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# Proteomic Identification of the MYST Domain Histone Acetyltransferase TIP60 as a Coactivator of the Myeloid Transcription Factor C/EBPα

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Direktor: **Prof. Dr. med. Wolfgang Hiddemann**

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an der Medizinischen Fakultät der Ludwig-Maximilians-Universität zu München, Germany

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# 1 ABBREVIATIONS

| | |
|---|---|
| ALL | Acute Lymphoid Leukemia |
| AML | Acute Myeloid Leukemia |
| AML1-ETO | Acute Myeloid Leukemia Eight Twenty One fusion protein |
| AR | Androgen Receptor |
| ATRA | All-Trans Retinoic Acid |
| BR-LZ | Basic Region-Leucine Zipper |
| bZIP | Basic Leucine Zipper |
| C/EBP | CCAAT enhancer binding protein |
| CBP | CREB Binding Protein |
| CDK | Cell Division Protein Kinase |
| CHAPS | 3-[(3-Cholamidopropyl) Dimethylammonio]-1-Propanesulfonate |
| ChIP | Chromatin Immunoprecipitation Assay |
| CHOP | C/EBP homologous protein |
| CK | Complex Karyotype |
| CML | Chronic Myeloid Leukemia |
| CREB | cAMP Response Element-Binding Protein |
| DBD | DNA Binding Domain |
| DHB | 2,5-Dihydroxy-Benzoicacid |
| DMEM | Dulbecco´s Modified Eagle Medium |
| DMSO | Dimethylsulfoxide |
| DTE | Dithioerythritol |
| DTT | Dithiothreitol |

| | |
|---|---|
| EDTA | Ethylenediamine Tetra-Acetic Acid |
| EGTA | Ethylene Glycol bis (2-aminoethyl ether)-N,N,N'N'-Tetra Acetic Acid |
| FAB | French American British Classification |
| FBS | Fetal Bovine Serum |
| G-CSFR | Granulocyte-Colony Stimulating Factor Receptor |
| GM-CSF | Granulocyte-Macrophage Colony Stimulating Factor |
| GMPs | Granulocyte/Macrophage Progenitors |
| GST | Glutathione-S-Transferase |
| HAT | Histone Acetyltransferase |
| HDAC | Histone Deacetyltransferase |
| hnRNP | Heterogeneous Nuclear Ribonucleoprotein |
| HSCs | Hematopoietic Stem Cells |
| HTATIP | Histone acetyltransferase TIP60 |
| IB | Immunoblot |
| IEF | Isoelectric Focussing |
| IP | Immunoprecipitation |
| IPTG | Isopropyl-beta-D-Thiogalactopyranoside |
| Ivt | In-vitro Translated |
| LC-MS/MS | Liquid Chromatography/Mass Spectrometry/Mass Spectrometry |
| MALDI-TOF | Matrix-Assisted Laser Desorption/Ionization Time-of-Flight |
| MCM5 | Mini Chromosome Maintenance 5 |
| M-CSF | Macrophage-Colony Stimulating Factor Receptor |
| MS | Mass Spectrometry |

| | |
|---|---|
| MYST | MOZ, Ybf2/Sas3, Sas2 and TIP60 |
| nano-HPLC/MS | nano–high-performance liquid chromatography/mass spectrometry |
| NK | Normal Karyotype |
| NP40 | Nonidet P-40 |
| pI | Isoelectric Point |
| PMF | Peptide Mass Fingerprinting |
| RB | Retinoblastoma protein |
| Rpm | Revolutions per Minute |
| RT | Room Temperature |
| SDS | Sodium Dodecyl Sulphate |
| TAD | Transactivation Domain |
| TBE | Tris-Borate EDTA |
| TIP60 | Tat Interactive Protein 60kD |
| TOF | Time of Flight |
| TRRAP | Transformation/transcription domain-associated protein |
| USF | Upstream Stimulatory Factor |
| WHO | World Health Organization |
| β-ME | β-Mercaptoethanol |
| 2D | 2 Dimensional Gel Electrophoresis |

# 2 INTRODUCTION

## *2.1 Hematopoiesis*

Hematopoiesis is an orderly process of tightly controlled expression of specific transcriptional regulators, growth factors, and growth factor receptors, the combination of which determines lineage commitment and maturation of blood cells. The hematopoetic cells of all lineages derive originally from a relatively small number of committed hematopoetic progenitors, which arise from even fewer hematopoietic stem cells [1]. The very first step in hematopoietic differentiation involves a commitment of the stem cell to one of two main lineages, *myeloid or lymphoid.* Subsequently, progeny of cells committed to each of these two lineages will differentiate further into the various cell types of the hematopoietic system. Pluripotential hematopoietic stem cells are an extremely rare population in the bone marrow (< 0.1 % of nucleated bone-marrow cells) [2], estimated to be approximately one per $10^5$ marrow cells which undergo a decision to either self-renew or remain pluripotent or to differentiate into immature but committed progenitor cells. The differentiation of HSCs to various hematopoietic lineages has been studied intensively and the mechanisms that regulate these processes provide important models for the regulation of cell fate determination. Granulocytes and monocytes, collectively called myeloid cells, are differentiated descendants from common progenitors derived from HSC in the bone marrow. The term *myeloid* is derived from the Greek word for marrow and, indeed myeloid cells constitute the dominant cellular population in bone marrow. Granulocytes and monocytes are key mediators of innate immunity and the inflammatory response.

A classical understanding of the development of mature blood cell types from HSCs is presented in **Figure 1.** This model incorporates findings from immunophenotyping, *in vitro* culture analyses, experimental bone manrow transplantation, and clinical experience. In this view, hematopoiesis is a relatively linear and hierarchical process whereby pluripotent HSCs undergo successive symmetric and asymmetric divisions to yield committed progenitor cells. The latter also possess stem cell-like properties, but exhibit a progressive restriction of cellular fate as they differentiate toward mature lymphoid, erythroid,

megakaryocytic, or myeloid cells. Committed progenitor cells proliferate to meet the enormous daily needs of blood cell production and through this hierarchical system, they ultimately yield the mature elements of blood [1,3]

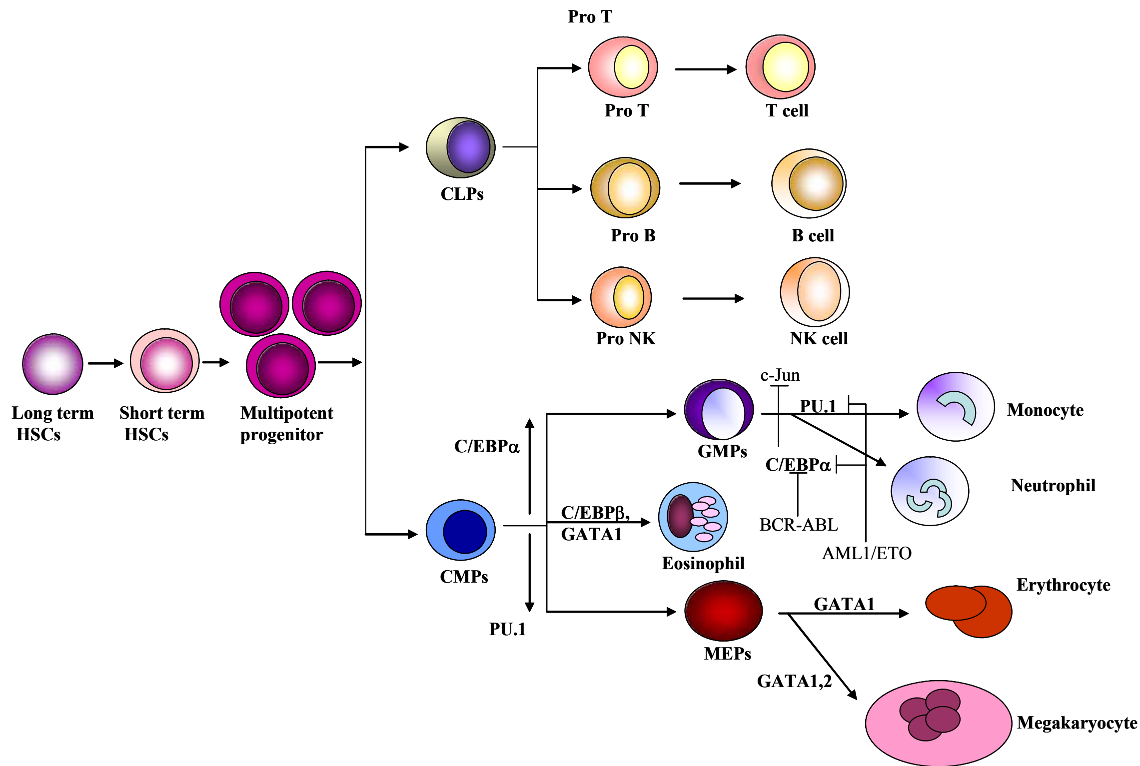



**Fig 1: Role of transcription factors in hematopoietic development:** Long-term and short-term haematopoietic stem cells (HSCs) provide long-term (more than 3 months) and short-term reconstitution in lethally irradiated mice. The common lymphoid progenitors (CLPs) gives rise to T and B cells, whereas the common myeloid progenitors (CMPs) gives rise to granulocyte/macrophage progenitors (GMPs) and megakaryocyte/erythroid progenitors (MEPs). Upregulation of the transcription factor PU.1 is essential for the transition from HSC to CLP, whereas downregulation of PU.1 is required for the differentiation of CMP to MEP. CCAAT/enhancer binding protein(C/EBP)α upregulation initiates the transition from CMP to GMP [4]. Adapted from Nature reviews, Tenen DG [2].

## 2.2 *Transcription factors involved in normal hematopoiesis*

Beside the role of growth factor receptors and their signaling intermediates, a network of transcription factors regulates the expression of a cell type-specific pattern of genes and directs the cells down the path from stem cells and early precursors to fully differentiated cells of the various lymphoid and myeloid lineages [5]. Transcription factors are sequence-specific DNA binding proteins with a variety of functions that include: (i) folding of the DNA molecule, (ii) the initiation of DNA replication and (iii) control of gene transcription. Key transcription factors regulate multiple aspects of hematopoietic differentiation - from lineage commitment through terminal maturation [6]. A number of transcription factors have been identified that play a role in the development of myeloid differentiation. Important information about the role of transcription factors in haematopoietic lineage development has been obtained from studies involving either targeted gene disruption or overexpression of these factors. Transcription factors are also the genes most commonly targeted by leukemia-associated genetic aberrations [7]. An acquired genetic aberration in a relevant transctiption factor can perturb normal hematopoietic development and lead to malignant disease. The study of factors mutated or altered in leukemia has thus led to the identification of new transcription factors involved in hematopoiesis **(Table 1).**

**Table 1: Transcription factors involved in hematopoiesis**

| Factor | Expression | Target Genes | Comments |
|---|---|---|---|
| AML1 | HSCs and most others | M-CSF receptor; T-cell receptor enhancer | Knockouts lack all definitive haematopoiesis; conditional knockouts develop moderate thrombocytosis |
| GATA1 | HSCs,CMPs,MEPs,not GMPs or lymphoid | Erythropoietin receptor and many others | Knockouts lack all mature erythroid cells |
| PU.1 | All progenitors; downregulated in erythroid and T-cells | Receptors for GM-CSF,G-CSF and M-CSF, and many others | Knockout leads to complete loss of macrophages and B cells, delayed development of T cells and granulocytes; the block at the HSC to CLP transition and at the CMP stage |
| | | | |

| C/EBPα | HSCs, CMPs, GMPs not in MEPs or lymphoid | Receptors for G-CSF,IL-6, E2F, c-MYC,; and primary granule proteins | Knockout results in complete loss of granulocytic maturation, block at the CMP to GMP stage, can induce granulocytic differentiation and block monocytic differentiation of multipotential cell lines. |
|---|---|---|---|
| C/EBPβ | most hematopoietic cells | G-CSF and others | Knockout shows not required for myeloid development but has role in macrophage activation, knock-in to C/EBPα locus rescues granulopoiesis; required for emergency granulopoiesis. |
| C/EBPε | Granulocytic and lymphoid cells | Secondary granule proteins | Knockout blocks terminal granulocyte maturation and function. |

Adapted from Nature Reviews Cancer [2],Tenen DG.

## *2.3 Transcription factor C/EBPα in myeloid differentiation*

The first C/EBP protein was identified in the laboratory of Steve McKnight as a heat-stable factor in rat liver nuclei that was capable of interacting with the CCAAT box motif present in several cellular gene promoters and a 'core homology' sequence found in certain viral enhancers [8]. CCAAT enhancer binding protein (C/EBP)α is the founding member of a family of related leucine-zipper transcription factors that play important roles in myeloid differentiation **(Fig. 2)**. There are six members of the C/EBP family: α, β, δ, ε, γ, and CHOP. All C/EBP isoforms share substantial sequence identity (>90%) in the C-terminal 55–65 amino acid residues, which contains the bZIP domain. This domain consists of a basic amino acid-rich DNA-binding region followed by a dimerization motif termed the 'leucine zipper' [9]. Multiple protein isoforms are generated from each CEBP gene through the use of alternative splicing and alternative translational initiation sites. Because some of these isoforms exclude the transcription activation domains, heterodimerization can yield a large numbers of complexes with different functional properties [10-12]. Such interactions are

likely to have a profound influence on the regulation of gene transcription. In contrast with the bZIP domain, the N-termini of the C/EBP proteins are quite divergent (< 20 % sequence identity), except for two short subregions that are conserved in most members. These subregions have been shown to represent the activation domains. The major exception is CHOP, which lacks an activation domain and, therefore, represses gene transcription by forming inactive heterodimers with other C/EBP members [13].

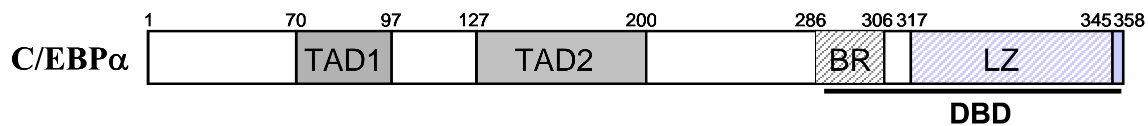



**Fig 2**: The diagram shows the C/EBPα transactivation domains, TAD1, and TAD2 which were defined by Friedman *et al.* [11]. The basic zipper (bZip) domain (DNA Binding Domain; DBD) mediates DNA binding as well as homo- and heterodimerization with other C/EBP proteins.

During differentiation, the fate of a given cell depends as much on the signals that it receives as on its unique response to those signals. So the lineage-restricted transcription factors do not appear to exercise monopoly in differentiation function. None of the C/EBP factors is restricted to myeloid cells, for they are also expressed in other hematopoietic lineages, in liver cell, adipocytes, and other tissues. High-levels of C/EBPα, β, and δ expression is found in granulocytes, monocytes, and eosinophils [14-16]. C/EBPα is the predominant C/EBP factor in immature granulocytes [15,17]. C/EBPα is also abundant in early myeloid cells where it binds and activates key myeloid target genes. In contrast, C/EBPε is found predominantly in maturing granulocytes and T lymphocytes [18]. CHOP is expressed only in granulocytes that are subjected to stress, such as DNA damage [19]. **Figure 3** depicts the temporal expression of various C/EBP proteins during granulopoiesis. The expression of C/EBPα mRNA and protein in early myeloid cells increases up to three fold following induction of granulocytic differentiation by retinoic acid in myeloid cell lines; in contrast, it is rapidly downregulated during monocytic differentiation. These changes in expression are also seen in normal human granulocytes [14] and in analysis of single, primary human hematopoietic cells [20]. Induction of C/EBPα in primary human $CD34^+$ cells, leads to

granulocytic differentiation and inhibits erythrocyte differentiation [21]. Mouse C/EBPα $^{-/-}$ HSCs express elevated levels of the polycomb gene Bmi-1, which can enhance HSC self-renewal [4] and these cells demonstrate a competitive advantage over wild type HSCs [4,22]. This indicates that C/EBPα might normally serve to limit HSC self-renewal. Novel targets of C/EBPα in HSCs such as Id1, a transcriptional repressor of erythrocyte differentiation, have been identified by oligonucleotide arrays, suggesting additional mechanisms by which C/EBPα induces granulopoiesis and blocks other differentiation pathways in early hematopoietic progenitors [21].

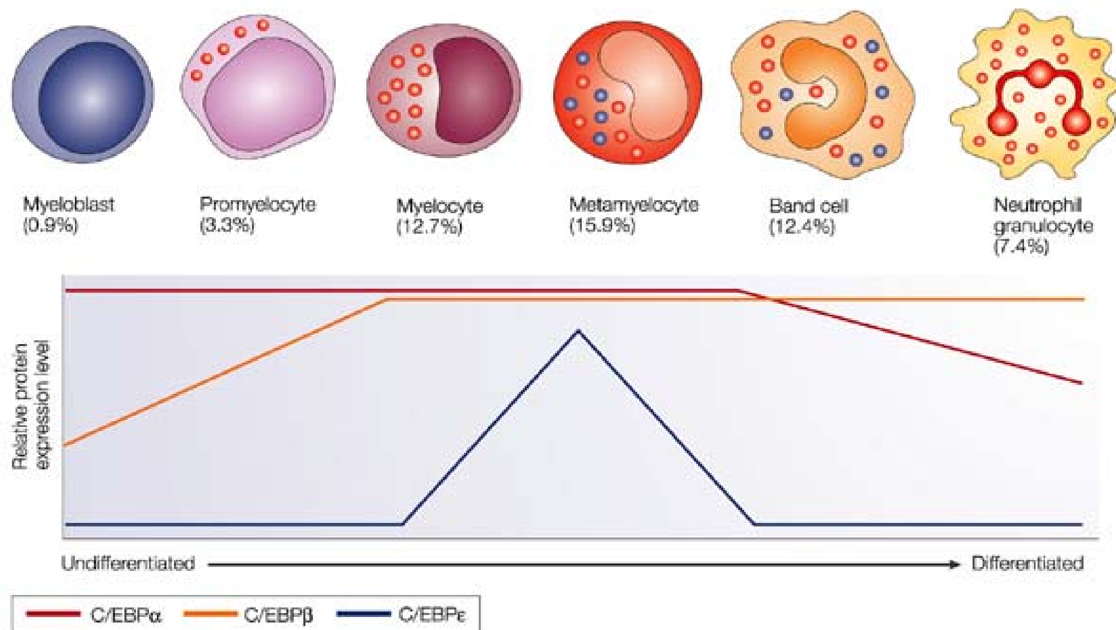



**Fig 3: Expression pattern of C/EBP proteins during granulopoiesis:** Granulopoiesis is the formation of mature neutrophil granulocytes from immature myeloblasts through a differentiation process that involves the cessation of cellular proliferation concomitant with the sequential synthesis of a number of enzymes and structural proteins that are contained in the different types of granules of the granulocyte. The figure shows the relative contribution of C/EBPs to the granulocytic compartment as percent of total nucleated bone-marrow cells, as well as the expression pattern of the C/EBPα, C/EBPβ and C/EBPε

proteins [23-25] during granulocyte differentiation. Adapted from Nature Reviews Cancer [25], Nerlov.

Binding sites for the C/EBPs are present in the promoter regions of numerous genes that are expressed in myeloid cells like G-CSFR (Granulocyte-Colony Stimulating Factor Receptor), myeloperoxidase, neutrophil elastase [26-28]. The human *CEBPA* promoter lacks a C/EBP recognition sequence. However, it can still be activated by C/EBPα, which acts indirectly via stimulation of the DNA-binding activity of USF (Upstream Stimulatory Factor), which in turn interacts with a site present in the proximal promoter region [29]. These studies suggest a role for C/EBPα in myeloid cell development. C/EBPα is also involved in the regulation of cell proliferation [30]. Recently it was reported that C/EBPα causes an acceleration of cell proliferation after being post-translationally modified (phosphorylated) [31]. There are numerous reports demonstrating that C/EBPα activity is regulated at the level of post-translational modifications [31-33].

## *2.4 Acute Myeloid Leukemia (AML)*

Acute myeloid leukemia (AML) can be defined as an accumulation of immature myeloid cells in the bone marrow and blood resulting from dysregulation of normal proliferation, differentiation, and apoptosis. Lineage-specific transcription factors have been identified as key regulators in these differentiation programs [34]. AML is the most common type of leukemia in adults and occurs in approximately one third of newly diagnosed patients with malignant hematological disease.

The fundamental biological feature of the malignant cells in AML is their ability to proliferate continuously with an aberrant or arrested differentiation [35]. The French-American-British classification, introduced approximately 25 years ago [36,37] remains the foundation on which the morphologic diagnosis of AML and ALL is based **(Table 2, Fig. 4)**.

**Table 2: The French-American-British classification of AML**

| FAB Subtype | Description | Comments |
|---|---|---|
| M0 | Undifferentiated | Myeloperoxidase negative, Myeloid markers positive; |
| M1 | Myeloblastic without maturation | Some evidence of granulocytic differentiation |
| M2 | Myeloblastic with maturation | Maturation at or beyond the promyelocytic stage of differentiation; can be divided into those with an AML1-ETO fusion and those without |
| M3 | Promyelocytic | APL; most cases have t(15;17) PML-RARα or another translocation involving RARα |
| M4 | Myelomonocytic | Cases with 11q23/MLL translocations were observed. |
| M4E0 | Myelomonocytic with bone marrow eosinophilia | Characterized by inversion of chromosome 16 involving CBFβ, which normally forms a heterodimer with AML1 |
| M5 | Monocytic | Cases with 11q23/MLL translocations were observed more frequent than in M4 [38]. |
| M6 | Erythroleukemia | Excess of myeloblasts in an erythroid dominant marrow. |
| M7 | Megakaryoblastic | GATA1 mutations in patients with Down's syndrome. |

Adapted from Nature Reviews Cancer [2,37]

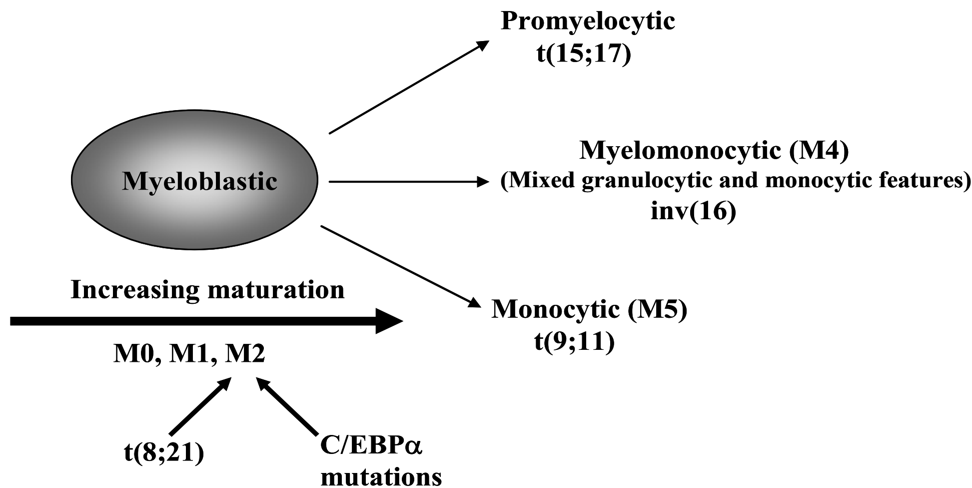



**Fig 4:** Differentiation stages of acute myeloid leukemias that affect the granulocytic and monocytic lineages, according to the FAB classification. The least differentiated myeloblastic leukemia is categorized as M0. Myeloblastic leukemia with some maturation is categorized as M1, and with more maturation as M2. The t(8;21) or mutations of C/EBPα are found in M2 leukemia. Acute promyelocytic leukemia (M3) is associated with the t(15;17). Myelomonocytic leukemia (M4) has both granulocytic and monocytic characteristics. The inv(16) is strictly associated with an M4 leukemia with increased eosinophils. Monocytic leukemia (M5) is associated in some cases with a t(9;11) leading to an MLL/AF9 fusion protein. Adapted from Nature Medicine [39], Hiebert, S.

The classification of AML has recently been revised by a group of pathologists and clinicians under the auspices of the World Health Organization (WHO). While elements of the French-American-British classification were retained (i.e., morphology, immunophenotype, cytogenetics and clinical features), the WHO classification incorporates more recent discoveries regarding the genetics and clinical features of AML in an attempt to define entities that are biologically homogeneous and that have prognostic and therapeutic relevance [36,40]. Each criterion has prognostic and treatment implications but, for practical purposes, antileukemic therapy is similar for all subtypes.

A large number of diverse translocations have been described in AML [41]. The most frequent are the t(8;21): AML1-ETO, t(15;17): PML-RARα, inv(16): CBFB-MYH11 and

t(9;11): MLL-MLLT3, which, together with their variants, are found in approximately 40 % of all AMLs [42]. Many other chromosome translocations have been described in AML, including t(3;5): NPM/MLF1, t(6;9): DEK-CAN, t(16;21): AML1-MTG16, t(7;11): NUP98-HOXA9, which are present, however, in less than 10 % of cases. Random chromosome aberrations have been described in 30 % of AML cases, whereas 20 % of cases displays a normal karyotype [42]. Regardless of subtype, AML is characterized by a defect in the normal process of maturation that converts a myeloid precursor cell into a mature white blood cell. Cytogenetically, AML is probably the most extensively analyzed human neoplastic disease. Numerous chromosomal aberrations specific for AML have been identified, and in many instances, molecular genetic investigations have identified the genes that are affected by these translocations. The study of these genes has elucidated their contribution to the neoplastic process and led to the discovery of signal transduction pathways and transcription factor networks relevant to leukemogenesis. The majority of genes that have been cloned from these breakpoints are known to be involved in cell proliferation, cell death, or cell differentiation. These so-called "oncogenes" are critical components in the multifactorial pathway leading to the development of a malignancy. The more common aberrations have been associated with clinical characteristic, and are now being used as diagnostic and prognostic markers [43]. Studying chromosome abnormalities/rearrangements in leukemia has been a very successful approach for identifying genes responsible for these disorders. Chromosomal rearrangements result in the rearrangement of genes at the location of the breakpoints. The identification of genes at the chromosomal breakpoints is performed by a variety of techniques including positional cloning which is aided by FISH (Fluorescence *in situ* hybridization) breakpoint mapping. By analyzing chromosomal abnormalities at the molecular level new genes that might play a role in AML can be identified.

The genes and fusion genes identified at chromosomal translocation breakpoints in AML can also lead to the development of new treatment strategies. One of the first examples of "targeted leukemia therapy" is acute promyelocytic leukemia (APL). In this leukemia subtype the discovery that the RARα gene is involved in the gene rearrangement resulting

from the t(15;17) is very intriguing, given the clinical response of patients with APL to all-trans retinoic acid [44].

In the past few years, a number of studies have pointed to the dominant role of lineage-specific transcription factors in normal hematopoietic differentiation [26,45,46]. These studies predicted that the function of these transcription factors might be disrupted in AML. Recent studies have confirmed this hypothesis, showing that a number of cases of AML are associated with small mutations in the coding regions of these lineage-specific transcription factors or these factors are inhibited by direct protein-protein interactions [25,47-50]. In addition, in many cases of chromosomal translocations the resulting translocation fusion proteins disrupt the expression and/or function of lineage-specific factors [50,51]. In fact, the most common finding in AML and ALL is the aberrant expression of transcription factors or the production of an abnormal hybrid transcription factor [50-52]. A selection of hematopoietic transcription factors often associated with AML is given in **Table 3**.

**Table 3: Transcription factors associated with AML**

| **Factor** | **Subtype** | **Mutation** |
|---|---|---|
| AML1 | FAB M0 | Often biallelic mutations |
| AML1 | FAB M2 | t(8;21) AML1-ETO and AML1/EVI1 |
| GATA1 | FAB M7 associated with Down's syndrome | Amino terminal mutations similar to those seen with C/EBPα |
| PU.1 | M0, M4, M5, M6 | Mutations were generally heterozygous: DBD, PEST, and TAD domain of PU.1 [53]. |
| C/EBPα | M1, M2 few M4 | Mutation not associated with t(8;21); amino-terminal dominant negative |
| C/EBPα | M2 with t(8;21) | No mutation; |

| | | |
|---|---|---|
| | | downregulation of C/EBPα at the RNA level |
| C/EBPα | CML myeloid blast crisis | No mutation; downregulation at the protein level |
| C/EBPα | APL | No downregulation; loss of DNA binding in cell lines; C/EBPβ and C/EBPε mediate ATRA response in AML. |
| CBFβ | M4e | Inversion of chromosome 16 involving CBFβ, which normally forms a heterodimer with AML1. |

Adapted from Nature Reviews, Cancer [2], Tenen, DG

## *2.5 C/EBPα and Cancer*

C/EBPα-/- mice show a block in hematopoietic differentiation, with an accumulation of myeloblast and an absence of mature granulocytes, similar to what is observed in humans with AML [26]. The expression of C/EBPα, on the other hand, in leukemic cell lines leads to granulocytic differentiation [14,26,50]. Expression of C/EBPα is downregulated in patients with the t(8;21) AML1-ETO translocation [50]. C/EBPα mutations are found in a significant number of AML patients with a normal karyotype [47,54,55].

Furthermore, the roles of C/EBPα in HSCs and for stage- and lineage-specific decisions in granulopoiesis have suggested that C/EBPα might be a tumor suppressor gene. Recent results suggest that the exact expression level of such lineage-specific transcription factors might be of utmost importance. For example, reduction in the expression of the PU.1 gene to 20 % of normal levels in mice leads to the development of AML, whereas a 50 % or an absent expression of PU.1 will result in a different phenotype characterized by accumulation of an abnormal precursor pool retaining responsiveness to G-CSF with

disruption of M-CSF and GM-CSF pathways [56]. A similar strong association between leukemogenesis and hypomorphic transcription factor function may apply to C/EBPα as well, since C/EBPα mutations in AML patients do not occur as bi-allelic null mutations [47,57].

About 7.3% of AML samples carry heterozygous CEBPA mutations. In contrast to wild type C/EBPα, the mutant proteins cannot induce neutrophil differentiation when expressed in bipotential myeloid precursor cells [14,47]. Interestingly, in cases with an N-terminal C/EBPα mutation, a dominant negative isoform of C/EBPα, the p30 protein, which lacks the N-terminal transactivation domain but retains the C-terminal DNA-binding domain, is still expressed from the mutated allele [58,59]. The expression of truncated or other mutant C/EBPα proteins probably facilitates AML development by imposing a differentiation block and disrupting normal cell cycle exit. The hyperproliferating mutant cells may be at increased risk of acquiring other oncogenic mutations that are necessary for transformation and leukemogenesis [60]. Knock-in mice with a targeted mutation in the C/EBPα basic region (C/EBPαBRM2 mutation) that specifically inhibits C/EBPα–E2F interaction showed increased capacity of bone marrow myeloid progenitors to proliferate. Furthermore the mice were predisposed to a granulocytic myeloproliferative disorder resulting in the transformation of the myeloid compartment of the bone marrow. This indicates that disrupting the cell cycle regulatory function of C/EBPα is sufficient to initiate AML-like transformation of the granulocytic lineage [61].

**C/EBPα function is impaired in specific subgroups of AML by either of the following:**

1) Mutations of the CEBPA gene in acute myeloid leukemia as described above.

2) Transcriptional modulation of C/EBPα by AML1-ETO both *in vitro* and *in vivo* [50] or by FLT3/ITD signaling *in vitro* in 32D cells [62]. Interestingly, other leukemic fusion proteins involving CBF family members such as the AML1–MDS1–EVI1 or the CBFB–MYH11 fusion have not been shown to suppress C/EBPα mRNA [63,64].

3) Epigenetic mechanisms: C/EBPα promoter hypermethylation has been reported in two out of 23 AML-M2 patients [65]. Recently, the C/EBPα upstream region and not the core

promoter region has been shown to be hypermethylated in 12 out of 15 lung cancer cell lines and 81 out of 120 primary lung tumors associated with low or absent C/EBPα expression [66].

4) Posttranscriptional regulation of C/EBPα: C/EBPα mutations are not found in CML but expression of C/EBPα protein is not detectable in primary cells from patients with CML [51]. The C/EBPα mRNA is clearly present in CML samples. The expression of C/EBPα was found to be suppressed at the translational level by interaction of the poly(rC)-binding protein hnRNP E2 with C/EBPα mRNA [67].

C/EBPα mRNA levels remain unchanged in AML with t(3;21) encoding the AML1–MDS1–EVI1 fusion gene (AME) where the expression of AME regulates the rate of mRNA translation and suppresses C/EBPα protein in a conditional cell line model and in AML patient samples. Calreticulin, an RNA-binding protein which is strongly activated in AME patients interacts with stem loop structures of C/EBPα and C/EBPβ mRNAs. This leads to inhibition of translation of C/EBP proteins *in vitro* and *in vivo* [68]. Furthermore, inhibition of calreticulin by siRNA restores C/EBPα levels [63,64]. Interestingly, posttranscriptional suppression of C/EBPα by activated calreticulin was also observed by the CBFB–MYH11 leukemic fusion protein [64]. These observations indicate that the down regulation of C/EBPα activity by one of several mechanisms is necessary for the decreased differentiation and increased proliferative capacity of cancer cells, specifically in those tissues where C/EBPα controls normal differentiation.

5). Post translational modifications regulating C/EBPα activity: Phosphorylation of C/EBPα at serine 21 is mediated by extracellular signal-regulated kinases 1 or 2 (ERK1/2), which recognize serine 21 of C/EBPα as a substrate through an FXFP docking motif. This phosphorylation induces a conformational change in C/EBPα such that the transactivation domains of two C/EBPα molecules within a dimer move further apart and this favors monocyte differentiation by blocking granulopoiesis. Activated Ras appears to act on serine 248 of the C/EBPα transactivation domain [32]. In liver tumor cells, the activation of the PI3K/Akt pathway blocks the growth inhibitory activity of C/EBPα through the PP2A-

mediated dephosphorylation of C/EBPα on serine 193, leading to a failure of C/EBPα to interact with and inhibit cdks and E2F. Mutations of serine 193 abolish the ability of C/EBPα to cause growth arrest [69]. Active JNK1 inhibits ubiquitination of C/EBPα possibly by phosphorylating its DBD and prolongs C/EBPα protein half-life [70].

