML: acute myeloid leukemia; CEBPA: CCAAT-enhancer binding protein alpha; KLF4: Krüppel-like factor-4; HSC: hematopoietic stem cells; PBMC: peripheral blood mononuclear cells.

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Key Points

• p53 function is inactivated in leukemic blast cells.

• CEBPA gene expression in myeloid cells can be activated by p53 and KLF4 through distal downstream gene regulatory elements.
Abstract

Purpose: In acute myeloid leukemia (AML), the transcription factors CEBPA and KLF4 as well as the universal tumor suppressor p53 are frequently deregulated. Here, we investigated the extent of dysregulation, the molecular interactions and the mechanisms involved.

Experimental Design: 110 AML patient samples were analyzed for protein levels of CEBPA, KLF4, p53 and p53 modulators. Regulation of CEBPA gene expression by KLF4 and p53 or by chemical p53 activators was characterized in AML cell lines.

Results: We found that CEBPA gene transcription can be directly activated by p53 and KLF4 suggesting a p53-KLF4-CEBPA axis. In AML patient cells, we observed a prominent loss of p53 function and concomitant reduction of KLF4 and CEBPA protein levels. Assessment of cellular p53 modulator proteins indicated that p53 inactivation in leukemic cells correlated with elevated levels of the nuclear export protein XPO1/CRM1 and increase of the p53 inhibitors MDM2 and CUL9/PARC in the cytoplasm. Finally, restoring p53 function following treatment with cytotoxic chemotherapy compounds and p53 restoring non-genotoxic agents induced CEBPA gene expression, myeloid differentiation and cell cycle arrest in AML cells.

Conclusions: The p53-KLF4-CEBPA axis is deregulated in AML, but can be functionally restored by conventional chemotherapy and novel p53 activating treatments.
Translational relevance

The transcription factors CEBPA and KLF4 act as key regulators of normal differentiation and suppress malignant transformation, whereas in acute myeloid leukemia (AML), the function of CEBPA, KLF4 and of the tumor suppressor p53 are frequently suppressed. Our study indicates that CEBPA gene transcription can be directly activated by p53 and KLF4 suggesting a p53-KLF4-CEBPA axis. We observed in primary AML samples a characteristic loss of p53 function and a concomitant reduction in KLF4 and CEBPA protein levels. Importantly, restoring p53 function following treatment with cytotoxic chemotherapy together with p53 activating non-genotoxic agents induced CEBPA gene expression and initiated myeloid differentiation and cell cycle arrest in AML cells. Our data pave the way towards therapeutic concepts combining conventional chemotherapy and novel p53 activating treatment based on the observation that conventional chemotherapy together with novel p53 activating treatment can restore CEBPA function.
Introduction

CCAAT/enhancer binding protein alpha (CEBPA) is a lineage-specific transcription factor expressed in hematopoietic stem and myeloid progenitor cells (1). CEBPA functions as a differentiation factor by directly targeting lineage-specific genes. CEBPA activates granulocyte- and eosinophil-specific genes while inhibiting erythroid- and lymphoid-specific factors. In addition, CEBPA acts as a potent cell cycle inhibitor. Cell cycle arrest and terminal differentiation are coupled processes, and the ability of CEBPA to mediate both may provide an explanation for its ability to act as a switch between uncommitted proliferating cells and differentiated cell cycle-arrested cells. The mechanisms by which CEBPA mediates cell-cycle arrest have been extensively investigated, and they involve the ability of CEBPA to upregulate the CDKN1A gene encoding the cyclin-dependent kinase inhibitor p21/WAF1/Cip1, its interaction with the cyclin-dependent kinases CDK2 and CDK4, and the CEBPA mediated repression of the E2F complex (2). CEBPA function is commonly deregulated in AML patients due to genomic mutations, suppressed transcriptional and/or translational expression, and altered protein degradation (3). Moreover, CEBPA is a DNA damage-inducible p53-regulated mediator of the G1 checkpoint in keratinocytes, but not in fibroblasts (4).

Similarly, Krüppel-like factor-4 (KLF4/GKLF) is a lineage-specific transcription factor expressed in a monocyte-restricted and stage-specific pattern during myelopoiesis, and it functions to promote monocyte differentiation (5) (6). Alike CEBPA, KLF4 can arrest cell proliferation via induction of the CDKN1A gene, and it represents a potent p53 mediator thus acting as a tumor suppressor (7). KLF4 expression is down regulated in NPM1 mutant AML by miR10a (8), and it can be repressed by CDX2 and HDAC1 (9) (10) (11).

The tumor suppressor p53 has been dubbed the guardian of the genome (12) reflecting its crucial role in dealing with all kinds of stressful conditions that can damage DNA. Physiologically, p53 induces cell cycle arrest to enable repair of damaged components or, in
p53 exerts its cell cycle arrest and apoptotic functions in the nucleus, but it is shuttled to the cytoplasm where it can be retained, re-shuttled to the nucleus or destroyed (14). The tumor suppressor \( p53 \) is inactivated in a wide variety of cancers, either by mutation or by dysregulation, in particular via induction of the \( p53 \) inhibitor \( MDM2 \) or by disturbing the nucleocytoplasmic shuttling of \( p53 \), either by dysregulation of the transporter proteins \( importin \) and \( exportin \) or induction of the cytoplasmic retention protein \( CUL9/PARC \). In AML cells, the incidence of \( p53 \) mutations is low; however, dysregulation of \( p53 \) function in AML appears to be frequent event (15).

