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# Proteomics of AML1/ETO Target Proteins: AML1–ETO Targets a C/EBP–NM23 Pathway

**Sheo Mohan Singh · Arun Kumar Trivedi · Savita Lochab · Maximilian Christopeit · Wolfgang Hiddemann · Gerhard Behre**

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**Abstract**
*Introduction* The rational design of targeted therapies for acute myeloid leukemia (AML) requires the discovery of novel protein pathways in the systems biology of a specific AML subtype. We have shown that in the AML subtype with translocation t(8;21), the leukemic fusion protein AML1–ETO inhibits the function of transcription factors PU.1 and C/EBPα via direct protein–protein interaction. In addition, recently using proteomics, we have also shown that the AML subtypes differ in their proteome, interactome, and post-translational modifications.
*Methods* We, therefore, hypothesized that the systematic identification of target proteins of AML1–ETO on a global proteome-wide level will lead to novel insights into the systems biology of t(8;21) AML on a post-genomic functional level. Thus, 6 h after inducible expression of AML1–ETO, protein expression changes were identified by two-dimensional gel electrophoresis and subsequent mass spectrometry analysis.
*Results* Twenty-eight target proteins of AML1–ETO including prohibitin, NM23, HSP27, and Annexin1 were identified by MALDI-TOF mass spectrometry. AML1–ETO upregulated the differentiation inhibitory factor NM23 protein expression after 6 h, and the NM23 mRNA expression was also elevated in t(8;21) AML patient samples in comparison with normal bone marrow. AML1–ETO inhibited the ability of C/EBP transcription factors to downregulate the NM23 promoter. These data suggest a model in which AML1–ETO inhibits the C/EBP-induced downregulation of the NM23 promoter and thereby increases the protein level of differentiation inhibitory factor NM23.
*Conclusions* Proteomic pathway discovery can identify novel functional pathways in AML, such as the AML1–ETO–C/EBP–NM23 pathway, as the main step towards a systems biology and therapy of AML.

**Keywords** AML · Proteomics · AML1–ETO · NM23 · C/EBP · Cancer biology

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## Introduction

The rational design of targeted therapies for cancer and leukemia requires the discovery of novel target proteins and protein pathways in cancer cells, and the determination of their functional relevance in the systems biology of a specific cancer type. Acute myeloid leukemia (AML) with translocation t(8;21)(q22;22) is characterized by the fusion protein AML1–ETO, which is produced by translocation of the *AML1* (*RUNX1/PEBPα/CBFA2*) gene on chromosome 21 to the

*ETO* (*MTG8*) gene on chromosome 8. This fusion protein consists of the N terminus of AML1 (1–177 amino acids) fused to the full length of ETO [1, 2]. Expression of AML1–ETO is detected in 12% of all AML cases and 40% of the FAB subtype M2-AML patients [3–5]. In murine AML models, AML1–ETO alone is not sufficient to induce leukemia [6]. However, in the presence of additional mutations introduced by using a strong mutagen *N*-ethyl-*N*-nitrosourea (ENU), 55% of the transgenic mice develop AML, and the other 45% develop T-lymphoblastic leukemia [7]. In another model, AML1–ETO induces AML-M2 resembling leukemia in cooperation with an activated receptor tyrosine kinase TEL/PDGFβR in all 19 mice transplanted [8]. Other studies have shown that AML1–ETO can lead to malignant transformation of fibroblasts and myeloid cells [9–11]. AML1–ETO promotes the expansion and self renewal capacities of human CD34+ hematopoietic stem cells, the cell type in which the translocation is physiologically found [12, 13]. AML1–ETO is reported to have dominant negative or dominant positive effects on AML1 activity, thus disrupting the normal function of AML1 in hematopoiesis [10, 14–18].

We have recently reported that AML1–ETO upregulates c-Jun expression via the JNK signal transduction pathway, which demonstrates another mechanism of AML1–ETO-induced positive effects on its target proteins [19]. We have previously shown that AML1–ETO blocks myeloid transcription factor C/EBPα by downregulating its mRNA, protein, and DNA binding activity in t(8;21) myeloid leukemia [20], and inactivates the myeloid master regulator PU.1 by direct protein–protein interaction in myeloid differentiation [21]. Our group has routinely applied proteomic approach to analyze proteomic profile of various AML subtypes, target, and interacting protein partner identification in AML and APL cells [22–26]. Because AML1–ETO disrupts and blocks the normal function of myeloid transcription factors and requires other cooperating factors to induce leukemia, we hypothesized that the systematic identification of AML1–ETO target proteins on a global proteome-wide level might lead to novel insights into the pathogenesis and systems biology of AML1–ETO-induced leukemia on a post-genomic functional level.