C/EBPα expression is known to be downregulated in lung cancer [71], hepatocarcinomas [10,72] and squamous cell carcinomas [73,74]. In addition to regulating terminal differentiation, C/EBPα can be induced by stress signals that inhibit cell proliferation during DNA repair, which could contribute to its role in tumor suppression [60].

## *2.6 Importance of protein-protein interactions in AML: C/EBPα Network*

### *C/EBPα is part of a network of interacting proteins*

Proteins never act in isolation; instead, they combine to form multiprotein complexes that function as "molecular machines." In the last several years, a plethora of C/EBPα-interacting proteins have been identified, which may explain the multitude of C/EBPα functions **(Fig.5).**

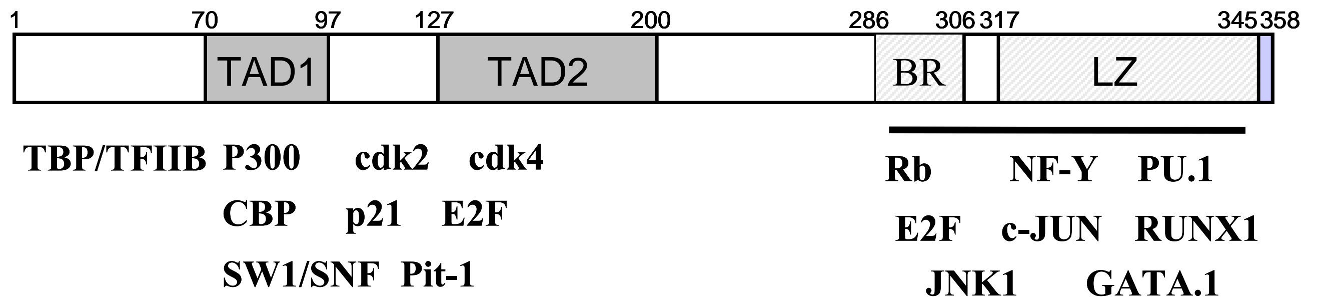



**Fig.5:** Schematic representation of C/EBPα. TAD1 and TAD2: trans-activation domain 1 and 2; BR: basic region; LZ: leucine zipper. Proteins known to physically interact with specific regions on C/EBPα are shown [45,70,75-85].

Recently, we and other groups have shown that the activity of C/EBPα in various processes like growth arrest, differentiation and proliferation depends in part, upon the protein

partners interacting with C/EBPα. We have shown that C/EBPα functionally inactivates PU.1 by displacing c-Jun, the coactivator of PU.1. This leads to the inhibition of cell fates specified by PU.1 and directs cell development to the granulocyte lineage [81]. C/EBPα downregulates c-Jun expression and its transactivation capacity to promote granulocytic differentiation [45]. Furthermore, phosphorylation of serine 248 of C/EBPα via Ras signalling enhances the activity of C/EBPα to induce granulocytic differentiation [32]. C/EBPα interacts directly with CDK2 and CDK4 and arrests cell proliferation by inhibiting these kinases [76]. Aging switches the C/EBPα pathway of growth arrest in liver from cdk inhibition to repression of E2F transcription [86-88]. The retinoblastoma protein interacts directly with C/EBPα [79]. Ser193-dephosphorylated C/EBPα interacts with retinoblastoma protein (Rb) independently on E2Fs and sequesters Rb, leading to a reduction of E2F-Rb repressors and to an acceleration of proliferation. P300 acts as a co-activator of C/EBPα [77]. C/EBPα has also been shown to interact with the SWI/SNF chromatin-remodeling complex during the regulation of differentiation-specific genes [84]. C/EBPα fails to suppress proliferation in SWI/SNF-defective cell lines after knock-down of SWI/SNF core components or after deletion of the SWI/SNF interaction domain in C/EBPα. C/EBPα interacts directly with the E2 SUMO-conjugating enzyme Ubc9 and can be SUMOylated *in vitro* using purified recombinant components [89]. C/EBPα recruits the coactivator CBP and triggers its phosphorylation [78]. The t(8;21) fusion product, AML1-ETO, associates with C/EBPα, inhibits C/EBP-α-dependent transcription, and blocks granulocytic differentiation [50]. C/EBPα interacts specifically with p21 [82] and Cdk2 [76] to directly inhibit the Cdk enzymatic activity required for cell cycle progression. Interaction of JNK1 with C/EBPα prolongs C/EBPα protein half-life leading to its enhanced transactivation and DNA-binding capacity [70].

All these findings demonstrate the crucial role of C/EBPα protein interactions in the myeloid differentiation program. Perturbations of interacting partners of a particular protein could contribute to the differentiation block in AML [90]. In addition, post translational modification of C/EBPα might be important in both normal and leukemic hematopoiesis. Furthermore, factors regulating C/EBPα activity could become targets for therapeutic intervention.

## 2.7 *Proteomics of interacting proteins*

The study of protein-protein interactions has provided an immense insight into human biology. A protein role is reflected in its interaction with other proteins. Therefore, the identification and analysis of multiprotein complexes is a mechanism to better understand protein function and cell regulation. Since errors in protein-protein interactions can manifest as human disease, the identification of protein-protein interactions holds great potential for the definition of new targets for therapeutic intervention [91]. Protein interactions can be determined by a combination of methods that exploits the high affinity nature of protein-protein interactions to capture protein complexes and the application of ultrasensitive protein identification techniques.

Interacting proteins can be identified by using methods like the yeast two hybrid screen [92], identification of affinity purified proteins by mass spectrometry, genetic interactions and phage display or by bioinformatic methods which are based on the assumption that homologous sequences interact with similar proteins [93].

**Affinity purification coupled with mass spectrometry**

The emergence of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry within the past decade has greatly simplified the ability to identify proteins using an approach termed proteomics. Proteomics generally involves separating a mixture of proteins in a given system using techniques such as 2D gel electrophoresis (here proteins are separated by isoelectric point focusing in the first dimension and subsequently by their molecular weight in the second dimension) followed by staining to detect protein bands. These bands are then excised, digested in-gel with a protease (Trypsin), and subjected to mass spectrometry. Each protein exhibits a specific digestion pattern, which is manifested on MALDI as a specific mass fingerprint. This allows the identification of unknown proteins by comparing their digestion pattern to a database containing the proteolytic masses of known proteins. A variety of databases and algorithms have been constructed and developed to search for corresponding fragments within an organism's

genome [94]. These techniques lead to the identification of protein complexes directly on a proteome-wide scale.

A key contribution to the identification of interacting proteins in stable complexes in cellular systems is provided by affinity-based approaches. The basic idea is to express the protein of interest with a suitable tag (such as the Glutathione S-transferase) to be used as a bait to fish its specific partners out of cellular extracts [95]. Individual components within the multi-protein complex can then be identified by mass spectrometry. Using a combination of affinity purification with two-dimensional and/or one-dimensional electrophoresis followed by mass spectrometry-based identification, we reported novel interacting partners of C/EBPα as shown in **Fig. 6**.

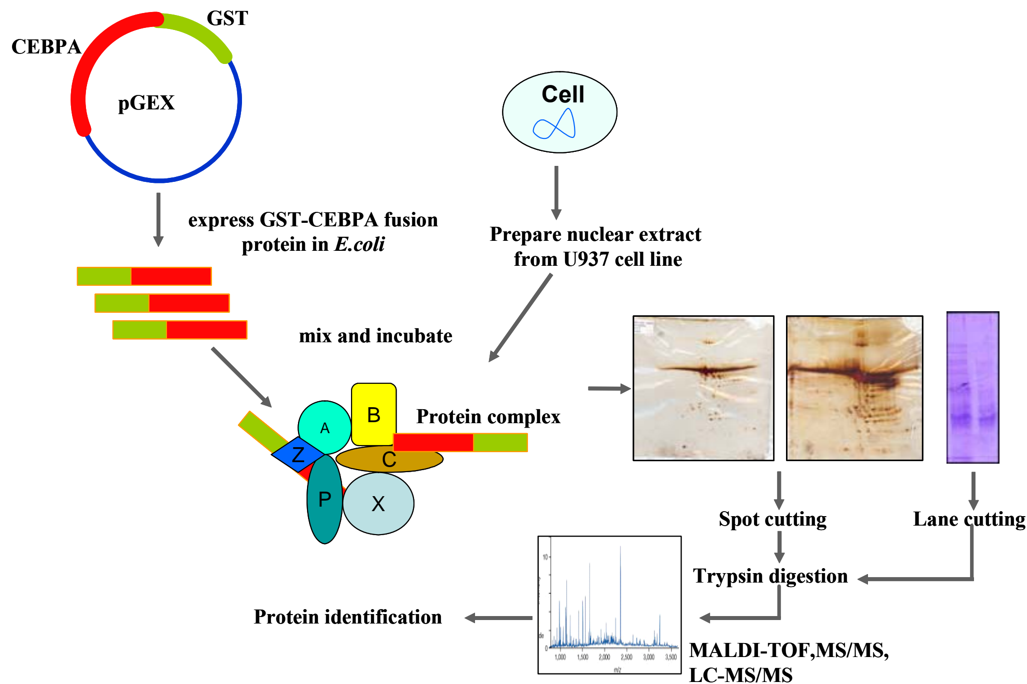



**Fig 6:** The proteomics approach employed to detect interacting proteins of the myeloid transcription factor C/EBPα.

## *2.8 Protein identification*

### 2.8.1 By peptide mass fingerprinting (PMF)

In this technique, the mass of peptides from a tryptic-digested protein are determined using MALDI-TOF mass spectrometry. Here, no information about peptide sequence is

generated but the set of measured peptides for that protein is characterized and can serve as a fingerprint that enables its identification [96]. Proteins are identified by a statistically significant overlap between the experimentally determined and theoretically predicted peptide masses. Peptide mass fingerprinting works by the statistical rationale that although a single peptide mass might correspond to many different peptides in many different proteins, it is extremely unlikely that the same set of peptide masses would be found in a number of different (random) proteins by chance. The advantages of the technique are that it is experimentally simple to perform, very sensitive, fast and that the results are usually straightforward to interpret. The downsides are the statistical limitations imposed by the method. These include the requirement that the majority of the coding sequence (> 80%) of a protein has to be present in a database and that a sufficiently high number of peptides must be detected in the experiment. For the analysis of protein complexes, this means that PMF can only be used in conjunction with 1D or 2D gels as an initial protein separation step [97].

### 2.8.2 By tandem mass spectrometry

An alternative approach that overcomes some of the limitations of peptide mass fingerprinting (PMF) uses a combination of partial peptide sequence and mass information for protein identification (tandem MS or MS/MS). In this technique, the mass of a particular peptide is measured first, and then the peptide is isolated from the mixture (within the mass spectrometer) and subjected to collisions with inert gas molecules. These collisions result in cleavage of the peptide along the peptide backbone and create a set of fragments that differ in length by one amino acid each. The masses of the fragments can again be measured within the mass spectrometer to produce a series of signals which correspond in mass to adjacent amino-acid residues in the sequence. Quite often, only a part of the sequence can be read from the sequence. However, this stretch of consecutive sequence is locked within the peptide by the masses of the fragments that define the beginning and the end of the determined sequence. Information on peptide sequence, peptide mass and fragment mass can be queried simultaneously against a database in which the fragmentation patterns of all peptides derived from all proteins in that database are

computed and compared to the experimentally determined spectrum in order to identify the underlying protein [98,99]. Each analyzed peptide independently identifies a given protein provided that this peptide sequence is unique. Analysis of many peptides of the digested protein can confirm the identification of a protein or identify a different protein that happens to be part of the mixture. Even a consecutive sequence read of three or four amino acids from a single partially sequenced peptide can be sufficient for protein identification.

We have combined tandem mass spectrometry with nano-LC (liquid chromatography) peptide separation [100]. NanoLC-MS is much more amenable to automation, provides enhanced sequence coverage of a protein (for example, analysis of post-translational modifications) and generally allows more efficient handling of complex mixtures especially if the relative quantities of proteins in the sample are very different. NanoLC-MS provides a sufficient dynamic range to allow the identification of proteins that constitute as little as 2–5% of the total protein mixture [97].

The above described strategies were applied to the identification of the protein partners of C/EBPα. Since C/EBPα plays a critical role in the activation of multiple signaling pathways, determination of its interacting proteins and those that form the complex of proteins within its 'interactome' may provide novel insights into its function, as well as reveal potential targets for novel therapies. Moreover, the identification of protein partners interacting with a given protein will lead to the description of cellular mechanisms at the molecular level.

## *2.9 TIP60*

It has become increasingly evident that the regulation of gene expression and hence cellular differentiation involve a fine balance of histone acetylation and deacetylation mediated by transcription factors [101]. Reports have suggested that C/EBPα uses these mechanisms for transcriptional regulation [77,102]. In our efforts to identify interacting partners of C/EBPα, we identified the histone acetyltransferase TIP60. The identification of TIP60 as a novel interacting partner of C/EBPα is intriguing because TIP60 has been shown to be an

interacting partner of MYC and E2F. In these interactions, TIP60 is recruited by MYC and E2F to the promoter of target genes [103,104]. In addition, TIP60 is known both as a coactivator [105] and corepressor [106] for a number of different transcription factors. Furthermore, TIP60 is part of the NuA4 DNA repair complex [107]. Based on these findings, we selected the C/EBPα - TIP60 interaction for further characterization.

## *2.10 MCM5*

Among others, we also identified the cell cycle regulator protein MCM5 as an interacting protein of C/EBPα. MCM5 is a member of the minichromosome maintenance (MCM) family of proteins. Initiation of DNA replication requires the function of MCM gene products, which participate in ensuring that DNA replication occurs only once during the cell cycle. MCM5 has been found to be upregulated in a human leukemia cell line that is resistant to doxorubicin, an anthracycline anticancer agent [108].

# 3 MATERIALS

## 3.1 *Biological material*

### 3.1.1 Bacteria

- *Escherichia coli* DH5α
- *Escherichia coli* BL21

### 3.1.2 Patient samples

Leukemia patient samples analysed by microarray were referred to the Laboratory for Leukemia Diagnostics, Department of Internal Medicine III, Hospital Grosshadern, for routine cytomorphologic and cytogenetic analyses. All samples were from the time of diagnosis and selected for mononuclear cells by Ficoll gradient separation. Microarray analysis was performed as described [109].

### 3.1.3 Mammalian Cell lines

- 293T (human embryonic kidney fibroblast cell line)
- U937 (human myeloid cell line)
- K562 C/EBPα-ER (β-estradiol inducible C/EBPα, Kind gift from D G Tenen)

## 3.2 *Cell culture media*

50 ml heat-inactivated FBS (56ºC, 30 min), sterile filtered.
5 ml penicillin/streptomycin solution (10,000 U penicillin/ml, 10 mg/ml, respectively)
RPMI 1640 or DMEM to complete 500 ml volume

## 3.3 *Chemicals, Commercial solutions*

**Table 4a:**

| Reagents | Suppliers |
|---|---|
| β-Estradiol | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |

| | |
|---|---|
| 2,5-Dihydroxy-Benzoic Acid | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| 2-Hydroxy-5-Methoxy-Benzoic Acid | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| Acetic Acid | Merck, Darmstadt, Germany |
| Acetonitrile | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| Acetyl Co A | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| Ammonium bicarbonate | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| Ammonium persulfate | Fluka-Sigma Chemie GmbH, Steinheim, Germany |
| Ampicillin sodium salt | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| Amplify solution | Amersham Biosciences, U.K. |
| Aprotinin | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| Boric acid | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| Bradford assay buffer | Biorad Laboratories, Germany |
| Bromophenol blue | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| Cesium chloride | MP Biomedicals Inc., Ohio, USA |
| Chloroform | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| Chymostatin | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| Colloidal Commassie Blue G-250 | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| Deoxycholate sodium salt | Merck, Darmstadt, Germany |
| Deoxynucleotide Triphosphates (dNTPs) | Promega Corporation, Madison, USA |
| Deoxyribonuclease I, Amplification grade | Invitrogen Life Technologies, Karlsruhe, Germany |
| Dialysis bag | Carl Roth GmbH, Karlsruhe, Germany |
| Dimethyl Sulfoxide | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| Dithioerythritol (DTE) | Merck, Darmstadt, Germany |
| Dithiothreitol (DTT) | Merck, Darmstadt, Germany |
| DNA fish sperm | SERVA Electrophoresis GmbH, Heidelberg, Germany |
| ECL detection kit | Amersham Biosciences, Uppsala, Sweden |
| ECL hyperfilm | Amersham Biosciences, Uppsala, Sweden |
| Ethanol | Merck, Darmstadt, Germany |
| Ethylene Glycol bis (2-aminoethyl ether)-N,N,N'N'-Tetra acetic Acid | Merck, Darmstadt, Germany |
| Ethylenediamine Tetra-Acetic Acid (EDTA) | Merck, Darmstadt, Germany |
| Fetal Bovine Serum | Gibco BRL, Life Technologies. Paisley, Scotland |
| Formaldehyde | Merck, Darmstadt, Germany |
| Giemsa’s solution | Merck, Darmstadt, Germany |
| Glass beads, 150-212 µm | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |

| | |
|---|---|
| Glycerol | MP Biomedicals Inc., Ohio, USA |
| Glycine | ICN Biomedicals GmbH, Eschwege, Germany |
| Glycogen | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| GST sepharose beads 4B | Amersham Biosciences, Uppsala, Sweden |
| HEPES | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| IGEPAL® CA-630 | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| Immobilin dry strips (IPG strips, pH 3-10) | Amersham Biosciences, Uppsala, Sweden |
| Immobilon-P, PVDF (0.45µm) | Millipore. Billerica, Massachusetts, USA |
| Isopropyl-beta-D-thiogalactopyranoside (IPTG) | Biomol GmbH, Hamburg, Germany |
| Leupeptin hydrochloride | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| Lipofactamine™ transfection reagent | Invitrogen Life Technologies, Karlsruhe, Germany |
| Lithium Chloride | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| Luria Agar | Gibco-BRL, Paisley, Scotland |
| Luria Broth Base | Gibco-BRL, Paisley, Scotland |
| Lysozyme | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| May-Grünwald's eosine-methylene blue solution modified | Merck, Darmstadt, Germany |
| Methanol | Merck, Darmstadt, Germany |
| Milk powder | Merck, Darmstadt, Germany |
| Nonidet® P 40 (NP40) | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| Orange G | MP Biomedicals Inc., Ohio, USA |
| P81 Phosphocellulose squares | Upstate, Biomol GmbH, Hamburg, Germany |
| Penicilin/Streptomycin | PAN Biotech GmbH, Aidenbach, Germany |
| Pepstatin A | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| Phenol-chloroform | Carl Roth GmbH, Karlsruhe, Germany |
| Phenylmethanesulfonyl fluoride (PMSF) | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| Phosphatase inhibitor Cocktail I | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| Phosphatase inhibitor Cocktail II | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| PLUS™ reagent | Invitrogen Life Technologies, Karlsruhe, Germany |
| PolyFect® Transfection Reagent | Qiagen GmbH, Hilden, Germany |
| Ponceau S | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| Potassium ferricyanide | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| Propidium iodide | Calbiochem, San Diego, USA |
| Protease Inhibitor Cocktail | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| Protein agarose beads A/G | Roche Molecular Diagnostics, Germany |

| | |
|---|---|
| Proteinase K | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| Puromycin | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| Puromycin | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| QIAshredder™ | Qiagen GmbH, Saint Louis, USA |
| Resolyte buffer (Rehydration buffer) | Amersham Biosciences, Uppsala, Sweden |
| Restriction enzymes and buffers | New England Biolabs, Frankfurt, Germany |
| Retionic acid (ATRA) | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| Rnasein | Promega, Mannhein, Germamy |
| Rotiphorese® Gel 30 (37,5:1) | Carl Roth GmbH, Karlsruhe, Germany |
| Rotiphorese® NF-Acrylamid/Bis 40 % (19:1) | Carl Roth GmbH, Karlsruhe, Germany |
| RPMI 1640 | PAN Biotech GmbH, Aidenbach, Germany |
| S.O.C. Medium | Invitrogen Life Technologies, Karlsruhe, Germany |
| SeeBlue®Plus | Invitrogen life technologies, Karlsruhe, Germany |
| Silver nitrate | Merck, Darmstadt, Germany |
| Sodium acetate | Sigma-Aldrich, Steinheim, Germany |
| Sodium azide | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| Sodium carbonate | Merck, Darmstadt, Germany |
| Sodium chloride | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| Sodium Dodecyl Sulphate (SDS) | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| Sodium fluoride | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| Sodium hydroxide | Merck, Darmstadt, Germany |
| Sodium orthovanadate | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| Sodium pyrophosphate tetrabasic decahydrate | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| Sodium thiosulfate | Merck, Darmstadt, Germany |
| Taq DNA Polymerase | New England Biolabs, Frankfurt, Germany |
| Taq DNA Polymerase | Qiagen GmbH, Hilden, Germany |
| TEMED | Carl Roth GmbH, Karlsruhe, Germany |
| Trifluoroacetic acid | Merck, Darmstadt, Germany |
| Trifluoroacetic Acid (TFA) | Merck, Darmstadt, Germany |
| TRIS – (hydroxymethl)-aminomethane | ICN Biomedicals GmbH, Eschwege, Germany |
| Triton X-100 | Merck, Darmstadt, Germany |
| Trypan Blue solution | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| Trypsin Gold, Mass Spectrometry Grade | Promega Corporation, Madison, USA |
| Trypsin/EDTA | PAN Biotech GmbH, Aidenbach, Germany |
| Tween®20 | Carl Roth GmbH, Karlsruhe, Germany |

| | |
|---|---|
| Tween-20 | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |
| Urea Plus | Amersham Biosciences, Uppsala, Sweden |
| α-cyano-5-hydroxy cinnamic acid (4-HCCA) matrix | Sigma-Aldrich Chemie GmbH, Steinheim, Germany |

## *3.4 Kits*

**Table 4b:**

| **Kits** | **Suppliers** |
|---|---|
| Cell Line Nucleofector Kit V | AMAXA GmbH, Cologne, Germany |
| Dual-Luciferase® Reporter Assay System | Promega Corporation, Madison, USA |
| Endofree®Plasmid Maxi Kit | Qiagen GmbH, Hilden, Germany |
| HiSpeed Plasmid Maxi Kit | Qiagen GmbH, Hilden, Germany |
| MALDI Plate Cleaning Kit | Amersham Biosciences, Uppsala, Sweden |
| QIAprep Spin Miniprep Kit | Qiagen GmbH, Hilden, Germany |
| QIAquick Gel Extraction Kit | Qiagen GmbH, Hilden, Germany |
| ThermoScript™ RT PCR System Plus Platinum® Taq DNA Polymerase | Invitrogen Life Technologies, Karlsruhe, Germany |
| TNT® T7/SP6 Coupled Reticulocyte Lysate System | Promega Corporation, Madison, USA |
| TRIzol® Reagent | Invitrogen Life Technologies, Karlsruhe, Germany |
| ZipTip with 0.2 µL C18 resin | Millipore. Billerica, Massachusetts, USA |

## *3.5 Radioactive Substances*

**Table 4c:**

| **Radioactive Substances** | **Suppliers** |
|---|---|
| [$^{3}$H] Acetyl coenzyme A | MP Biomedicals Inc. Irvine, USA |
| L-$^{35}$S Methionine | MP Biomedicals Inc. Irvine, USA |
| α-32P-dCTP | Amersham Biosciences, Braunschweig, Germany |

## *3.6 Markers/Ladders*

**Table 4d:**

| **Markers/Ladders** | **Suppliers** |
|---|---|
| DNA 100bp ladder | New England Biolabs, Frankfurt, Germany |
| DNA 1kb ladder | Invitrogen Life Technologies, Karlsruhe, Germany |
| DNA PCR marker | New England Biolabs, Frankfurt, Germany |

| | |
|---|---|
| Protein marker | Amersham Biosciences Braunschweig, Germany |

## *3.7 Labwares*

**Table 4e:**

| Labwares | Suppliers |
|---|---|
| 2-D gel apparatus | Bio-rad, Germany |
| Blotting paper | Schleicher and Schüll, Stuttgart, Germany |
| Cell culture material | Costar, Cell star, Nunc, Sarstedt, Greiner |
| Filters | Millipore |
| IPGphore | Amersham Biosciences Braunschweig, Germany |
| Nunc Cryotube™ vials | Nalge Nunc International, Denmark |
| Pipette Tipps | Star Labs (K &K labordarf), Munich, Germany |
| Plastic matreial (tubes etc) | Eppendorf, Greiner, Falcon, Munich, Germany |
| Reaction tubes | Eppendorf, Costar, Germany |
| Ultra centrifuge tubes | Beckman |
| X-ray films | Kodak (Biomax) |
| Automatic Gel-stainer Hoefer | Hoefer Pharmacia Biotech, San Francisco, USA |

## *3.8 Antibodies*

**Table 5.**

| Antibody | Isotype | Supplier |
|---|---|---|
| C/EBPα (14aa) | Rabbit Polyclonal IgG; Primary | Santa Cruz Biotechnology |
| TIP60 (K-17) | Goat Polyclonal IgG; Primary | Santa Cruz Biotechnology |
| TIP60 (N-17) | Goat Polyclonal IgG; Primary | Santa Cruz Biotechnology |
| Anti-GFP | Rabbit Polyclonal IgG; Primary | Invitrogen, Molecular Probes |
| Pan-Acetyl (C2) | Goat Polyclonal IgG; Primary | Santa Cruz Biotechnology |
| Normal IgG | Goat | Santa Cruz Biotechnology |
| Normal IgG | Rabbit | Santa Cruz Biotechnology |
| Anti-acetyl Histone H3 | Rabbit Polyclonal IgG | Upstate, Biomol GmbH |
| Anti-acetyl Histone H4 | Rabbit antiserum | Upstate, Biomol GmbH |
| MCM5 | Rat Monoclonal IgG; Primary | Alloys Scheppers, GSF, Munich |
| PU.1 | Rabbit Polyclonal IgG; Primary | Santa Cruz Biotechnology |
| β-tubulin (H-235) | Rabbit IgG | Santa Cruz Biotechnology |
| Anti-human CD11b (Mac-1) | Mouse Monoclonal IgG1; APC | BD Pharmingen |
| Anti-human CD11b (Mac-1) | Mouse Monoclonal IgG1; PE | BD Pharmingen |

| | | |
|---|---|---|
| Anti Goat IgG | Donkey IgG; HRP conjugated; Secondary | Santa Cruz Biotechnology |
| Anti Rat IgG | Goat IgG; HRP conjugated; Secondary | Santa Cruz Biotechnology |
| Anti Rabbit IgG | Goat IgG; HRP conjugated; Secondary | Santa Cruz Biotechnology |
| Anti Rabbit IgG | Donkey IgG; HRP conjugated; Secondary | Amersham Biosciences |

### *3.9 Plasmid Constructs*

In this article we used GST (pGEX4T, expressing a GST fusion protein in bacteria, Amersham, UK), GST-DBD, GST-C/EBPα (kind gift from Dr. Claus Nerlov); pCDNA3-human C/EBPα was previously described [47], GST-TIP60, YFP-N1-TIP60 (pEYFP-N1, expressing a yellow fluorescence fusion protein in mammalian cells, Clontech, USA), YFP N1-TIP60 (-HAT), pCDNA3 TIP60, p(C/EBP)2TK (luciferase reporter plasmid with two CCAAT binding sites), pTK (luciferase reporter plasmid, Promega, USA), pRL null, pGAL4 luc, pC/EBPα GAL4DBD (vector expressing a GAL4DNA activation domain fusion protein , Clontech, USA), GAL4 VP16 (yeast activator GAL4 fused to a highly acidic portion of the herpes simplex virus protein VP16 and is potent transcriptional activator on pGAL4 luc promoter), and PCMV5 (Stratagene, USA)

### *3.10 Buffers*

Buffers provided with the kits were used in the case of plasmid isolation, protein expression, RNA isolation, and polymerase chain reaction. The other buffers used are listed below.

| | |
|---|---|
| Electrophoresis buffer (SDS-PAGE) | 25 mM Tris/HCl, [pH 8.3]<br>250 mM Glycine<br>0.1 % SDS |
| Gel fixer solution | 50 % Methanol<br>10 % Acetic acid |
| Staining solution | 30 % Methanol<br>10 % Acetic acid<br>0.25 % Coomassie-Blue R-250 |

| | |
|---|---|
| Destaining solution | 30 % Methanol<br>10 % Acetic acid |
| Gel fixer solution (2-D) | 50 % Methanol<br>12 % Acetic acid<br>500 µl formaldehyde (37%)/l |
| Washing solution (2-D) | 50 % Ethanol (three times, 20 min each) |
| Sensitizinng solution (2-D) | 200 mg/ml Sodium thiosulphate (1 min) |
| Staining solution (2-D) | 2 g/l Silver nitrate, 500 µl formaldehyde (37 %)/l<br>(20 min) |
| Destaining solution (2-D) | 50 % Methanol<br>12 % Acetic acid |
| Storage solution (2-D) | 1 % Acetic acid |
| Electrophoresis buffer (Tris-Glycine) | 250 mM Tris/HCl, [pH 8.3]<br>1.9 M Glycine<br>10 mM EDTA |
| Tris-Borate buffer | 0.89 M Tris<br>0.89 M Boric acid<br>0.5 M EDTA |
| SDS-PAGE gel loading dye (2×) | 125 mM Tris/HCl, [pH 6.8]<br>4 % SDS<br>10 % ß-Mercaptoethanol<br>30 % Glycerol<br>0.004 % Bromophenol blue |
| Western Stripping solution | 0.1 M ß Mercaptoethanol<br>2 % SDS<br>1 M Tris, [pH 6.8] |
| TE buffer | 50 mM Tris/HCl, [pH 8.0]<br>1 mM EDTA |
| 10× Orange G dye | 40 % Sucrose<br>0.2 % Orange G |
| RIPA lysis buffer | 50 mM Tris, [pH 8.0]<br>150 mM NaCl |

5 mM EDTA
1.0 % NP40
0.5 % Na-deoxycholate
Protease-phosphatase inhibitors (freshly added)

# 4 METHODS

## *4.1 Cell Culture Techniques*

### 4.1.1 Mammalian Cell Culture

Adherent human embyronic kidney fibroblast cells 293T were cultured in DMEM and suspension U937, human myeloid cell line, monoblastic cells were cultured in RPMI (PAA, Cölbe, Germany). Both supplied with 10% FBS (Invitrogen/GIBCO, Germany) and antibiotics (Penicillin/Streptomycin, GIBCO,Germany). Cells were culturd at 37ºC in a 10% CO2 humid atmosphere. K562 ER-C/EBPα (Erythroleukemia cells stably transfected with a C/EBPα-ER fusion vector) were grown in RPMI without Phenol red (PAA, Cölbe, Germany) supplemented with 10% Charcoal treated FBS (Hyclone, Greiner, Nürtingen, Germany) with Puromycin (10 μg/ml) as a selection marker.

### 4.1.2 Transient Transfection of 293T Cells

Cells plated in 24 well plates (for luciferase assay) and in 10 cm plates (for protein lysate) at 70- 80% confluence were transfected with 1.2 μg (24 well) to 10 μg (protein lysate) of total plasmid using Polyfect transfection reagent (Qiagen) or Lipofactamine-PLUS reagent (Invitrogen), respectively, according to manufacturers' instructions. Cells were harvested 24 h after transfection.

### 4.1.3 Transient Transfection of U937 Cells

Cells were grown at density 1 $\times 10^5$ cells/ml a day before transfection. Cells were transfected with total 1 μg plasmid DNA using Nucleofactor Kit V according to the manufacturer's protocol (AMAXA). Cells were harvested after 24 h.

#### 4.1.4 Treatment of U937 with Retionic acid

U937 cells were induced to differentiate at $1 \times 10^5$ cells/ml by incubation with 1 µM all-trans retinoic acid for 60 h.

#### 4.1.5 K562 ER-C/EBPα treatment with β-estradiol

Cells were induced to differentiate at $5 \times 10^5$ cells/ml by incubation with 5 µM β-estradiol.

### 4.2 *Firefly and Renilla Luciferase Reporter Gene Assays*

Two different reporter systems were used. One for analyzing the transcriptional properties of proteins and protein domains which were fused to the GAL4 DNA binding domain (DBD). Here, the reporter plasmid pGAL4TK Luc, which contains 5 GAL4 DBD binding sites and a TK (Herpes simplex virus thymidine kinase) minimal promoter followed by the firefly luciferase reporter gene, was used. The other reporter system used a reporter plasmid with two C/EBPα binding sites upstream of the firefly luciferase gene p(C/EBP)2TK. To control for transfection efficiency, the pRL-null plasmid, which encodes the Renilla luciferase under the control of a minimal promoter, was used. In order to analyze the significance of protein-protein interactions on the transcriptional activity of C/EBPα, reporter gene assays were performed in which C/EBPα or C/EBPα GAL4DBD were cotransfected with TIP60 and/or its HAT mutant in HEK 293T cells. 293T cells were transfected using Polyfect (Qiagen) and U937 cells using nucleofactor Kit V (AMAXA) according to the manufacturer's instructions. Firefly luciferase activities from the constructs pGal4-luc, pTK, and p(C/EBP)2TK and *Renilla* luciferase activity from the internal control plasmid pRL-null were determined 24 h after the initiation of the transfection protocols using the dual-luciferase reporter assay system (Promega). Firefly luciferase activities were normalized to the *Renilla* luciferase values [110]. The DNA concentrations of the reporter constructs and expression plasmids used for Polyfect transfections were 0.1 µg of pGal4-luc, pTK, or p(C/EBP)2TK, 0.01 µg of the internal control plasmid pRL-null, 0.1 µg of the expression plasmids for C/EBPα and 0.05 µg, 0.1 µg, 0.25 µg for pYFP-N1-TIP60(w),

pYFP-N1-TIP60(-HAT) and 0.1 µg, 0.2 µg, 0.3 µg for C/EBPα-GAL4DBD. The same concentrations of the empty expression vectors were used as controls.