In AML as well as in other hematologic malignancies, \( CEBPA, KLF4 \) and \( p53 \) can be deregulated. The extent of dysregulation, the molecular interactions and mechanisms involved in this dysregulation are unclear. We investigated whether \( KLF4 \) and \( p53 \) can activate \( CEBPA \) gene expression, and we assessed the extent of dysregulation in the protein levels of the three transcription factors and of cellular \( p53 \) modulators in primary AML patient samples.
Materials and Methods

CEBPA reporter plasmids

Highly conserved non-coding elements of the CEBPA gene (HCNEs) were defined using the genome browser genome.ucsc.edu. HCNEs were PCR amplified from human genomic DNA using primers with restriction sites XhoI (5’) and HindIII (3’) for all HCNEs, with the exception of SacI (5’) and BamHI (3’) for the 33kb3’ and 31kb3’ HCNEs, and BamHI (5’ and 3’) for the 6kb3’ HCNE, and they were cloned into pCR4-Topo (Invitrogen, Carlsbad, USA) before being transferred to the pGL4.23 firefly luciferase reporter plasmid (Promega, Madison, WI, USA). The renilla luciferase (Rluc) plasmid was used for normalization.

HCNEs were located on the NCBI 36/hg18 assembly at chr19:38443446-38443899 (454bp, 38kb3’), chr19:38446041-38446632 (592bp, 36kb3’), chr19:38449792-38450687 (895bp, 33kb3’), chr19:38451402-38451655 (254bp, 31kb3’), chr19:38452815-38453385 (571bp, 30kb3’), chr19:38463178-38463622 (360bp, 19kb3’), chr19:38464933-38465373 (441bp, 17kb3’), chr19:38472626-38473335 (730bp, 9kb3’), chr19:38475809-38476523 (715bp, 6kb3’), chr19:38478999-38479794 (796bp, 3kb3’), and chr19:38485160-38486494 (1334bp, 1kb5’). All HCNEs were analyzed for transcription factor binding sites using MatInspector software (Genomatix, Munich, Germany).

A smaller construct pGL4.23-36kb3 (180bp, 36kb3’) encoding the region flanking the putative p53 binding sites in the 36kb3’ HCNE was created by internal SacI deletion in pGL4.23-36kb3’ (592bp, 36kb3’). To test the p53 binding sites in the 36kb3’ HCNE, one or both sites were mutated by site-directed mutagenesis to create pGL4.23-36kbM12 using primer pairs p53_mut_F (5’-CCTCTGAATTCCCGCATCTATCCAC-3’) and p53_mut_R (5’-GTGGATAGATGCAGGGCGAATTCACTGAGG-3’), and to create pGL4.23-36kbM1 using primer pairs p53-mut F2 (5’-CCTCTGCATGCCCGCATCTATCCAC-3’) and p53_mut_R2 (5’- GTGGATAGATGCAGGGCGAATTCACTGAGG-3’). Oligonucleotide primers were supplied by Microsynth, Rotkreuz, Switzerland).

p53-KLF4-CEBPA in AML.
To test the KLF4 binding sites in the 9kb3' HCNE, a partial construct pGL4.23-9kb3C (190bp, 9kb3') was created by internal SacI deletion in pGL4.23-9kb3' (730bp, 9kb3'). The other 540 bp sequence was PCR amplified using primers with restriction sites XhoI (5') and HindIII (3') and subcloned into pGL4.23 to create pGL4.23-9kb3N (540bp, 9kb3'). The 730bp 9kb3' region encoded four KLF4 sites, while the 190bp 9kb3' and the 540bp 9kb3' constructs encoded two KLF4 sites.

**Luciferase reporter assays**

CEBPA-firefly luciferase reporter and renilla luciferase reference plasmids together with pcDNA3.3_KLF4 (16) and pcDNA_TP53 expression plasmids were transfected into H1299 cells using Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA). After 24 hours, cells were lysed and luciferase activity was assessed using the Dual-Luciferase-Reporter Assay system (Promega, Madison, WI, USA) on a microplate reader Infinite 200 (Tecan, Männedorf, Switzerland). Assays were performed in at least three independent experiments. Statistical analysis was done with GraphPad Prism software using two-tailed t-tests. Data are depicted in column bar graphs plotting mean with SD values. The TP53 expression plasmid was provided by Carol Prives and the KLF4 expression plasmid by Derrick Rossi (Addgene #26815).

**CEBPA-luc-BAC stable cell lines**

The CEBPA-BAC transgene P391 was created by insertion of a luciferase-puromycin cassette into RP11-270I13 (Gene Bridges, Heidelberg, Germany). P391 BAC DNA was prepared from the E. coli HS996 bacterial culture grown in LB medium with Cm (15ug/ml), linearized by SgfI digestion, and subsequently used to generate stable cell lines. Leukemic HL60 cells were transfected with linearized P391 BAC DNA (4ug/10⁶ cells) using the Nucleofector® Kit V Program X-001 (Lonza, Cologne, Germany). Cells were cultured for 24 hours before addition of puromycin (0.5ug/ml). Colonies emerged after 10 to 14 days. Single cell colonies were
expanded, and P391-HL60 cells were tested by transfection with pcDNA3.3_KLF4 (16), pcDNA_TP53 or pcDNA-AML1-ETO (17) expression plasmids (2.5μg plasmid per 2x10^5 cells) and renilla reference plasmid using Transit2020 transfection reagent (Mirus Bio LLC, USA). After 24 hours, cells were lysed and luciferase activity was assessed using the Dual-Luciferase-Reporter Assay system (Promega, Madison, WI, USA) on a plate reader (Tecan, Männedorf, Switzerland). Assays were performed at least in three independent experiments. Statistical analysis was done with GraphPad Prism software using two-tailed t-tests. Data are depicted in column bar graphs plotting mean with SD values.