## Materials and Methods

*Cell Culture* Human myeloid cell line U937 stably transfected with AML1–ETO cDNA under the control of human metallothionein promoter in the expression vector pPC18 (U937Z/A-E) was kindly provided by Dr. P. G. Pelicci (Instituto Europeo di Oncologia, Milan, Italy). Parental cell line U937 was purchased from DSMZ (ACC5) and used as a control throughout this study. A tet-off-inducible U937 cells stably transfected with AML1–ETO under the control of tet-responsive transcriptional repressor tTA (U937T/A-E) and U937 cells containing the empty vector tTA (U937T) was kindly provided by Dr. D. E. Zhang (Scripps Research Institute). These cell lines were cultured as described previously [19].

*Two-Dimensional Gel Electrophoresis To Identify AML1–ETO Target Proteins* U937Z/A-E cells were induced with Zn for 6 or 12 h, and samples were lysed in urea lysis buffer (7 M urea, 2 M thio-urea, 4% CHAPS, 50 mM dithiothreital, 2.5 mM EDTA, and 2.5 mM EGTA). Lysed cells were ultracentrifuged for 90 min at 50,000 rpm at 21°C. In the first dimension, 350 μl of lysed samples was separated on Immobiline™ dry strip pH 3–10 (Amersham Biosciences, Cat. 17-1234-01) by isoelectric focusing on IPGPhor machine (Amersham) as recommended. The separated protein strips were reduced in urea buffer containing 2% DTE and alkylated in urea buffer containing 2.5% iodoacetamide. The strips were loaded and separated on 12% SDS-PAGE in the second dimension. The gels were stained with colloidal Coomassie blue stain (Sigma, Cat. B-0770) as recommended. Differential expression pattern of proteins in uninduced U937Z/A-E and Zn-induced U937Z/A-E gels was done using the ProteomWeaver Software (Definiens). These protein spots were excised and digested overnight with 50 ng trypsin in 50 mM $NH_4HCO_3$, pH 8.0. Digested peptides were eluted in 70% acetonitrile and lyophilized using speed vac. Peptides were resuspended in 5 μl of 10% acetonitrile, 0.1% TFA, and mixed 1:1 with 2,5-dihydroxybenzoic acid matrix solution. Further, mass spectrometry was performed as described previously [23, 26]. The probability-based Mowse score for peptide mass fingerprinting (PMF) database search hits as between 65 and 227; protein scores greater than 62 were significant ($p<0.05$). Twenty-eight target proteins of AML1–ETO were identified. Two-dimensional gel electrophoresis and mass spectrometry were performed from three independent experiments, and only proteins identified in each of those three experiments were considered to be target proteins of AML1–ETO.

*Western Blot Analysis* U937Z/A-E cells were induced with 100 μM $ZnSO_4$ for 6 h, lysed in RIPA buffer, and analyzed for AML1–ETO expression by Western blot as described before [19, 21]. Anti-ETO (Cat. sc9737), anti-NM23-H2 goat polyclonal antibody (sc14790), and anti-β-tubulin rabbit polyclonal (Cat. sc9104) antibodies were purchased from Santa Cruz, and anti-prohibitin antibody (Cat. MS-261-P) was from NeoMarkers, and were used as recommended and described earlier [19, 27].

*Patient Samples and Affymetrix* Patient samples were referred to the Laboratory for Leukemia Diagnostics, Department of Internal Medicine III, Hospital Grosshadern,

for routine cytomorphologic and cytogenetic analyses. At the time each AML patient was diagnosed, mononuclear cells from the bone marrow aspirate with more than 90% blast cells were purified by Ficoll gradient separation. Total RNA was isolated and processed as described before [28]. Standard affymetrix software (Microarray Suite, Version 5.0) and the HG-U133A set of normalization controls were used for data analysis (mask file online available, www.affymetrix.com). As recommended by the manufacturer, 100 human maintenance genes served as a tool to normalize and scale the data prior to performing data comparisons [28]. Expression signal intensities are given as absolute numbers. The error bars indicate the s.e.m.; *n* indicates the number of patient samples analyzed in each subgroup. AML patient samples included FAB M2 patients with translocation t(8;21), M2 with normal karyotype (M2-NK), M2 with complex karyotype (M2-CK), M3 with t(15;17), M3 variants (M3v), M4eo inversion 16, t(11q23), AML complex karyotype, AML normal karyotype, normal karyotype with FLT+, and normal karyotype with MLL-PTD+.