## *4.3 Immunoblotting*

SDS PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis) was carried out according to standard procedures. Proteins were transferred to PVDF membranes (Millipore) at 110 V for 90 min. Membranes were incubated in blocking solution (TBS, 0.1 % Tween-20, 5 % w/v milk powder) for at least 1 hour, followed by incubation in blocking solution containing the appropriate antibody (see Materials) for at least 1 hour at RT or overnight at 4ºC. After washing 3 times for 10 min at RT with TBS containing 0.1 % Tween-20 and 2.5 % w/v milk powder, the membranes were incubated in blocking solution containing the appropriate, HRP-conjugated secondary antibody for at least 1 h. After washing 3 times for 15 min at RT with TBS containing 0.1 % Tween-20, protein bands were detected by enhanced chemoluminescence (ECL; Amersham) according to the manufacturer's protocol.

## *4.4 Preparation of Nuclear Extracts*

Cells were washed with PBS and then subjected to lysed with Buffer A (20 mM Tris/HCl [pH 8.0], 10 mM NaCl, 3 mM $MgCl_2$, 0.1 % NP40, 10 % glyceol, 0.2 mM EDTA, 1 mM DTT and proteases-phosphatase inhibitors cocktail) for 15 min on ice with occasional mixing. Nuclei were pelleted by centrifugation in a tabletop centrifuge at 2,000 rpm for 5 min at 4°C. The proteins were then extracted from the nuclei by incubation at 4°C with snap freeze-thawing three times in buffer C (20 mM Tris/HCl [pH 8.0], 400 mM NaCl, 20 % glyceol, 0.2 mM EDTA, 1 mM DTT, and proteases-phosphatase inhibitors cocktail). Nuclear debris was pelleted by centrifugation at 14,000 × *g* for 15 min at 4°C, and the supernatant extract was aliquoted, snap frozen, and stored at -70°C or used directly for the experiments.

## *4.5 GST-Pull Down*

For the pull down experiments the U937 cell line ws used. These cells can be induced to differentiate from their immature state to cells resembling more morphologically and

functionally mature monocytes, macrophages, and granulocytes. Nuclear extract from U937 cells was prepared as described above. Equal amounts of bacterially purified proteins were incubated with 1 mg of nuclear extract (volume made up to 1 ml with NETN buffer) for 3 h at 4°C. After pull down, protein bound beads were washed 3 times with NETN buffer at 10 rpm on a rotating shaker for 5 minutes at 4°C. Beads were lysed in urea lysis buffer (66 % Urea plus one, 1 % DTE, 4 % CHAPS, 2.5 mM EDTA, 2.5 mM EGTA) to completely denature the interacting proteins which were then applied for 1-D and 2-D separation in a pH range of 3-10. In addition, $S^{35}$-Methionine labelled *in-vitro* translated C/EBPα was pulled down with GST-fusion protein TIP60 by incubating for 3 hours, separated on 12 % SDS PAGE and detected by autoradiography using Kodak films.

## *4.6 Co-Immunoprecipitation*

Co-immunoprecipitation is an *in-vitro* biochemical assay which was used to detect *in-vivo* interactions. For TIP60, 293T cells were transfected at a density of $1\times 10^6$ cells in 10 cm plates. The cells were transfected with Polyfect with expression plasmids of human C/EBPα and pYFP-N1-TIP60. 24 h after transfection, cells were scraped off with a cell scraper. Cells were washed with PBS and then subjected to lysis for nuclear extracts as described above. 300 µg nuclear extracts was incubated with 40 µl of 50 % protein G agarose beads slurry and precleared using a goat isotype IgG antibody (2 µg) with mild agitation at 4°C. The supernatants were then added to the Protein G agarose beads preincubated with antibodies against TIP60 protein (Santa Cruz; sc-5725 [N-terminal] and sc-5727 [C-terminal], 1 µg each, mixed together, as we observed only weak precipitation using only one antibody) for 4 h followed by extensive washes in coimmunoprecipitation buffer (50 mM Tris/HCl [pH 7.5], 150 mM NaCl, 0.5 % NP-40, 0.25% sodium deoxycholate, and proteinase-phosphatases inhibitor cocktail). The corresponding IgG goat served as a control. Immunoprecipitated proteins were heated at 56°C for 90 min in 2× SDS loading buffer and then boiled at 95°C for 5 minutes before being loaded on the SDS PAGE. Western blot analysis was performed with a rabbit anti-C/EBPα polyclonal antibody and a rabbit anti-GFP monoclonal antibody.

In order to immunoprecipitate MCM5, U937 cells were lysed in RIPA buffer. 600µg of U937 lysate were used to co-immunoprecipitate MCM5 using an C/EBPα antibody in the Co-IP buffer (50 mM Tris/HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 5 % glycerol, 0.25 % NP-40, and proteinase inhibitor cocktail). Incubation was for overnight at 4ºC followed by extensive washes in Co-IP buffer. We used 5 0 µl slurry of protein A agarose beads and 2 µg of the anti C/EBPα antibody. Denatured proteins were subsequently separated on 12 % SDS PAGE and immunoblotted with rat monoclonal anti-MCM5 and anti C/EBPα antibody.

## *4.7 GST-Purification*

Full length C/EBPα and TIP60 were cloned is in frame with the Glutathione-S-Transferase (GST) in the pGEX bacterial expression vector (Amersham Biosciences, Germany). Fusion proteins were expressed in the BL21 *E. coli* bacterial strain after 0.5 mM IPTG induction for 2 hours. The bacterial pellet was lysed in NETN buffer (150 mM NaCl, 20 mM Tris/HCl [pH 8.0], 1 mM EDTA [pH 8.0], 0.1 % NP40 and protease inhibitors cocktail) and sonicated, followed by protein purification using immobilised Glutathione Sepharose 4B beads (Amersham Biosciences, USA). Sepharose beads bound with GST proteins were washed 4 times with NETN buffer on a rotating shaker at 4°C for 10 minutes each and then lysed in 2× SDS sample loading buffer (125 mM Tris [pH 6.8], 4 % SDS, 20 % glycerol, 10 % 2 β-mercaptoethanol and 0.03 % bromophenol blue). Subsequently proteins were separated on 12 % SDS PAGE  and visualised by Coomassie blue staining.

## *4.8 Interaction assay with radiolabelled proteins*

C/EBPα was *in vitro* transcribed and translated in the presence of [$^{35}$S] methionine by using the T7 coupled reticulocyte lysate system (Promega) according to the manufacturer's instructions. GST purification for TIP60 was performed as described above.

## *4.9 Histone Acetyltransferase (HAT) Assay*

HAT assays were performed similarly to previously published protocols [111]. Cell culture and GST protein purification was performed as described above. For the filter binding

assay, acetylation reactions contained 1 µg of recombinant *Drosophila* histones (Kind gift from Axel Imhof) or GST-C/EBPα, 0.25 µCi of [$^3$H] acetyl-CoA, (Amersham; e.g. 0.2 to 0.4 µl of 2-10 Ci/mmol) with 100 ng (1 µl) GST-TIP60 in 10 mM Tris [pH 8.0], 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF, 10 mM sodium butyrate, and 5 % glycerol in a total 50 µl volume. The yeast Hat1 protein (kind gift from Axel Imhof) was used as positive control for the acetylation reactions. Reactions were incubated at 37°C for 30 min, spotted onto phosphocellullose p81 filters and allowed to air dry. Purified histones (provided by Axel Imhof) were used as positive control for acetylation reaction by GST-TIP60 and by yeast Hat1 protein. Dry filters were washed three times for 5 min at RT with 50 mM sodium carbonate/bicarbonate buffer ($Na_2CO_3/NaHCO_3$ **)** [pH 9.2], and then rinsed in acetone to remove unincorporated radioactive acetyl-CoA. The filters were air dried. The filters were then placed in scintillation vials, and 2 mls of scintillation fluid was added. After incubating the vials at RT for 10 min, the amount of incorporated radioactivity was determined using a liquid scintillation counter.

For non-radiaoactive acetylation experiments, 5 µM non-radioactive acetyl-CoA was used. In the Western blot acetylation assays, reactions were separated by 10 % SDS-PAGE, proteins were transferred onto a PVDF (0.22 µM pore size) and then probed with antibodies specific to acetylated lysines (Pan acetyl, Santa Cruz Biotechnology, USA).

## *4.10 Determination of DNA concentration*

The DNA concentration was determined either by spectrophotometry or by comparing different dilutions of DNA with a standard amount of DNA in an agarose gel.

## *4.11 FACS (Florescence activated cell sorting) analysis*

U937 cells were induces with retinoic acid for 60 h, followed by FACS analysis. For each flow cytometry analysis, $4\times10^6$ cells were washed twice in washing buffer (plain RPMI, 1 % FBS) and resuspended in 100 µl of washing buffer with 6 µl of the Cd11b APC or PE labelled antibody. Incubation was performed at 4ºC for 30 min. Cells were washed and resuspended in washing buffer with PI (propidium iodide, 0.1 %) and were analyzed by flow cytometry on a BD FACS Calibur System (BD Biosciences, Palo Alto, CA).

### *4.12 Semi-quantitative RT-PCR*

Expression of *TIP60, CEBPA* and *CEBPE were* assayed by RT-PCR in induced (U937 + RA, 1 µM, CD11b$^+$) and uninduced (U937, C11b$^-$) flow-sorted cells (BD FACSVantage SE System; BD Biosciences, Palo Alto, CA). Total RNA was isolated using Trizol reagent (GIBCO BRL) and treated with DNase I (amp grade) to remove contaminating genomic DNA. First strand cDNA was synthesized from 1 µg total RNA using the thermoScript RT-PCR system (all reagents from Invitrogen GmbH, Karlsruhe, Germany). Equal amounts of cDNA originating from 50 ng starting RNA were loaded on to an Ethidium bromide gel to quantitate the RNA before amplification. The annealing temperature was 60°C for *CEBPA and CEBPE* and 52°C for *TIP60*. The number of PCR cycles for each gene was chosen to stop the reaction in the logarithmic phase of amplification (22 cycles for *β-tubulin*, 30 cycles for *TIP60, CEBPA and CEBPE*).

Primer sequence for *TIP60*:

TIP60_Ex7_Sense 5'- CACATCGTGGGCTACTTCT-3'

TIP60_Ex9_ASsense 5'- TGTTTTCCCTTCCACTTTGG -3'

Expected size: 150 bp

Primer sequence for CEBPA [112]

CEBPA_Sense 5'- AAGGTGCTGGAGCTGACCAG -3'

CEBPA_ASense 5'- AATCTCCTAGTCCTGGCTCG -3'

Expected size: 255 bp

Primer sequence for CEBPE [112]

CEBPE_Sense 5'- AGTCTGGGGAAGAGCAGCTTC -3'

CEBPE_ASense 5'- ACAGTGTGCCACTTGGTACTG -3'

Expected size: 305 bp

Primer sequence for *β-tubulin*:

β-actin _Sense 5'-CTTCAACACCCCAGCCAT-3'

β-actin _ASense 5'-TAATGTCACGCACGATTTCC-3'

Expected size: 285 bp

### *4.13 Expression analysis of TIP60 and C/EBPα in leukemia samples*

Total mRNA was isolated from patient samples, processed and analyzed on the Affymetrix HG-U133A and HG-U133B chips as described before [109]. The .CEL file data from the samples used in the comparison were normalized together according to the procedure described by Huber et al., [113]. Normalized expression data were then analyzed with the R software package and the "boxplot" function (www.r-project.org). Expression signal intensities are given on a logarithmic scale. Ten samples from each leukemic subgroup as well as ten normal bone marrow samples were included in the analysis. The leukemic subgroups were: chronic myeloid leukemia (CML), AML with the CBF/MYH11 fusion gene (AML_M4), with the PML/RARA fusion gene (AML_M3), and AML with the AML1/ETO fusion gene (AML_M2).

### *4.14 Chromatin Immunoprecipitation (ChIP Assay)*

The ChIP assay is a technique used to map the location of modified histones and other DNA interactiong proteins in the genome. In these experiments antibodies are used that recognise and bind to the protein of interest, not only in free solution, but also when contained in chromatin. The ChIP assay is composed of two steps — first, *in vivo* formaldehyde cross-linking of whole cells which stabilizes protein-protein and protein-DNA interactions, followed by immunoprecipitation of protein-DNA complexes with specific antibodies from sonicated extracts followed by a reversal of the cross-linking, and amplification of precipitated DNA **(Fig. 7)**.

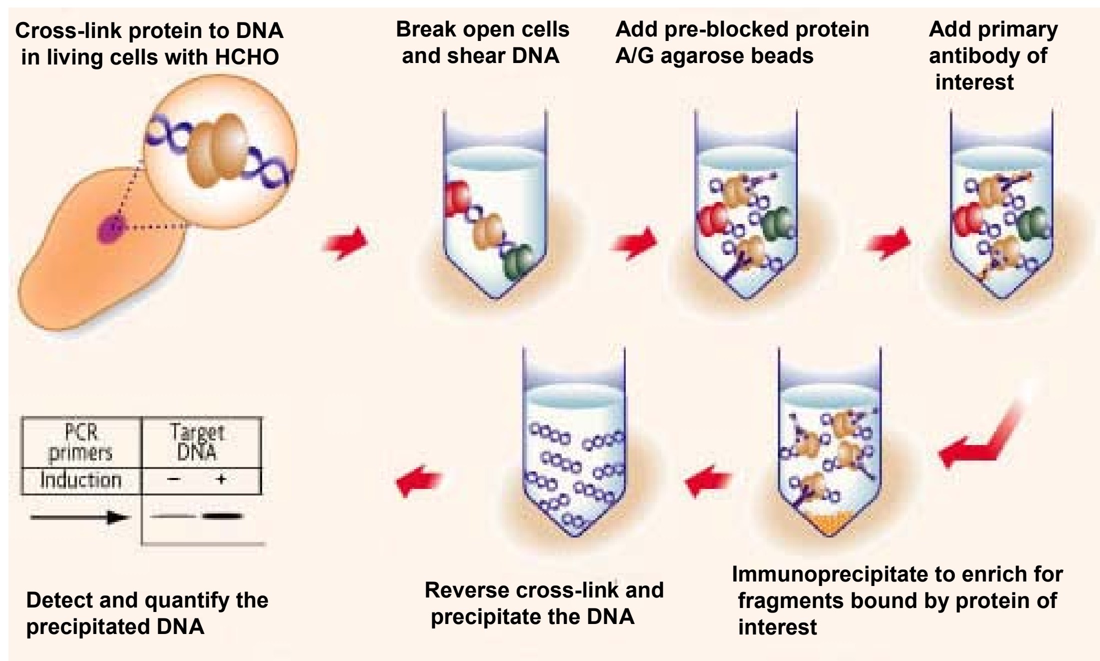



**Fig 7:** General principle of Chromatin ImmunoPrecipitation assay

Suspension cultures of human K562 C/EBPα-ER cells induced with β-estradiol (5 µM, 6 h) were used to prepare cross-linked extracts. Formaldehyde (37 %) was added directly to the cultured cells (approx. $5 \times 10^5$ cells/ml, 150 ml medium), i.e. four confluent flasks of K562 ER-C/EBPα cells per time-out for ChIP (triplicates) to a final concentration of 1 % . Then the cells were gently fixed at RT for 9 min on a roller. Fixation was stopped by the addition of glycine to final concentration of 0.125 M, and the cells were then immediately transferred on ice. Cells were rinsed twice with PBS at 4ºC. PBS was aspirated completely, and the harvested cells were either stored at -80ºC or used directly for lysis in 10 ml of sonication buffer (Buffer A: 50 mM HEPES-KOH [pH 7.9], 140 mM NaCl, 1 mM EDTA [pH 8.0], 1% Triton, 0.1% Sodium deoxycholate, 1 mM PMSF) with the addition of glass beads. Cross linked material was sonicated (Branson Sonifer) in an ice-ethanol bath to an average DNA length of 500-1000 bp (4 min total time, 0.5 sec on, 1 sec off, 30% amplitude). Samples were centrifuged for 10 min at 3500 rpm to remove glass beads. Lysates were collected and adjust to 0.5 % sarcosyl and 1.42 g/$cm^3$ CsCl and run on a CsCl gradient (Beckman SW 41 swinginng bucket rotor) at 38,000 rpm for 36 h at 18ºC as described previously [114]. After centrifugation, the centrifuge tube was punctured at the

bottom with a syringe and fractions of 500-800 µl were collected. An aliquot from these fractions were adjusted to NaCl concentration 500 mM and incubated for 2 h at 65ºC to remove cross-links and were checked on a eithidum bromide stained 0.8% agarose gel for DNA size. Fractions in the size range of 500 - 1.5 kb were pooled and dialysed overnight against buffer D (Dialysis buffer: 5% glycerol, 1 mM EDTA, 10 mM Tris/HCl [pH 8.0], 1 mM PMF, 4ºC). The optical density (O.D.) of the samples was measured, and then the samples were stored at -80ºC. These fractions represent the cross linked whole cell extracts ready for immunoprecipitation. OD 1.5 fraction (1.5 $A_{260}$) was adjusted to buffer A in a total volume of 1 ml. As a pre clearing step, 120 µl of slurry (equilibrated with buffer A, 30 µl of protein G sephrose, 500 ng/ml BSA, and salmon sperm DNA [sonicated to an average size of 2-5kb] 10 µg/ml) was added and incubated for 2 h at 4ºC. Protein G sephrose was removed by centrifugation at 2000 rpm. Precleared lysates were then used for immunoprecipitation with the appropriate antibodies (10 µg of C/EBPα, TIP 60 [K-17]. IgG Goat, IgG Rabbit and 4 µl of anti-acetylated histone H3 and anti acetylated histone H4). After immuno-precipitation (8 h), antibody bound complexes were isolated by adding protein G. Washed two times, 1 ml each with buffer A, (with 500 mM NaCl for anti-acetylated histone H3 and anti acetylated histone H4 antibodies; 350 mM for C/EBPα and TIP60 antibodies), buffer B (10 mM Tris/HCl [pH 8.0], 250 mM LiCl, 0.5 % IGEPAL CA-630, 0.5 % Sodium deoxycholate, 1 mM EDTA), TE buffer (10 mM Tris/HCl [pH 8.0], 1 mM EDTA) and finally with buffer E (1 %SDS, 0.1 M $NaHCO_3$). After extensive washing, complexes were eluted from the beads, cross-links were reversed. It is important to revert the cross-linked from a certain volume (usually 10% of that used for immunoprecipitation) after the the optical density determineation. This will be needed as the input control. Samples were adjusted to 500 mM NaCl and incubated for overnight at 65ºC to reverse the cross-links 6 µg of proteinase K was added and samples were adjusted to 100 mM Tris/HCl [pH 6.8], 50 mM EDTA, and incubated at 56ºC for 1 h. DNA was extracted by phenol-chloroform extraction and precipitated. The samples were finally resuspended in 30 µl of $ddH_20$. For the detection of immunoprecipitated C/EBPα promoter region, and as a control the C/EBPα coding region, PCR reactions (27 cycles) were performed with 4 µl of resuspended DNA in the presence of 1 µCi α32-dCTP and separated on PAGE. Primers used for ChIP were described previously [115].

| | |
|---|---|
| CEBPA_Prom_F | 5’ ACTTCGG TACCGCTACCGACCACGTGGGCG 3’ |
| CEBPA_Prom_R | 5’ GTGAACTCGAGC ACCTCCGGGTCGCGAATGG 3’ |
| CEBPA_cds_F | 5’ATTCACTCGAGGATCCCCATGAGCGCGCTGAAGGG GCTG 3’ |
| CEBPA_cds_R | 5’ GTGAACT CGAGGTACCTCACGCGCAGTTGCCCAT 3’ |

### *4.15 2D-Gel Electrophoresis*

GST and GST-C/EBPα were incubated with nuclear extracts of the myelomonocytic cell line U937 in NETN buffer as described above in the GST purification section. Beads with their associated proteins from nuclear extract were lysed in urea lysis buffer for 1h at RT on a rotating shaker. Lysed beads were passed through an RNA qiashredder (Quiagen, Germany), and resulting supernatant containing dissolved proteins was centrifuged for 50 minutes at 50,000 rpm at 22°C to remove DNA and other cellular debris. For the first dimension, 350 µl of dissolved proteins were separated on an immobiline$^{TM}$ dry strip pH 3-10 by isoelectric focusing (IEF). The reduction and alkylation of separated proteins was carried out in urea buffer containing 2 % DTE and 2.5 % iodoacetamide. Failure in maintaining the proteins in a reduced state can lead to artefactual spots and/or streaking due to the formation of disulphide bridges [116]. In many cases, streaking in the basic gradient is a consequence of cysteine oxidation. Artefactual modification of proteins during sample preparation and 2-D can mask functionally significant events within the cell and can hinder protein identification by MS. Several types of protein modifications have been reported to occur during electrophoretic separation, and among them alkylation seems to occur most often. Alkylation of different amino acid residues (most often of cysteine) can be caused by free immobiline monomers, free unpolymerised acrylamide, N-substituted acrylamide in the acrylamide gel or cross-linkers [117]. An attractive alternative to reducing the cysteine residues and a possible solution to the described problems is to covalently block these residues either irreversibly, by alkylation, or reversibly, by oxidation to mixed disulphides [118]. After reduction and alkylation of 1D strips, proteins were separated in the second dimension using 12 % SDS PAGE on the basis of their size (relative molecular weight). 2D gels were silver stained to visualise the protein spots. In silver staining, treatment with

glutaraldehyde (cross-linking and sensitizing agent) was omitted. Instead the gel was sensitized by sodium thiosulfate.

## *4.16 1D SDS-PAGE*

1-D SDS-PAGE of the GST pull down samples (pull down were carried as described before) with 1 mg of U937 nuclear extracts was carried out according to the Laemmli method. The beads were dissolved in a modified Laemmli buffer (3 % SDS, 10 % glycerol, 0.1 % bromophenol blue, 100 mM Tris-HCl, [pH 6.8]. Proteins were denatured by incubation at 100ºC for 5 min, and were separated on a 12 % bis-tris polyacrylamide gel. Protein lanes were visualised by Coomassie R- 250 staining.

## *4.17 Destaining protocol for Trypsin in-gel digestion*

To promote efficient enzymatic cleavage and to reduce the background for subsequent mass spectrometric analysis, any excessive stain must be carefully removed. Differentially expressed silver stained protein spots from 2-D gel electrophoresis were excised with cut pipette tip and were transferred to a 0.65 ml polypropylene Eppendorf tube. Excess water was removed and samples were washed briefly with 100 mM $NH_4HCO_3$. For destaining, a volume of destaining solution (0.2 g potassium ferricyanide [$K_3Fe(CN)_6$] in 100 ml of 100 mg/l sodium thiosulphate) was added sufficient to cover the gel pieces and incubated on a shaker until the gel was completely destained, typically 15-30 min. The gel pieces turned bright yellow and were washed twice with 100 mM $NH_4HCO_3$, for 10 minutes and twice with milli-Q water for 5 min, until the yellow color was no longer visible. Excess liquid was removed, and the gel was washed for 5 min with 50 % acetonitrile (ACN)/ 50 % 50 mM $NH_4HCO_3$, and then 5 min with ACN for dehydration. Tubes were vortexed during incubation. The ACN was removed and the samples were air dried for 10 min or put into a speedvac for 1 min at RT.

The protein lanes from the Coomassie stained gel was excised and thencut into small pieces with a scalpel on a glass plate. The gel pieces were transferred to a 0.65 ml polypropylene eppendorf tube and were washed briefly with 100 mM $NH_4HCO_3$ to adjust the pH. 50 % acetonitrile (ACN)/ 50 % 50 mM $NH_4HCO_3$ were added and the tubes were incubated in a

vortexer for 10 min. The liquid was replaced several times until the gel pieces appeared colourless. Excess liquid was then removed and the gel pieces were incubated with 100 % ACN for dehydration. Finally, the ACN was removed, and the samples were either air dried or evaporated briefly in a speedvac. The gel pieces were then processed for reduction and alkylation. The gel pieces were covered with 150 µl 10 mM DTT in 100 mM $NH_4HCO_3$, vortexed, spun briefly and incubated for 1 h at 56ºC. The tubes were cooled to room temperature and the DTT solution was removed and replaced with 150 µl of 50 mM of idoacetamide in 100 mM $NH_4HCO_3$. Tubes were vortexed, spun briefly, and incubated for 45 min in a dark place at room temperature. The idoacetamide was then removed. Gel pieces were washed with 100 mM $NH_4HCO_3$ for 5 min and then twice with 50 % ACN/ 50 % 50 mM $NH_4HCO_3$ for 5 minutes with vortexing. The gel pieces were dehydrated with ACN for 5 min. The ACN was removed and the gel pieces were dried in a speedvac. After this procedure, the gel pieces were noticeably shrunken and appeared white.

## *4.18 Trypsin In-Gel Digestion protocol for MALDI-TOF, TOF/TOF and for LC-MS/MS*

The gel pieces were swollen in digestion buffer containing 12.5 ng/µl trypsin (Promega) in 50 mM $NH_4HCO_3$ in an ice-cold bath. After 45 min, the supernatant was removed and replaced with 5-10 µl of the same buffer but without trypsin to keep the gel pieces wet during enzymatic cleavage (37ºC overnight). Peptides were extracted with 10-15 µl of 20 mM $NH_4HCO_3$. Samples were spun down, sonicated for 5 min in a water bath. Peptide enriched supernatant was collected. Gel pieces were washed three times with 2 % formic acid in 50 % ACN (incubated for 20 min each time) at room temperature. Supernatant were collected and dried down in speedvac. For LC-MS/MS lyophilized peptides were dissolved in 20 % acetonitrile (ACN) and 0.1 % trifluoroacetic acid (TFA).

## *4.19 Separation of Peptides by 1D nano-LC*

Peptide separations were performed on a nano-HPLC system (UltiMate, LC Packings, Sunnyvale, USA). A 20 ml aliquot of each sample was injected into a C18 RP trapping column (300 mm×65 mm, flow rate 30 ml/min) using a Famos autosampler. After elution onto a nano-RP column (C18, 75 mm×615 cm, flow rate 200 nl/min), the peptides were

separated with a gradient from 5 to 80 % ACN in 0.1 % TFA in 120 min. Fractions were collected at 30-s intervals directly on a 192-well MALDI target plate using a spotting robot (Probot, LC Packings). The eluent from the capillary column was mixed with a matrix solution consisting of 2 mg/ml $\alpha$-Cyano-4-hydroxycinnamic acid (CHCA) matrix.

### *4.20 Sample Preparation for Mass Spectrometry*

$\alpha$-Cyano-4-hydroxycinnamic acid (CHCA) matrix preparation for MALDI-TOF: CHCA saturated solution (8 mg) was mixed in 200 µl of solvent solution (50 % ACN and 0.1 % TFA). This mixture was vortexed for 5-10 min, spun down, and one part of this CHCA saturated supernatant was mixed with three part of solvent solution. This constituted the matrix solution. 0.5 µl of matrix solution together with 0.5 µl of the sample was applied onto anchorChip plate (Bruker Daltonics, Leipzig, Germany) and samples were air dried and used for mass spectrometry.

### *4.21 Mass Spectrometry*

Mass spectra were obtained on a Bruker REFLEX III mass spectrometer (Bruker Daltonics, Leipzig, Germany). MALDI peptide spectra were calibrated using several matrix ion peaks (or standard keratin peaks) as internal standards. Proteins were identified by a MASCOT database search (Matrix Sciences). Mass spectra of some spots were also analysed by an AB4700 MALDI-TOF/TOF (Applied Biosystems, Darmstadt, Germany) operating in reflectron mode with an ion source pressure of ~0.5 µ torr. After a 400-ns time-delayed ion extraction period, the ions were accelerated to 20ºkV for TOF mass spectrometric analysis. A total of 600–1000 laser shots were acquired and signal averaged for MS/MS analysis and data were analyzed by the GPS explorer software (AB 4700 Inc., USA) using a Swiss/Prot protein database for Mascot search assuming (1) monoisotopic peptide masses, (2) cysteine carbamidomethylation, (3) variable oxidation of methionine, (4) a maximum of one missed trypsin cleavage and (5) a mass accuracy of 60 ppm. or better. The four highest intensity peaks were selected in a range between *m/z* 1500 and 2500 for MS-MS peptide sequencing in an interpretation method. A molecular weight search (MOWSE) score > 53 was assumed to indicate a significant match.

The GPS Explorer 2 software reports two different scores: the MASCOT best ion score, the highest score of a single peptide, and a total ion score, the sum of all peptide scores of one protein. The significance level for a peptide score should usually be higher than 20 and for a protein score higher than 40–50. Because different database searches have different MASCOT significance levels due to different database sizes and different numbers of masses submitted for a search, scores cannot be compared directly. For this reason, the software calculates a confidence interval from MASCOT protein scores or ion scores, and the MASCOT significance level for each search is defined as the 95 % confidence level. Therefore, the total ion score confidence level is also a reliable and comparable parameter for the significance of a database search as shown in **Table 7** of nanoLC coupled with MALDI-TOF/TOF.

# 5 AIM OF THE STUDY

Mechanisms underlying the inactivation of C/EBPα function in acute myeloid leukemia are poorly understood. Recent findings suggest that protein-protein interactions play a pivotal role in normal functioning of lineage-specific factors. Hence, the objective of the present study is to identify and characterize interacting proteins of C/EBPα which might modulate C/EBPα protein function. For this purpose we used the GST fused C/EBPα and performed GST-pull down assays for isolating the interacting proteins and further identifying them by mass spectrometry.

# 6 RESULTS

## *6.1 Purification of GST-tagged proteins*

C/EBPα–interacting proteins were identified using co-purification of complexes with GST affinity tags, coupled with protein identification by mass spectrometry. A GST-C/EBPα fusion protein and a GST only control were used in this study. The Coomassie blue stained gel shows the expression and purification of GST-C/EBPα and GST **(Fig. 8)**. The functionality of the GST-C/EBPα fusion protein was assayed by pulling down the known C/EBPα interacting protein PU.1 using the same buffer condition that were later used in the pull down experiments for the identification of unknown proteins in nuclear extracts.

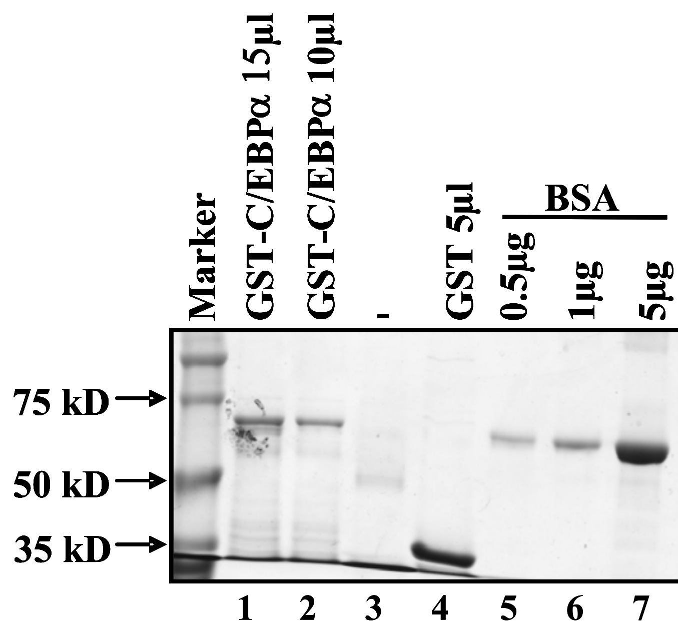



**Fig 8:** Coomassie-stained gel showing the expression of GST-C/EBPα (lane 1, 2) and the GST protein (lane 4). Known amount of BSA were loaded in lane 5 (0.5 µg), lane 6 (0.1 µg), and lane 7 (5 µg) to show the relative expression of the purified tagged proteins.

## *6.2 Screening for C/EBPα interacting proteins in the myeloid cell line U937*

### 6.2.1 2-D gel electrophoresis of interacting proteins

The selection of an appropriate cell system is crucial for studies on C/EBPα which is an important transcription factor in the myeloid compartment. The U937 myeloid cell line has been successfully used to investigate C/EBPα and/or myeloid development. These cells are committed precursors and can give rise to granulocytic cells. Putative protein interacting partners of C/EBPα were identified by using a pull-down assay in which a GST-tagged C/EBPα fusion protein was incubated with lysates containing nuclear proteins (1 mg) from the myeloid U937 cell line **(Fig. 9)**. Equal amounts of GST and GST-C/EBPα were used for the pull down. Protein spots that were specific for the GST-C/EBPα fusion protein were analyzed by 2-D gel electrophoresis, and identified by matrix-assisted laser desorption/ionization time of flight mass spectrometry/MALDI-TOF (Bruker), or MALDI-TOF/TOF (Applied Biosystems, Darmstadt, Germany).