**Patient samples**

A cohort of 110 consecutive AML patients diagnosed and treated at the University Hospital, Bern, Switzerland between 2002 and 2012 was included in this study. Informed consent from all patients was obtained according to the Declaration of Helsinki, and the studies were approved by decisions of the local ethics committee of Bern, Switzerland. Mutational screening for NMP1, CEBPA and FLT3, as well as conventional karyotype analysis of at least 20 metaphases were performed for each patient. Peripheral blood mononuclear cells (PBMCs) and bone marrow mononuclear cells (BMMCs) were collected at the time of diagnosis before initiation of treatment.

**Measurement of protein expression by ELISA**

Nuclear and cytoplasmic proteins were extracted from Ficoll-separated mononucleated cells (Lymphoprep, Axis-Shield, Oslo, Norway). Cell pellets were lysed in 100μl HEPES buffer containing 10mM KCl, 1.5mM MgCl2, 1% NP40, 1mM DTT, and 10mM PMSF. After centrifugation, supernatants containing cytoplasmic proteins were collected. Pelleted nuclei were extracted in 100μl HEPES buffer containing 420mM NaCl, 1.5mM MgCl2, 25% Glycerol, 0.2mM EDTA, and 1mM PMSF. A280 nanodrop measurements were used to
determine total protein concentration equivalents. Active p53 protein levels were assessed by colorimetric assay (OD450) using the TF-Detect Human p53 Activity Assay kit (GeneCopoeia, Rockville, MD, USA). CEBPA, KLF4, MDM2, CUL9, XPO1, LMNA and GAPDH protein levels were measured by colorimetric assay (OD450) using Sandwich ELISA kits supplied by USCN Life Science Inc, Houston, TX, USA. Measurements for nuclear proteins (p53, CEBPA, KLF4, MDM2, XPO1) were normalized with nuclear LMNA, and measurements for cytoplasmic proteins (MDM2, CUL9) were normalized with cytoplasmic GADPH levels. Statistical analysis was performed using Mann-Whitney-test on GraphPad Prism software. Data are depicted as box and whiskers graphs plotting Tukey range.

AML cell lines

OCI-AML3 (AML-M4, DNMT3A mut R882C, NPM1 mut, TP53wt), MOLM-13 (AML-M5, t(9;11), FLT3-ITD, TP53wt), ML2 (AML-M4, t(6;11)(q27;q23), TP53wt), ME-1 (AML-M4eo, inv16, TP53mut/null) and HL60 (AML-M2, MYC amp, TP53del/null) cells were supplied by the Leibniz Institute DSMZ, German Collection of Microorganisms and Cell Cultures. AML cells were grown in RPMI 1640 (SIGMA-ALDRICH, St. Louis, USA) supplemented with 20% fetal bovine serum (FBS, Biochrom GmbH, Germany). ME-1 cells were transfected with pcDNA3.3_KLF4 (16) and pcDNA_TP53 expression plasmids using Nucleofector® Kit V (Lonza, Cologne, Germany).

Cytotoxicity Assays

AML cells were treated with the genotoxic compounds cytarabine (SIGMA-ALDRICH, St. Louis, MO, USA) and idarubicin (Selleck Chemicals, Houston, TX, USA) in equimolar concentrations. To activate p53, cells were treated with the MDM inhibitor Nutlin-3A (Tocris Bioscience, Bristol, UK) and the exportin inhibitor Leptomycin-B (Biovision Inc., Milpitas, CA, USA). Cell viability was determined using the MTT-based in vitro Toxicology assay
Measurement of protein levels by Western Blot

Total protein extracts were prepared by RIPA lysis. 100ug total protein extracts were PAGE separated, transferred to nitrocellulose membrane and stained against mouse anti GAPDH (G8795, SIGMA-ALDRICH, St. Louis, MO, USA) and mouse anti-human p53 (sc-126, Santa-Cruz Biotechnology, Dallas, TX, USA), followed by IRDye® 680RD goat anti-mouse IgG (LI-COR Biotechnology, Germany). Membranes were scanned and quantified on LI-COR Odyssey Infrared Scanner (LI-COR Biotechnology, Germany).

Measurement of mRNA expression by qPCR

RNA was extracted from AML cells and quantified using qPCR. The RNA extraction kit was supplied by Macherey-Nagel, Düren, Germany. Reverse transcription was done with MMLV-RT (Promega, Madison, WI, USA). Real-time PCR was performed on the ABI7500 Real-Time PCR Instrument using ABI universal master mix (Applied Biosystems, Austin, TX, USA) and gene specific probes Hs00269972_s1 (CEBPA), Hs00355782_m1 (CDKN1A), Hs00167918_m1 (GCSFR), and Hs02758991_g1 (GAPDH). Measurements for CEBPA were normalized with GAPDH values (ddCt relative quantitation). Assays were repeated in at least three independent experiments. Statistical analysis was done with GraphPad Prism software using two-tailed t-tests. Data are depicted in column bar graphs plotting mean with SD values.