## Electrophoretic Mobility Gel Shift and Luciferase Promoter Assay

Electrophoretic mobility gel shift assay (EMSA) was performed as previously described. For promoter assays human kidney 293T cells were transfected using Lipofectamine reagent (Invitrogen) in 24 well plates as described earlier [29, 30], and myeloid U937 and K562 cell lines were transfected using the effectene kit (Qiagen) as described before [31, 32]. The plasmid constructs used in the transfection assays were NM23h1-luc promoter (kindly gifted by Dr. Dirk Eick, GSF, Munich), human C/EBPα, C/EBPβ C/EBPδ, and AML1–ETO plasmids, which have been previously described [21, 30]. As an internal control plasmid for cotransfection assays, the pRL null (pRLO) construct driving the *Renilla* luciferase gene (Promega) was used as described [33] earlier. Firefly luciferase and *Renilla* luciferase activities were measured in Turner Designs Luminometer using Dual Luciferase Reporter Assay System (Promega). Results are given as means+s.e.m. of three independent experiments.

## Results

*Identification of AML1–ETO-Regulated Target Proteins by 2D Gel Electrophoresis and MALDI-TOF Mass Spectrometry* For the purpose of identifying AML1–ETO-regulated proteins, a myeloid cell line system was used where AML1–ETO is stably transfected in U937 cells under Zn-inducible metallothionein promoter (U937Z/A-E) or under the tet-off-inducible promoter (U937T/A-E) [19, 27]. AML1–ETO protein expression is induced when Zn is added in the medium (Fig. 1a, lane 5) or when tetracycline is removed from the medium (Fig. 1b, lane 4). In vitro translated AML1–ETO was used as a positive control (Fig. 1a, b, lane 1). For identification of AML1–ETO target proteins, AML1–ETO expression was induced in U937Z/A-E cells with Zn for 6 or 12 h, cells were lysed in urea lysis buffer, and proteins were separated by 2D gel electrophoresis. The gels were stained with colloidal Coomassie blue stain (Fig. 1c, d). After comparing uninduced U937Z/A-E cells (gel not shown) with Zn-induced U937Z/A-E cells (Fig. 2) using the ProteomWeaver software (Definiens), differentially expressed protein spots (marked with arrows) were excised and digested with trypsin. The proteins were identified by PMF generated by MALDI-TOF mass spectrometry and MASCOT database search (Tables 1 and 2). The probability-based Mowse score for PMF database search hits were between 65 and 227, and protein scores greater than 62 were significant ($p < 0.05$). As a control, U937 cells were also treated with Zn, lysed in urea lysis buffer, separated on 2D gels, and compared with U937Z/A-E gels (data not shown). We were able to identify new target proteins of AML1–ETO whose function is not yet known, for example, cDNA clone IMAGE 4104570, cDNA clone IMAGE 3905254, hypothetical protein FLJ35908 fragment, and uncharacterized hematopoietic stem/progenitor cells protein MDS032. We also identified other promising target proteins of AML–ETO, e.g. NM23-H1, HSP27, peptidil-prolyl *cis–trans*

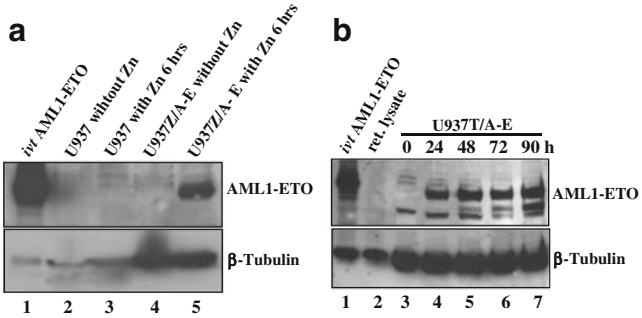


**Fig. 1** Western blot analysis for inducible AML1–ETO expression. **a** U937Z/A-E cells were induced with 100 μM $ZnSO_4$ for 6 h, lysed in RIPA buffer, and analyzed for AML1–ETO expression by Western blot. U937 cells were also induced and used as control. In vitro translated AML1–ETO (*lane 1*) was used as a positive control. As seen in *lane 5*, AML1–ETO expression is increased after Zn induction. **b** U937T/A-E cells were induced by removing tetracycline from the medium for 24, 48, 72, and 94 h. Cells were lysed in RIPA buffer and blotted for AML1–ETO. U937T cells containing the empty vector were used as a control. AML1–ETO expression level was increased after 24 h of tet-off induction (*lane 4*) and was continuously detectable even after 90 h of induction (*lanes 5–7*)