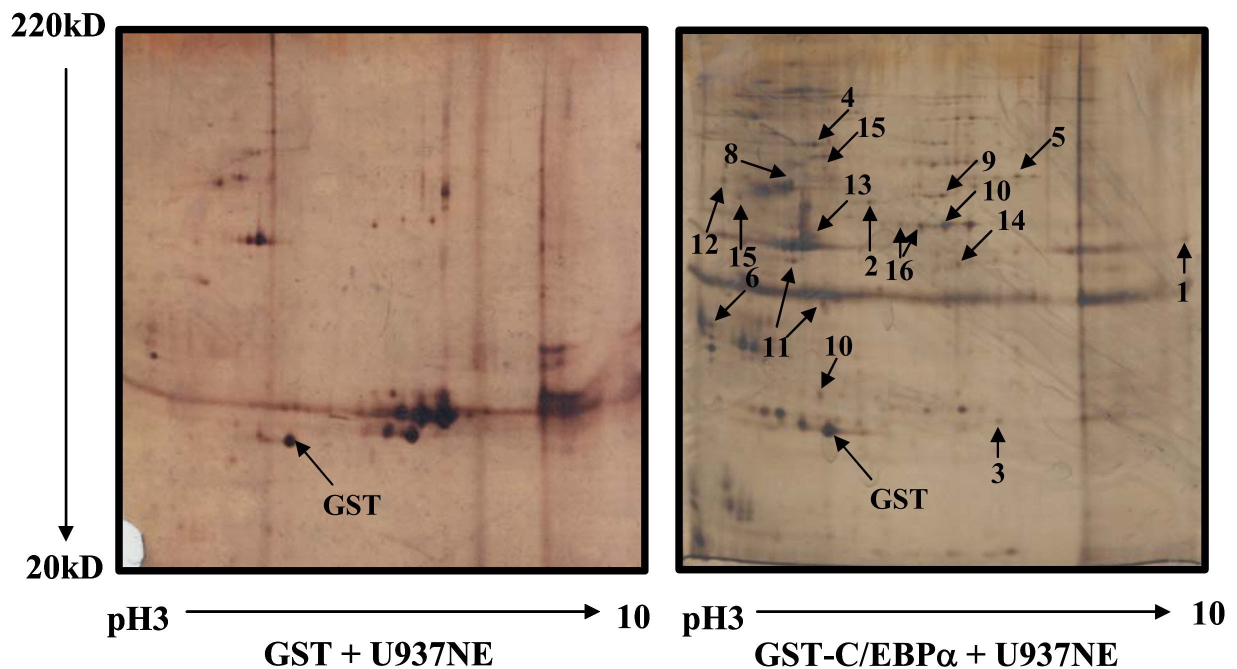



**Fig 9:** 2D gel electrophoresis for the identification of C/EBPα interacting proteins. U937

nuclear extracts were incubated with GST or the GST-C/EBPα fusion protein. Arrows in the silver stained 2D gel on the right (pH 3-10, 12 % SDS PAGE) indicate proteins specifically interacting with C/EBPα.

### 6.2.2 1D SDS PAGE of differentially interacting proteins

**A**fter thet GST pull down the samples were also directly subjected to 12 % SDS-PAGE (without 2-D gel electrophoresis) and stained with Coomassie blue. The complete lanes containing the interacting proteins were trypsin digested and peptides were separated by reverse-phase nano liquid chromatography and identified by MALDI-TOF/TOF.

Using the 2D gel approach, 19 potentially interacting proteins were identified: 7 with the MALDI-TOF (Bruker) machine and 12 with a MALDI-TOF/TOF (Applied Biosystems) **(Table 6).** We identified 11 additional proteins using the 1-D nano-LC approach **(Table 7)**. In summary, **we identified a total of 30 potential C/EBPα interacting proteins of which 4 were known previously as a binding partner of C/EBPα**. Among these known interacting partners of C/EBPα are pRB (retinoblastoma susceptibility protein), hnRNP, E2F4, and C/EBPα itself **(Table 6 and 7)**. Because the identified proteins included most reported C/EBPα interacting proteins, the reliability and efficiency of this approach appears quite high.

### *6.3 List of C/EBPα interacting proteins identified by 2D SDS PAGE and MS or MS/MS*

**Table 6:**

| Spot No. | Accession No. | Mr. (kD) | Score | Gene name | Description |
|---|---|---|---|---|---|
| 1 | Q92993 | 60.0 | 62 | *HTATIP* | HIV-1 Tat interactive protein 60kDa; TIP60 |
| 2 | Q13696 | 62.3 | 77 | *ACF7* | Actin cross linking family protein |
| 3 | Q9UKD2 | 27.5 | 74 | *MRT4* | mRNA turnover protein 4 homolog |
| 4 | Q96FE8 | 68.3 | 62 | *EWSR1* | Similar to Erwing Sarcoma protein |
| 5 | Q01844 | 68.4 | 70 | *EWS* | RNA-binding protein EWS |
| 6 | Q9HB58 | 78.5 | 59 | *SP110* | Transcriptional Coactivator sp110 |
| 7# | AAA82555 | 40.9 | 66 | *RB1* | Retinoblastoma Suspectibility Protein fragment |
| 8* | P62995 | 33.6 | 92 | *SFRS10* | Arginine/serine-rich splicing factor 10; TRA2B |
| 9* | Q9BZG1 | 29.0 | 61 | *RAB34* | Ras related protein Rab 34 |
| 10* | Q9Y6K8 | 22.0 | 57 | *AK5* | Adenylate kinase isoenzyme; KAD5 |
| 11* | P18669 | 28.6 | 261 | *PGAM1* | Phosphoglycerate mutase 1 |
| 12* | P33992 | 82.2 | 63 | *MCM 5* | DNA replication licensing factor; CDC46 homolog |
| 13* | P61812 | 48.5 | 69 | *TGFB 2* | Transforming growth factor beta-2 |
| 14* | Q14683 | 143.2 | 64 | *SMC1L1* | Structural maintenance of chromosome 1-like protein; SMC1A |
| 15#* | P07910 | 33.6 | 135 | *HNRPC* | Heterogeneous nuclear ribo-nucleoproteins; hnRNPC1/C2 |
| 16* | P23246 | 76.1 | 129 | *SFPQ* | Polyprimidine tract binding protein associated splice factor; PSF |
| 17* | P14136 | 49.8 | 71 | *GFAP* | Glial fibrillary acidic protein |
| 18* | Q14168 | 64.5 | 54 | *MPP2* | MAGUK p55 subfamily member 2 |
| 19* | Q6N069 | 101.4 | 55 | *NARG1L* | NMDA receptor-regulated 1-like protein; NARGL |

***Mass spectra were acquired using a Proteomics Analyzer 4700 (MALDI-TOF/TOF) mass spectrometer**

**# Same protein or family members were previously shown as an interacting partner of C/EBPαX**

### *6.4 List of C/EBPα interacting proteins identified by the 1D nano-LC-MS/MS method*

**Table 7:**

| **No.** | **Accession No.** | **Mr. (kD)** | **Peptide count** | **Total ion score confidence level*, %** | **Gene name** | **Description** |
|---|---|---|---|---|---|---|
| 1# | P49715 | 37.7 | 1 | 99.95 | *CEBPA* | CCAAT/enhancer-binding protein alpha; C/EBPα |
| 2 | Q99583 | 62.3 | 2 | 99.86 | *MNT* | Max-binding protein MNT; ROX |
| 3 | Q8TCU5 | 126.6 | 2 | 99.73 | *GRIN3A* | Glutamate [NMDA] receptor subunit 3A; NMD3A |
| 4 | Q16513 | 104.7 | 3 | 98.66 | *PKN2* | Protein-kinase C related kinase 2 |
| 5 | Q99996 | 455.7 | 2 | 97.777 | *AKAP9* | A-kinase anchor protein 9 |
| 6 | Q8IXJ9 | 165.4 | 3 | 97.47 | *ASXL1* | Putative Polycomb group protein |
| 7 | P17655 | 80.6 | 1 | 95.91 | *CAN2* | Calpain-2 catalytic subunit; M-calpain |
| 8 | Q9GZS1 | 53.9 | 1 | 95.83 | *PRAF1* | DNA-directed RNA polymerase I-associated factor 53kDa subunit; RPF53 |
| 9 | Q15906 | 40.7 | 1 | 95.52 | *VPS72* | Component of the NuA4 histone acetyltransferase complex which contains catalytic subunit TIP60 |
| 10 | P49792 | 358.1 | 2 | 95.38 | *RANBP2* | Nuclear pore complex protein Nup358; RBP2 |
| 11# | Q16254 | 44.2 | 1 | 95.37 | *E2F4* | Transcription factor E2F4 |

***MASCOT significance level for each search is defined as the 95% confidence level.**

**# Known interacting partners of C/EBPα**

## 6.5 Detailed Analysis of the Interaction of C/EBPα with Selected Putative Partner Proteins.

### 6.5.1 MCM5 interacts with C/EBPα *in vivo*

To confirm the interaction between C/EBPα and MCM5, co-immunoprecipitation and GST pulldown experiments were performed. Endogenous MCM5 could be co-immunoprecipitated with C/EBPα antibodies from U937 RIPA lysates. The membrane was then stripped and immunoblotted with a C/EBPα antibody showing the immunoprecipitated C/EBPα **(Fig. 10 A)**. Additionally, a GST pull down experiment was performed with U937 nuclear extracts using GST-C/EBPα and GST only as a control. Immunoblotting with an MCM5 antibody showed that GST-C/EBPα beads selectively retained MCM5 **(Fig. 10 B)**.

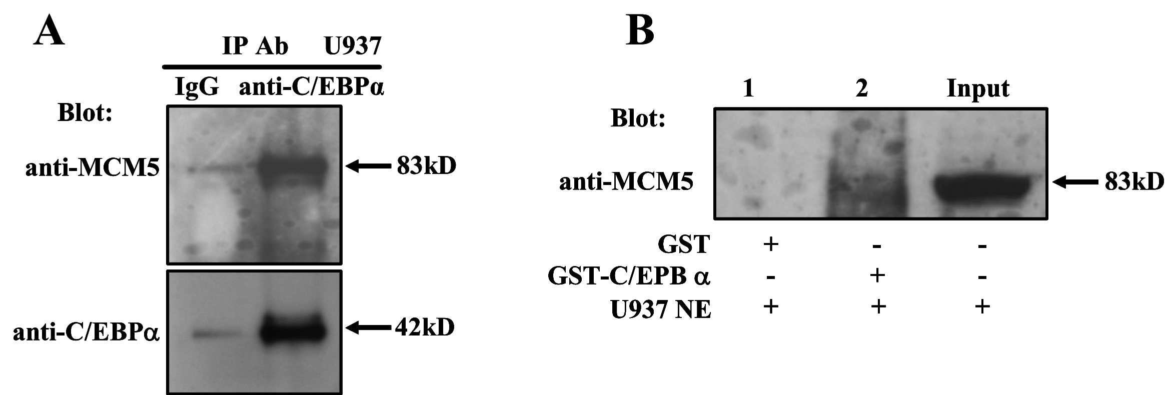



**Fig 10: C/EBPα physically interacts with MCM5:** Coimmunoprecipitation of endogenous MCM5 and C/EBPα from U937 RIPA lysates with C/EBPα antibody and control IgG. Western blot analysis was performed using an anti-MCM5 (upper panel) and an anti C/EBPα antibody (lower panel) for the same membrane. **B**. A GST pull-down assay was performed with U937 nuclear extracts (Input) incubated with equal amounts of bacterially expressed GST alone as a control (lane 1) and GST-C/EBPα (lane 2). Western blot analysis was performed by using anti-MCM5 antibody.

### 6.5.2 TIP60 interacts with C/EBPα directly

To confirm the physical interaction between C/EBPα and the proteins identified in our initial pull-down assay, we conducted co-immunoprecipitation and an *in vitro* GST pull-down assay for the histone aceyltransferase TIP60. To show Tip60 interaction with C/EBPα, GST-TIP60 was incubated with *in vitro* translated [$^{35}$S] methionine labelled C/EBPα. GST-TIP60 was able to retain C/EBPα **(Fig. 11 A, Lane 5)**. *In vitro* translated C/EBPα did not bind to GST beads alone **(Fig. 11 A, Lane 3)**. Unprogrammed reticulocyte lysate with [$^{35}$S] methionine incubated with GST-TIP60 served as a further control **(Fig. 11 A, Lane 4)**. We next asked if the interaction between TIP60 and C/EBPα occurs *in vivo*. 293T cells were transfected with expression plasmids for C/EBPα and YFP-tagged TIP60. The lysates of the transfected cells were immunoprecipitated with two TIP60-specific antibodies. Then immunoblotting of the precipitate with C/EBPα and GFP antibodies was performed **(Fig. 11 B)**. C/EBPα was specifically co-immunoprecipitated with TIP60-specific antibodies but not with the IgG control **(Fig. 11 B, panel-I)**. Detection of YFP-TIP60 under these conditions using an anti-GFP antibody served as a control **(Fig. 11 B, panel-II).**

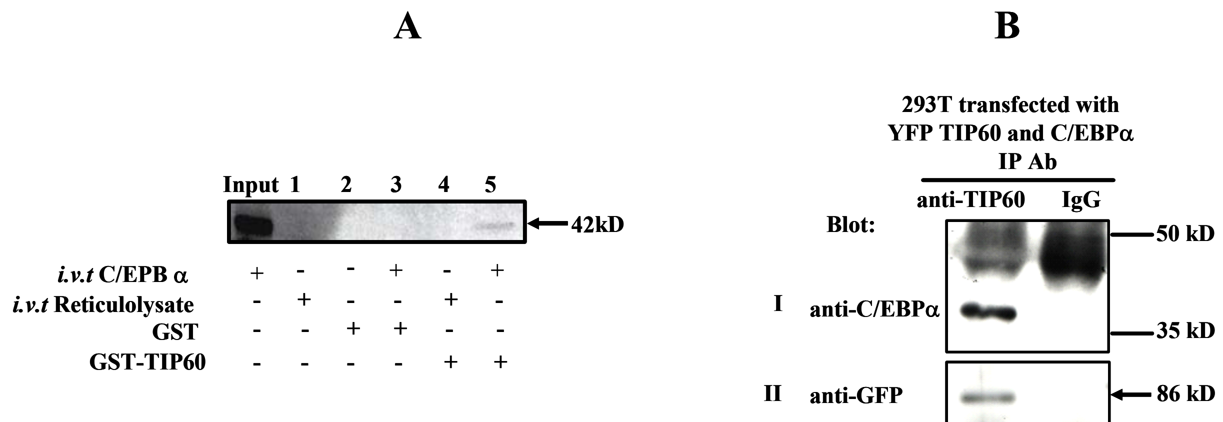



**Fig 11: C/EBPα physically interacts with TIP60 *in vitro* and *in vivo***: **A** GST pull-down assay was performed with [$^{35}$S]-methionine-labelled *in vitro*-translated C/EBPα and bacterially expressed GST-TIP60. Equal amounts of bacterially expressed GST-TIP60

(lane 5) or GST alone (lane 3) were incubated with *in vitro* translated C/EBPα. Only GST-TIP60 (lane 5) was able to retain C/EBPα. **B**. Coimmunoprecipitation of C/EBPα and TIP60 from nuclear extracts of transiently transfected 293T cells with TIP60 antibodies and an IgG control. Western blot analysis was performed by using anti-C/EBPα (panel I) and with anti-GFP antibody (panel II) on the same membrane.

## *6.6 TIP60 increases the transactivation capacity of C/EBPα*

Transient transfection assays were performed in the HEK293T and U937 cell lines using a luciferase reporter plasmid with a minimal TK promoter containing two CCAAT sites to test the ability of TIP60 to modulate C/EBPα-mediated transcriptional activation. Reporter gene expression was determined 24 h after transfection. Transfection of a TIP60 expression construct significantly enhanced the ability of C/EBPα to transactivate the reporter plasmid up to 25-fold in a dose-dependent manner **(Fig. 12 A)**. In control experiments, no effect of TIP60 on C/EBPα activity was observed when a reporter plasmid without CCAAT sites was used. While C/EBPα alone was able to transactivate the CCAAT site containing reporter up to 5-fold, TIP60 alone (without coexpression of C/EBPα) had no effect on luciferase activity, indicating that TIP60 specifically mediates up-regulation of the reporter through interaction with C/EBPα. Similar results were obtained in the myeloid U937 cell line **(Fig. 12 B)**. Thus, the association of C/EBPα with TIP60 potentiates the transactivation activity of C/EBPα.

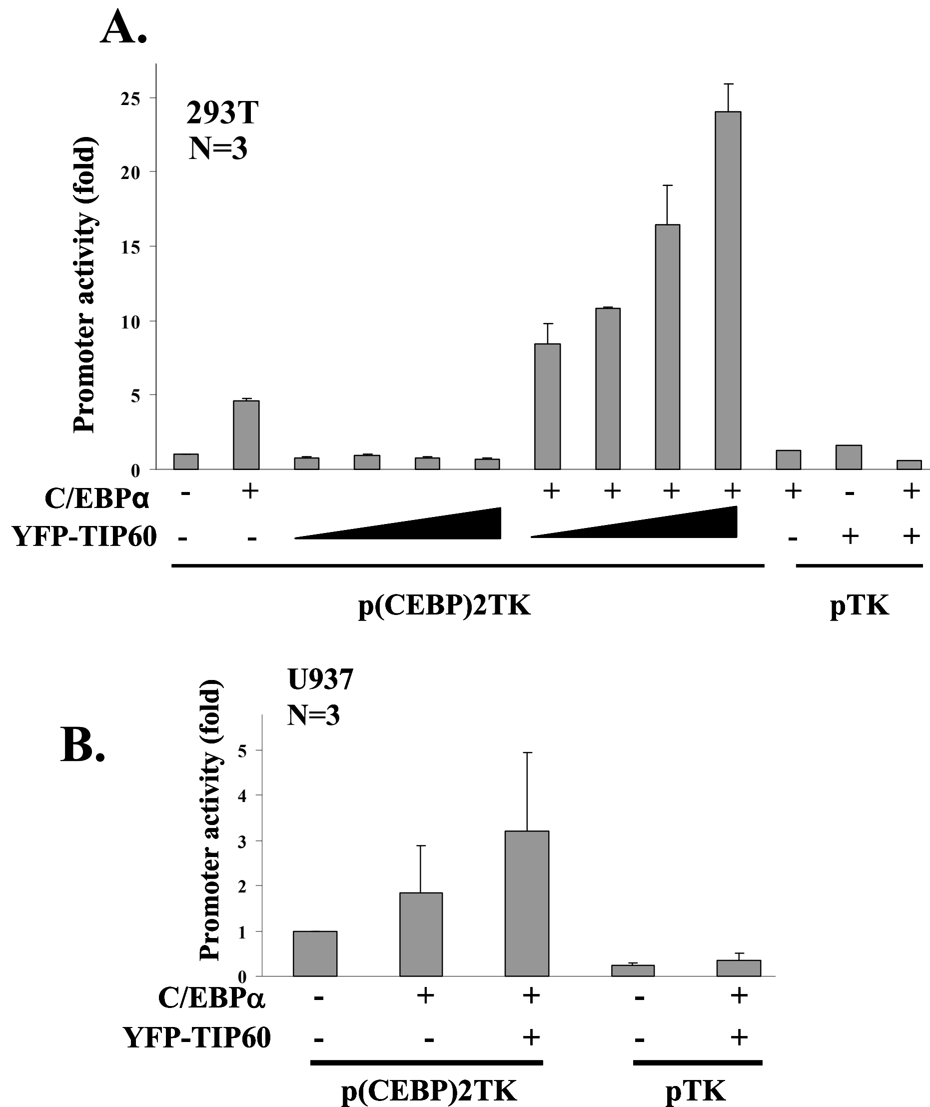
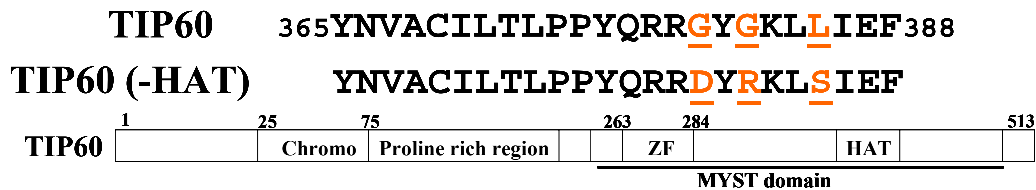



**Fig 12: TIP60 enhances the transactivation capacity of C/EBPα.** Transient transfection of 293T (**A**) and U937 cells (**B**) were performed with a reporter construct containing a minimal TK promoter with two CEBP binding sites p(CEBP)2TK or a reporter without CEBP sites (pTK) and expression plasmids for C/EBPα and YFP-TIP60. The four different amounts of YFP-TIP60 transfected in **A** were 0.05 µg, 0.1 µg, or 0.25 µg. The activity obtained for the p(CEPB)2TK plasmid without C/EBPα transfection was set as one and fold changes of normalized relative firefly luciferase activities are shown.

## 6.7 *A functioning TIP60 HAT domain is required for the co-operativity with C/EBPα*

TIP60 is a histone aceyltransferase (HAT). Therefore, we asked if the histone aceyltransferase activity of TIP60 is required for increasing the transcriptional activity of C/EBPα. To address this, we used site-directed mutagenesis to alter three critical amino acids in the acetyl co-enzyme A binding domain of the TIP60 HAT domain which resulted in a TIP60 protein lacking histone acetyltransferase activity; TIP60(-HAT) **(Fig. 13 A)** [106]. While YFP-TIP60 cotransfected with C/EBPα was able to increase luciferase activity 5-fold compared to C/EBPα alone, cotransfection of the YFP TIP60 (-HAT) expression plasmid with C/EBPα resulted in no change in transativation capacity compared to C/EBPα transfected alone **(Fig. 13 B)**. Increasing the concentration of the YFP TIP60(-HAT) was unable to change the transactivation potential of C/EBPα. YFP TIP60(-HAT) alone had no effect on the CCAAT binding site-containing reporter plasmid. These results demonstrate that the acetyl transferase activity of TIP60 is required for increasing the transcriptional activity of C/EBPα.

**A.**



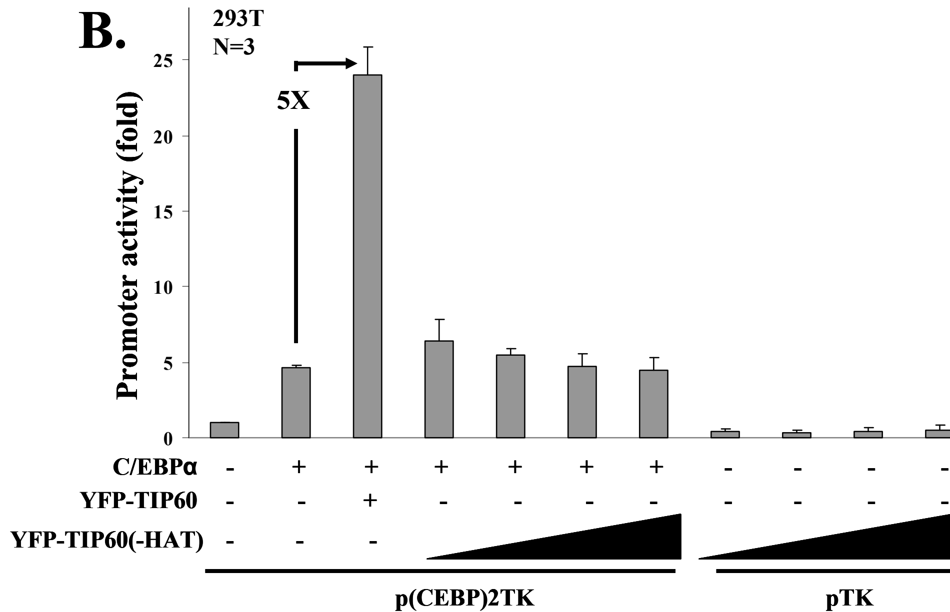



**Fig 13: TIP60(-HAT) does not enhances the transactivation capacity of C/EBPα.** **A**. Amino acid sequence of the core acetyl transferase domain of TIP60 with the mutated amino acids highlighted, and bar diagram of TIP60 with conserved protein domains. **B**. Transient transfection of 293T cells with the p(CEBP)2TK reporter construct and expression plasmids for C/EBPα, YFP-TIP60, and YFP-TIP60(-HAT). The four different amounts of YFP-TIP60(-HAT) transfected were 0.05 μg, 0.1 μg, 0.2 or 0.25 μg. The activity obtained for the p(CEPB)2TK plasmid without C/EBPα transfection was set as one and fold changes of normalized relative firefly luciferase activities are shown. YFP-TIP60 increases the transactivation up to 5 fold in a dose dependent manner while YFP-TIP60(-HAT) has no effect on C/EBPα transactivation.

## *6.8 The TIP60 HAT domain mutant protein is expressed at similar levels compared to wild type TIP60 in 293T*

We have shown that a functioning HAT domain of TIP60 is required for the co-operativity with C/EBPα on the CCAAT binding site containing reporter plasmid. However, the lack of increased transactivation after transfection of the HAT domain mutant TIP60 expression plasmid could be due to low expression of the YFP-TIP60(-HAT) protein. We therefore compared the levels of protein expression of the YFP-

TIP60 (wild) and YFP-TIP60(-HAT) plasmids transfected in 293T cells. There was no difference in the expression of these two proteins when detected with an anti-GFP antibody (Fig. 14). The same membrane was also blotted with an anti-β-tubulin antibody as a loading control **(Fig. 14)**.

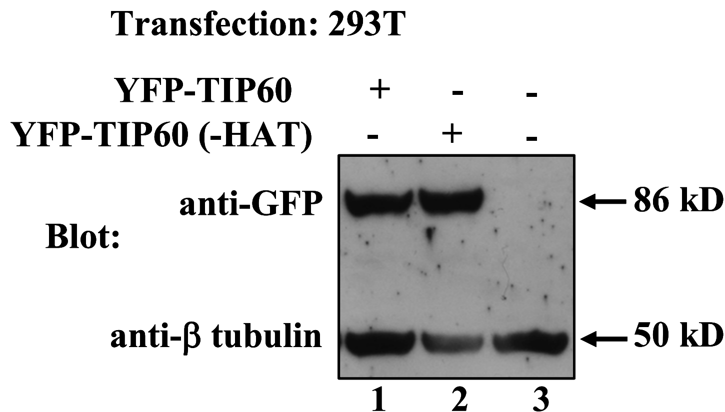
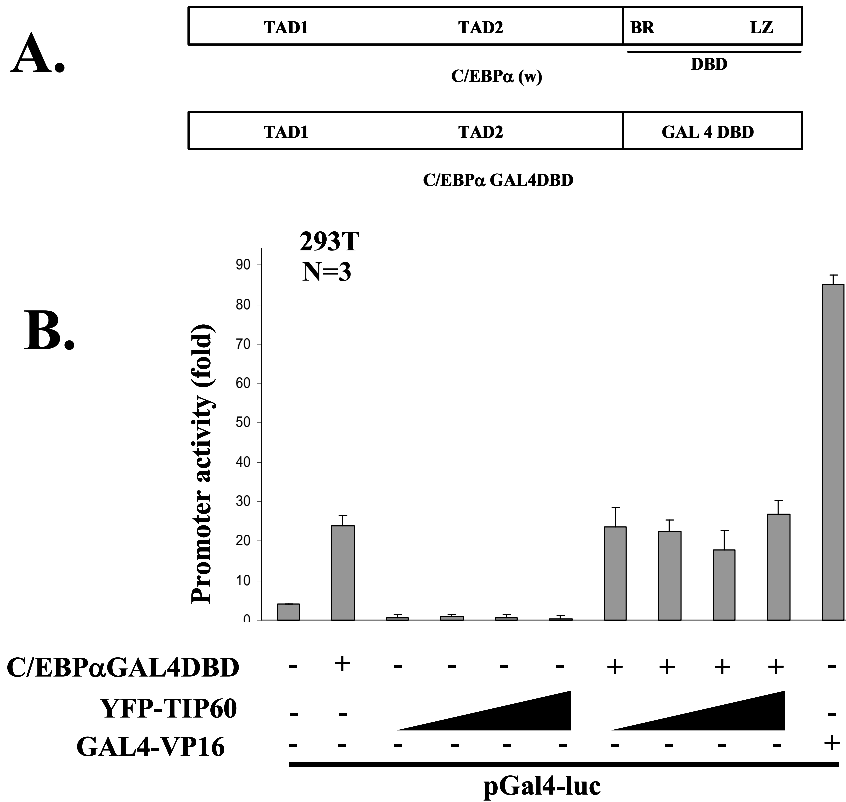



**Fig 14**: Western blot showing equal expression of YFP-TIP60 (lane 1), and YFP-TIP60(-HAT) (lane 2) in 293T cells.

## *6.9 Coactivation by TIP60 depends on the DNA binding domain of C/EBPα*

The DNA binding of C/EBPα is required for its transactivation potential [47,119] and we showed that the HAT activity of TIP60 is crucial for increasing the transactivation activity of C/EBPα **(Fig. 13)**. We therefore asked if TIP60-mediated increased transactivation potential is specific to C/EBPα or whether it is a general effect of TIP60 overexpression. To address this, we used the C/EBPα-Gal4DBD construct, in which the Gal4-DNA binding domain is fused to the transcriptional activation domain 1 and 2 (TAD1 and TAD2) of C/EBPα and replaces the DNA binding domain of C/EBPα (basic region/leucine zipper domain) **(Fig. 15 A)** [120]. C/EBPα-Gal4DBD transactivates a Gal4-UAS-luciferase reporter plasmid 33.5 fold as expected **(Fig. 15 B)**. However, cotransfection of TIP60 does not lead to an increased transactivation. These results indicate that the coactivator function of TIP60 requires the C/EBPα DNA binding domain. We, then performed a pull down assay with GST-C/EBPα,

GST-DBD (of C/EBPα), and GST alone with the nuclear extracts of 293T cells transfected with YFP-TIP60. Our results indicate that GST-DBD and GST-C/EBPα interact with TIP60 **(Fig. 15 C, Lane 1, 2)**. However, no interaction was observed when the GST protein only was incubated with nuclear extracts of YFP-TIP60 expressing cells **(Fig. 15 C, Lane 3)**.



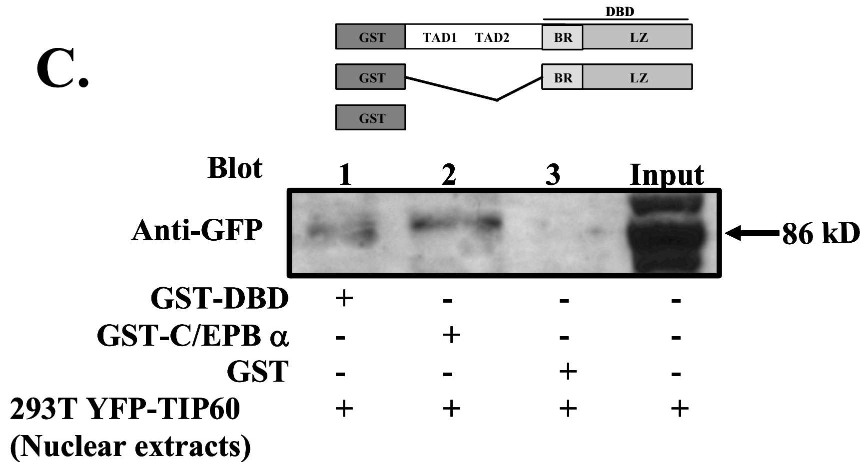



**Fig 15: Co-activation by TIP60 depends on the DNA binding domain of C/EBPα.** **A)** Diagram of the protein domain structure of C/EBPα and the C/EBPα-GAL4DBD construct, in which the basic region and leucine zipper of C/EBPα are replaced by the DNA binding domain of the yeast transcription factor GAL4. **B)** Transient transfection of 293T cells using a luciferase reporter plasmid containing Gal4DNA-binding sites only (pGal4-luc). The C/EBPαGAL4DBD is able to transactivate the reporter plasmid. However, coexpression of YFP-TIP60 does not lead to increased activation. GAL4-VP16 was used as a positive transactivation control. The normalized firefly luciferase activity obtained for the pGal4-luc plasmid without C/EBPαGAL4DBD transfection was set as one and fold changes are shown. **C)** GST pulldown showing physical interaction between YFP-TIP60 and GST-C/EBPα and GST-DBD (the DNA binding domain of C/EBPα fused to GST). YFP-TIP60 was transiently expressed in 293T cells. YFP-TIP60 was detected with an anti-GFP antibody after the pulldown experiment and in the cell lysate. GST-DBD and GST-C/EBPα interact with YFP-TIP60 (lane 1 and 2). GST alone does not retain YFP-TIP60 (lane 3).

## *6.10 TIP60 is found at the endogenous C/EBPα promoter in vivo*

Since the TIP60 and C/EBPα interaction could be shown to greatly change the transcriptional activation potential of C/EBPα, we examined whether this interaction

also occurs *in vivo* at the endogenous C/EBPα promoter. To this end, chromatin immunoprecipitation (ChIP) [121] experiments were performed **(Fig. 16)**. Chromatin was extracted from K562ER-C/EBPα cells and subjected to immunoprecipitation with antibodies directed against C/EBPα and TIP60. C/EBPα activation (nuclear translocation) can be induced in K562ER-C/EBPα cells with β-estradiol. The presence of the C/EBPα promoter DNA fragment, which contains the C/EBPα binding sites, in the immunoprecipitated chromatin fraction was detected by PCR amplification of a 280 bp region from the human C/EBPα promoter [122]. Our results revealed that even in uninduced conditions some endogenous TIP60 and C/EBPα are present at the C/EBPα promoter **(Fig. 16 Panel I Lane 7, 8)**. This is a first report showing the occupancy of the C/EBPα promoter by TIP60 *in vivo*. Since K56ER-C/EBPα cells can form granulocytes after induction with β-estradiol, we examined if induction of differentiation affects the C/EBPα and/or TIP60 binding at the C/EBPα promoter. After induction with β-estradiol both precipitation with anti TIP60 and anti C/EBPα antibodies led to a stronger amplification of the C/EBPα promoter amplicon **(Fig. 16 Panel II Lane 7, 8)**. Immunoprecipitation using an isotype-matched IgG was used as a negative control **(Fig. 16 Lane 4, 5)**. No amplification of an amplicon from the C/EBPα coding was found after using C/EBPα and TIP60 antibodies in the ChIP experiment (negative control) **(Fig. 16 Lane 10, 11)**. The induction of differentiation by β-estradiol was verified by analyzing the expression of CD11b, a marker of myeloid differentiation. Thus, C/EBPα and TIP60 associate *in vivo* in the context of chromatin and are more abundant on the C/EBPα promoter when cells are induced towards granulocytic differentiation.