Antibodies and flow cytometry

Staining for cell surface markers was performed using CD11b-PE Cy7 (Biolegend, San Diego, CA, USA) in FACS staining buffer for 30 min. at 4C and AnnexinV-FITC p53-KLF4-CEBPA in AML.
(ImmunoTools, Germany) in AnnexinV buffer for 15 minutes followed by 7AAD cell viability staining (BD BioSciences, NJ, USA). For cell cycle analysis, cells were fixed in 1% PFA/PBS overnight at 4°C. Staining of intracellular antigens was achieved on fixed cells using Ki67-PE from eBioscience (San Diego, CA, USA) in permeabilization buffer for 30 minutes at 4°C, followed by DNA staining using DAPI (Roche, Switzerland). Data were analyzed using FlowJo and Kaluza software (Beckmann Coulter, USA).

**Chromatin immunoprecipitation (CHIP)**

OCI-AML3 cells were treated with conventional induction therapy, Nutlin-3A or Leptomycin B for 24 hours before fixation in paraformaldehyde. Chromatin was prepared using the CHIP-IT express enzymatic kit (Active Motif Europe, Rixensart, Belgium). Immunoprecipitation was performed using rabbit-anti-human KLF4 (sc-2069) and mouse anti-human p53 (sc-126X, Santa-Cruz Biotechnology, Dallas, TX, USA). Isotype antibodies served as control. Input and CHIP DNA samples were purified using Wizard PCR cleanup system (Promega, Madison, WI, USA) and analyzed by qPCR using ABI universal master mix (Applied Biosystems, Austin, TX, USA) and CEBPA gene specific probes. For the 36kb3’ HCNE, the probes were 36kb-F (5’-GTACACCTCCTGGCCCTTGA-3’), 36kb-R (5’-GCTAACCTGGCTGGGGACGTCGCGGCGGCG-3’) and 36kb-qPCR (5’-FAM-GCCTCTGGGAGGTAGAGGATGG-3’). For the 9kb3’ HCNE, the probes were 9kb-F (5’-GCTAACCTGGCTGGGGACGTCGCGGCGGCG-3’), 9kb-R (5’-GCTAACCTGGCTGGGGACGTCGCGGCGGCGG-3’), and 9kb-qPCR (5’-FAM-GGTCCAAGGAGCGCTTTGTCCT-TAMRA-3’). CHIP qPCR data were normalized applying the percent input method.
Results

**CEBPA activation by p53 and KLF4 in AML cells**

In human AML cells, CEBPA, KLF4 and p53 are frequently deregulated. However, the extent of dysregulation, the molecular interactions and mechanisms leading to dysregulation largely remain to be elucidated. We hypothesized that the CEBPA gene is a p53 and KLF4 downstream target in myeloid cells. Supporting this hypothesis, we found that both p53 and KLF4 were able to induce cellular CEBPA mRNA expression in AML cells in a dose dependent manner (Fig. 1A). To study the activation of the CEBPA gene in a different genomic setting, we created a comprehensive CEBPA-BAC transgene with a luciferase reporter which was transfected into HL60 cells. Similar to the endogenous CEBPA gene, we found that the CEBPA-BAC transgene expression was induced by KLF4 and p53 (Fig. 1B) and repressed by AML1-ETO (17). Induction was consistently higher in stably transfected BAC transgenic cells as they most likely carry multiple copies of the reporter gene.

To define the p53 and KLF4 responsive sites in the CEBPA gene, eleven highly conserved non-coding elements (HCNEs) including the promoter of the CEBPA locus were cloned into luciferase reporter plasmids and tested for activation by p53 and KLF4. The HCNEs located 36kb and 9kb downstream of the CEBPA encoding gene sequence selectively mediated a strong response to p53 and KLF4, respectively (Fig. 1C). Direct activation of CEBPA gene expression by p53 was demonstrated by complete loss of activation in the 36kB3’ HCNE constructs mutated in both half-sites of the predicted p53 binding site, with minor activation still residing in the constructs with only one mutated half-site (Fig. 1D). To define the KLF4 responsive sites, deletion constructs of the 9kB3’ HCNE were created and tested for activation by KLF4. Maximum transcriptional activation was observed in the 9kB HCNE driven reporter containing four predicted KLF4 sites indicating that KLF4 proteins bound to multiple sites may cooperate. In the reporter constructs with only two predicted
sites, KLF4 activation was retained in the N-terminal region of the 9kb' HCNE indicating that the major KLF4 sites are located in this region (Fig. 1E). To confirm the functionality of the presumed binding sites, leukemic cells were treated with conventional cytotoxic drugs and non-genotoxic p53 activating compounds, and transcription factor occupancy was tested by chromatin immunoprecipitation. These experiments consistently identified p53 occupancy on the CEBPA 36kb' HCNE (Fig. 1F), and KLF4 occupancy on the 9kb' HCNE (Fig. 1G).