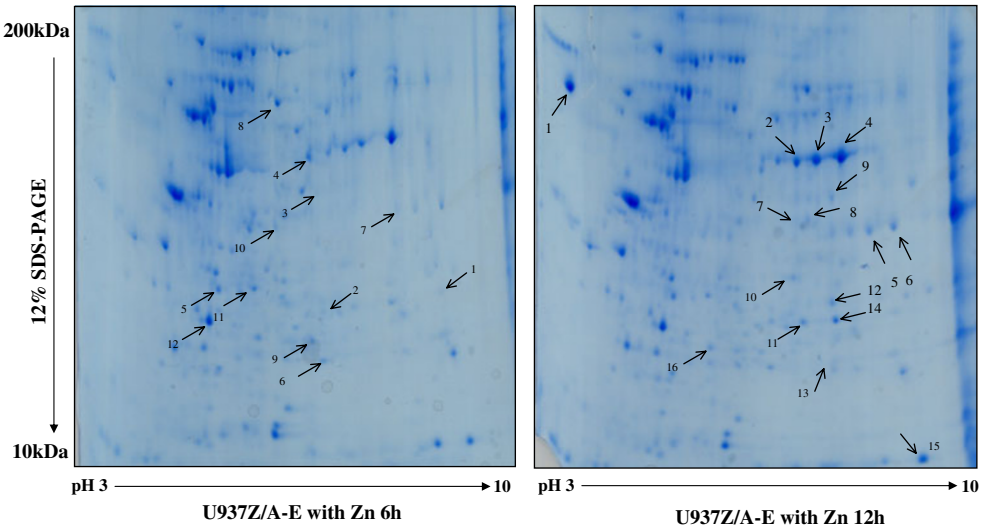


**Fig. 2** Two-dimensional gel electrophoresis to identify AML1–ETO target proteins. U937Z/A-E cells were induced with Zn for 6 or 12 h, and samples were lysed in urea lysis buffer, and lysates were separated in one- and two-dimensional gel electrophoresis and were stained with colloidal Coomassie blue stain (Sigma, Cat. B-0770) to visualize the protein spots. As a control, U937 cells were also induced with Zn, lysed in urea lysis buffer, separated on 2D gels, and compared with U937Z/A-E gels (data not shown)

isomerase A, prohibitin, thioredoxin peroxidase, etc. (Tables 1 and 2). Concordant data were observed with both the inducible cell lines.

NM 23 (nucleoside diphosphate kinase A) is a tumor metastatic process-associated protein reported to be involved in a variety of cellular processes such as cell proliferation, differentiation and development, signal transduction, and G protein-coupled receptor endocytosis [34]. In mouse myeloid leukemia M1 cells, NM23 was identified as a differentiation inhibitory factor [35]. NM23 has been previously shown to correlate with increased cell proliferation in leukemia patient samples and in the leukemic cell line HL60 [36], and in addition, NM23 is expressed at higher levels in CD34+ proliferating hematopoietic progenitors than in the differentiating cells [37]. Another isoform DR-NM23, which is highly homologous to the NM23-H1 and NM23-H2 gene, was cloned from chronic myelogenous leukemia blast crisis primary cells [38]. Overexpression of DR-NM23 inhibits granulocytic differentiation and induces apoptosis in 32Dcl3 myeloid cells [39]. NM23 inhibits the induction of differentiation of mouse myeloid leukemia cells M1 and human erythroleukemia cells HEL, KU812, and K562 [40–44]. The amount of NM23-H1 mRNA and protein decreases during the induced differentiation of promyelocytic leukemia cells HL-60 by TPA or DMSO into monocytic or granulocytic lineage, respectively [45]. Elevated serum levels of NM23 protein has been implicated in AML, CML, ALL, MDS,

**Table 1** AML1–ETO-regulated proteins identified 6 h after Zn induction

| Description | Acc. no. | Mass (Da) | Up/down |
|---|---|---|---|
| *Homo sapiens* cDNA clone IMAGE 4104570 | gi11156428 | 30,383 | Down |
| *Homo sapiens* cDNA clone IMAGE 3905254 | gi10407098 | 34,977 | Down |
| cDNA FLJ11534 FIS, clone HEMBA1002679 | Q9HAJ5 | 71,672 | Down |
| Phosphoglycerate kinase | KIHUG | 44,985 | Down |
| Prohibitin | I52690 | 29,843 | Up |
| Uncharacterized hematopoietic stem/progenitor cells protein MDS032 | Q9NZ43 | 29,440 | Up |
| mRNA for DNA helicase Q1 | BAA07200 | 74,392 | Down |
| TRIP230 | 015154 | 228,116 | Up |
| Nucleoside-diphosphate kinase-NM23-H1g | A33386 | 17,309 | Up |
| dUTP pyrophosphatase | G02777 | 17,908 | Down |
| HSP27 | E980237 | 22,427 | Up |
| HSTROPCR (Tropomyosin) | CAA28258 | 28,185 | Down |