TIP60 is known to acetylate histones H3 and H4 and C/EBPα has been shown to alter the acetylation of histone H3 [123]. We therefore examined whether the histone acetylation at the C/EBPα promoter would change upon C/EBPα-TIP60 recruitment. Antibodies against acetylated histones H3 and H4 were used to precipitate the cross-linked chromatin derived from uninduced and induced cells. Abundant levels of acetylated H3 and H4 histones were found to be present at the C/EBPα promoter after

induction with β-estradiol **(Fig. 16 Panel II Lane 1, 2)**. Whereas no H3 or H4 acetylation at the C/EBPα promoter was found in the uninduced conditions **(Fig. 16 Panel I Lane 1, 2)**. These data strongly suggest that TIP60 is involved in histone acetylation at the human C/EBPα promoter.

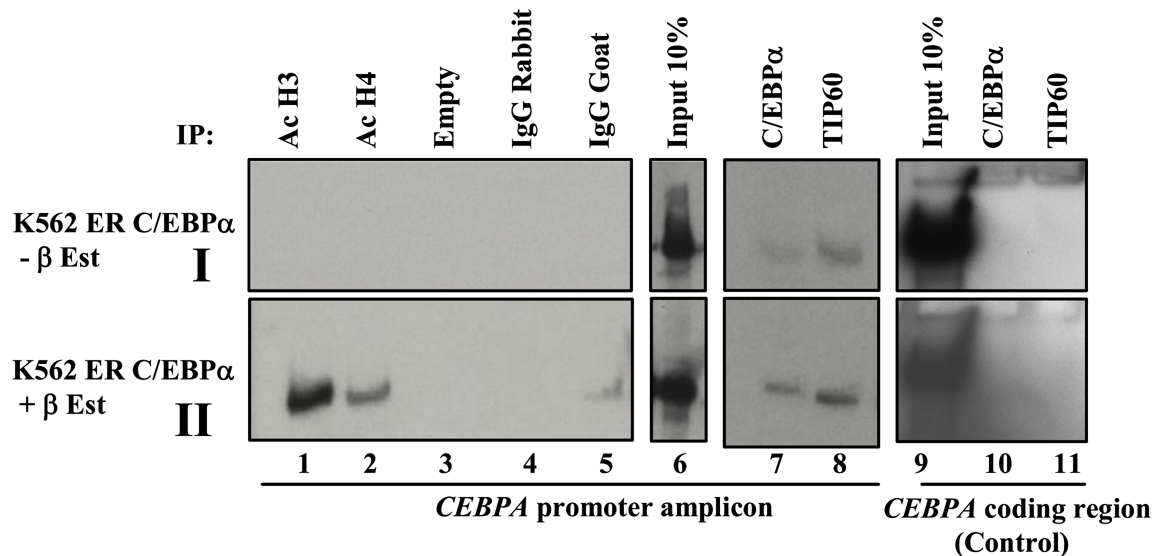
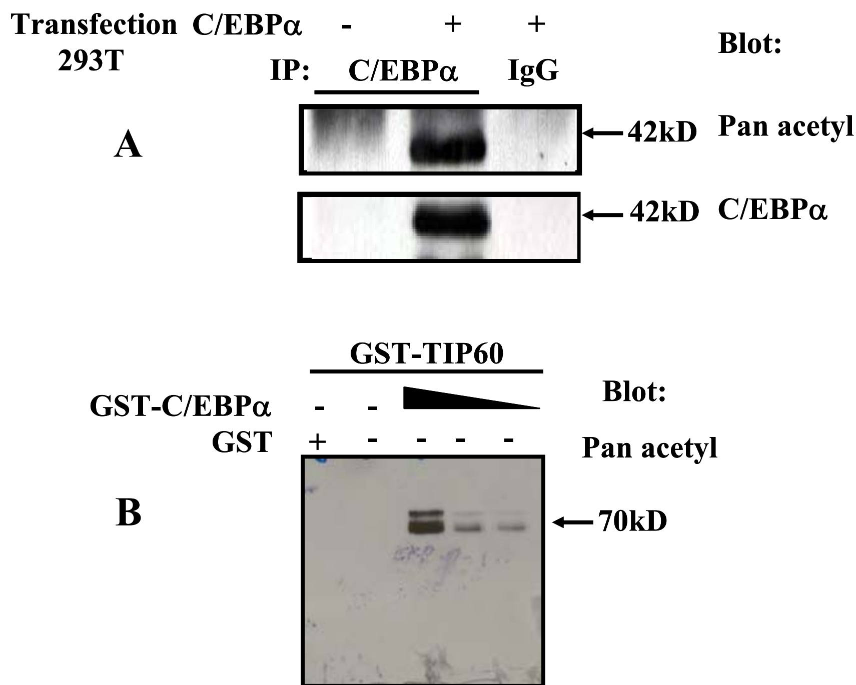



**Fig 16: TIP60 is recruited to the human C/EBPα promoter *in vivo* with concomitant increase in histone H3 and histone H4 acetylation after induction of granulocyte differentiation in K562ER-C/EBPα cells**. ChIP assay was performed on logarithmically-growing uninduced and β–estradiol induced K562ER-C/EBPα cells (6 hr after induction). An increase in the acetylation of H3 and H4 histones (Lane 1, 2) and an increase in C/EBPα and TIP60 binding at the C/EBPα promoter were observed in induced K562ER-C/EBPα cells (Lane 7, 8). Input (Lane 6): PCR performed on total chromatin. Immunoprecipitations with antibody isotype controls are shown in lane 4 and 5. PCR performed on chromatin immunoprecipitated using C/EBPα and TIP60 antibodies with primers for an amplicon in the C/EBPα coding region served as a control (Lane 9, 10, 11).

## *6.11 C/EBPα becomes acetylated in vivo and in vitro*

C/EBPα interacts with a number of histone aceyltransferases. However, acetylation of C/EBPα has not been reported so far. We inquired whether C/EBPα is subject to

acetylation. 293T cells were transfected with C/EBPα expression plasmid and nuclear extracts were prepared in the presence of the HDAC (Histone deacetylase) inhibitor, TSA (10 mM). Immunoprecipitation was carried out using C/EBPα antibody and blotted with Pan acetyl antibody (Santa cruz biotechnology). The same membrane was stripped and blotted with an anti C/EBPα antibody to demonstrate the immunoprecipitated C/EBPα **(Fig. 17 A)**. This is, to our knowledge, the first report showing C/EBPα can become acetylated. Furthermore, a non-radioactive *in vitro* acetylation reaction was performed to analyze whether TIP60 would be capable of acetylating C/EBPα. GST-TIP60 was incubated with GST-C/EBPα and GST as control with non-radioactive acetyl CoA, followed by SDS PAGE and probed with anti-acetyl antibody. These experiments clearly showed that C/EBPα becomes acetylated by TIP60 *in vitro* **(Fig. 17 B)**. However, in a P81 filter binding HAT assay, we observed no acetylation of GST-C/EBPα **(Fig. 17 C)** in the preseence of GST-TIP60.



**C**

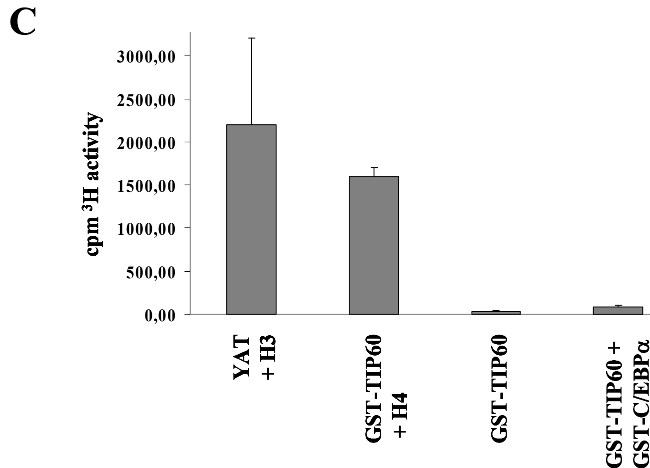



**Fig 17: C/EBPα acetylation: A.** Co-immunoprecipitation of C/EBPα from nuclear extracts of transiently transfected 293T cells with C/EBPα antibody and an IgG control. Western blot analysis was performed using an anti-acetyl antibody (upper panel) and an anti-C/EBPα antibody (lower panel) on the same membrane. **B**. An *in vitro* non-radioactive acetylation assay was performed with GST-TIP60 incubated with decreasing amounts of GST-C/EBPα and GST as control. Only GST-C/EBPα and not GST became acetylated in the presence of GST-TIP60. Western blot analysis was performed using an anti-acetyl antibody. **C.** HAT activity was monitored in the radioactive P81-filter binding assay: Yeast acetyl transferase (YAT) with histone H3 was used as a positive control. TIP60 is known to acetylate histone H4. GST-TIP60 alone was used as a negative control.

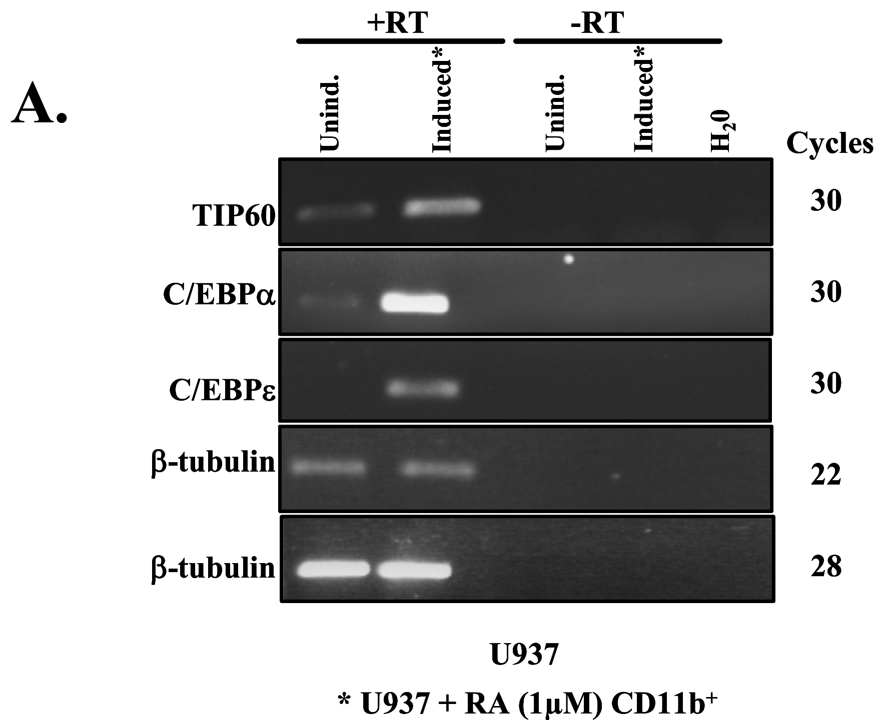
## *6.12 TIP60 expression in AML*

### 6.12.1 TIP60 mRNA expression is higher in differentiated U937 cells

TIP60 expression was analyzed by a semi quantitative RT-PCR assay in RNA isolated from FACS-sorted differentiated $CD11b^+$ U937 cells after 60 hours of treatment with retinoic acid. Higher TIP60 mRNA expression was found in committed granulocytic cells compared to undifferentiated $CD11b^-$ U937 cells. C/EBPα and C/EBPε transcript levels were used as positive controls for the induction of differentiation and β-tubulin levels were used as RNA quality controls **(Fig. 18 A)**.

### 6.12.2 TIP60 and C/EBPα expression levels correlate in different leukemia subtypes

To obtain a first glimpse of what the role of TIP60 in the development of AML might be, we analyzed the TIP60 mRNA expression in a microarray dataset from AML patients with defined leukemia subtypes **(Fig. 18 B)**. Our data suggest that there is a correlation between the expression levels of C/EBPα and TIP60 in normal bone marrow, CML and AML subtypes with the AML1/ETO (AML_M2), the CBFB/MYH11 (AML_M4) and the PML/RARA (AML_M3) fusion genes.




**U937**

*** U937 + RA (1µM) CD11b⁺**

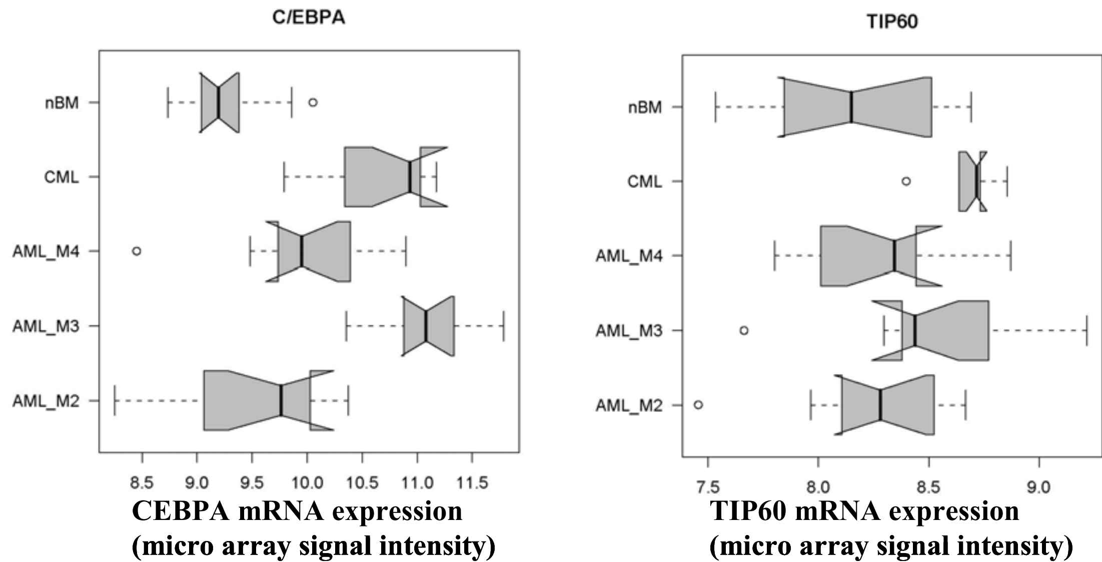



**Fig 18: TIP60 mRNA expression in differentiated cells and in AML subtypes. A.** Expression of TIP60, C/EBPα, C/EBPε analysed by semiquantitative RT PCR in CD11b-negative, retinoic acid untreated U937 cells and in retinoic acid treated, CD11b-positive U937 cells. **B.** Boxplots of mRNA expression levels (micro array signal intensity values) of C/EBPα and TIP60 in normal bone marrow, CML and 3 AML subtypes (AML_M4: AML with CBFB/MYH11 fusion; AML_M3: AML with PML/RARA fusion; AML_M2: AML with AML1/ETO fusion). The normalized and variance-stabilized expression values are shown on a logarithmic scale (approximately log 2). Dark bar represents median, boxes give the 25 to 75 % quantile range (inter quantile range: IQR), whiskers represent the 1.5 fold IQR, small circles represent outlier. If the "notches" of two distributions do not overlap, it is considered strong evidence that their medians differ.

# 7 DISCUSSION

## 7.1 C/EBPα interacting proteins

The C/EBPα protein is of great interest because of its involvement in myeloid differentiation and leukemia. C/EBPα is abundant in early myeloid cells where it binds to the promoters of key myeloid target genes [124], including its own promoter [29]. Ectopic C/EBPα expression induces granulocytic differentiation and blocks erythroid differentiation of human CD34$^+$ cells [21]. The identification of novel interacting partners of C/EBPα might lead to novel therapeutic approaches that specifically target leukemogenic mechanisms which depend on C/EBPα loss of function mutations [125]. C/EBPα interacting proteins have previously been identified by 1-D SDS PAGE coupled with mass spectrometry in prostate cancer cell lines [126] , using GST-C/EBP-DBD in myeloid cell line [70] and by yeast two hybrid screens [127]. Here, we describe two approaches, one based on 2-D gel electrophoresis and the other on 1-SDS PAGE/nano LC, to identify novel C/EBPα interacting proteins which will help in elucidating the complicated protein interaction network underlying normal C/EBPα function.

Several novel C/EBPα interacting proteins were identified by this approach (**Table 6 and 7**). These are proteins involved in DNA repair and cell cycle: SMC1, MCM5; Proteins involved in chromatin modification and remodeling VPS72 (which is a part of NuA4 complex containing TIP60), TIP60 and ASXL1; the Ras superfamily member RAB34; the MAX binding protein MNT; the nuclear pore complex protein RANBP2; the splice factors SFPQ and TRA2B; metabolic enzymes like KAD5 and PGAM1 and many other proteins including proteins like hnRNP, RB, E2F4, and C/EBPα, which had already been shown to interact with C/EBPα directly [51,80,86]. To confirm the protein interaction results found by mass spectrometry, we have confirmed the interaction between C/EBPα and MCM5 and TIP60 and further characterized the functional significance of the interaction between C/EBPα and TIP60.

Our two approaches, the 2-D and the 1-D/nano LC, revealed different C/EBPα interacting protein. This result is most likely due to the inherent differences in these two approaches which affect the coverage and the confidence of the protein identification procedure. In the 2-D gel approach, complex protein samples were separated over a larger defined pH range, increasing the resolution for each protein when compared to a 1-D SDS-PAGE gel. Furthermore, in contrast to the 2-D analyses, where differentially expressed spots were visually identified and manually excised, the identification of proteins from the 1-D gel was independent of their visualization and allowed for the identification of proteins with high isoelectric points.

Using these two approaches we identified a large number of known and novel C/EBPα interacting proteins. We could confirm the interaction of C/EBPα and two other proteins, namely MCM5 and TIP60, by alternative methods. Furthermore, we concentrated our efforts on analyzing the functional significance the interaction between C/EBPα and TIP60.

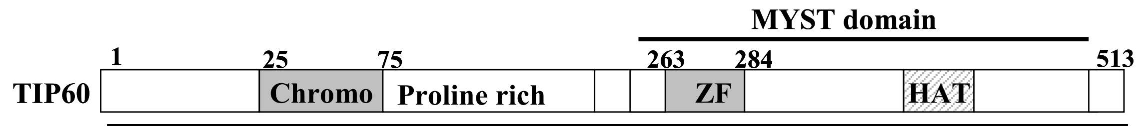
The identification of TIP60 as a novel interacting partner of C/EBPα was intriguing because TIP60 has been shown to be an interacting partner of a numbr of other transcription factors in mammalian cells and has been shown to function as a coactivator or a corepressor. TIP60-mediated repression might be effected through the recruitment of histone deacetylase HDAC7 [128] or independent of HAT activity [106,129]. TIP60-mediated activation is generally thought to require the HAT activity of TIP60, in particular through the acetylation of histones on target promoters. Transcription factors themselves might be substrates for TIP60-mediated acetylation [130].

TIP60 is also a very interesting protein because it might affect higher-order chromatin structure. The eukaryotic chromatin structure is very complex, highly dynamic and regulates virtually all DNA-associated processes, like transcription, replication and DNA repair [131]. Chromatin is also the basis of epigenetic inheritance, by which the differential expression state of genes can be transmitted through successive cellular generations [132]. There are two known broad classes of enzymes that regulate chromatin [133,134]. First the chromatin remodeling enzymes that uses ATP hydrolysis to modify the histone

composition or positioning of nucleosomes, without introducing covalent modifications of histones. Second, histone-modifying enzymes modify chromatin by covalently adding a variety of chemical moieties to specific histone residues. These enzymes are highly specifc and belong to a variety of protein families like histone acetyltransferases (HATs), deacetylases (HDACs), methyltransferases, demethylases, kinases, ubiquitin ligases and others. Chromatin–modifying enzymes exist as a part of large macromolecular complexes that exercise various functions like enzymatic activity, substrate specificity, chromatin association, and site specific recruitment by DNA-bound transcription factors. TIP60 is a HAT and is recruited by many transcription factors to a number of target promoters, where it participates in histone acetylation and transcriptional activation. It is interesting to note that members of the MYST domain histone acetyltransferase family are also involved in long lasting modifications of chromatin functions such as dosage compensation of the Drosophila X chromosome and therefore might play a special role in more long lasting epigenetic modifications which might be associated with permanent lineage decisions in the hematopoietic differentiation program. This is the first report of the interaction of the transcription factor C/EBPα interacting with a MYST family member protein.

## *7.2 Structure and function of the histone acetyltransferase TIP60*

Acetyltransferases are enzymes that catalyse the transfer of acetyl groups from acetyl coenzyme A to either the α-amino group of N-terminal amino acids or the ε-amino group of internal lysine residues. Lysine acetyltransferases fall into several categories, one of which is the MYST family, named after its founding members: MOZ, Ybf2/Sas3, Sas2 and TIP60. MYST family members function in a broad range of biological processes, such as gene regulation, dosage compensation of the *Drosopila* X chromosome, DNA damage repair and tumourigenesis [135]. Although MYST proteins seem to have diverse cellular roles, all family members are characterised by the highly conserved MYST acetyltransferase domain and most MYST enzymes exist as the catalytic subunits of multiprotein complexes.




**TFs: APP, Fe65, CREB, E2F, Myc, NF-κB, P53, PIX2, SRF, STAT3, UBF, Tbx, TEL, ZEB, TCF-β-catenin**

**Non-TFs: ATM, PIRH2, ETA, IL9-a, Nuclear receptors (AR,ER)**

**Fig 19:** Schematic diagram of TIP60 protein domains: ZF (Zinc finger), HAT (Histone acetyltransferase domain), MYST (MOZ, Ybf2/Sas3, Sas2 and TIP60). Proteins known to interact physically with TIP60 are shown.

TIP60 was originally isolated as a HIV-1 Tat interactive protein [136] **(Fig. 19)**. The *HTATIP* gene encoding TIP60 is located at 11q13.1 and consists of 14 exons. Alternative splicing results in the expression of at least three splice variants, TIP60 isoform 1, TIP60 isoform 2 (TIP60α) and TIP60 isoform 3 (TIP60β, PLA2 interacting protein, PLIP). The best characterised splice variant is isoform 2. Isoform 1 arises from translation of intron 1 and encodes for a protein with potentially distinct functions from isoform 2 [137]. Isoform 3 (TIP60β) results from the exclusion of exon 5 that encodes a proline-rich region [138] and appears to have similar properties as TIP60α [139]. TIP60 isoforms are expressed at relatively low levels in a broad variety of tissues and cells and exhibit cell type specific functions [129]. *HTATIP* homologues have been identified in various organisms, including *G. gallus*, *M. musculus* and *D. melanogaster*, which encode proteins that share considerable homology with human TIP60 (57–99 %) [140-142].

The TIP60 isoform 2 (TIP60α) is a 513 amino acid protein (58 kDa), which contains an N-terminal chromodomain and a C-terminal conserved MYST domain **(Fig. 19)** Chromodomains are present in many chromatin regulatory proteins and are thought to mediate interactions with methylated histone lysines or RNA molecules, although in the case of TIP60 the chromodomain may have yet unidentified functions [135,143]. The MYST domain is the catalytic domain and contains a short sequence (residues 335–404, ‘conserved HAT domain’), which binds to acetyl coenzyme A and the substrate and which is structurally conserved in other acetyltransferase families. The MYST domain

also contains a characteristic Cys-Cys-His-Cys zinc finger, which is essential for acetyltransferase activity and is required for protein–protein interactions [128,129,144].

Shortly after its discovery, it became evident that TIP60 possesses histone acetyltransferase (HAT) activity. Recombinant TIP60 acetylates core the histones H2A (Lys5), H3 (Lys14) and H4 (Lys5, Lys8, Lys12 and Lys16) *in vitro*; [145,146]. As part of a stable multiprotein complex, TIP60 can also modify histones assembled into nucleosomes *in vitro*; in this case, TIP60 selectively targets nucleosomal H2A and H4 [147]. Recent evidence from *D. melanogaster* indicates that TIP60 can also acetylate modified histone variants, such as phospho-H2Av at Lys5 [140]. TIP60 functions as transcriptional coactivator or corepressor, connecting a number of different factors to the basal transcriptional machinery.

Apart from histones, cellular TIP60 can acetylate transcription factors, such as androgen receptor (AR), the upstream binding transcription factor (UBF), the myelocytomatosis oncogene c (CMYC) [130,148,149] and the kinase Ataxia Telangiectasia mutated (ATM) [150].

## *7.3 Reported functions of TIP60*

**Nuclear receptor coactivation and involvement in prostate cancer:**

TIP60 predominantly coactivates and interacts with class I nuclear recpetors (NR) [151], although there have been reports of TIP60-dependent NR corepression [152]. TIP60 directly acetylates the androgen receptor (AR) and this acetylation is essential for TIP60-dependent AR coactivation. TIP60 forms a trimeric complex with histone deacetylase 1 (HDAC1) and AR on AR-responsive promoters and competes with HDAC1 most likely by inducing changes in AR acetylation status [148].

AR signalling is central to normal prostate development and carcinogenesis. The fact that TIP60 is involved in AR signalling suggests that it may be important for prostate cancer development. In metastatic prostate cancer cells, this function of TIP60 is lost, expression of TIP60 protein is decreased and TIP60 localisation is more diffuse [153].

**Involvement of TIP60 in MYC function:**

The TRRAP–TIP60 complex acts as a dual cofactor for the MYC transcription factor: The recruitment of the acetyltransferase complex TRRAP-TIP60 is important for the ability of CMYC to regulate promoters and enhance MYC transactivation efficiency independently of MYC acetylation [103] In addition, TIP60 can directly acetylate MYC which results in increased stability of the MYC protein [130].

**TIP60 and amyloid-β precursor protein signaling and NF-κB signaling**

Although subcellular localization data show that TIP60 is mainly a nuclear protein, there is also some evidence that suggests that TIP60 might have a role in signal transduction in the cytoplasm. TIP60 forms a protein complex in the cytoplasm with the intracellular tail fragment of the amyloid-beta precursor protein (APP) and the nuclear adapter protein Fe65. APP is a widely expressed cell surface protein involved in the pathophysiology of Alzheimer's disease [154-157]. This complex stimulates histone acetylation [158] and coactivates gene promoters which are linked to apoptosis and neurotoxicity [159]. However, recent evidence have cast doubt on the exact role of TIP60 in this complex as, in some cases, TIP60 appears to be redundant or even acts as a Fe65 corepressor [156,160].

The transcription factor nuclear factor kappa light chain gene enhancer in B cells (NF-κB) is a homo- or heterodimeric transcription factor that consists of members of the Rel family (Rel-A/p65, REL-B, c-Rel, p50 and p52) and controls processes, such as immunity, inflammation, proliferation and apoptosis. TIP60 acts as a coactivator of certain NF-κB-regulated genes like *KAI1* [161,162].

**Involvement of TIP60 in E2F-mediated transcription:**

E2F proteins can either activate or repress transcription. E2F1 recruits the TIP60 complex (Tip60, TRRAP, p400, Tip48, and Tip49) to target promoters *in vivo*. This recruitment is responsible for the H4 acetylation observed in several E2F responsive genes [104]. In addition, E2F, in cooperation with the transcription factors specificity protein 1/3 (Sp1 and Sp3), recruits TIP60 to the *MYCN* promoter [163].

**Involvement of TIP60 in other transcriptional processes:**

Tip60 was first identified as an interaction partner for the HIV-1 Tat protein and was shown to increase Tat transactivation of the HIV-1 promoter [136]. TIP60 has subsequently been reported to coregulate several other transcription factors. UBF, a ribosomal specific transcription factor actively involved in ribosomal gene transcription, is acetylated by Tip60 and coactivated by TIP60 within the nucleolus at sites of active rDNA transcription [149]. Jade-1 has two zing finger motifs called plant homeodomains (PHD). It is involved in renal cancer. TIP60 interacts with Jade-1 and is believed to be responsible for Jade-1-dependent H4 acetylation [164]. TIP60 is also reported to coactivate transactivation of the serum response factor (SRF) gene by the T-Box 2 and 5 (Tbx2 and Tbx5) transcription factors in cardiac cells thus modulating the expression of SRF-dependent cardic genes [165].

**TIP60 as a corepressor of transcription:**

TIP60 has not only been shown to be a transcriptional co-activator, but also been shown to be a transcriptional co-repressor depending on the proteins that it interacts with. TIP60 functions as a transcriptional coactivator for androgen receptor [148,166], NF-κB [161], UBF [149] and c-Myc [103] but also as co-repressor for ETV6 [144] and STAT3 [128]. This co-repressor function of TIP60 can be further enhanced by co-transfection of HDAC7. TIP60 usually induces gene repression via the recruitment of other complexes, for example, deacetylases. Another example of a transcription factors corepressed by TIP60 are the cAMP response element binding protein (CREB) [106,128]. CREB is a transcriptional activator stimulated by hormone and growth factor dependent phosphorylation by protein kinase A (PKA). TIP60 corepresses CREB by direct binding to CREB and repressing CREB stimulation by PKA [106]. STAT3 activity is modulated by a TIP60/HDAC7 complex [128]. Furthermore, TIP60 represses gene expression by cooperating with transcriptional repressors. Zinc finger E box (ZEB) binding protein is a repressor that associates with TIP60 and whose repressor activity is stimulated by TIP60 in certain cell types [129]. The translocation E26 transforming-specific leukaemia gene (TEL, ETV6) is a putative tumour suppressor gene and transcriptional repressor disrupted by a number of

chromosomal translocations in haematological malignancies [167]. TIP60 directly interacts with ETV6 and function as corepressor in ETV6-mediated transcription repression., possibly by stimulating the interaction between ETV6 and its corepressor Switch independent 3 (Sin3) and Silencing Mediator for retinoid and thyroid Receptor (SMRT)/NCoR [144].

**TIP60 is involved in DNA repair and apoptosis**

The TIP60 histone aceyltransferase complex also exhibits ATPase, DNA helicase, and structural DNA binding activities. Ectopic expression of mutated TIP60 lacking the histone acetyltransferase activity in HeLa cells results in cells with defective double stranded DNA break repair. Furthermore, the mutated TIP60 expressing cells were resistant to apoptosis [147].

## *7.4 C/EBPα and TIP60 interaction*

The direct physical interaction between C/EBPα and TIP60 was demonstrated by a GST pull down assay. The C/EBPα-TIP60 interaction was shown to occur *in vivo* by co immunoprecipitation and chromatin immunoprecipitation assay **(Fig. 11 A and Fig. 16)**. In reporter gene assays C/EBPα and TIP60 synergistically cooperated to stimulate transcription from a C/EBPα responsive promoter in a dose dependent manner **(Fig. 12)**. It could also been shown that the acetyltransferase activity of the MYST domain of TIP60 is essential for this co-activation activity. An acetyltransferase dead mutant of TIP60 showed no co-activation and was unable to cooperate with C/EBPα in enhancing the transcription of the luciferase reporter gene **(Fig. 13, A, B)**. These findings imply that TIP60 is directly responsible for the transcriptional coactivation observed and that this is not due to the recruitment of other activators by TIP60 to the promoter complex. Replacement of the DNA binding domain of C/EBPα with the yeast Gal4 DNA binding domain leads to a loss of TIP60 coactivator function with respect to C/EBPα **(Fig. 15 A, B).** The DNA binding domain of C/EBPα interacts directly with TIP60 **(Fig. 15 C)** suggesting that the basic region/leucine zipper domain of C/EBPα is critical for TIP60 recruitment.

Acetylation of transcription factors is a widely known phenomenon. Dynamic acetylation of non-histone proteins has multiple effects on cellular function. Acetylation increases the DNA binding affinity (GATA-2 acetylation by P300 and GCN5 [168]) or decreases the DNA binding affinity (acetylation of transcription factor YY1 by P300 and PCAF [169]). Acetylation increases (androgen receptor by TIP60 [148]) or decreases (Hypoxia-inducible factor 1, HIF-1, by arrest defective 1 protein, ARD1 [170]) the transcriptional activation. It might increase the protein stability (MYC acetylation by TIP60 [130]) or decrease the protein stability (HIF-1 acetylation by ARD1 [170]); might promote protein-protein interaction (STAT3 acetylation by P300/CBP [171]) or disrupts protein-protein interactions (Hsp90 hyperacetylation, results its dissociation from an essential cochaperone p23 [172].

TIP60 is known to acetylate non histone proteins such as UBF [149], MYC [130] and ATM [150]. Acetylation of C/EBPα by TIP60 has not been shown so far. To determine whether TIP60 has a role in C/EBPα acetylation, *in vitro* HAT assays were performed followed by Western blot. Evidence of acetylation was provided by sole use of acetylated-lysine-specific antibody **(Fig 17 A, B)**. However, we were unable to confirm that C/EBPα is directly acetylated by TIP60.by other methods like $^{3}$H *in vitro* HAT assays. Thus our results did not show conclusively that C/EBPα is acetylated. However our data do not rule out the possibility that C/EBPα is acetylated by TIP60. Alternative strategies to demonstrate C/EBPα acetylation by TIP60 such as HAT assays with cleaved the GST tag cleaved off from fusion protein, *in vivo* labeling or mass spectrometry are required to confirm our findings.