**Reduced protein levels of CEBPA and KLF4 in AML cells**

The nuclear protein levels of the transcription factors CEBPA and KLF4 were determined in samples from AML patients at diagnosis and from healthy volunteers. Clinical characteristics of the AML study cohort are summarized in Table I, and in further details in a supplemental Table S1. We found that the median levels of nuclear CEBPA protein were reduced by 20% (p=0.03) in AML patients versus PBMCs from healthy volunteers (Fig. 2A). The lowest CEBPA protein levels were found in FLT3-ITD and NPM1 mutant AML samples (p=0.002) and in normal CD34+ selected hematopoietic stem cells (HSCs) (p<0.0001), with median reductions of 50% compared to normal PBMCs. With no apparent differences in CEBPA levels between AML blasts in this cohort and normal HSCs, we investigated whether differing stages of differentiation might account for reduced CEBPA levels. As a surrogate for the degree of differentiation in leukemic cells, CEBPA levels were assessed in subgroups according to the FAB classification. Following this approach, significantly reduced CEBPA levels were found in the minimally differentiated M0 and M1 FAB subtypes as well as in AML that progressed from MDS (Supplementary Fig. S1A). Consistently higher CEBPA levels were present in the more differentiated AML subtypes M3 and M4.

Likewise, KLF4 protein was present at very low levels in HSCs with a median 67% reduction compared to normal PBMCs (p<0.0001). Median levels of nuclear KLF4 protein were reduced by 21% (p=0.3) in AML patients compared to normal PBMCs (Fig. 2B), with a
52% reduction in FLT3-ITD and NPM1 mutant samples (p=0.04). With respect to FAB subtypes, significant reduction of KLF4 levels was found in the M0 and M4 subtypes (Supplementary Fig. S1B), and there was a correlation of CEBPA and KLF4 levels (Spearman coefficient r = +0.472; p<0.0001). In summary, CEBPA and KLF4 protein levels were concomitantly reduced in AML cells, with significant reduction in the least differentiated M0 subtype and in FLT3-ITD and FLT3/NPM1 double mutant AML.

**Inactivation of the p53 pathway in AML cells**

To further assess the p53 pathway dysregulation in AML cells, functionally active p53 levels as well as the nuclear protein levels of MDM2 and XPO1/CRM1 and the cytoplasmic protein levels of CUL9/PARC were determined in samples from AML patients and healthy volunteers. We detected a strong inactivation of p53 function in leukemic cells. The median levels of functional nuclear p53 protein were reduced by 84% in AML patients (p<0.0001) versus healthy PBMCs, with a 98% reduction in FLT3-ITD and NPM1 mutant samples (p<0.0001) (Fig. 2C). Normal HSCs had a 99% reduced p53 level compared to normal PBMCs (p=0.004). With respect to FAB classification, minimum p53 levels were found in M0 cells, followed by M4, M5 and progressed MDS (Supplementary Fig. S1C), with significant reductions also in M1, M2 and therapy-related AML, whereas AML-M3 samples expressed distinctly higher levels. Finally, we observed a correlation of p53 and KLF4 levels (Spearman r = +0.29; p=0.008), and of p53 and CEBPA levels (r = +0.22, p=0.018).

We also assessed levels of the p53 inhibitor MDM2, the nuclear export protein XPO1/CRM1 and the cytoplasmic retention protein CUL9/PARC. The median levels of nuclear XPO1 protein were increased by 50% in AML samples (p=0.048) versus normal PBMCs, with a maximum (90%) increase in NPM1 mutant AML (p=0.002) (Fig. 2D), which was 20% more than detected in normal HSCs (p= 0.2). Again, higher XPO1 levels were present in the least differentiated M0 subtype (Supplementary Fig. S1D). While nuclear
MDM2 protein levels were reduced by 20% in AML cells (p=0.4) versus normal PBMCs (data not shown), the cytoplasmic MDM2 protein levels were increased six-fold in AML samples (p=0.0005), and twelve-fold in FLT3 and NPM1 single mutant subtypes (p=0.003) which is twice above the levels in normal HSCs (p=0.4) (Fig. 2E). Maximum cytoplasmic MDM2 levels were detected in samples from AML progressing from MDS, compared to levels in normal HSCs (p=0.003) (Supplementary Fig. S1E). The median levels of cytoplasmic CUL9 protein were increased five-fold in AML samples (p=0.004) versus normal PBMCs, and more than seven-fold in FLT3-ITD mutant samples (p=0.01) which is two-fold more than observed in normal HSCs (p=0.008) (Fig. 2F). In summary, p53 appears to be broadly inactivated in AML cells, with lowest levels in the FLT3-ITD and NPM1 mutant subsets and in the least differentiated M0 subtype. At the same time, we observed induction of the cytoplasmic protein levels of MDM2 and CUL9/PARC, and a substantial increase in the nuclear XPO1/CRM1 protein levels across various AML subtypes.