Identification of AML1–ETO target proteins by MALDI-TOF mass spectrometry. After comparing gels for uninduced U937Z/A-E (gel not shown) and Zn-induced U937Z/A-E cells using the ProteomWeaver Software (Definiens), differentially expressed protein spots were excised, digested with trypsin, and identified using mass spectrometry and MASCOT database search. A total of 28 target proteins of AML1–ETO (12 from 6 h and 16 from 12 h Zn-induced condition) are identified. Two-dimensional gel electrophoresis and mass spectrometry were performed from three independent experiments, and only proteins identified in each of those three experiments were considered to be target proteins of AML1–ETO

**Table 2** AML1–ETO-regulated proteins identified 12 h after Zn induction

| Description | Acc. no. | Mass (Da) | Up/down |
|---|---|---|---|
| Calreticulin precursor | A37047 | 48,283 | Up |
| Alpha enolase | ENOA_HUMAN | 47,350 | Up |
| Alpha enolase | ENOA_HUMAN | 47,350 | Up |
| Alpha enolase | ENOA_HUMAN | 47,350 | Up |
| Glyceraldehyde-3-phosphate dehydrogenase | CAA25833 | 36,202 | Up |
| Glyceraldehyde-3-phosphate dehydrogenase | CAA25833 | 36,202 | Up |
| Annexin I | LUHU | 38,918 | Up |
| Hypothetical protein FLJ35908 (fragment) | Q8NA27 | 65,077 | Up |
| Alpha-complex protein 1 | S58529 | 37,987 | Up |
| Proteasome alpha 1 subunit, isoform 2 | AAH02577 | 29,822 | Up |
| Triosephosphate isomerase | TPIS_HUMAN | 26,807 | Up |
| Phosphoglycerate mutase | PMHUYB | 28,900 | Up |
| Probable thioredoxin peroxidase | A46711 | 22,324 | Up |
| Triosephosphate isomerase | TPIS_HUMAN | 26,807 | Up |
| Peptydil-prolyl-*cis-trans* isomerase A | CYPH_HUMAN | 18,098 | Up |
| Glutathion *S*-transferase P1C human | Q15690 | 23,583 | Up |

Identification of AML1–ETO target proteins by MALDI-TOF mass spectrometry. After comparing gels for uninduced U937Z/A-E (gel not shown) and Zn-induced U937Z/A-E cells using the ProteomWeaver Software (Definiens), differentially expressed protein spots were excised, digested with trypsin, and identified using mass spectrometry and MASCOT database search. A total of 28 target proteins of AML1–ETO (12 from 6 h and 16 from 12 h Zn-induced condition) are identified. Two-dimensional gel electrophoresis and mass spectrometry were performed from three independent experiments, and only proteins identified in each of those three experiments were considered to be target proteins of AML1–ETO

malignant lymphomas, and neuroblastma [43, 44, 46–51]. In another study, the cell surface expression of NM23-H1 and H2 was decreased during in vitro erythroid and granulocytic differentiation [43, 46]. Tumor suppressor p53, which is involved in the G1 cell cycle check point, regulates NM23 differentially depending on the cell type. p53 upregulates the expression of NM23-H1 at mRNA and protein level in MCF7 and J7B cells; in contrast, p53 downregulates Nm23-H1 in RKO and H1299 cells [52]. Thus, differentiation inhibitory factor NM23 seems to be a key factor in leukemogenesis. Therefore, we chose NM23 to further characterize its role in AML–ETO-mediated leukemogenesis.

*The Protein Level of NM23 Is Increased After AML1–ETO Induction and NM23 mRNA Expression Is Elevated in t(8;21)-AML Patient Samples* To further confirm the proteomics data, AML1–ETO expression was induced in U937T/A-E cells, and the expression of NM23 protein was analyzed by Western blotting. AML1–ETO increases the NM23 protein level (Fig. 3a, lanes 3–5) in comparison with empty vector U937T cells (Fig. 3a, lane 1) and U937T/A-E cells at 0 h (Fig. 3a, lane 2). Since the NM23 expression is decreased during induced differentiation of leukemic cell lines MEG-01 and HL60 [36, 45], these data suggest that one possible mechanism of AML1–ETO-induced leukemia could be via the increase of NM23 protein level.

To rule out false positives, it was important to confirm more than one target by an independent method. Therefore, we analyzed the prohibitin expression by Western blotting to further validate the proteomics data. Induction of AML1–ETO expression increased the prohibitin protein level (Fig. 3b, lane 4). We further analyzed the NM23 mRNA expression in AML patient samples ($n$=199) by Affymetrix oligonucleotide microarrays (Fig. 4). NM23 expression was elevated in all AML patient subgroups analyzed in comparison with normal bone marrow mononuclear cells (Fig. 4a).