## *7.5 C/EBPα recruits TIP60 to chromatin*

C/EBPα is known to bind and cooperate with numerous chromatin modifying coactivators to stimulate transcription. It is also known that C/EBPα stimulates the acetylation of nucleosomal histones by CBP [102]. DNA binding of C/EBPα in adipocytes is associated with the recruitment of the coactivators CBP and p300 and abundant acetylation of histones [173]. C/EBPα also interacts with the SWI/SNF complex to induce proliferation arrest through the regulation of differentiation-specific genes [174]. Furthermore, expression

of C/EBPα led to the enhanced acetylation of histone H3 at peri-centromeric chromatin [123].

The association of TIP60 with the C/EBPα promoter *in vivo* under physiological conditions, and the recruitment of C/EBPα and TIP60 to the C/EBPα promoter during granulocytic differentiation indicates that the formation of a CEBPα-TIP60 complex on the C/EBPα promoter might be a key event during granulocytic differentiation. Our results showed that TIP60 and C/EBPα interact *in vivo* at the C/EBPα promoter concomitant with an increase in H3 and H4 acetylation **(Fig. 16)**. It could very well be that the primary targets of TIP60 acetylation in this context are the histones in the promoter regions of C/EBPα regulated genes. We do not know whether histone acetylation is a prerequisite or a consequence of the C/EBPα-TIP60 interaction. So far, this is the first report showing occupancy of the C/EBPα promoter by TIP60 *in vivo*.

Whether TIP60 functions as a tumor suppressor gene or oncogene is unknown at the present time and might be highly context dependent. TIP60 could influence tumor progression either positively or negatively, depending on whether we consider it as a coactivator for TFs that promote (e.g. Myc, E2F) or suppress tumorigenesis (e.g. p53, C/EBPα). In addition, competition or sequestration of TIP60 by various transcription factors such as MYC may constitute a mechanism of negative cross talk between different signaling pathways.

TIP60 is a part of the TRRAP complex [133]. It is thus very likely that other members of the TIP60 complex are also recruited to the C/EBPα promoter. TIP60 may therefore serve as a platform for the assembly of multiprotein complexes. The role of signaling pathways that control the association of TIP60 with C/EBPα might be equally important in controlling tumor progression.

## *7.6 C/EBPα and TIP60 m RNA expression in AML*

Higher expression levels of TIP60 mRNA in retinoic acid induced differentiated U937 CD11b$^{+}$ cells compared to undifferentiated U937 CD11b$^{-}$ cells suggest that higher TIP60

expression is associated with myeloid differentiation similar to what is observed for C/EBPα expression **(Fig. 18 A)**. In order to get a first glimpse at the role of TIP60 in the pathogenesis of AML, we examined TIP60 and C/EBPα mRNA expression in a microarray data set from AML patients with defined leukemia subtypes. Interestingly, TIP60 and C/EBPα showed high expression levels in CML compared to normal bone marrow and similar expression trends in three AML subtypes **(Fig. 18 B)**. The significance of these observations for the development of AML and for granulocytic differentiation has to be analyzed in future experiments.

Interestingly, TIP60 and two other MYST domain HATs (MOZ and MORF) have been shown to interact with other leukemia-relevant proteins like ETV6 [144,175] or to participate in leukemia-associated chromosomal translocations (e.g. the MOZ/TIF2, MOZ/P300 and MORF/CBP) [176-178]. All MYST family members share a region of similarity of approximately 240 amino acids in length containing the canonical acetyl CoA binding site found in most acetyl transferases [179]. MYST family members are also implicated in transcriptional silencing in yeast (SAS2 and YBF2/SAS3), and in dosage compensation in *Drosophila* (MOF). A novel gene product, hMOF, which exhibits significant similarity to the *Drosophila* dosage compensation regulator Mof (males absent of the first) has been found in humans [180]. MYST acetyltransferase proteins differ from other acetyltransferase: 1). Involvement in DNA repair 2). Long lasting changes for dosage compensation. The presence of MYST homologs in organisms as diverse as yeast and humans suggests that these proteins function in one or more important cellular processes.

These observations and findings further confirm the functional synergism between C/EBPα and TIP60 and suggest that TIP60 might be an important player in leukemogenesis. It is now well known that changes in chromatin structure, which corresponds to the epigenetic memory of a cell, play an essential role in cellular differentiation processes and lineage choices. The findings here give a glimpse of the complex and intricate network of interactions between TIP60 and transcription factor that regulate myeloid cell development. It is therefore tempting to speculate that the MYST domain histone acetyltransferase TIP60 is crucial in converting the transcriptional decisions initiated by C/EBPα into long-lasting epigenetic chromatin changes. In

summary, our findings that TIP60 interacts both physically and functionally with the myeloid transcription factor C/EBPα **(Fig. 20)** will lead to further studies to define the role of TIP60 protein complexes in the process of hematopoietic cell differentiation and leukemogenesis.

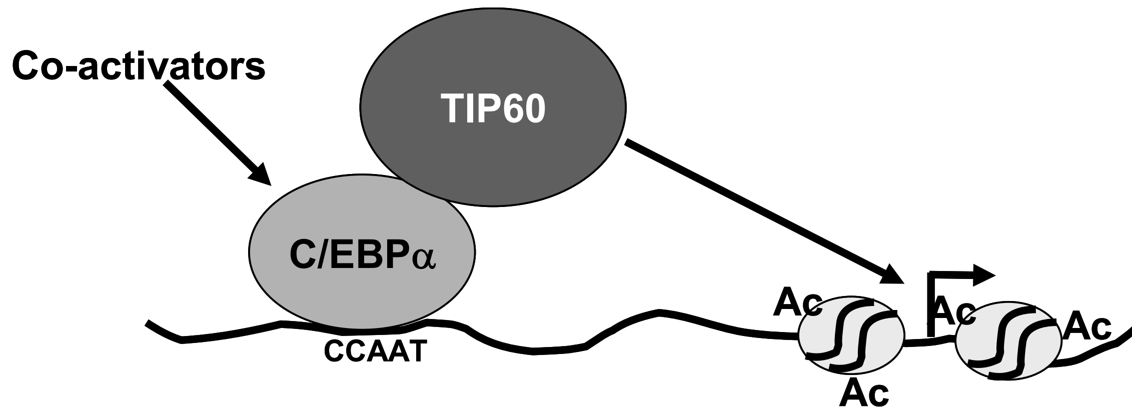



**Fig 20: MODEL** Direct recruitment of TIP60 by C/EBPα may serve to modulate C/EBPα transactivation potential by aiding disruption of local chromatin structure thereby facilitating C/EBPα access to its CCAAT DNA binding sites and providing easier access for other transcription factors at the promoters of target genes.

# 8 SUMMARY

The transcription factor C/EBPα is a key player in granulopoiesis and leukemogenesis. In the present study, we sought to identify C/EBPα interacting proteins. A glutathione-S-transferase-C/EBPα fusion protein was used to pull down interacting proteins from U937 nuclear extracts. These proteins were analyzed by 2-D gel electrophoresis or 1-D nano LC and identified by mass spectrometry. The interaction between C/EBPα and two novel interacting partners, the cell cycle regulator protein MCM5 and the MYST domain histone aceyltransferase TIP60, was confirmed by using pull-down and co-immunoprecipitation experiments. TIP60 was able to markedly enhance C/EBPα mediated transcriptional activation in reporter gene assays, suggesting that TIP60 is a co-activator of C/EBPα. This co-activator function of TIP60 was dependent on its intact histone aceyltransferase domain and on the C/EBPα DNA binding domain. TIP60 was found to be associated with the human C/EBPα promoter *in vivo* in a chromatin immunoprecipitation assay with a concomitant increase in histone H3 and H4 acetylation. Furthermore, we observed a lower expression of TIP60 mRNA in undifferentiated U937 $CD11b^-$ cells compared to retinoic acid induced differentiated U937 $CD11b^+$ cells suggesting that higher TIP60 expression is associated with myeloid differentiation. Correlated expression between C/EBPα and TIP60 was also observed in certain leukemia subtypes. These findings point to a functional synergism between C/EBPα and TIP60 in myeloid differentiation and suggests that TIP60 might be an important player in leukemogenesis.

# 9 ZUSAMMENFASSUNG

Der Transkriptionsfaktor C/EBPα spielt eine wichtige Rolle bei der Entwicklung von Granulozyten sowie bei der Leukämieentstehung. Das Ziel dieser Arbeit war, Proteine zu identifizieren, die mit C/EBPα interagieren. Ein Glutathione-S-Transferase-C/EBPα-Fusionsprotein wurde verwendet, um interagierende Proteine aus Extrakten der Zelllinie U937 zu binden. Diese Proteine wurden mittels 2-D Gelelektrophorese oder chromatographisch getrennt und anschließend im Massenspektrometer identifiziert. Die Interaktion von C/EBPα mit zwei neuen Interaktionspartnern, dem Zell-Zyklus-Regulator MCM5 sowie der MYST Domänen Histon-Acetyltransferase TIP60, wurde mit Hilfe von *Pull-down-* und Koimmunpräzipitations-Experimenten bestätigt. TIP60 konnte die Transkriptions-Aktivierung eines Reportergens durch C/EBPα deutlich verstärken, was darauf hinweist, dass TIP60 ein Co-Aktivator von C/EBPα ist. Für diese Co-Aktivation werden eine intakte Histon-Acetyltransferasedomäne von TIP60 sowie die DNA-Bindungsdomäne von C/EBPα benötigt. Nach Chromatin Immunpräzipitation war TIP60 mit dem humanen C/EBPα Promotor *in vivo* assoziiert, wobei gleichzeitig die Histone H3 und H4 eine erhöhte Acetylierung aufwiesen. Darüber hinaus fand sich eine verminderte Expression der TIP60 mRNA in undifferenzierten U937 $CD11b^-$ Zellen im Vergleich zu U937 $CD11b^+$ Zellen, deren Differenzierung mit Retinolsäure induziert wurde, was auf einen Zusammenhang zwischen der Expression von TIP60 und der myeloiden Differenzierung hinweist. Es wurde auch eine Korrelation der Expressionshöhen von C/EBPα und TIP60 in bestimmten Formen von Leukämie beobachtet. Zusammengefasst weißen die Ergebnisse auf einen funktionellen Synergismus zwischen C/EBPα und TIP60 während der myeloiden Differenzierung hin, was den Schluss zuläßt, dass TIP60 eine wichtige Rolle bei der Leukämieentstehung spielen könnte.

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# 11 ACKNOWLEDGEMENTS

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I want to thank my parents, who taught me the value of hard work by their own example and my lovely sisters Teena and Daizy. They rendered me enormous support during the whole tenure of my research. I am thankful to my friends, at home, and elsewhere in the world for their dedication and advice.

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# 12 CURRICULUM VITAE

**Deepak Bararia**

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***RESEARCH EXPERIENCE***

***2001 -present*** ***Ph.D. Human Biology, Klinikum Grosshadern, Ludwig Maximillians Universität, Germany.***

"Proteomic Identification and Biological Characterization of C/EBPα multiprotein Complex".

Work involves systematic identification and characterization of proteins at a global proteome-wide level. We hypothesized that the identification and functional characterization of all C/EBPalpha interacting proteins will lead to novel insights into the systems biology of C/EBPalpha. C/EBPalpha interacting proteins were identified by two-dimensional gel electrophoresis and MALDI-TOF mass spectrometry. We were able to identify known as well as novel interacting proteins of C/EBPalpha which include proteins involved in DNA repair, cell cycle; chromatin modification and remodelling; metabolic enzymes. We have further characterized the interaction of C/EBPalpha with HIV-1Tat interactive Protein (TIP60)

***1999-2001*** ***Master of Biotechnology (65.2%)***
***All India Institute of Medical Sciences, New Delhi, India.***

M.Sc. Dissertation. Title of the thesis: "Mutation Specific Polymerase Chain Reaction to study sequence variation in *dhfr* and *dhps* genes of *Plasmodium falciparum* and their correlation with antifolate resistance.

**Summary:**
The work involved detection of point mutations at various codon sites of dihydrofolate reductase gene and dihydrofolate synthetase gene, in the field isolates of *Plasmodium falciparum* from India. These point mutations were correlated with the reported antifolate resistance. Sequencing and analysing representative PCR products confirmed the mutations.

*1996-1999* ***Bachelor of Science (Microbiology)(64.9%)***
***Delhi University, Delhi, India***

*1995* **Senior Secondary School Examination, C.B.S.E. India.** ***(71.2%)***

*1993* **Secondary School Examination, C.B.S.E. India.** ***(77%)***

## ACHIEVEMENTS AND AWARDS

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*2006* **Oral Presentation: Fifth Scientific Symposium of the Department of Medicine III, University Hospital Grosshadern, LMU Munich.**
*Herrsching.*
Proteomic identification of Tip60, a histone acetyltransferase, as binding partner and coactivator of C/EBPα.

*2006* **Oral presentation at Deutsche Gesellschaft für Humangenetik e.v.**
Proteomic identification of Tip60, a histone acetyltransferase, as binding partner and coactivator of C/EBPα.

**Bararia D,** Trivedi AK, Peerzada AA, Hiddemann W , Behre G, Bohlander S

*2006* **Oral presentation at 32nd Annual Meeting of the European Group for Blood an Marrow Transplantation 22nd Meeting of the EBMT Nurses Group 5th Meeting of the EBMT Data Management Group Hamburg, Germany**
Proteomic identification of histone acetyltransferase TIP60 as a coactivator of myeloid transcription factor C/EBPalpha
**Bararia D,** Trivedi AK, PeerZada AA, Christopeit, H. Behre, G. Behre

*2004* **Oral presentation at Deutsche Gesellschaft für Hämatologie und Onkologie (DGHO) Innsbruck, Austria, 2004.**
Proteomic identification of Tip60, a histone acetyltransferase, as binding partner and coactivator of C/EBPα whose expression is downregulated in patients with acute myeloid leukemia

**Bararia D,** Trivedi AK, Peerzada AA, Hiddemann W, Behre G

*2003* **Poster presentations at Deutsche Gesellschaft für Hämatologie und Onkologie (DGHO), Basel, Switzerland. "**Proteomic Identification and Biological Characterization of C/EBPα multiprotein Complex".

*2003* Poster presentations at American Society of Hematology (ASH) San Diego,USA. "**Proteomic Identification and Biological Characterization of C/EBPα multiprotein Complex".**

***2002*** Participant in on line Bioinformatic course, Organised by s*star.org

***2001*** Cleared ICMR (Indian Centre for Medical Research and Education) JRF (Junior research fellowship) examination May 2001, Govt. of India.

***2001*** Poster presentations at AIIMS, Institute Day, 2001.
MALARIA: A major global health problem.
PCR-molecular biology technique.

***1999-2001*** Recipient of Scholarship during M.Biotechnology Course sponsored by Department of Biotechnology (D.B.T.), India.

***1996-1999*** Stood first in B.SC (H) Microbiology (Swami Shraddhanand College, University of Delhi)

***1998*** Third prize in Inter-College Quiz Contest. Organized by Biochemical Society, Department of Biochemistry, (University of Delhi).

***1997*** First prize in the Inter-College Quiz Contest sponsored by AMI (Association of Microbiologist of India), Delhi, Unit-II.

***PUBLICATIONS***

***2006*** Trivedi AK, **Bararia D**, Christopeit M, Peerzada AA, Singh SM, Kieser A, Hiddemann W, Behre HM, Behre G.

Proteomic identification of C/EBP-DBD multiprotein complex: JNK1 activates stem cell regulator C/EBPalpha by inhibiting its ubiquitination. **ncogene. 2006 Sep 18**

***2006*** Abdul A Peer Zada, John A Pulikkan, **Deepak Bararia**, Mulu Geletu, Arun K Trivedi, Mumtaz Y Balkhi, Wolfgang D Hiddemann, Daniel G Tenen, Hermann Behre, Gerhard Behre.

Proteomic discovery of Max as a novel interacting partner of C/EBPα: a Myc/Max/Mad link. **(In press: Leukemia)**

***2004*** Anwar Ahmed, **Deepak Bararia**, Sumiti Vinayak, Mohammed Yameen, Sukla Biswas, Vas Dev, Ashwani Kumar, Musharraf A. Ansari, and Yagya D. Sharma.

*Plasmodium falciparum* Isolates in India Exhibit a Progressive Increase in Mutations Associated with Sulfadoxine-Pyrimethamine Resistance. **Antimicrob. Agents Chemother. 2004. 48: 879-889**

## ***ACADEMIC LABORATORY EXPERIENCE***

Proteomics and MALDI-TOF, LC/MS/MS

Basic laboratory techniques, Tissue culture techniques, Cloning, Real Time-PCR, Southern blots, EMSA, *in-vitro* kinase reaction, Proteosomal assay, DNA sequencing, Probe preparation, Western Blot, Chromatin immunoprecipitations, , Immunoprecipitations methods.

## ***EXTRA-CURRICULAR ACTIVITIES***

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***1999-2001*** Organiser for various Seminars at Department of Biotechnology A.I.I.M.S.

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ORIGINAL ARTICLE

# Proteomic identification of the MYST domain histone acetyltransferase TIP60 (HTATIP) as a co-activator of the myeloid transcription factor C/EBPα

D Bararia[1], AK Trivedi[2], AA Peer Zada[2], PA Greif[1], MA Mulaw[1], M Christopeit[2], W Hiddemann[1], SK Bohlander[1,3] and G Behre[2,3]

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**The transcription factor C/EBPα (CEBPA) is a key player in granulopoiesis and leukemogenesis. We have previously reported the interaction of C/EBPα with other proteins (utilizing mass spectrometry) in transcriptional regulation. In the present study, we characterized the association of the MYST domain histone acetyltransferase Tat-interactive protein (TIP) 60 (HTATIP) with C/EBPα. We show in pull-down and co-precipitation experiments that C/EBPα and HTATIP interact. A chromatin immunoprecipitation (ChIP) and a confirmatory Re-ChIP assay revealed *in vivo* occupancy of the C/EBPα and GCSF-R promoter by HTATIP. Reporter gene assays showed that HTATIP is a co-activator of C/EBPα. The co-activator function of HTATIP is dependent on its intact histone acetyltransferase (HAT) domain and on the C/EBPα DNA-binding domain. The resulting balance between histone acetylation and deacetylation at the C/EBPα promoter might represent an important mechanism of C/EBPα action. We observed a lower expression of HTATIP mRNA in undifferentiated U937 cells compared to retinoic acid-induced differentiated U937 cells, and correlated expression of CEBPA and HTATIP mRNA levels were observed in leukemia samples. These findings point to a functional synergism between C/EBPα and HTATIP in myeloid differentiation and suggest that HTATIP might be an important player in leukemogenesis.**
*Leukemia* advance online publication, 31 January 2008; doi:10.1038/sj.leu.2405101
**Keywords:** C/EBPα; HTATIP; mass spectrometry; AML

## Introduction

Transcription factors strongly influence cellular lineage commitment during hematopoiesis, and the enforced expression of some transcription factors can alter the fate of developing hematopoietic progenitor cells.[1] The CCAAT/enhancer-binding protein alpha (C/EBPα, CEBPA) is a tissue-specific transcription factor expressed in the liver, differentiating adipocytes and myelomonocytic cells that was shown to regulate hematopoietic activity.[2,3] C/EBPα-knockout mice have a block in granulocyte differentiation in the fetal liver and in newborns.[4] C/EBPα acts as a tumor suppressor gene in acute myeloid leukemia (AML).[5] Inactivation of C/EBPα is an important event in AML and overexpression of C/EBPα leads to differentiation and growth arrest in AML.[6] Thus, C/EBPα has essential functions in the regulation of cell proliferation and differentiation. Recent data from our laboratory indicated that the differentiation and proliferation functions of C/EBPα involve direct protein–protein interactions.[7–10] Furthermore, C/EBPα is known to cooperate with the histone acetyltransferases (HATs) p300 and CREB-binding protein (CBP) to activate transcription.[11,12]

Thus, C/EBPα functions within a large network consisting of different protein–protein interactions to regulate gene expression and hence cellular differentiation by maintaining a fine balance between histone acetylation and deacetylation. However, in-depth studies involving protein–protein interactions of C/EBPα with HATs are lacking.[11,13]

In our efforts to identify interacting partners of C/EBPα utilizing mass spectrometry-based proteomics,[7,8] we identified TIP60 (HTATIP; Tat-interactive protein, 60 kDa), a MYST family HAT. The identification of TIP60 as a C/EBPα interacting partner is intriguing because of the role of TIP60 in transcriptional activation, DNA repair and histone acetylation. TIP60 functions as a co-activator of many key cellular proteins such as nuclear hormone receptors, β-catenin and nuclear factor-κB.[14]

In the present study, we provide evidence for the physical association of TIP60 with C/EBPα and the consequences of this association for the transcriptional function of C/EBPα, and for the correlated expression between C/EBPα and TIP60 in certain subtypes of leukemia and during differentiation of U937 cell line. Our data might provide a useful framework for elucidating the role of TIP60 in the biology of leukemia in general and in novel C/EBPα functions in particular.

## Materials and methods

### Cell culture, plasmids and reporter constructs

The HEK293T, U937 and K562 C/EBPα-ER (estrogen receptor) cell lines were cultured as described previously.[7] The pEYFP (yellow fluorescent protein)-N1-TIP60 and pEYFP-N1-TIP60 (-HAT) plasmids have been described.[15] TIP60 was subcloned from pcDNA3-TIP60 to obtain the pGEX-4T3-TIP60 (GST-TIP60) expression plasmid. The pcDNA3-C/EBPα, pcDNA6-C/EBPα-$His_6$, pTK, p(C/EBP)2TK, pGAL4-luc and pGAL4DBD-VP16 plasmids were described previously.[7,9] As an internal control plasmid for co-transfection assays, the pRL-TK (thymidine kinase) construct driving a *Renilla* luciferase gene (Promega, Madison, WI, USA) was used. The C/EBPα-GAL4DBD expression plasmid was kindly provided by A Friedman. GST, GST-C/EBPα and GST-C/EBPα DBD (the basic region and the leucine zipper of C/EBPα; amino acid 270–358, fused to GST) plasmids were kind gifts from C Nerlov. GST-C/EBPα 1–97 (fragment

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containing transactivation domain 1) and GST-C/EBPα 98–262 (fragment containing transactivation domain 2) of C/EBPα were constructed by PCR amplification from C/EBPα-$His_6$ and cloned into the *Eco*RI/*Xho*I sites of pGEX4 T.1 (Amersham Biosciences, GE Healthcare, Uppsala, Sweden). Details of plasmid construction are available upon request.

### *Reporter gene assays*

Reporter assays using transient transfection of 293T cells have been described before.[7] The DNA amount of the reporter constructs and expression plasmids used for transfections were 0.1 μg for pTK, p(CEBP)2TK and pGAL4-luc; 0.01 μg for pRL-TK; 0.1 μg for the expression plasmids for C/EBPα and 0.05, 0.1, 0.2 or 0.3 μg for pEYFP-N1-TIP60 (or pcDNA3-TIP60) or pEYFP-N1-TIP60(-HAT) and 0.1, 0.2 or 0.3 μg for C/EBPα-GAL4DBD. The total DNA amount of pre-transfection was normalized with the corresponding empty expression vectors.

### *Glutathione* S*-transferase pull down, gel electrophoresis and mass spectrometry*

The proteomics methodology was used essentially as described recently by our group.[7,8] The separation of peptides by one-dimensional nano reverse-phase liquid chromatography and their identification by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF/TOF), including the use of the GPS Explorer software for protein identification, were performed as described.[16]

### *Co-precipitation*

293T cells were transfected with expression plasmids for YFP-TIP60, YFP-EV (empty vector) and $His_6$-tagged C/EBPα as described.[17] For co-immunoprecipitation, nuclear extracts were prepared from 293T cells transfected with C/EBPα and YFP-TIP60 expression plasmids and were used for pull down with antibodies against TIP60 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-5725 (N terminal) and sc-5727 (C terminal), 1 μg each, mixed together). The protocol used for co-immunoprecipitation was described before.[8] The following antibodies were used for subsequent immunodetection: rabbit anti-C/EBPα polyclonal antibody (sc-61; Santa Cruz), rabbit anti-green fluorescent protein (GFP) monoclonal antibody (Invitrogen, Molecular Probes, Karlsruhe, Germany) and mouse anti-$His_6$ (1 922 416, Roche, Indianapolis, IN, USA).

Chromatin immunoprecipitation (ChIP) assay, Re-ChIP, real-time PCR quantification of genomic DNA ChIP, cell sorting, semiquantitative reverse transcriptase-PCR and expression analysis of HTATIP/CEBPA in leukemia samples are described in detail in the Supplementary Information.

## Results

### *Proteomic identification of TIP60 as an interacting partner of C/EBPα*

We have previously reported the use of mass spectrometry-based proteomics in identifying C/EBPα-interacting proteins *in vitro* as well as *in vivo*.[7,8] In the present study, we extended our approach to include full-length C/EBPα. A glutathione *S*-transferase (GST) C/EBPα fusion protein was used to pull down C/EBPα-interacting proteins from nuclear extracts of the myeloid cell line U937 prepared under similar experimental conditions as described previously[7] (Supplementary Figure 1a). Protein spots specific for the GST-C/EBPα pull down (Figure 1a) were excised from the gels and identified by MALDI-TOF/TOF. Additionally, pre-purified GST pull-down samples (pre-cleared with GST protein) were incubated with the GST-C/EBPα bait and subjected to 12% SDS-polyacrylamide gel electrophoresis (PAGE) followed by nano reverse-phase liquid chromatography and subsequent peptide identification by MALDI-TOF/TOF (Figure 1b). Twenty-nine candidate interacting proteins were identified using these approaches (Supplementary Table 1). No major overlap was found among the proteins identified by the two approaches used, probably reflecting the differences in the separation techniques used and the fact that only a subset of potential interactors was identified by either approach. However, confirming the validity of these approaches several known C/EBPα interacting partners, such as pRB (retinoblastoma susceptibility protein), hnRNP, E2F4 and C/EBPα itself, were among the proteins identified.

TIP60 was identified as one of the interacting partners of C/EBPα. Because of its function as a HAT involved in many biological processes, we chose to concentrate on the analysis of the TIP60–C/EBPα interaction.

### *Confirmation of C/EBPα and TIP60 interaction*

To confirm the interaction of TIP60 with C/EBPα, we performed co-precipitation and GST pull-down assays. The GST-TIP60 fusion protein was able to retain *in vitro*-translated [$^{35}$S]methionine-labeled C/EBPα (Figure 2a, lane 5), whereas GST alone was not (Figure 2a, lane 3). We next sought to characterize this potential interaction in a myeloid cell line. As previously reported, endogenous TIP60 could not be immunodetected by available TIP60 antibodies. Therefore, 293T cells transfected with expression constructs for $His_6$-tagged C/EBPα and YFP-TIP60 or YFP alone were followed by a Nickel-NTA ($Ni^{2+}$-nitrilotriacetate) Agarose purification of the His-tagged C/EBPα protein and western blotting using anti-GFP and anti-His antibodies. This experiment showed that YFP-TIP60 but not YFP was recovered with C/EBPα (Figure 2b, compare lanes 3 and 4). Similarly, co-immunoprecipitation assays revealed that C/EBPα co-immunoprecipitates with TIP60 antibodies but not with immunoglobulin G control (Supplementary Figure 1b). Confocal microscopy also revealed colocalization of TIP60 and C/EBPα in the nucleus (data not shown). Taken together, these experiments showed that C/EBPα and TIP60 do interact, confirming the result of the proteomic interaction screen.

### *TIP60 increases the ability of C/EBPα to transactivate a minimal TK promoter*

To test the ability of TIP60 to modulate C/EBPα-mediated transcriptional activation, transient transfection assays were performed in the HEK293T cell line using a luciferase reporter plasmid with a minimal TK promoter containing two CCAAT sites.[8] Co-transfection of a TIP60 expression construct enhanced the ability of C/EBPα to transactivate the reporter plasmid fivefold compared to C/EBPα alone in a dose-dependent manner (Figure 2c). In control experiments, no effect of TIP60 on C/EBPα activity was observed when a reporter plasmid without CCAAT sites was used. TIP60 alone had no effect on luciferase activity, indicating that TIP60 specifically mediates upregulation of the reporter through its interaction with C/EBPα.

### *HAT activity of TIP60 is required for its cooperativity with C/EBPα*

We next investigated whether the HAT activity of TIP60 is required to increase the transcriptional activation capacity of

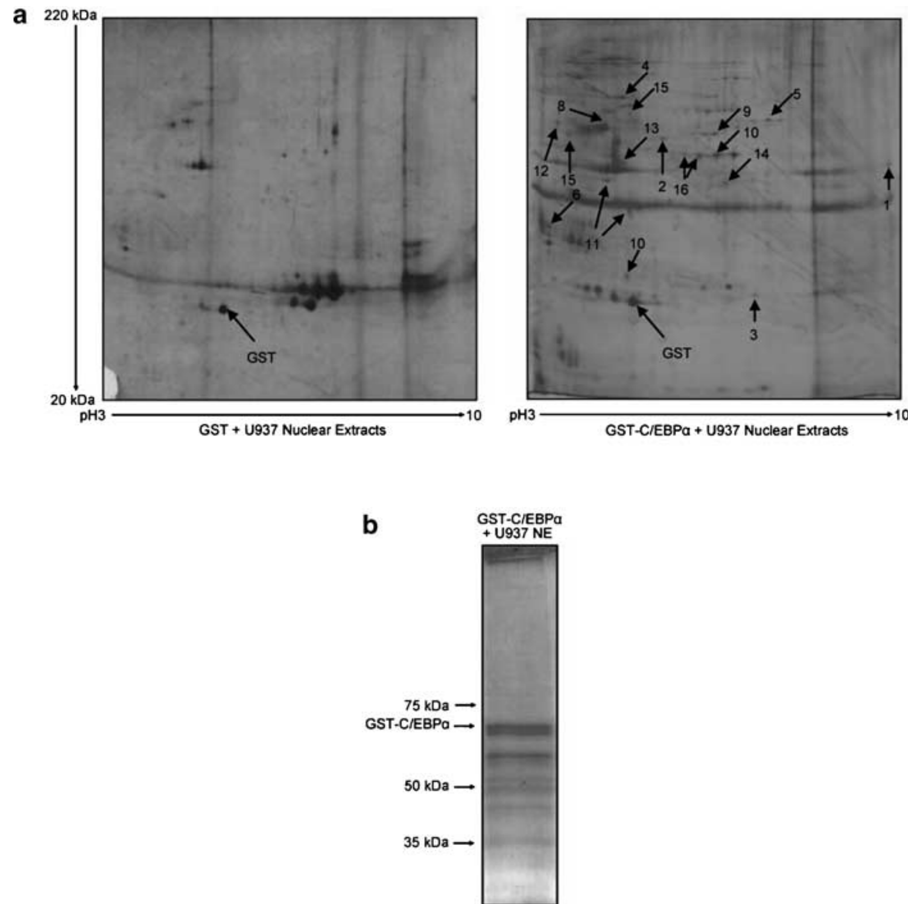


**Figure 1** Mass spectrometry-based proteomics identifies putative proteins of the C/EBPα multiprotein complex. (**a**) Silver-stained two-dimensional gel showing differentially expressed proteins after glutathione *S*-transferase (GST) pull down from nuclear extracts of U937; protein spots that were excised and subsequently analyzed by mass spectrometry are marked with arrows. (**b**) Coomassie-stained SDS-polyacrylamide gel electrophoresis (PAGE) gels after GST pull down from U937 nuclear extracts using GST-C/EBPα. Nuclear extracts were pre-cleared thrice by incubation with GST protein that retained all the proteins unspecifically interacting with both matrix and the GST protein. The pre-cleared extract was then incubated with the GST-C/EBPα bait. After several wash steps to remove unbound proteins, the complex components were eluted from the beads, separated by two-dimensional SDS-PAGE and stained with colloidal Coomassie; proteins identified are listed in Supplementary Table 1.

C/EBPα. While YFP-TIP60 co-transfected with C/EBPα is able to increase the luciferase activity fivefold compared to C/EBPα alone, co-transfection of the YFP-TIP60(-HAT) expression plasmid failed to affect the transactivation capacity of C/EBPα (Figure 2d). YFP-TIP60 and YFP-TIP60(-HAT) were expressed at equal levels (Figure 2e). Furthermore, we show that co-transfection of C/EBPα with TIP60 does not alter the C/EBPα protein levels to rule out an increase in the reporter gene activity due to increased C/EBPα protein levels (Figure 2f).

### *Co-activation by TIP60 depends on the DNA-binding domain of C/EBPα on a GAL4-responsive promoter*

The DNA-binding domain (DBD) of C/EBPα is required for its transactivation potential.[18] To investigate whether the TIP60-mediated increase in the transactivation capacity of C/EBPα requires the DBD of C/EBPα, a C/EBPα-Gal4DBD construct was used, in which the DBD of C/EBPα is replaced by the Gal4-DBD. C/EBPα-Gal4DBD transactivates a Gal4-$UAS_5$ (pentameric upstream-activating sequence) luciferase reporter plasmid 35-fold (Figure 3a). However, co-transfection of TIP60 did not lead to increased transactivation. Gal4DBD-VP16 was used as a positive control in these experiments. These results indicate that the co-activator function of TIP60 requires the C/EBPα-DBD.

To investigate the protein domains involved in the C/EBPα–TIP60 interaction, we performed GST pull-down experiments using GST-tagged C/EBPα DBD and C/EBPα 1–97. 293T cells were transfected with pcDNA-TIP60 and 24 h after transfection, nuclear extracts were incubated with the GST-tagged proteins as shown in Figure 3b (upper panel). Immunoblot analysis using TIP60 antibody revealed that TIP60 interacts with GST-C/EBPα, GST-C/EBPα DBD and GST-C/EBPα 1–97. Equal amounts of GST-tagged proteins were used in these experiments (Figure 3b, lower panel, Supplementary Figure 1c). Taken together, we conclude that both the DBD and the fragment containing the transactivation domain of C/EBPα are sufficient on their own for interaction with TIP60.