**Restoring p53 and CEBPA function by conventional induction therapy and by non-genotoxic p53 activators in AML cells.**

Based on the results above indicating that p53 can directly activate CEBPA gene expression, we investigated whether treating AML cells with p53 activators enhances CEBPA activity. Indeed, we observed that CEBPA can be functionally induced by conventional chemotherapy induction treatment as well as by non-genotoxic p53 activators in OCI-AML3 cells (Fig. 3), but also in MOLM-13 and ML-2 cells (data not shown). While conventional genotoxic induction therapy induces p53 activation via the DNA damage response (18), Nutlin-3A works by blocking the function of the p53 inhibitor MDM2 (19), and Leptomycin-B by inhibiting the p53 nuclear export (20). Consequently, we tested conventional cytotoxic compounds, Nutlin-3A and Leptomycin-B, alone or in combination, in AML cells. Cells were collected after treatment for 24 hours. We observed an increase of total cellular p53 protein p53-KLF4-CEBPA in AML.
(Fig. 3A), with highest levels in Nutlin-3A and Leptomycin-B treated cells. There was also an induction of CEBPA gene expression (Fig. 3B) and transcriptional activation of the CEBPA target genes CDKN1A (Fig. 3C) and GCSFR (Fig. 3D), indicating induction of cell cycle arrest and granulocyte differentiation, respectively. In addition, these treatments led to induction of the p53 inducible pro-apoptotic gene NOXA (Fig. 3E), with highest levels following combination treatment. Reflecting our measurement after 24 hours, it is possible that NOXA gene expression reached higher levels at earlier time points (21), so the observed expression levels may be at the tail-end of induction.

The combination treatment was found to be more effective than single compound treatment, in particular with respect to induction of CDKN1A and NOXA expression where maximum levels were detected following the combination treatment of conventional therapy together with Nutlin-3A. The transcriptional activation effects appeared to be additive for CEBPA and GCSFR expression, and synergistic for CDKN1A and NOXA gene expression, respectively.

Along with the increase of total cellular p53 protein, we also found elevated p53 DNA binding activity (Fig. 3F), with highest levels following Leptomycin-B and combination treatments. Apoptosis and differentiation were induced as demonstrated by an increase of AnnexinV positive cells (Fig. 3G) and CD11b induction (Fig. 3H). Moreover, the proportion of cells in cell cycle arrest and exit were induced (Fig. 3J); whereas conventional induction treatment alone or in combination induced cell cycle arrest in G2/S and cell cycle exit, p53 activation by Nutlin-3A and Leptomycin B induced cell cycle arrest in the G1 phase. Flow cytometry data have been summarized in the supplementary Fig. S2 and in Table S2.

**Sensitivity of AML cells to conventional induction treatment and p53 activators.**

In vitro cytotoxicity assays were performed to determine the sensitivity of AML cells to conventional induction therapy, Nutlin-3A and Leptomycin-B alone or in combination. All
treatment regimens led to a dose-dependent loss of cell viability (Fig. 4). While the combination of conventional induction therapy and Nutlin-3A had synergistic effects on cell viability in all tested doses (Fig. 4A), any combination with Leptomycin-B appeared to have antagonistic effects at low dosages, but synergistic effects at higher dosages (Fig. 3G and 3H). Finally, we performed cytotoxicity assays using AML and normal bone marrow samples, and we observed that normal bone marrow cells appeared to be significantly less sensitive to the treatment regimens described above than AML bone marrow cells (Fig. 4D).

In summary, our data indicate that CEBPA can be functionally restored by p53 activators in AML cells and that combination treatment may be more effective than single agents. We present evidence for a $p53$-$KLF4$-$CEBPA$ pathway which is frequently inactivated in AML cells. While p53 function is strongly repressed, the KLF4 and CEPBA protein levels are also substantially and concomitantly reduced in AML blast cells. CEBPA gene expression can be directly activated by p53 and KLF4, and we have evidence that the p53-KLF4-CEBPA axis may be restored in AML cells following treatment with genotoxic chemotherapy and inhibitors of MDM2 and XPO1. Supposedly, the p53-KLF4-CEBPA axis may also be restored in AML cells by treatment with FLT3 or Cullin inhibitors as summarized in Fig. 5.
Discussion

CEBPA gene expression is regulated by transcription factors including the Wnt signal mediator LEF1 (22), NFKB (23), AML1/RUNX1 (24) and SPI1/PU.1 (25). Here, we identified the tumor suppressors p53 and KLF4 as novel transcriptional activators of the CEBPA gene thereby suggesting a core activation pathway consisting of p53, KLF4 and CEBPA that is blocked in AML. Inactivation of the p53-KLF4-CEBPA axis in leukemic cell leads to loss of cell cycle control, inadequate response to apoptotic signals and loss of myeloid differentiation. Moreover, we showed that CEBPA gene expression can be restored in AML cells by treatment with conventional chemotherapy or novel p53 activating compounds.

CEBPA restricts hematopoietic stem cell renewal and can trigger myeloid differentiation (1). CEBPA gene mutations are present in 7-12% of newly diagnosed AML (26). In addition, there is an apparent loss of detectable CEBPA protein in our AML cohort, with lowest levels in the undifferentiated subtypes M0 and M1 and in AML that progressed from preceding MDS, with particularly low levels in FLT3-ITD and NPM1 mutant samples.