Furthermore, we also analyzed HSP27 mRNA expression in AML patients as another target and found that HSP27 mRNA level was highly elevated in comparison with normal bone marrow cells. (Fig. 4b). These data correlate with the reports published by other groups where NM23 expression is higher in AML patients than in normal bone marrow. Taken together, our data suggest that NM23 is a target of AML–ETO. It is interesting to note that so far the molecular mechanism of how NM23 expression is regulated in AML or how NM23 blocks granulocytic differentiation is not known. Most of the studies have focused on analyzing mRNA or protein levels of NM23 in AML patient samples and correlating it to prognosis. Therefore, further, we sought to investigate the mechanism on how AML1–ETO might regulate NM23.

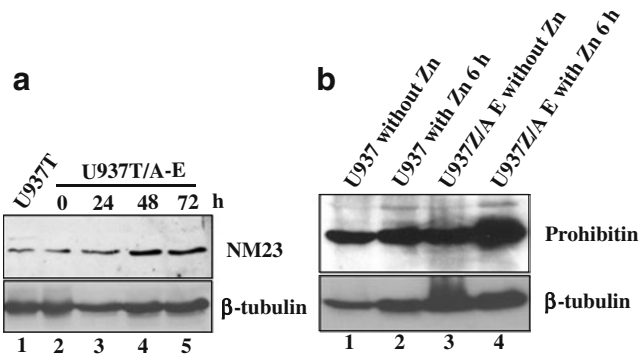


**Fig. 3** AML1–ETO upregulates the protein expression of NM23. **a** U937T/A-E cells were induced by removing tetracycline from the medium for 24, 48, and 72 h. Cells were lysed in RIPA lysis buffer; 80 μg protein was separated on 12% SDS-PAGE and blotted for NM23 with anti-NM23-H2 goat polyclonal antibody (Santa Cruz, Cat. sc14790). NM23 expression was upregulated in AML1–ETO-induced cells (*lanes 3–5*) in comparison with empty vector U937T cells (*lane 1*) and U937T/A-E cells at 0 h (*lane 2*) of *upper panel*. The same blot was stripped and probed for β-tubulin for loading control (*lower panel*). **b** Western blot analysis for prohibitin from U937Z/A-E cells using anti-prohibitin antibody shows prohibitin is upregulated in with AML1–ETO expression after 6 h (*upper panel*). The same blot was stripped and probed for β-tubulin for loading control (*lower panel*)

*AML1–ETO Upregulates NM23 by Blocking the Ability of CCAAT Enhancer Binding Proteins (C/EBP) to Downregulate the NM23 promoter* NM23 and AML1–ETO are involved in a block of granulocytic differentiation [20, 39, 53], and C/EBPα induces granulocytic differentiation [54, 55]. We hypothesized that C/EBPs may downregulate NM23 expression, and AML1–ETO might inhibit this function in order to increase the protein level of NM23 as seen in Fig. 3a. We, therefore, transiently transfected human kidney 293T or myeloid U937 and K562 cells with NM23h1-luc promoter and C/EBPα (Fig. 5a), C/EBPβ (Fig. 5b), or C/EBPδ (Fig. 5c). Twenty-four-hour post-transfection, the promoter-luciferase activity was measured, which shows that C/EBPα downregulated NM23 promoter twofold (Fig. 5a), C/EBPβ fourfold (Fig. 5b), and C/EBPδ fivefold (Fig. 5c). When AML1–ETO was cotransfected with C/EBPα, C/EBPβ, or C/EBPδ, the downregulation of NM23 promoter by C/EBPs was blocked. Next, we also performed EMSA where we used GCSF receptor promoter as positive control (Fig. 5d, left panel) for *ivt* C/EBPα and human NM23 promoter oligo (Fig. 5d, right panel) as probe, which shows that C/EBPα indeed binds and downregulates hNM23 promoter. To our knowledge, this is the first report where we show that C/EBP proteins downregulate the NM23 promoter, and these data for the first time link AML1–ETO and C/EBP to regulate NM23. As NM23 is overexpressed in AML and blocks granulocytic differentiation, downregulation of NM23 might be an important step to induce myeloid/leukemic cell differentiation.