### *TIP60 occupies the endogenous C/EBPα and GCSFR promoters* in vivo

We next investigated whether the C/EBPα–TIP60 interaction also occurs *in vivo* at the C/EBPα promoter and the GCSFR promoter, which is known to be a target of C/EBPα.[19,20] To address this, we performed quantitative radioactive and non-radioactive ChIP experiments in K562 cells that express conditionally active C/EBPα-ER chimaera (Figure 4). These cells undergo granulocytic differentiation when treated with β-estradiol (data not

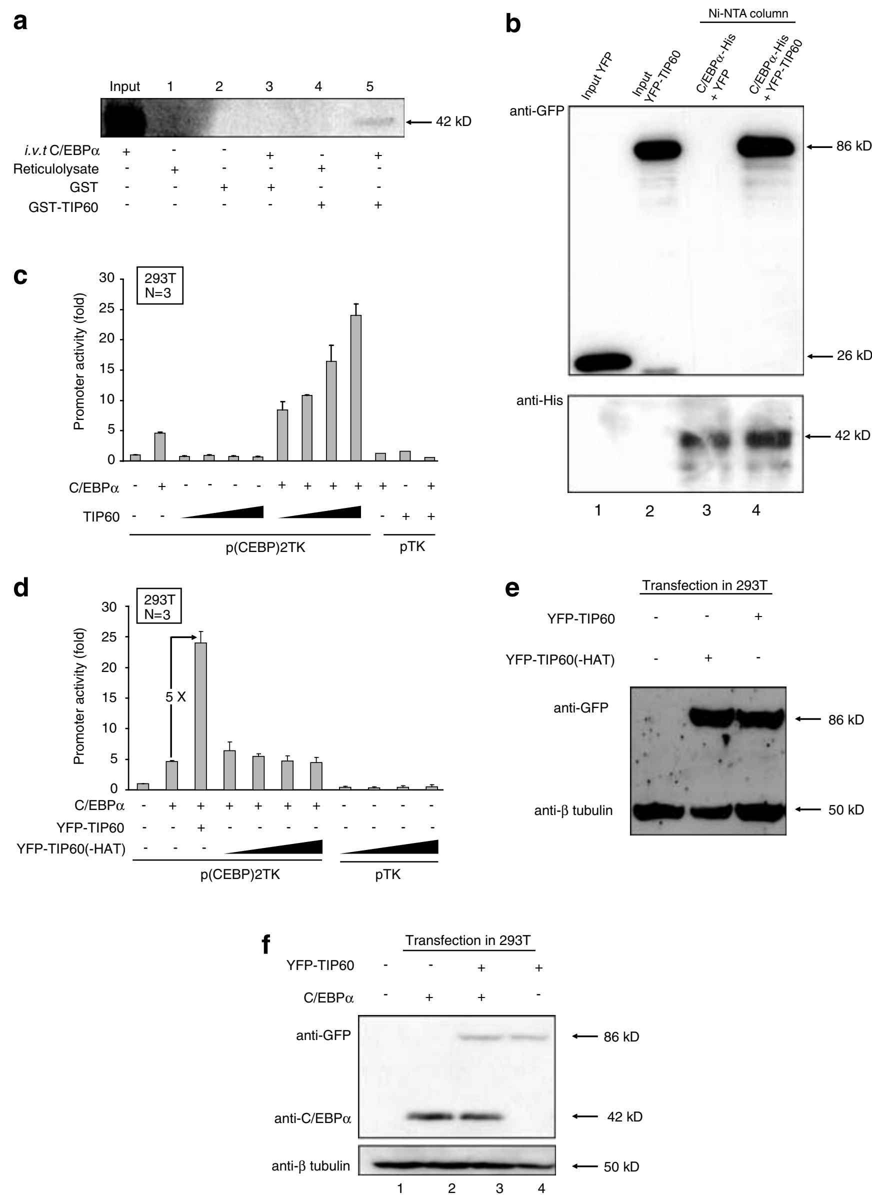


**Figure 2** C/EBPα interacts with TIP60. (**a**) Glutathione *S*-transferase (GST) pull-down assay was performed with [$^{35}$S]methionine-labeled *in vitro*-translated C/EBPα and bacterially expressed GST-TIP60. Only GST-TIP60 (lane 5) was able to retain C/EBPα. (**b**) 293T cells were transfected with C/EBPα-His$_6$ and yellow fluorescent protein-empty vector (YFP-EV) or YFP-TIP60 as indicated and then harvested in co-immunoprecipitation buffer and incubated with Nickel-NTA agarose beads to precipitate His-tagged C/EBPα protein. Eluted proteins were analyzed by western blotting. The upper half of the western blot was probed with a green fluorescent protein (GFP) antibody and the bottom half with a His antibody as indicated. Western blot analysis was performed on the same membrane. (**c**) TIP60 increases the ability of C/EBPα to transactivate a minimal p(CEBP)2TK promoter. Luciferase assays were performed in 293T cells lacking endogenous C/EBPα. Cells were transiently transfected with a reporter construct containing a minimal thymidine kinase (TK) promoter with two CEBP-binding sites p(CEBP)2TK or a reporter without CEBP sites (pTK) and expression plasmids for C/EBPα and YFP-TIP60 (or pcDNA-TIP60). The activity obtained for the p(CEPB)2TK plasmid without C/EBPα transfection was set as one and fold changes are shown. (**d**) The increase in the C/EBPα transactivation activity depends on the histone acetyltransferase (HAT) domain of TIP60: Transient transfections were performed in 293T cells with the p(CEBP)2TK reporter construct and expression plasmids for C/EBPα, YFP-TIP60 and YFP-TIP60(-HAT). The activity obtained for the p(CEPB)2TK plasmid without C/EBPα transfection was set as one and fold changes are shown. The pRL-TK *Renilla* luciferase construct was co-transfected to normalize for transfection efficiency. Experiments were performed in triplicate and luciferase activity was measured in duplicate for each experiment. Error bars represent s.d. (**e**) Western blot showing equal expression of YFP-TIP60 (lane 1) and YFP-TIP60(-HAT) (lane 2) in 293T cells. (**f**) TIP60 co-transfection does not alter C/EBPα protein level: 293T cells were transfected with expression plasmids for C/EBPα-His$_6$ and YFP-TIP60. C/EBPα levels were determined by western blotting. Cells transfected with C/EBPα in the absence of TIP60 (lane 2) served as the source for determining the C/EBPα level.

shown). Antibodies against TIP60 and C/EBPα were used to precipitate the crosslinked chromatin. Our results showed that even in the uninduced condition (0 h) some endogenous TIP60 and C/EBPα are present at the C/EBPα promoter (Figures 4a and b). After induction with β-estradiol, immunoprecipitation with anti-TIP60 antibodies (N/C terminal) led to a strong enrichment of the GCSFR promoter amplicon and a significant enrichment for the CEBPA promoter amplicon (Figure 4a). Immunoprecipitation using the C/EBPα antibody led to stronger amplification of the GCSFR and CEBPA promoter amplicons (Figure 4b). The

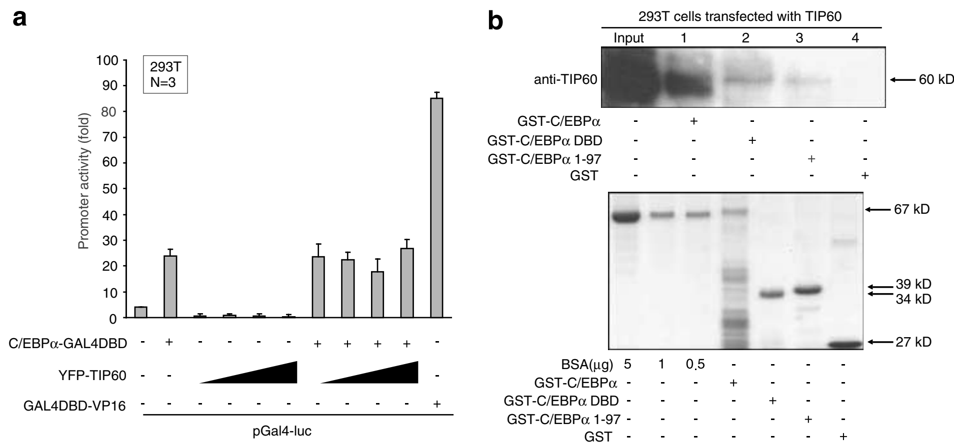


**Figure 3** Co-activation by TIP60 requires the DNA-binding domain (DBD) of C/EBPα and in GST pull down assays, TIP60 interacts with GST-C/EBPα DBD and GST-C/EBPα 1–97. (**a**) Transient transfection of 293T cells using a luciferase reporter plasmid containing Gal4DNA-binding sites only (pGal4-luc). GAL4DBD-VP16 was used as a positive transactivation control. The activity obtained for the pGal4-luc plasmid without C/EBPα-GAL4DBD transfection was set as one and fold changes are shown. The pRL-TK *Renilla* luciferase construct was co-transfected to normalize for transfection efficiency. Experiments were performed in triplicates and luciferase activity was measured in duplicate for each experiment. Error bars represent s.d. (**b**) (Upper panel) GST pull down showing physical interaction between pcDNA-TIP60 and GST-C/EBPα, GST-C/EBPα-DBD and GST-C/EBPα 1–97. GST-C/EBPα, GST-C/EBPα-DBD and GST-C/EBPα 1–97 interact with TIP60 (lanes 1–3). GST does not retain TIP60 (lane 4). (Lower panel) Coomassie-stained gel showing bacterially expressed and purified GST-C/EBPα, GST-C/EBPα-DBD, GST-C/EBPα 1–97 and GST proteins; bovine serum albumin was used for quantification.

specificity of the binding of TIP60 and C/EBPα to the promoter region of C/EBPα could be demonstrated by a no-antibody control and by the fact that an amplicon from the C/EBPα coding region could not be amplified after precipitation (Supplementary Figure 1d). Thus, C/EBPα and TIP60 associate *in vivo* in the context of chromatin and are more abundant on the C/EBPα promoter when cells are induced toward granulocytic differentiation.

### *Differentiation induction leads to increased histone acetylation at the C/EBPα and GCSFR promoters* in vivo *concomitant with TIP60 recruitment*

Our results indicated TIP60 occupancy at the C/EBPα promoter. Therefore, we hypothesized that histone acetylation at the C/EBPα promoter might be influenced by TIP60 recruitment to chromatin. We therefore performed a ChIP experiment using antibodies against acetylated histones H3 and H4 to precipitate the crosslinked chromatin derived from uninduced and induced K562 C/EBPα-ER cells. As measured by quantitative PCR, abundant levels of acetylated H4 and H3 histones were found to be present at the C/EBPα and GCSFR promoter loci in K562 C/EBPα-ER cells 6 h after the addition of β-estradiol (Figures 4c and d). Immunoprecipitation using an isotype-matched immunoglobulin G was also used to serve as a negative control (data not shown).

To verify the TIP60–C/EBPα interaction at the GCSFR promoter amplicon, a Re-ChIP experiment was performed. After β-estradiol treatment, crosslinked nuclear lysates were first immunoprecipitated with anti-C/EBPα, and then the immunoprecipitate was subjected to a second immunoprecipitation with anti-TIP60 antibodies. The amount of GCSFR promoter DNA was then assayed by quantitative real-time PCR. The second immunoprecipitation showed an enrichment of over 100-fold of the GCSFR promoter compared to the uninduced samples (Figure 4e). This clearly shows a strong increase of TIP60 at the GCSFR promoter when the cells are induced toward granulocytic differentiation. In summary, we conclude that TIP60 might play an important role in the regulation of histone acetylation and deacetylation at the C/EBPα promoter.

### *HTATIP and CEBPA expression levels correlate in retinoic acid-induced myeloid differentiation in U937 cells as well as in certain leukemia subtypes*

To analyze the level of TIP60 mRNA upon granulocytic differentiation, we used the myelomonocytic U937 cell line that can be induced to differentiate toward more mature granulocyte cells by retinoic acid treatment as assessed by the expression of the cell surface differentiation marker CD11b and morphology (data not shown). TIP60 expression was analyzed by a semiquantitative reverse transcriptase-PCR assay on RNA isolated from differentiated $CD11b^{+}$ U937 cells by fluorescence-activated cell sorting after 60 h of treatment with retinoic acid. Compared to undifferentiated $CD11b^{-}$ U937 cells, higher TIP60 mRNA expression was observed in differentiated U937 cells. As expected, C/EBPα and C/EBPε transcript levels were increased upon myeloid differentiation. β-actin levels were used for RNA quality control (Figure 5a).

To obtain a preliminary assessment whether this correlated expression would also be found in myeloid leukemias, we analyzed the mRNA expression of TIP60 and CEBPA in a microarray data set from a small cohort of patients with defined leukemia subtypes (CML: chronic myeloid leukemia; AML_M2: AML-M2 with the AML1/ETO fusion; AML_M3: AML-M3 with the PML/RARA fusion; AML_M4: AML-M4eo with the CBFB/MYH11 fusion) and from normal bone marrow samples (Figure 5b). A Pearson's two-tailed correlation analysis showed that there was a significant positive correlation between the expression levels of HTATIP and CEBPA when considering all samples ($n=50$) together ($r=0.606$, $P=0.00000314$). In view of the low sample number ($n=10$) in the individual subgroups, this correlation reached significance at the 95% confidence interval only in the group with the AML1/ETO fusion gene (AML_M2) ($r=0.703$, $P=0.023$). Studies in larger patient cohorts are expected to clarify this issue.

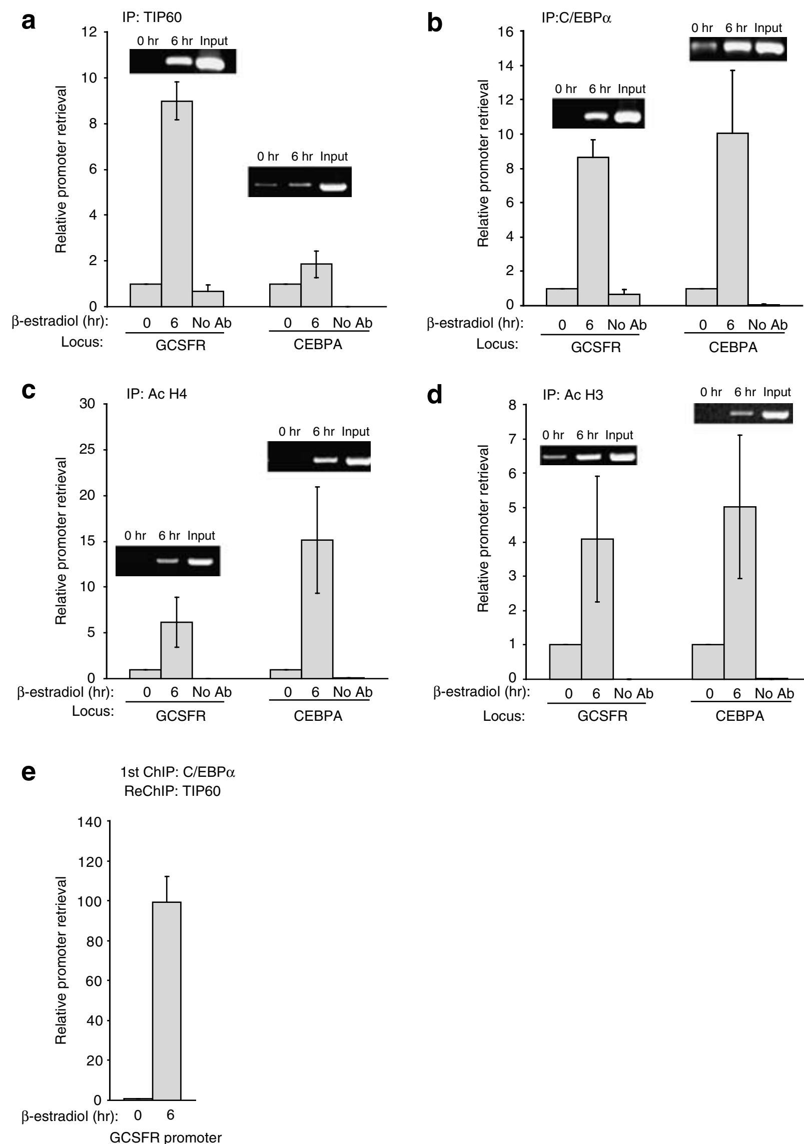


**Figure 4** TIP60 associates with C/EBPα target loci. Chromatin immunoprecipitation (ChIP) analysis of quiescent (0 h) and stimulated (6 h) K562 C/EBPα-ER cells using antibodies against TIP60 (**a**), C/EBPα (**b**), acetylated histone H4 (**c**) and acetylated histone H3 (**d**). Two promoters were analyzed: GCSFR and CEBPA. (**a**, **b**) C/EBPα and TIP60 colocalize on the GCSFR and CEBPA loci when stimulated with β-estradiol. Ethidium bromide-stained gels of conventional PCR products after ChIPs are shown in small insets. (**e**) Re-ChIP was carried out using the indicated antibodies on extracts from the quiescent and stimulated (6 h) K562 C/EBPα-ER cells. The data are representative of three experiments with similar results. Error bars represent s.d.

## Discussion

Protein–protein interactions play key roles in all cellular processes and functions. Identifying and characterizing such protein interactions is therefore required to understand these processes at a molecular level. To our knowledge, TIP60 is the first MYST domain family member identified as an interaction partner of C/EBPα. This finding might be of potential importance with regard to the functions of C/EBPα, in particular since there are sparse data regarding C/EBPα and histone acetylation.

While we were partially successful in finding known C/EBPα interaction partners using different proteomic approaches (two-dimensional gels vs SDS-PAGE LC-MS/MS), we also identified several novel putative C/EBPα-interacting proteins, thereby giving new dimensions to C/EBPα functions. Future technological advances in the proteomics field should overcome the discrepancies found with the different proteomic approaches. Nevertheless, we report several additional putative C/EBPα-interacting proteins to be characterized in future studies (Supplementary Table 1).

The TIP60–C/EBPα interaction is intriguing given the fact that TIP60 is a MYST domain HAT. This family of HATs has been linked to mediate long-lasting epigenetic changes of chromatin (for example, *mof* in *Drosophila* is responsible for X chromosome dosage compensation[21]). Additionally, the acetyl transferase activity of TIP60 on nucleosomal histones in the presence of other factors has been reported, and TIP60 can function both as a transcriptional co-activator or as a co-repressor, connecting a number of different factors to the basal transcriptional machinery.[14]

TIP60 is involved in oncogenesis and other disease processes. Upregulation of TIP60 has been recently linked to the promotion of epithelial tumorigenesis, Alzheimer's disease and progression

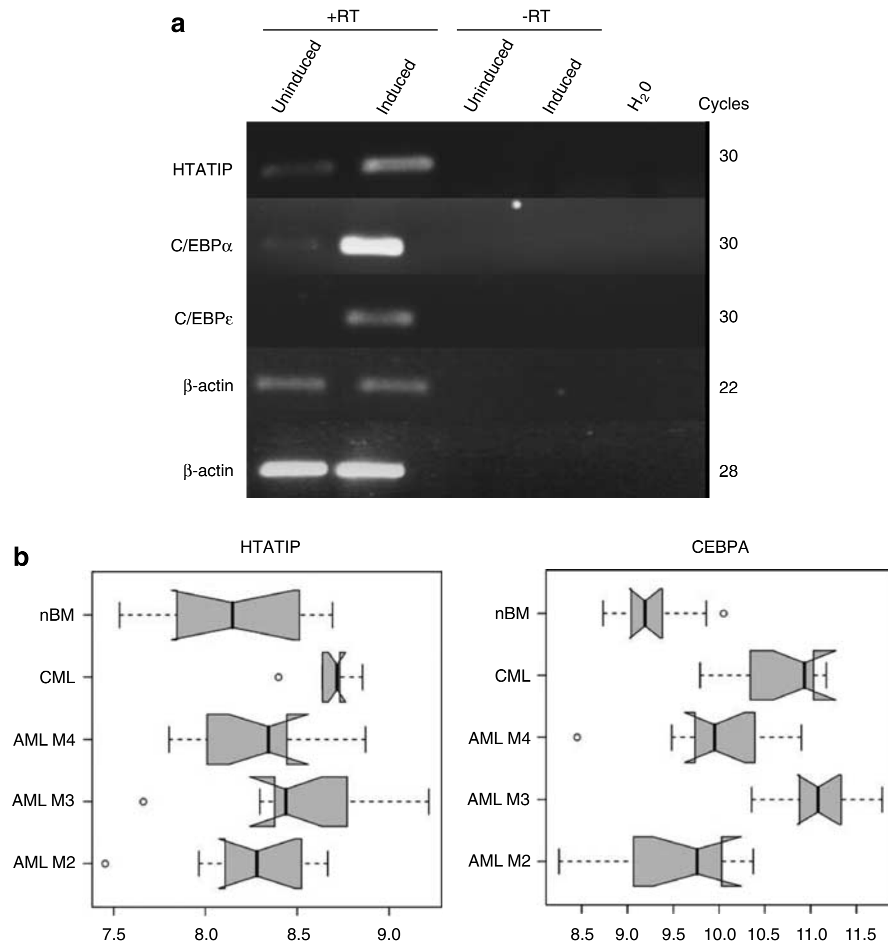


**Figure 5** TIP60 mRNA expression upon retinoic acid-induced U937 cell differentiation and in acute myeloid leukemia (AML) subtypes. (**a**) Expression of TIP60 (HTATIP), C/EBPα and C/EBPε was analyzed by semiquantitative reverse transcriptase-PCR in $CD11b^-$, retinoic acid untreated U937 cells and in retinoic acid treated, $CD11b^+$ U937 cells by fluorescence-activated cell sorting after 60 h. (**b**) Boxplots of mRNA expression levels (microarray signal intensity values) of C/EBPα and TIP60 in normal bone marrow, chronic myeloid leukemia (CML) and three AML subtypes (AML_M4: AML with CBFB/MYH11 fusion; AML_M3: AML with PML/RARA fusion; AML_M2: AML with AML1/ETO fusion). The dark bar represents median, boxes give the 25–75% quantile range (interquantile range: IQR), whiskers represent the 1.5-fold IQR and small circles represent the outlier. If the 'notches' of two distributions do not overlap, it is considered strong evidence that their medians differ. Correlation (Pearson's two-tailed correlation analysis) was found to be statistically significant at 95% confidence interval for AML_M2 subtype between CEBPA and HTATIP ($r=0.703$, $P=0.023$).

of prostate cancer cells to hormone independence and resistance to chemotherapy.[14] Thus, it is possible that upregulation of TIP60 in leukemia confers to the leukemic clone a survival advantage. It should be noted that TIP60 and two other MYST domain HATs (MOZ and MORF) have been shown to interact with other leukemia-relevant proteins like ETV6[15,22] or to participate in leukemia-associated chromosomal translocations (for example, the MOZ/TIF2, MOZ/P300 and MORF/CBP).[23] It was recently shown that TIP60 acts as a tumor suppressor gene in a mouse model.[24] However, we are unaware of any reports of chromosomal aberrations or mutations that directly target TIP60.

Interestingly, we observed that TIP60 can interact with the DBD as well as with the transactivation domain containing portions of C/EBPα (aa 1–97, Figure 3b; and aa 98–262, data not shown). These fragments contain transactivation domains of C/EBPα. Thus, TIP60 could be an important transcriptional co-regulator for C/EBPα functions by acting in large multiprotein complexes with other factors that also associate with C/EBPα. This might be particularly true for proteins such as E2F, which have been shown to interact with both TIP60[25] and C/EBPα.[26]

TIP60 can directly acetylate and stabilize non-histone proteins.[14] Our efforts to detect acetylation of C/EBPα by TIP60 failed (data not shown). The fact that C/EBPα and TIP60 are recruited to the C/EBPα and GCSFR promoters during granulocytic differentiation *in vivo* under physiological conditions (Figure 4) suggests that TIP60 enhances the transactivation capacity of C/EBPα on these promoters. This has been reported for MYC.[27] It is possible that the primary targets of TIP60 acetylation in this context are the histones at C/EBPα-dependent genes. In fact, we show that there is increased histone acetylation when the cells are induced toward granulocytic differentiation concomitant with recruitment of C/EBPα and TIP60 at the GCSFR promoter. However, at the moment it is not clear whether histone acetylation is a prerequisite or a consequence of the C/EBPα–TIP60 interaction or whether this histone acetylation is a direct result of the TIP60 HAT activity.

The increase in expression of both C/EBPα and TIP60 during retinoic acid-induced differentiation of U937 cells suggests a

role for TIP60 in myeloid differentiation. Expression microarray data from patients with defined leukemia subtypes showed an overall significant positive correlation between TIP60 (HTATIP) and C/EBPα mRNA levels (Figure 5b), suggesting the role of TIP60 in leukemogenesis. However, the different levels of TIP60 expression in the AML subtypes could also be a reflection of the differentiation status of the AML blasts rather than being important for leukemogenesis. Nevertheless, TIP60 might be critical in regulating the promoters of myeloid differentiation-specific genes.

In summary, our observations and findings indicate a functional synergism between two tumor suppressor proteins involved in hematological malignancy. TIP60 may be an integral part of multiprotein complexes, which convert the transcriptional decisions initiated by C/EBPα into long-lasting epigenetic chromatin changes. Finally, our data provide a framework for investigating the mechanisms and signaling pathways that control the interaction of TIP60 with C/EBPα, for example, by examining the phenotype of mice with tissue-specific knockouts of these two genes.

## Acknowledgements

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)

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ORIGINAL ARTICLE

# Proteomic identification of C/EBP-DBD multiprotein complex: JNK1 activates stem cell regulator C/EBPα by inhibiting its ubiquitination

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**Functional inactivation of transcription factors in hematopoietic stem cell development is involved in the pathogenesis of acute myeloid leukemia (AML). Stem cell regulator C/enhancer binding protein (EBP)α is among such transcription factors known to be inactive in AML. This is either due to mutations or inhibition by protein–protein interactions. Here, we applied a mass spectrometry-based proteomic approach to systematically identify putative co-activator proteins interacting with the DNA-binding domain (DBD) of C/EBP transcription factors. In our proteomic screen, we identified c-Jun N-terminal kinase (JNK) 1 among others such as PAK6, MADP-1, calmodulin-like skin proteins and ZNF45 as proteins interacting with DBD of C/EBPs from nuclear extract of myelomonocytic U937 cells. We show that kinase JNK1 physically interacts with DBD of C/EBPα *in vitro* and *in vivo*. Furthermore, we show that active JNK1 inhibits ubiquitination of C/EBPα possibly by phosphorylating in its DBD. Consequently, JNK1 prolongs C/EBPα protein half-life leading to its enhanced transactivation and DNA-binding capacity. In certain AML patients, however, the JNK1 mRNA expression and its kinase activity is decreased which suggests a possible reason for C/EBPα inactivation in AML. Thus, we report the first proteomic screen of C/EBP-interacting proteins, which identifies JNK1 as positive regulator of C/EBPα.**
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**Keywords:** JNK1; C/EBPα; ubiquitination; AML and proteomics

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## Introduction

C/enhancer binding protein (EBP)α belongs to a family of transcription factors that homo- and hetero-dimerize via their conserved C-terminal leucine zipper domains and bind DNA as dimers through the adjacent basic regions (b-Zip) (Landschulz *et al*., 1989). C/EBPα and PU.1 are two major regulators of hematopoietic stem cell development. Unlike PU.1, which governs transcription of a wide spectrum of myeloid-specific genes, C/EBPα has a more specific function in granulopoietic stem cell development (Scott *et al*., 1992; Muller *et al*., 1995). C/EBPα$^{-/-}$ knockout mice show no mature granulocytes, whereas other hematopoietic lineages are not affected (Zhang *et al*., 1997). Recent findings have demonstrated a direct link between myeloid leukemogenesis and certain transcription factors including C/EBPα.

Mutations in C/EBPα leading to its disruption have recently been shown in acute myeloid leukemia (AML) (Pabst *et al*., 2001b; Gombart *et al*., 2002; Frohling *et al*., 2004; Perrotti *et al*., 2004). In a recent study, Smith *et al*. (2004) have shown a C/EBPα mutation in inherited AML, where multiple members were affected by AML associated with an identical mutation in CEBPA. Several studies have emphasized that protein–protein interactions of transcription factors may contribute to leukemogenesis (Westendorf *et al*., 1998; Pabst *et al*., 2001a; Reddy *et al*., 2002; Tenen, 2003; Vangala *et al*., 2003). Modified properties of a multiprotein complex resulting from variable interacting partners are likely to be involved with all three hematopoietic checkpoints: cell proliferation, differentiation and apoptosis; impairment of each of which may induce leukemia. Hence, an important emerging concept is that not only relative expression of transcription factors is important, but that protein–protein interactions among various transcription factors are crucial (Sieweke and Graf, 1998). Potential antagonistic protein interactions leading to a block in C/EBPα function in AML have been well implicated in recent findings (Pabst *et al*., 2001a; Vangala *et al*., 2003; Zheng *et al*., 2004). Interestingly, C/EBPα also functions via direct protein–protein interactions in normal stem cell development

(Behre et al., 2002a; D'Alo et al., 2003; Muller et al., 2004); PU.1 and c-Jun inactivation (Rangatia et al., 2002; Reddy et al., 2002), E2F repression (Porse et al., 2001), and cdk2 and cdk4 inhibition (Wang et al., 2001) by C/EBPα are all accompanied by direct protein–protein interactions. Moreover, different protein partners of C/EBPα in young and old mice livers itself demonstrate the importance of protein–protein interactions during ageing (Iakova et al., 2003).

Owing to its role in differentiation, anti-proliferation and apoptosis, functional inactivation of C/EBPα is central to the pathogenesis of AML. However, molecular mechanisms behind this are poorly understood except for some cases (Pabst et al., 2001b; Perrotti et al., 2002; Zheng et al., 2004). Recent advances in mass spectrometry have revolutionized the analysis of the proteome of a cell by simplifying the analytical protocol and increasing the sensitivity of detection by several orders of magnitude. Global high throughput mass spectrometry-based functional proteomic approaches could lead to new insights into the network of C/EBPα-interacting proteins relevant for stem cell development and AML therapeutics (Mann et al., 2001; Cristea et al., 2004; Balkhi et al., 2006). Therefore, to evaluate the significance and role of proteins interacting with C/EBPα and modulating its activity, we analysed the C/EBPα multiprotein complex using mass spectrometry-based proteomics.

## Results

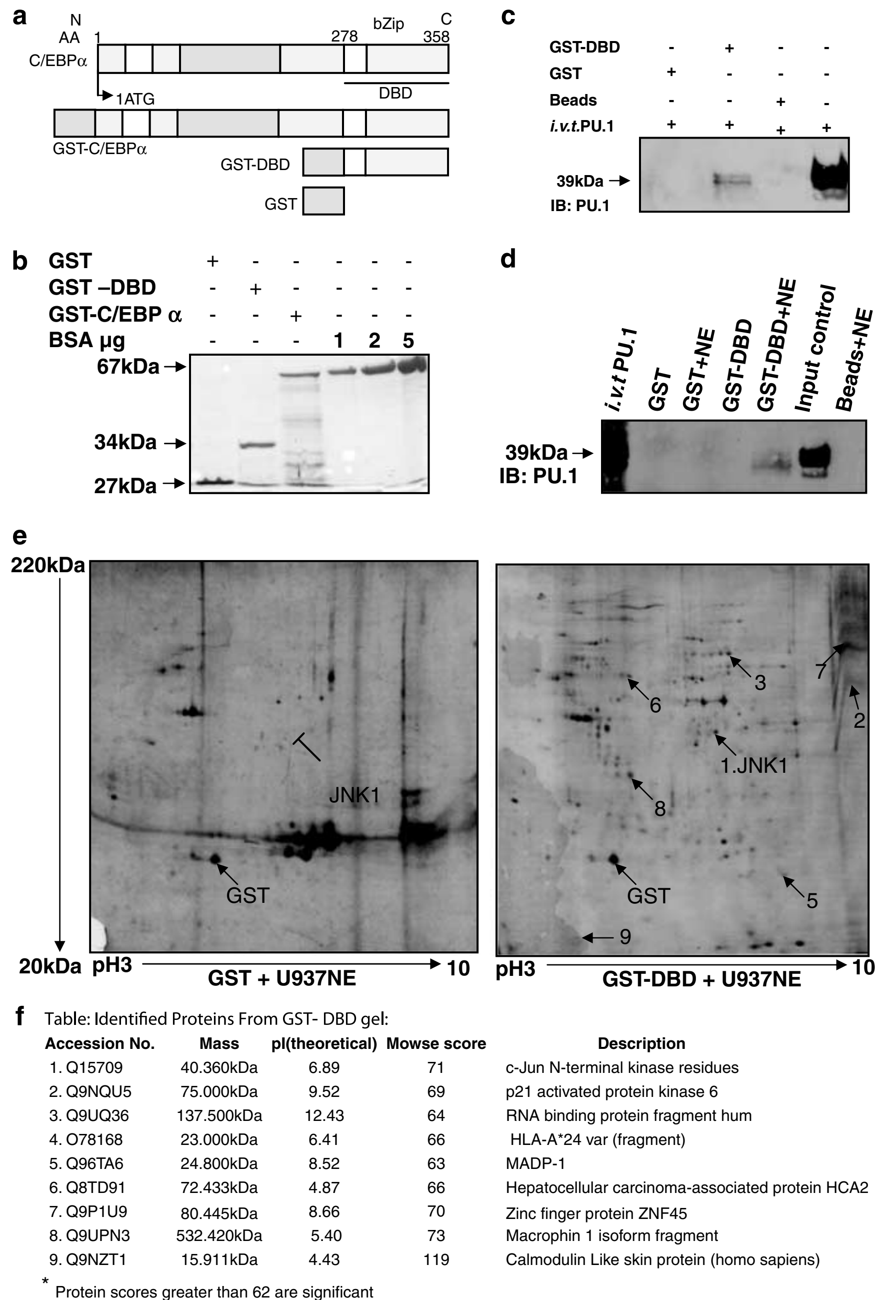
*Proteomic identification of the C/EBP-DBD multiprotein complex by 2D gel electrophoresis*

In order to identify co-activator proteins interacting with C/EBPα and modulating its activity in stem cell development and AML, a mass spectrometry-based proteomics approach was applied. The DBD of C/EBPs contains a conserved leucine zipper region, which is necessary for interaction with other proteins; therefore, we used glutathione-*S*-transferase (GST) fused with DBD to identify C/EBP-interacting proteins. Figure 1a depicts wild-type C/EBPα with conserved domains of C/EBP family proteins and GST-fused constructs, which we used for our investigation in this study. We purified GST fusion proteins from bacterial strain *Escherichia coli* after inducing with 0.5 mM isopropyl-thiogalactopyranoside (IPTG). The commassie-stained gel picture shows expression of GST fused with DNA-binding domain (DBD) of C/EBP (GST-DBD), GST-C/EBPα and GST alone (Figure 1b); Different amount of commercially available bovine serum albumin was resolved together to quantify the respective volume of GST fusion proteins to be used in further assays. We confirmed the physical activity of bacterially purified fusion proteins by assessing their interaction with *in vitro*-translated (*ivt* PU.1) and endogenous PU.1 from U937 nuclear extract (NE); PU.1 is known to interact with C/EBPα in its DBD (Reddy et al., 2002) (Figure 1c–d). Next, we performed GST pull down assay from NE of myelomonocytic cell line U937. Proteins from NE bound with fusion proteins on GST sepharose beads were lysed in sample buffer and subsequently separated by isoelectric focussing (IEF) in first dimension followed by second-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Silver staining was performed to visualize differentially interacting proteins from 2D gels (Figure 1e). Protein spots present only in GST-DBD gel were excised and analysed by matrix-assisted laser desorption/ionization–time of flight (MALDI–TOF) mass spectrometry for further identification; as control we identified GST as a common spot from both gels. Identified protein spots interacting with the DBD region of C/EBP are listed in Figure 1f. Note that for large number of protein spots the mowse score was insignificant and hence could not be identified. This is presumably due to small spot size (protein amount), silver interference and low sensitivity of mass spectrometer.

