Inactivation of the p53–KLF4–CEBPA Axis in Acute Myeloid Leukemia

Katja Seipel¹, Miguel Teixeira Marques¹, Marie-Ange Bozzini¹, Christina Meinken¹, Beatrice U. Mueller¹, and Thomas Pabst²

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: One hundred ten 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.

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 functions 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 investigated whether normalization.

Materials and Methods

CEBPA reporter plasmids

Highly conserved noncoding elements of the CEBPA gene (HCNE) were defined using the genome browser genome.ucsc.edu. HCNEs were PCR-amplified from human gDNA using primers with restriction sites XhoI (5′) and HindIII (3′) for all HCNEs, with the exception of SacI (5′) and BamHI (3′) for the 33-kb 5′ and 3-kb 3′ HCNEs, and BamHI (5′) and SacI (3′) for the 6-kb 3′ HCNE, and they were cloned into pCRA-Topo (Invitrogen) before being transferred to the pGL4.23 firefly luciferase reporter plasmid (Promega). The Renilla luciferase (Rluc) plasmid was used for normalization.

HCNEs were located on the NCBI 36/bgn18 assembly at chr19:38443446–38443899 (454 bp, 38-kb 3′), chr19:38446041–38446632 (592 bp, 36-kb 3′), chr19:38449792–38450687 (895 bp, 33-kb 3′), chr19:38451402–38451655 (254 bp, 31-kb 3′), chr19:38452815–38453385 (571 bp, 30-kb 3′), chr19:38463178–38463622 (360 bp, 19-kb 3′), chr19:38464933–38465373 (411 bp, 17-kb 3′), chr19:38472626–38473335 (730 bp, 9-kb 3′), chr19:38475809–38476523 (715 bp, 6-kb 3′), chr19:38478999–38479794 (796 bp, 3-kb 3′), and chr19:38485160–38486494 (1,334 bp, 1-kb 5′). All HCNEs were analyzed for transcription factor–binding sites using MatInspector software (Genomatix).

A smaller construct pGL4.23-36-kb3′ (180 bp, 36-kb 3′) encoding the region flanking the putative p53-binding sites in the 36-kb 3′ HCNE was created by internal SacI deletion in pGL4.23-36-kb3′ (592 bp, 36-kb 3′). To test the p53-binding sites in the 36-kb 3′ HCNE, one or both sites were mutated by site-directed mutagenesis to create pGL4.23-36-kbM12 using primer pairs p53_mut_F (5′-CTTCTGAATTCCCGCATCTATC-3′) and p53_mut_R (5′-GTGGATAGATGC GGAAATTC-GAGG-3′) and to create pGL4.23-36-kbM1 using primer pairs p53-mut_F (5′-CCCTGATGCTCGCCGATTCCGAGG-3′) and p53_mut_R2 (5′-GTGGATAGATGCGGAAATTC-GAGG-3′). Oligonucleotide primers were supplied by Microsynth.

To test the KLF4-binding sites in the 9-kb 3′ HCNE, a partial construct pGL4.23-9-kb3C (190 bp, 9-kb 3′) was created by internal SacI deletion in pGL4.23-9-kb3′ (730 bp, 9-kb 3′). 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 pGL4.23-9-kb3N (540 bp, 9-kb 3′). The 730-bp 9-kb 3′ region encoded four KLF4 sites, whereas the 190-bp 9-kb 3′ and the 540-bp 9-kb 3′ 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 HI299 cells using Lipofectamine2000 (Invitrogen). After 24 hours, cells were lysed and luciferase activity was assessed using the Dual-Luciferase-Reporter Assay System (Promega) on a plate reader (Tecan). 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-purocytomycin cassette into RP11-270T13 (Gene Bridges). P391 BAC DNA was prepared from the E. coli H9S96 bacterial culture grown in LB medium with Cm (15 μg/mL), linearized by Sgfl digestion, and subsequently used to generate stable cell lines. Leukemic HL60 cells were transfected with linearized P391 BAC DNA (4 μg/10^9 cells) using the Nucleofector Kit V Program X-001 (Lonza). Cells were cultured for 24 hours before addition of puromycin (0.5 μg/mL). Cultures were expanded to 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 (2.5 μg plasmid per 2 × 10^5 cells) and Renilla reference plasmid using Transit2020 transfection reagent (Minus Bio LLC). After 24 hours, cells were lysed and luciferase activity was measured using the Dual-Luciferase-Reporter Assay System (Promega) on a plate reader (Tecan). 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 patients with AML diagnosed and treated at the University Hospital Bern (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. Mutational screening for NPM1, CEBPA, and FLT3, as well as conventional karyotype analysis of at least 20 metaphases were performed for each patient. Peripheral blood mononuclear cells (PBMC) and bone marrow mononuclear cells
(BMMC) 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). Cell pellets were lysed in 100 μL HEPEs buffer containing 10 mmol/L KCl, 1.5 mmol/L MgCl₂, 1% NP40, 1 mmol/L diithiothreitol (DTT), and 10 mmol/L PMSF. After centrifugation, supernatants containing cytoplasmic proteins were collected. Pelleted nuclei were extracted in 100 μL HEPEs buffer containing 420 mmol/L NaCl, 1.5 mmol/L MgCl₂, 25% glycerol, 0.2 mmol/L EDTA, and 1 mmol/L PMSF. Ape200 Panodrop 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). 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. Measurements for nuclear proteins (p53, CEBPA, KLF4, MDM2, CUL9) were normalized with nuclear LMNA, and measurements for cytoplasmic protein levels (MDM2, CUL9) were normalized with cytoplasmic GAPDH levels. Statistical analysis was performed using the 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, DNA13Amut R882C, NPM1mut, TP53wor), MOLM-13 (AML-M5, t(9;11), FLT3-ITD, TP53wor), ML2 (AML-M4, t(6;11)(q27;q23), TP53wor), MEL1 (AML-M4e, inv16, TP53mut/null), and HL60 (AML-M2, MYCAMP, 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) supplemented with 20% FBS (Biochrom GmbH). ME-1 cells were transfected with pcDNA3.3_KLF4 (16) and pcDNA_TP53 expression plasmids using Nucleofector Kit V (Lonza).