## Discussion

The proteomic approach is being routinely applied to the molecular analysis of various human diseases and in particular cancers such as breast, bladder, colorectal, stomach cancers, and so forth. However, few have been reported on the systematic identification of protein profile changes under a given condition of leukemia. In this study, we applied mass

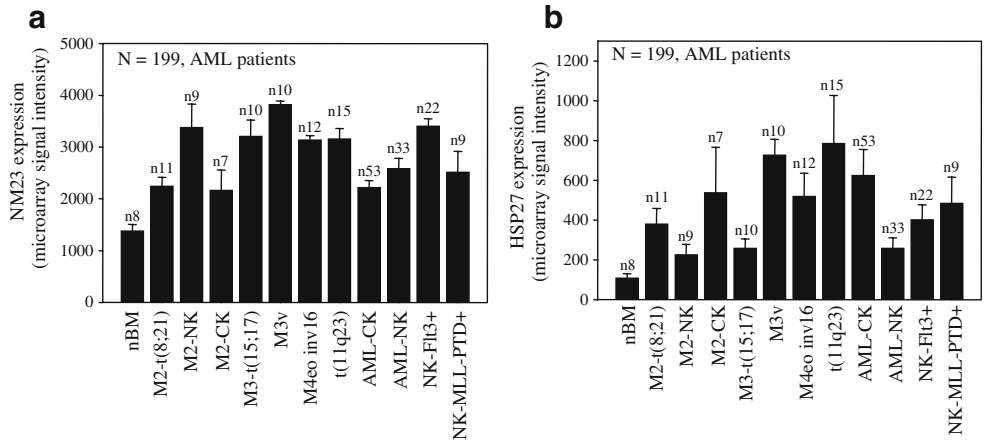


**Fig. 4** NM23 mRNA expression is increased in AML patient samples: **a** NM23 mRNA expression is increased in AML patient samples ($n$=199) in comparison with normal bone marrow (*nBM*) mononuclear cells ($n$=8). AML patient samples included FAB M2 patients with translocation t(8;21), M2 with normal karyotype (*M2-NK*), M2 with complex karyotype (*M2-CK*), M3 with t(15;17), M3 variants (*M3v*), M4eo inversion 16, t(11q23), AML complex karyotype, AML normal karyotype, normal karyotype with FLT+, and normal karyotype with MLL-PTD+. The *error bars* indicate the s.e.m.; $n$ the number of patient samples analyzed in each subgroup. **b** HSP27 mRNA expression is also increased in AML patients which further validates the proteomics data

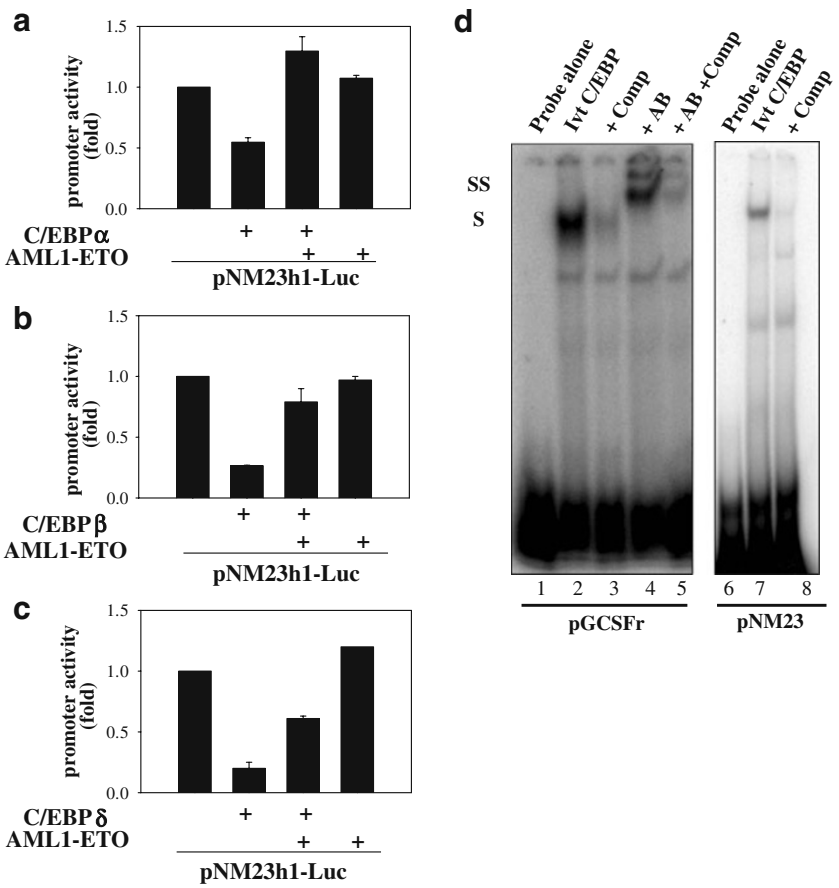


**Fig. 5** AML1–ETO blocks the C/EBP protein-mediated NM23 inhibition: Human kidney 293T cells were transfected with 0.2 μg NM23h1-luc promoter (provided by Dr. Dirk Eick, GSF, Munich), 0.1 μg C/EBPα (**a**), or 0.1 μg C/EBPβ (**b**) or 0.1 μg C/EBPδ (**c**) [30], and 0.2 μg AML1–ETO [21]. **d** EMSA analysis using radiolabeled probe and 6 μg of in vitro translated C/EBPα (*left panel*) showing binding of C/EBPα on GCSF receptor promoter served as positive control; subsequently, binding of human NM23 promoter was observed with same ivt C/EBPα. Results are representative of three separate experiments