Similar to CEBPA, KLF4 is a transcription factor restricting hematopoietic stem cell renewal and it induces myelo-monocytic differentiation (5) (6). KLF4 mediates the trans-activating effect of p53 on the CDKN1A promoter (7). In AML cells, the KLF4 gene is targeted by miRNA10a which is induced in the presence of mutant NPM1 function (8). KLF4 expression has also been shown to be activated by AML1/RUNX1 (27), whereas the fusion protein AML1-ETO / RUNX1-MTG8 encoded by the t(8;21) translocation inhibits both KLF4 and CEBPA gene expression (17). KLF4 gene mutations have not been reported so far in AML patients; however, we found an apparent loss of detectable KLF4 protein in our AML study cohort, again with lowest levels in the undifferentiated subtype M0 and in FLT3-ITD and NPM1 mutant AML, possibly due to overexpression of miR10A, Cdx2 or HDAC1 as suggested previously (8) (9) (10) (11).
The tumor suppressor p53 is rarely mutated in AML blasts, with the exception of secondary therapy-related AML (28). p53 function, however, is inactivated in the vast majority of AML samples, with a loss of activity of 84% in our AML cohort, again with lowest levels in the undifferentiated subtype M0, and a 98% loss in FLT3-ITD and NPM1 mutant samples. In summary, there appeared to be a correlated reduction in the levels of the three transcription factors p53, KLF4 and CEBPA in AML cells compared to normal PBMCs, with overall lowest levels in the M0 subtype and in FLT3/NPM1 double mutant cells; however, these levels were still higher than those observed in normal HSCs. This does not exclude the possibility that the reduced levels of these transcription factors may reflect, but not necessarily cause the increased proliferation and blocked differentiation of AML cells.

In contrast to CEBPA, KLF4 and p53, the nuclear XPO1 and the cytoplasmic protein levels of the p53 modulators MDM2 and CUL9 in AML cells were not only higher than in normal PBMCs, but also higher than in normal HSCs, indicating that MDM2 assisted p53 export from the nucleus by XPO1/CRM1 (14) and p53 retention in the cytoplasm by CUL9 (29) may be essential for the differentiation block in AML cells. Notably, XPO1 levels were highest in NPM1 mutant AML, with a 90% increase of XPO1/CRM1, and six- and twelve-fold induction of cytoplasmic CUL9/PARC and MDM2, respectively, in our cohort. NPM1 was reported to interact directly with the tumor suppressor p53 in the nucleus, and it enhances its stability and transcriptional activation function (15). Mutant NPM1 (NPMc), however, is exported to the cytoplasm (30). NPMc can no longer stabilize p53 in the nucleus, but may further inhibit p53 by cytoplasmic retention. Increased levels of nuclear XPO1 protein lead to increased cytoplasmic levels of NPMc and MDM2 (31) (14). Low levels of Mdm2 mediate mono-ubiquitination of p53 and result in nuclear export of p53, whereas high levels of Mdm2 mediate p53 poly-ubiquitynation and proteasome-mediated degradation in the nucleus. CUL9/PARC was identified to function similar to NPMc in order to retain p53 in the cytoplasm (29). Notably, CUL9 levels were highest in FLT3 mutant and FLT3/NPM1 double
mutant AML, with a seven-fold induction compared to normal PBMCs and twice the level observed in normal HSCs.

In addition to *MDM2*, *CUL9/PARC* and *XPO1/CRM1*, there may be other *p53* inhibitors deregulated in AML. Indeed, *MDM4*, another RING finger ubiquitin ligase that interacts with *MDM2* and inhibits *p53*, appears to be overexpressed in AML (32) (33). Moreover, it was shown that *p53* is suppressed by overexpression of the transcriptional co-activator *MNL* in some AML (34). Finally, *SIRT-1* also prevents *p53* activation in AML cells (35). Consequently, the list of *p53* regulators and, thus, of potential drug targets steadily continues to grow (36).

Recently, potent and selective small-molecule inhibitors of *MDM2* and *XPO1/CRM1* have been identified (19) (37). These studies have strengthened the concept that selective, non-genotoxic *p53* activation is a viable alternative to current cytotoxic chemotherapy, and first clinical studies with *MDM2* inhibitors have shown promising efficacy (36). Our data suggest that treatment with *p53* activators can reactivate the postulated pathway leading to induction of *CEBPA* gene expression and concomitant inhibition of cell proliferation as well as induction of cellular differentiation towards the granulocytic lineages. Indeed, we found that *CEBPA* as well as the *CDKN1A* and *GCSFR* genes are upregulated in AML cells treated with Nutlin-3A and Leptomycin B.

*CEBPA* induction was also seen in leukemic cells treated with KPT-220, another *XPO1/CRM1* inhibitor (37). This effect, however, may be forestalled in the presence of the AML1-ETO fusion protein in t(8;21) AML. Indeed, *CEBPA* gene expression was not induced by *XPO1* inhibition in Kasumi-1 cells which carry the translocation t(8;21) (37). *MDM2* and *XPO1* inhibitors are not the only small molecule compounds able to induce *p53*. Similar effects may be observed in cells treated with *FLT3* or *Cullin* inhibitors and with *p53* activating compounds such as PRIMA-1 (38) and APR-246 (39). Moreover, *KLF4* and *CEBPA* function may also be restored by compounds inhibiting NPMc function in myeloid
leukemic cells (40) suggesting that multiple options are available for therapeutic modulation of the p53-KLF4-CEBPA axis.

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**Authorship Contribution:** K.S. designed and performed research, acquired and analyzed data, and wrote the manuscript; M.T.M, M.B. and L.V. acquired data; B.U.M. analyzed data; and T.P. designed research, analyzed data and wrote the paper. All authors read and approved the report in its final version.