Cytotoxicity assays

AML cells were treated with the genotoxic compounds cytarabine (SIGMA-ALDRICH) and idarubicin (Sellect Chemicals) in equimolar concentrations. To activate p53, cells were treated with the MDM inhibitor Nutlin-3A (Tocris Bioscience) and the exportin inhibitor Leptomycin-B (Biovision Inc.). Cell viability was determined using the MTT-based in vitro toxicology assay (SIGMA-ALDRICH) with four repeat measurements per dosage. Data are depicted as scatter plots of mean and SD values. For bone marrow samples, three samples each of normal and AML bone marrow were analyzed. Statistical analysis was done on GraphPad Prism in grouped tables and significance calculated by two-way ANOVA.

Measurement of protein levels by Western blotting

Total protein extracts were prepared by RIPA lysis. One hundred micrograms total protein extracts was PAGE-separated, transferred to nitrocellulose membrane, and stained against mouse anti-GAPDH (GA8795; SIGMA-ALDRICH) and mouse anti-human p53 (sc-126; Santa-Cruz Biotechnology), followed by IRDye 680RD goat anti-mouse IgG (LI-COR Biotechnology). Membranes were scanned and quantified on LI-COR Odyssey Infrared Scanner (LI-COR Biotechnology).

Measurement of mRNA expression by qPCR

RNA was extracted from AML cells and quantified using the RNeasy kit (QIA). Reverse transcription was done with MMLV-RT (Promega). Real-time PCR was performed on the ABI7500 Real-Time PCR Instrument using ABI universal master mix (Applied Biosystems) and gene-specific probes Hs00269972_s1 (CEBPA), Hs00355782_m1 (CDK11A), 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

For cell surface markers was performed using CD11b-PE Cy7 (BioLegend) in FACS staining buffer for 30 minutes at 4 °C. For AnnexinV-FTTC (ImmunoTools) in AnnexinV buffer for 15 minutes followed by 7AAD cell viability staining (BD Biosciences). For cell-cycle analysis, cells were fixed in 1% paraformaldehyde (PFA)/PBS overnight at 4 °C. Staining of intracellular antigens was achieved on fixed cells using Ki67-PE from ebioscience in permeabilization buffer for 30 minutes at 4 °C, followed by DNA staining using DAPI (Roche). Data were analyzed using FlowJo and Kaluza software (Beckmann Coulter).

Chromatin immunoprecipitation

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). Immunoprecipitation was performed using rabbit-anti-human KLF4 (sc-2069) and mouse anti-human p53 (sc-126X, Santa-Cruz Biotechnology). Isotype antibodies served as control. Input and Chromatin immunoprecipitation (ChIP) DNA samples were purified using Wizard PCR Cleanup System (Promega) and analyzed by qPCR using ABI universal master mix (Applied Biosystems) and CEBPA gene-specific probes. For the 36-kb 3′ HCNE, the probes were 36-kb F (5′-GTACACCTCTGCGCCCTTGA-3′), 36-kb R (5′-GCTAAAGCTTGGGCGACGTTGGGGCC-3′) and 36-kb qPCR (5′-FAM-GAGTGGCGGAGAAGCTTGGAGGGCC-3′). For the 9-kb 3′ HCNE, the probes were 9-kb F (5′-CCTTCTGAGACTCTGAAAACTTGG-3′), 9-kb C (5′-GGAGGTTAGAGGTTGTA-GAAGA-3′), and 9-kb qPCR (5′-FAM-GGGATAGAGGTTGTA-GAAGA-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).