spectrometry based proteomic approach to profile the changes in the protein expression pattern of human myeloid leukemia cells U937 with inducible AML1/ETO expression after 6 h. Because the molecular mechanism of how AML1/ETO induces leukemia phenotype is not clearly understood, it could, therefore, facilitate the efforts to establish the molecular mechanism for pathogenesis of acute myeloid leukemia. This could further help to develop a more systematic approach for prognosis and subsequent diagnosis of the AML M2 subtype

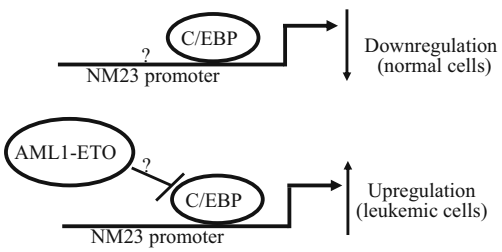


**Fig. 6** Model: AML1–ETO might block myeloid differentiation by blocking the ability of C/EBP proteins to downregulate the NM23 promoter. Our data suggest that C/EBP proteins downregulate NM23, which might be a prerequisite for normal cell growth and differentiation. AML1–ETO blocks this downregulation, thereby increasing the protein level of NM23, which might lead to a block in differentiation and increase in cell proliferation as seen in AML

where AML/ETO is reported to be overexpressed. Our results show that induced expression of AML1–ETO in U937A/E Zn-inducible cell line drastically changes the protein profile. We confirmed that NM23 is target of AML/ETO in Western blotting, and further validated the upregulation of prohibitin ruling out the possibility of identified protein being false positives. Although others have also shown that NM23 is target of AML/ETO, no one has thus far shown the biological relevance of NM23 being a target of AML1/ETO. Using microarray, we were able to show that NM23 is indeed overexpressed in various AML patient samples as compared with normal bone marrow from healthy volunteers. This further strengthens our mass spectrometry and Western blotting data of NM23 upregulation. Apart from NM23, HSP27 another target from identified list was upregulated at mRNA level in various AML samples. Although assessing expression of some of the identified proteins as down-regulated both at protein and mRNA level would had been ideal, however, none of them were interesting and hence not considered. Overexpression of NM23 in myeloid precursor 32Dcl3 cells blocks GCSF-induced granulocytic differentiation [39] and its expression increases in proliferating hematopoietic cells [36, 37], whereas overexpression of C/EBPα leads to granulocytic differentiation [54, 55] and downregulation of cell proliferation [56]. Furthermore, since

AML1/ETO disrupts myeloid differentiation by inhibiting C/EBPα, we hypothesized that C/EBPs may inhibit NM23 expression. Using luciferase promoter assay, we confirmed that C/EBPs do inhibit NM23 expression, and this inhibition was drastically overcome by cotransfection of AMl/ETO with C/EBPs proteins. C/EBPβ and δ had more inhibitory effect on NM23 promoter, which could be due to the presence of variable protein complexes at the target NM23 promoter. In addition to luciferase assay, we also performed EMSA using NM23 promoter, which shows that C/EBPα indeed binds to NM23 promoter and could possibly mediate its inhibitory action upon NM23 (Fig. 5d). Based on luciferase promoter assays, we assume that other C/EBP proteins, which were not included in the EMSA experiments, could bind more efficiently to the NM23 promoter and inhibit its expression. In AML patients, however, NM23 repression via C/EBPα is relieved due to functional inactivation of C/EBPα, which leads to enhanced NM23 expression and subsequent leukemic pathophysiology.

Based on our data, we propose a hypothetical model (Fig. 6) suggesting how AML1–ETO might block myeloid differentiation by blocking the ability of C/EBP proteins to downregulate the NM23 promoter. Our data suggest that C/EBP proteins downregulate NM23, which might be a prerequisite for normal cell growth and differentiation. AML1–ETO blocks this downregulation, thereby increasing the protein level of NM23, which might lead to a block in differentiation and increase in cell proliferation. Thus, proteomic pathway discovery can identify novel functional pathways in AML, such as the AML1–ETO–C/EBP–NM23 pathway, as key step towards gaining insights in to the AML systems biology and its therapeutics.

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