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References


Figure Legends

**Fig. 1** *CEBPA* gene transcriptional activation by KLF4 and p53.

*CEBPA* mRNA quantitation in ME-1 AML cells induced by KLF4 and p53. A total of 10μg plasmid DNA was nucleofected into 10⁶ cells (A). *CEBPA* reporter activity in P391-HL60 cells induced by KLF4 and p53 and suppressed by AML1-ETO (B). The linearized *CEBPA*-BAC P391 is shown schematically. Stars indicate significant p-values for t-tests in subgroup versus pcDNA-transfected control. *CEBPA* gene HCNE regulated luciferase reporter activity in H1299 cells induced by p53 and KLF4 (C). p53 induction of the *CEBPA* 36kb3’HCNE driven reporter is reduced in p53 single-site and lost in p53 double-site mutation (D). KLF4 induction of the *CEBPA* 9kb3’enhancer driven reporter containing four putative KLF4 binding sites is reduced in the deletion constructs (D). p53 occupancy on the CEBPA 36kb-3’HCNE (E) and KLF4 occupancy on the CEBPA 9kb3’ HCNE (F) in OCI-AML3 cells treated with cytarabin/idarubicin (CI, 100nM), with the MDM2 inhibitor Nutlin-3A (Nut, 2.5μM), or with the Exportin inhibitor Leptomycin B (LMB, 2ng/ml) for 24hrs.

**Fig. 2** Inactivation of CEBPA, KLF4 and p53 in AML blasts and normal HSCs.

Relative quantitation of nuclear protein levels for CEBPA (A), KLF4 (B) p53 (C) and XPO1(D) normalized to Lamin-A (LMNA), and cytoplasmic protein levels for MDM2 (E) and CUL9 (F) normalized to GAPDH. AML and normal samples are depicted in white and grey boxplots, respectively. AML samples have been grouped into FLT mutant (FLT3), NPM1 mutant (NPM), FLT3 and NPM1 double mutant (FLT3+NPM), and all AML (AML). Samples from healthy probands have been grouped into peripheral blood mononuclear cells (PBMC) and normal HSCs (CD34+). Whiskers indicate Tukey test range. Stars indicate significant p-values using Mann-Whitney tests in AML cells versus normal PBMCs. Hashtags indicate significant p-values in AML cells versus normal HSCs.

p53-KLF4-CEBPA in AML.  Seipel et al
Fig. 3  Induction of CEBPA activity in OCI-AML3 cells by conventional induction treatment and small molecule p53 activators.

Induction of p53 protein expression (A), CEBPA mRNA (B), CDKN1A mRNA (C), GCSFR mRNA (D), NOXA mRNA (E), p53 DNA binding activity (F), AnnexinV staining (G), CD11b staining (H) and cell cycle arrest (J) in OCI-AML3 (NPM1 mut, p53 wt) cells treated with cytarabin/idarubicin (CI, 100nM), the MDM2 inhibitor Nutlin-3A (Nut, 2.5μM), and the Exportin inhibitor Leptomycin B (LMB, 2ng/ml) for 24 hours. Cells cycle stage were defined as low in both Ki-67 and DAPI for G0, as high in Ki-67 and low in DAPI for G1, and as high in both Ki-67 and DAPI staining for G2/S/M.

Fig. 4  Sensitivity of AML cells to conventional induction treatment and small molecule p53 activators. Cell viability in OCI-AML3 cells treated for 24 hours with CI and Nut (A), CI and LMB (B), or Nut and LMB (D), respectively. CI was tested at 0, 100nM, 200nM, and 300nM CI; Nutlin-3A at 0, 2.5μM, 5μM, 7.5μM; and LMB at 0, 2, 8, 32 ng/ml in dosages 0, 1, 2, and 3, respectively. Cell viability in normal and AML bone marrow cells (D) treated with cytarabin/idarubicin (CI, 100nM), with the MDM2 inhibitor Nutlin-3A (Nut, 2.5μM), and with the Exportin inhibitor Leptomycin B (LMB, 2ng/ml) for 24hrs. Stars indicate significant p-values using Two-way Anova tests in normal versus AML bone marrow samples.

Fig. 5  Schematic representation of the TP53-KLF4-CEBPA axis (green) impaired by AML specific mutations (white) and altered protein levels of key regulators (red). Therapeutic strategies are indicated to reactivate the p53 tumor suppressor (blue) leading to induction of cell cycle arrest (black). Compounds used in this study include the XPO1 inhibitor Leptomycin-B and the MDM2 inhibitor Nutlin-3A.
Figure 4

A

B

C

D

**Figure 4:** Graphs showing cell viability (%).

**A:** Graph comparing cell viability with different treatments.

**B:** Graph comparing cell viability with different treatments.

**C:** Graph comparing cell viability with different treatments.

**D:** Bar graph showing cell viability with different treatments.

**Notes:**
- **A, B, C:** DMSO, CI, Nut, CI+Nut, LMB, CI+LMB, Nut+LMB.
- **D:** CI, Nut3A, LMB, CI+Nut, CI+LMB, Nut+LMB.
- **Normal BM** vs. **AML-BM**.
Figure 5
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MDS: myelodysplastic syndrome. *Favorable risk AML comprised t(8;21), t(15;17), inv16 and NK AML with NPM1mut or CEBPA mut and FLT3-ITD negative; intermediate risk comprised NK AML without any mutations or with FLT3-ITD and NPM1 mut; adverse risk AML comprised -5, -5q, -7, -7q, t(6;9), t(6;11), 11q23, NK with FLT3-ITD, or complex karyotype abnormalities.
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Inactivation of the p53-KLF4-CEBPA axis in acute myeloid leukemia.

Katja Seipel, Miguel Teixera Marques, Marie-Ange Bozzini, et al.

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