Figure 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 10 μg plasmid DNA was nucleofected into 10^6 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. /C3, significant P values for t tests in subgroup versus pcDNA-transfected control. CEBPA gene HCNEs regulated luciferase reporter activity in H1299 cells induced by p53 and KLF4 (C). p53 induction of the CEBPA 36-kb 3' HCNE-driven reporter is reduced in p53 single-site and lost in p53 double-site mutation (D). KLF4 induction of the CEBPA 9-kb 3' enhancer-driven reporter containing four putative KLF4-binding sites is reduced in the deletion constructs (D). p53 occupancy on the CEBPA 36-kb 3' HCNE (E) and KLF4 occupancy on the CEBPA 9-kb 3' HCNE (F) in OCI-AML3 cells treated with cytarabin/idarubicin (CI; 100 nmol/L), with the MDM2 inhibitor Nutlin-3A (Nut; 2.5 μmol/L), or with the exportin inhibitor Leptomycin-B (LMB; 2 ng/mL) for 24 hours.
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, 11 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 36-kb and 9-kb 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 36-kb 3′ 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 9-kb 3′ HCNE were created and tested for activation by KLF4. Maximum transcriptional activation was observed in the 9-kb 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 9-kb 3′ 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 ChIP. These experiments consistently identified p53 occupancy on the CEBPA 36-kb 3′ HCNE (Fig. 1F), and KLF4 occupancy on the 9-kb 3′ 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 patients with AML at diagnosis and from healthy volunteers. Clinical characteristics of the AML study cohort are summarized in Table 1 and in further details in a Supplementary Table S1. We found that the median levels of nuclear CEBPA protein were reduced by 20% ($P = 0.002$) versus healthy PBMCs, with a 98% reduction in M0 and M4 FAB subtypes as well as in AML that progressed from myelodysplastic syndrome (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 with normal PBMCs ($P < 0.0001$). Median levels of nuclear KLF4 protein were reduced by 21% ($P = 0.3$) in patients with AML compared with 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 cytoplastic protein levels of CIIL3/PARC were determined in samples from patients with AML 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 patients with AML ($P < 0.0001$) 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 with 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 ($r = +0.22$, $P = 0.018$).

### Table 1. Patient characteristics

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*Favorable risk AML comprised t(8;21), t(15;17), inv(6), and normal karyotype AML with NPM1mut or CEBPAmut 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), t(12;21), NK with FLT3-ITD, or complex karyotype abnormalities.

*P < 0.0001. Published OnlineFirst September 25, 2015; DOI: 10.1158/1078-0432.CCR-15-1054

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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 6-fold in AML samples (P = 0.0005) and 12-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 with levels in normal HSCs (P = 0.003; Supplementary Fig. S1E). The median levels of cytoplasmic CUL9 protein were increased 5-fold in AML samples (P = 0.004) versus normal PBMCs and more than 7-fold in FLT3-ITD mutant samples (P = 0.001) which is 2-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.

Figure 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), NPM-mutant (NPM), FLT3 and NPM double mutant (FLT3 + NPM), and all AML (AML). Samples from healthy probands have been grouped into PBMC and normal HSCs (CD34+). Whiskers indicate Tukey test range. *, significant P values using Mann-Whitney tests in AML cells versus normal PBMCs; #, significant P values in AML cells versus normal HSCs.

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

On the basis of 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 (Fig. 3A), with highest levels in Nutlin-3A- and Leptomycin-B-treated cells. 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 (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 proapoptotic 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.
Figure 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; 100 nmol/L), the MDM2 inhibitor Nutlin-3A (Nut; 2.5 μmol/L), and the exportin inhibitor Leptomycin-B (LMB; 2 ng/mL) for 24 hours. Cell-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–S–M, as high in both Ki-67 and DAPI staining for G2–S–M.
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 GSCFR 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 Annexin V–positive cells (Fig. 3G) and CD11b induction (Fig. 3H). Moreover, the proportion of cells in cell-cycle arrest and exit was induced (Fig. 3I), 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 cytometric data have been summarized in the Supplementary Fig. S2 and in Supplementary 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 H). 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 CEBPA 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.
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Discussion

CEBPA gene expression is regulated by transcription factors including the Wnt signal mediator LEF1 (22), NFκB (23), AML1/RUNX1 (24), and SP1/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 HSC renewal and can trigger myeloid differentiation (1). CEBPA gene mutations are present in 7% to 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 inFLT3-ITD and NPM1-mutant samples. Similar to CEBPA, KLF4 is a transcription factor restricting HSC renewal and it induces myelomonocytic 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 miR10A 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 patients with AML; 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 (8–11).

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 with 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 6- and 12-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 polyubiquitination and proteasome-mediated degradation in the nucleus. CUL9/PARC was identified to function similar to NPMc to retain p53 in the
cytoplasm (29). Notably, CIL19 levels were highest in FLT3-mutant and FLT3/NPM1 double mutant AML, with a 7-fold induction compared with 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 upregulated in AML (32, 33). Moreover, it was shown that p53 is suppressed by overexpression of the transcriptional co-activator MN1 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 toward 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 KPI-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 XPOI 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Seipel, B.U. Mueller, T. Pabst
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Inactivation of the p53–KLF4–CEBPA Axis in Acute Myeloid Leukemia

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