*C/EBPα physically interacts with JNK1* in vitro *and* in vivo

c-Jun N-terminal kinase (JNK) is an important member of mitogen-activated protein kinase (MAPK) family and regulates the activity of its physically associated substrates (Fuchs et al., 1997). Therefore, we chose JNK to study the biological relevance of its interaction with C/EBPα. We confirmed direct protein–protein interaction between GST-DBD and JNK1 by incubating GST-DBD with methionine-labelled *ivt* JNK1 for 90 min. After stringent washing, interaction between JNK1 and GST-DBD was observed (Figure 2a; lane 5). We next examined whether JNK1 interacts with full-length C/EBPα and performed GST pull down assay using GST-DBD, GST-C/EBPα and GST alone from 25 ng/ml anisomycin-induced (U937 radioimmunoprecipitation assay (RIPA*) and uninduced (U937 RIPA) cell lysates; anisomycin is a potent activator of JNK which acts on upstream activators of JNK (Bogoyevitch et al., 1995; Morton et al., 2003). Immunoblot against JNK1 showed direct interaction of JNK1 (Figure 2b; lanes 2, 6 and 8 with fast migrating lanes) and phospho-JNK1 (pJNK) (Figure 2b; lanes 1, 5 and 7 with slow migrating bands as compared to respective uninduced conditions) with GST-DBD and GST-C/EBPα. The higher binding affinity of GST-C/EBPα with JNK could be owing to favorable conformation of full-length wild-type C/EBPα protein as compared to shorter GST-DBD; GST-c-Jun served as positive control (Figure 2b). Note that, bands below 35 kDa, GST or degraded GST-fusion proteins show input in each lane. We also examined *in vivo* interaction of C/EBPα and JNK1 by co-immunoprecipitation of JNK1 from U937 RIPA lysates. Immunoblot against JNK1 (upper panel) followed by C/EBPα (lower panel) after stripping the same membrane confirmed *in vivo* interaction of JNK1 and C/EBPα in myeloid cells (Figure 2c).

As JNK interacts with GST-DBD, we wished to map down the region of C/EBPα interacting with JNK1 using




| Accession No. | Mass | pI(theoretical) | Mowse score | Description |
|---|---|---|---|---|
| 1. Q15709 | 40.360kDa | 6.89 | 71 | c-Jun N-terminal kinase residues |
| 2. Q9NQU5 | 75.000kDa | 9.52 | 69 | p21 activated protein kinase 6 |
| 3. Q9UQ36 | 137.500kDa | 12.43 | 64 | RNA binding protein fragment hum |
| 4. O78168 | 23.000kDa | 6.41 | 66 | HLA-A*24 var (fragment) |
| 5. Q96TA6 | 24.800kDa | 8.52 | 63 | MADP-1 |
| 6. Q8TD91 | 72.433kDa | 4.87 | 66 | Hepatocellular carcinoma-associated protein HCA2 |
| 7. Q9P1U9 | 80.445kDa | 8.66 | 70 | Zinc finger protein ZNF45 |
| 8. Q9UPN3 | 532.420kDa | 5.40 | 73 | Macrophin 1 isoform fragment |
| 9. Q9NZT1 | 15.911kDa | 4.43 | 119 | Calmodulin Like skin protein (homo sapiens) |

* Protein scores greater than 62 are significant

**Figure 1** 2D proteomic identification of the C/EBP-DBD multiprotein complex. (**a**) Schematic presentation of wild-type C/EBPα and different GST fusion proteins. (**b**) Commassie-stained gel shows bacterially purified GST-C/EBPα, GST-DBD and GST proteins; different amounts of commercially available purified bovine serum albumin was separated together to compare the equivalent amount of bacterially purified proteins. (**c**, **d**) Immunoblot against PU.1 antibody after GST pull down of PU.1 from *ivt* PU.1 and U937 NE shows interaction with GST-DBD; sepharose beads served as control. (**e**) Silver-stained 2D gel shows differentially expressed proteins after GST pull down from NE of U937; proteins excised and subsequently analysed by mass spectrometry are marked with arrow. (**f**) List of the 2D-gel separated C/EBP-DBD multiprotein complex identified by MASCOT search; protein scores greater than 62 are significant.

previously reported C/EBP-DBD mutants (Landschulz *et al*., 1989). 293T cells were transfected with different C/EBP-DBD mutants described in Materials and methods together with hemagglutinin (HA)-JNK; 24 h post-transfection RIPA extracts were prepared and JNK was immunoprecipitated using HA antibody. Immunoblot against HA and subsequently with C/EBPα antibody after stripping the same membrane shows JNK interaction with C/EBPα and various DBD mutants with variable intensity (Figure 2d). Taken together, this confirms that JNK1 directly interacts with DBD region of C/EBPα. Further studies using C/EBPα basic region deletion mutant could further narrow down the region of C/EBPα interacting with JNK1.

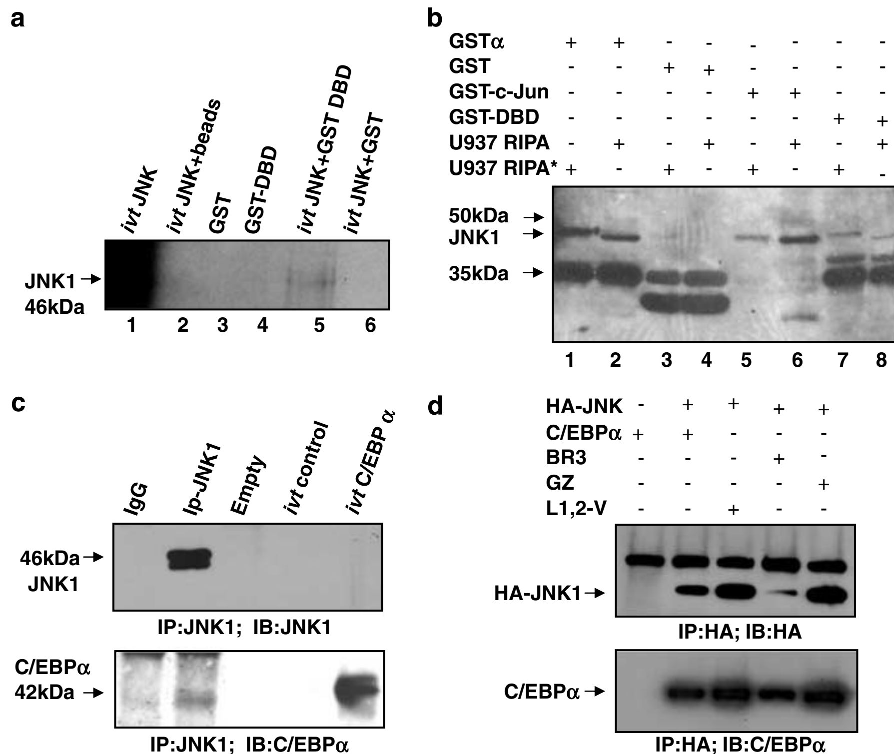



**Figure 2** C/EBPα physically interacts with JNK1 *in vitro* and *in vivo*. (**a**) GST pull down assay was performed from methionine-labelled *ivt* JNK1 incubated with equal amounts of bacterially purified GST-DBD (lane 5), GST (lane 6) and GST sepharose beads (lane 2); autoradiogram was developed after 36 h (**b**) Immunoblot against JNK1 after GST pull down of JNK1 from RIPA lysates of anisomycin-induced (U937 RIPA*) and -uninduced U937 cells shows *in vitro* interaction of JNK1 with GST-C/EBPα, GST-DBD and positive control GST-c-Jun. (**c**) C/EBPα and JNK1 interaction was confirmed *in vivo* in myeloid cells by immunoprecipitation of JNK1 from U937 RIPA lysates and blotting against JNK1 and C/EBPα antibody, respectively. (**d**) JNK1 interacts with different C/EBP-DBD mutants; GZ, (Leucine zipper replaced with leucine zipper of yeast GCN4 protein), point mutants BR3 (basic region mutant) and L1,and 2 form leucine zipper mutated to valine. 293T cells were transfected together with above mutants and HA-JNK and cells were harvested 24 h post-transfection. JNK1 was immunoprecipitated using an anti-HA antibody (upper panel), same membrane stripped and blotted with C/EBPα (lower panel). Results are representative of three independent experiments.

### *Active JNK increases C/EBPα protein stability by inhibiting its ubiquitination*

JNK1 regulates ubiquitination and hence protein stability of its physically associated substrates (Fuchs *et al*., 1997). Considering that C/EBPα levels are controlled post-translationally (Hattori *et al*., 2003; Subramanian *et al*., 2003), we determined the effect of JNK1 on the stability of former protein. Induced activation of JNK in acute promyelocytic NB4 cells with 25 ng/ml anisomycin significantly increased the expression of C/EBPα (Figure 3a.i, *ivt* C/EBPα-His, 46 kDa used as positive control; upper panel, lane 6) which is consistent with JNK activation as shown by phospho-c-Jun expression in a separate immunoblot of same lysates after indicated time points (3a.ii). A similar effect was observed with acute myelomonocytic HL60 cells when treated with 25 ng/ml of anisomycin (Figure 3b); 25 ng/ml anisomycin significantly activates JNK1 in HL60 cells (Terrance A Stadheim, 2002). We also assessed C/EBPα-inducible cell line K562C/EBPα-ER for JNK-induced C/EBPα protein stabilization, where C/EBPα-ER protein is active only when induced with 1 $\mu$M $\beta$-estradiol but with accelerated degradation (Cleaves *et al*., 2004). Immunoblot against C/EBPα shows anisomycin induction together with $\beta$-estradiol can reduce the degradation of C/EBPα-ER (Figure 3c). Additionally, MEKK1 (an upstream member of MAPK pathway that binds to JNK and thereby facilitates the receipt of signals from upstream inputs to activate it (Xu and Cobb, 1997)), with or without C/EBPα was expressed in C/EBPα-null 293T cells and protein levels were measured. MEKK1 transfection dramatically increased the amount of C/EBPα (Figure 3d; lane 4), an effect likely owing to JNK1 activation, as treatment of MEKK1- and C/EBPα-transfected cells with 20 $\mu$M JNK inhibitor SP600125 3 h post-transfection significantly reduced the amount of C/EBPα protein (Figure 3d; lane 5); SP600125 functions by directly inhibiting JNK (Han *et al*., 2001).

We next confirmed that JNK affects C/EBPα at protein level rather than its mRNA by treating U937 cells with 50 $\mu$g/ml cycloheximide (a chemical which inhibits new protein synthesis in eucaryotes by inhibiting peptidyl transferase) before the addition of 50 ng/ml anisomycin for different time points as indicated. Immunoblot against C/EBPα after resolving RIPA lysates on SDS–PAGE shows that C/EBPα protein degradation is reduced when treated together

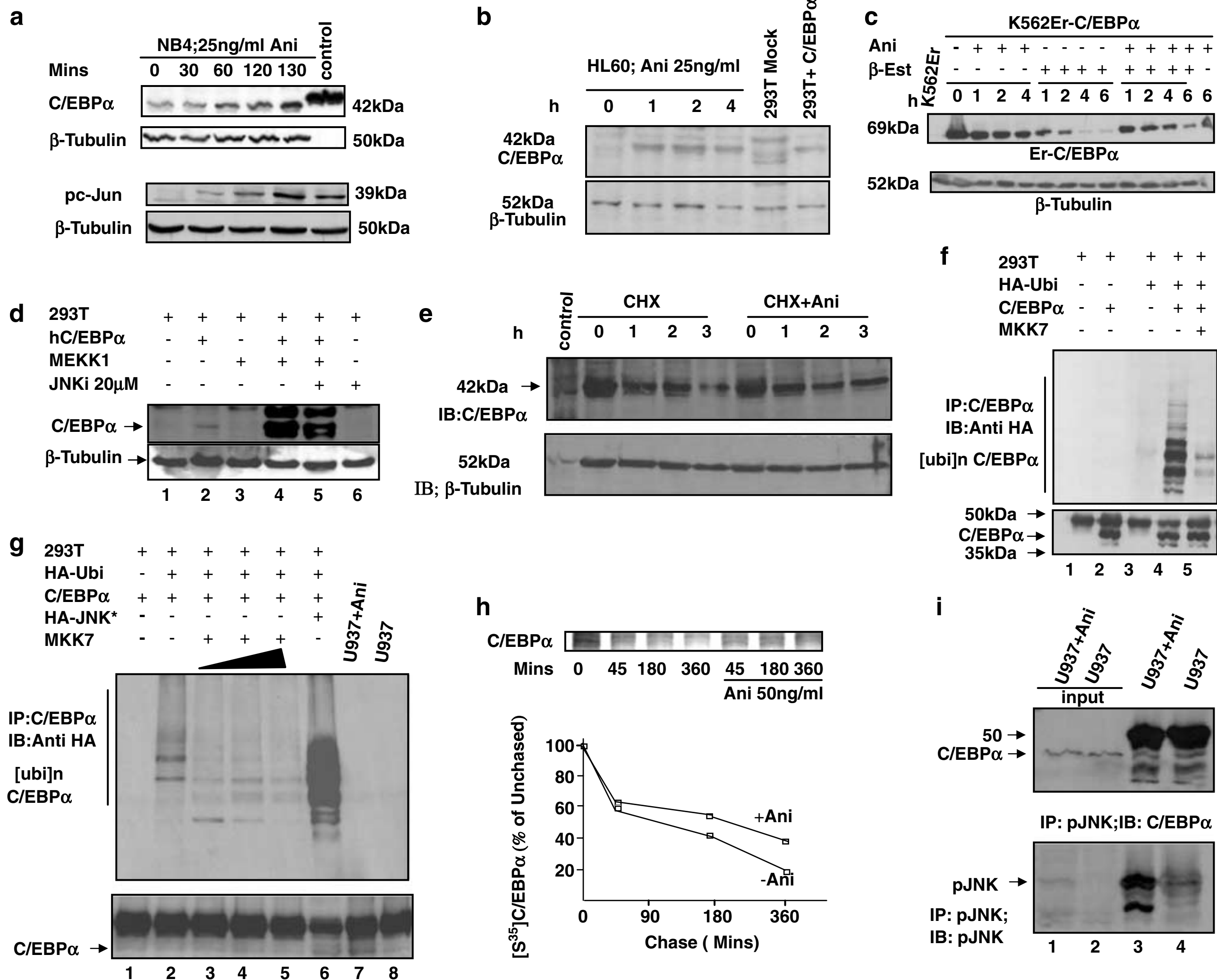



**Figure 3** Active JNK1 increases C/EBPα protein stability by inhibiting its ubiquitination. (**a**) Anisomycin-induced activation of JNK1 stabilizes C/EBPα protein; (i) NB4 cells were induced with 25 ng/ml anisomycin for different time points, RIPA lysates were resolved on SDS–PAGE and blotted with C/EBPα and β-tubulin antibody. (ii) Same lysates were separated on different gel and probed for pc-Jun as a measure of anisomycin-induced activation of JNK kinase and β-tubulin as loading control. (**b**) HL60 cells induced with 25ng/ml of anisomycin for different time points were resolved on SDS–PAGE. Immunoblot against C/EBPα shows significant increase in C/EBPα protein after 1 h as compared to uninduced cells. (**c**) β-Estradiol-inducible cell line K562C/EBPα-Er was treated with 25 ng/ml anisomycin for different time points and lysed in RIPA buffer. Immunoblot against C/EBPα antibody shows that the rate of degradation of C/EBPα is lower in anisomycin-induced cell lysates as compared to cells induced with β-estradiol alone; K562Er empty vector (extreme left, upper panel) is used as control. (**d**) MEKK1 co-transfection in 293T cells accumulates C/EBPα protein; 293T cells were transiently co-transfected with C/EBPα and MEKK1; 24 h post-transfection, cells were lysed and separated on SDS–PAGE. Immunoblot with C/EBPα antibody shows significant increase in C/EBPα protein (lane 4). MEKK1-induced increase in C/EBPα protein drastically decreased when these cells were treated with 20 μM JNK inhibitor SP600125 (lane 5). (**e**) JNK affects C/EBPα at protein level; U937 cells were treated with cycloheximide before treatment with anisomycin; whole-cell extract was resolved and immunoblotted with C/EBPα antibody. (**f**) *In vivo* HA-Ubi assay was performed as described in Materials and methods. Immunoblot with HA antibody shows that C/EBPα is significantly ubiquitinated (lane 4), whereas this ubiquitination was inhibited upon JNK activation by co-transfection of MKK7 (Upper panel, lane 5). The membrane was stripped and reprobed for C/EBPα. (**g**) Like in Figure 3f, *in vivo* HA-Ubi assay was performed by transfection of different plasmid constructs as indicated which shows that kinase-dead JNK mutant (HA-JNK*; lane 6) enhances C/EBPα ubiquitination. Lower panel probed with C/EBPα. (**h**) Anisomycin-induced JNK activation prolongs C/EBPα half-life; HL60 cells were labelled with methionine and chased with cold methionine for 0–6 h with or without anisomycin treatment. C/EBPα was immunoprecipitated from 250 μg protein, and analysed by autoradiography after separating on SDS–PAGE. Pulse chase result was quantified using AIDA software (Raytest, Germany) depicted in graph, represents values with error of <10%. Results are representative of three independent experiments. (**i**) pJNK interacts with C/EBPα *in vivo*; pJNK and C/EBPα interaction was confirmed *in vivo* in myeloid cells by immunoprecipitation of pJNK1 from anisomycin-induced and -uninduced U937 RIPA lysates. Membrane was immunoblotted against pJNK1 and C/EBPα antibody, respectively.

with anisomycin (Figure 3e). However, slow degradation of C/EBPα after 0 h may be attributed to JNK activation by cycloheximide itself (Kyriakis and Avruch, 1990). In addition, analysis of C/EBPα mRNA expression in anisomycin-induced and -uninduced U937 cells showed no increase in C/EBPα mRNA; rather was marginally downregulated (Supplementary Figure S1).

Further, as JNK activation stabilizes C/EBPα protein expression, we determined if activation of JNK1 inhibits JNK-targeted C/EBPα ubiquitination. Therefore, we performed *in vivo* ubiquitination assay by transiently transfecting 293T cells with HA-tagged Ubiquitin (HA-Ubi) alone, together with C/EBPα, and with C/EBPα and MKK7, respectively. Twenty-four hours post-transfection, cells were harvested in RIPA buffer. Immunoblot against HA after immunoprecipitation of C/EBPα shows that C/EBPα is significantly ubiquitinated when co-transfected with HA-Ubi (Figure 3f; lane 4), whereas this ubiquitination was inhibited in HA-Ubi-, C/EBPα- and MKK7-co-transfected cells where JNK1 is activated by MKK7 (Figure 3f; lane5). MKK7 directly interacts with JNK1 and activates it by receiving signals from upstream kinases (Moriguchi *et al*., 1997). Next, we demonstrate that inactive JNK indeed leads to enhanced C/EBPα ubiquitination; C/EBPα was co-transfected with varying amount of MKK7 (Figure 3g; lanes 3, 4 and 5) and kinase-dead JNK mutant (HA-JNK*; Figure 3g; lane 6) together with HA-Ubi; 24 h post-transfection, RIPA extract was prepared and separated on 10% SDS–PAGE. Immunoblot against HA antibody shows transfection of kinase-dead JNK with C/EBPα was sufficient to enhance ubiquitination of later. Note that in a separate experiment, activation of mutant JNK by co-transfection of MKK7 could marginally inhibit C/EBPα ubiquitination (Supplementary Figure S2).

Furthermore, if active JNK1 inhibits C/EBPα ubiquitination, it should enhance C/EBPα protein half-life. To answer this question, we performed pulse chase assay as described in Materials and methods. Autoradiogram developed after 2 days shows that C/EBPα protein half-life is prolonged (from 100 to ~160 min) when induced with anisomycin as compared to uninduced cells (Figure 3h). Next, to investigate if phospho-JNK interacts *in vivo* with C/EBPα to protect later from ubiquitination, we performed co-immunoprecipitation of phospho-JNK from anisomycin-induced and -uninduced RIPA lysates. Immunoblot against C/EBPα and subsequent phospho-JNK antibody confirms *in vivo* interaction of phospho-JNK with C/EBPα (Figure 3i). Alternatively, we also performed co-immunoprecipitation of C/EBPα, immunoblot with pJNK and later with C/EBPα after stripping the same membrane confirmed *in vivo* interaction of phsopho JNK and C/EBPα (Supplementary Figure S3). Taken together, these data suggest that under normal conditions, JNK1 interaction with C/EBPα targets its ubiquitination which is inhibited upon interaction of phospho-JNK with C/EBPα in response to chemicals or stress. To our knowledge, this is first report of modulation of C/EBPα ubiquitination by JNK1.

### *JNK1 phosphorylates C/EBPα*

The mechanisms that modulate ubiquitination by stress signals are: association with ancillary proteins, phosphorylation by specific kinases, and a combination of two (Fuchs *et al*., 1998). JNK1 is a stress-regulated kinase and C/EBPα is known to be post-translationally modified by small ubiquitin-like modifier, ubiquitin and phosphorylation (Mahoney *et al*., 1992; Behre *et al*., 2002b; Subramanian *et al*., 2003; Ross *et al*., 1999, 2004). Therefore, we hypothesized, under stress conditions JNK1 physically associate, phosphorylates C/EBPα and thereby inhibits its ubiquitination. To answer this, *in vitro* kinase assay performed using GST-C/EBPα as substrate and immunoprecipitated HA-JNK from transiently co-transfected 293T with MEKK1 as kinase shows JNK1 phosphorylates GST-C/EBPα (Figure 4a; lane 1). Next, we assessed phosphomodification of immunopurified C/EBPα from mammalian cells. For this, we again performed *in vitro* kinase assay using immunoprecipitated C/EBPα from transiently transfected 293T as substrate and immunoprecipitated HA-JNK and kinase-dead HA-JNK (HA-JNK*) from transiently co-transfected 293T together with MEKK1 as kinase. Wild-type JNK phosphorylated C/EBPα (Figure 4b; lane 3) whereas HA-JNK* could partially (Figure 4b; lane 5). GST-c-Jun was used as positive control whereas pRK5 empty vector-transfected cells were used as control for JNK immunoprecipitation (lane 1). As JNK interacts with GST-DBD, we then examined if JNK1 can also phosphorylate GST-DBD. *In vitro* kinase assay using GST-DBD as substrate and immunoprecipitated HA-JNK and HA-JNK*, respectively as kinase from transiently co-transfected 293T with MEKK1 shows GST-DBD can be phosphorylated by JNK whereas there is little or no phosphorylation with mutant JNK (Figure 4c). Collectively, these findings indicate that JNK1 does phosphorylate C/EBPα in DBD region. However, future work will be required to locate the amino-acid residue of C/EBPα being phosphomodified by JNK.

### *JNK1-induced increase in C/EBPα protein expression augments C/EBPα transactivation and DNA binding*

To assess if active JNK-induced increase in C/EBPα protein expression is also accompanied by an increase in C/EBPα transactivation activity, we transiently transfected 293T cells with a minimal thymidine kinase (TK) promoter containing two C/EBP sites cloned upstream of the luciferase reporter gene along with expression plasmids for hC/EBPα and MEKK1. Expression of the luciferase reporter gene was determined 24 h post-transfection. Transfection of MEKK1 expression construct significantly enhanced the ability of C/EBPα to transactivate a minimal C/EBP promoter almost fivefold, whereas this effect was drastically reduced to 1.5-fold when C/EBPα- and MEKK1-co-transfected cells were treated with 10 $\mu$M JNK inhibitor (SP600125) 3 h post-transfection. No significant change was observed in pTK-luc empty vector (Figure 5a). Because JNK1 enhances C/EBPα protein expression and its transactivation capacity, we next asked, if MEKK1-mediated activation of JNK1 increases DNA-binding capacity of C/EBPα. To investigate this, 293T cells were transiently transfected with expression plasmids for hC/EBPα and MEKK1 as indicated (Figure 5b). NE was

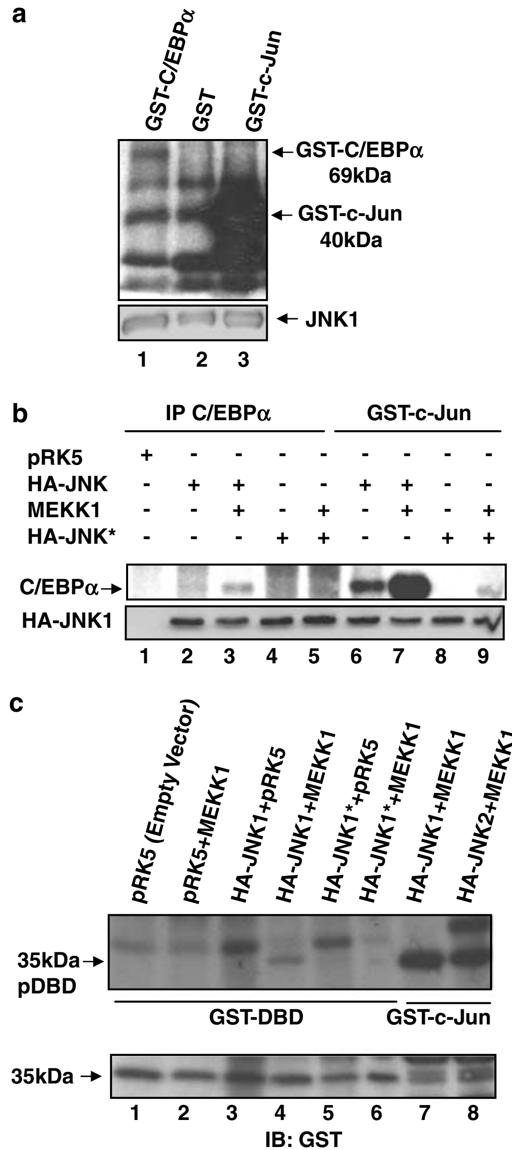
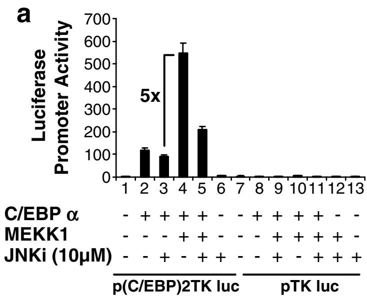



**Figure 4** JNK1 phosphorylates the DBD of C/EBPα. (**a**) JNK phosphorylates GST-C/EBPα (lane 1) and positive control GST-c-Jun in i*n vitro* kinase assay (lane 3). Same membrane probed with HA antibody shows presence of kinase in each lane (**b**) *In vitro* kinase assay shows phosphorylation of wild-type C/EBPα (lane 3, upper panel), whereas there is much less phosphorylation of C/EBPα with HA-JNK1* (kinase-dead HA-JNK; lane 5, upper panel); GST-c-Jun served as positive control. Same membrane was immunoblotted with JNK1 antibody (lower panel). (**c**) *In vitro* kinase assay shows phosphorylation of GST-DBD (lane 4, upper panel), whereas much less with mutant JNK1 (HA-JNK* lane 6); same membrane was stripped and probed with GST antibody to confirm the presence of substrate. GST-c-Jun (GST fused with 1–79 amino acids of c-Jun have mol wt. ~35 kDa) was used as control. Data are representative of three separate experiments.



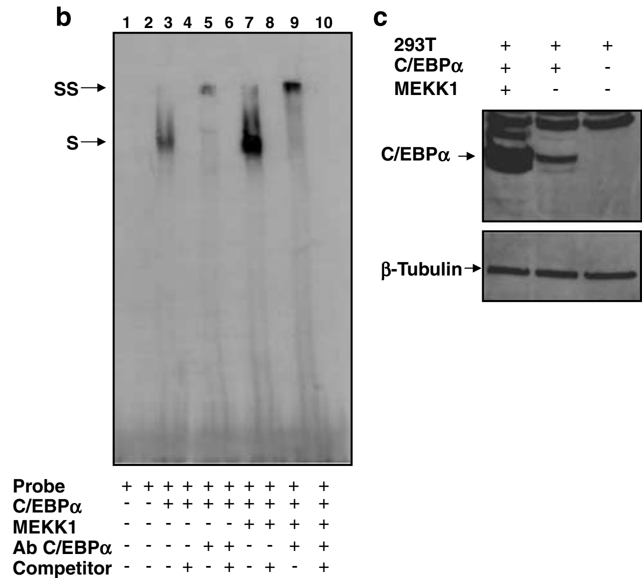




**Figure 5** JNK1-induced increase in C/EBPα protein expression augments C/EBPα trans-activation and DNA-binding activity: (**a**) 293T cells were transiently transfected with various expression plasmids as described in Materials and methods. MEKK1 enhances C/EBPα transactivity almost fivefold whereas JNK inhibitor (JNKi; SP600125) treatment under these conditions reduces it to 1.5-fold. (**b**) EMSA analysis using radiolabelled probe and 10 $\mu$g of NE from 293T cells transiently transfected with C/EBPα and MEKK1 (lane 7) shows enhanced C/EBPα DNA binding as compared to NE extract from cells transfected with C/EBPα alone (lane 3); no binding was observed in NE from mock-transfected cells (lane 2). Shifted complexes are indicated with an arrow (S; lanes 3 and 7); for competition analysis, 50-fold molar excess of unlabelled oligonucleotide probe was used (lanes 4, 6 and 10); super shift was observed with C/EBPα antibody (SS; lane 5 and 9). Results are representative of three separate experiments. (**c**) Western blot analysis for C/EBPα expression (and $\beta$-tubulin expression as loading) for the experiment is shown in (**b**).

prepared 24 h post-transfection and 10 $\mu$g of NE was used in electrophoretic mobility shift assay (EMSA) reaction together with labelled probe which is a 30-nucleotide oligomer corresponding to the C/EBPα-binding region on the granulocyte colony-stimulating factor (G-CSF) receptor promoter (Behre *et al.*, 2002b). Significant increase in C/EBPα DNA binding with probe was observed when NE from co-transfection of C/EBPα and MEKK1 was used as compared to NE from C/EBPα-transfected cells alone. Super shift with C/EBPα antibody was observed whereas no binding occurred when 50-fold excess of unlabelled probe was used as competitor. Figure 5c shows loading control for EMSA experiment. Taken together, these data suggest that active JNK-induced stabilization of C/EBPα protein indeed contributes to enhanced C/EBPα function.

*JNK1 is inactive and downregulated in certain AML subtypes*

In order to ascertain the correlation of C/EBPα and JNK1 interaction in myeloid cells, we assessed the JNK1 mRNA expression and its kinase activity in patients from different AML subtypes where C/EBPα is reported to be inactive. Affymetrix analysis shows that JNK1 mRNA expression is downregulated in AML patients as compared to the normal bone marrow mononuclear cells (Figure 6a) from healthy volunteers. Total mRNA was isolated and processed as described before (Schoch *et al*., 2002). Standard affymetrix software (Micro array Suite, Version 5.0) and the HG-U133A set of normalization controls were used for data analysis. As recommended by the manufacturer, 100 human maintenance genes served as a tool to normalize and scale the data before performing data comparisons. AML patient samples included French-American-British M2 patients with translocation t(8;21), normal karyotype, complex karyotype, M3 with t(15;17), M4eo inversion 16 (inv 16) and mixed lineage leukemia (MLL). As amount of mRNA expression of a gene does not always correspond to its protein expression and moreover our data suggests that active JNK is required for C/EBPα activity, we therefore analysed phospho-JNK protein expression. Patient samples (M2 with t(8;21), M3 with t(15;17), MLL and chronic myeloid leukemia (CML)) were lysed in RIPA buffer and 50 μg of protein was resolved on 8%

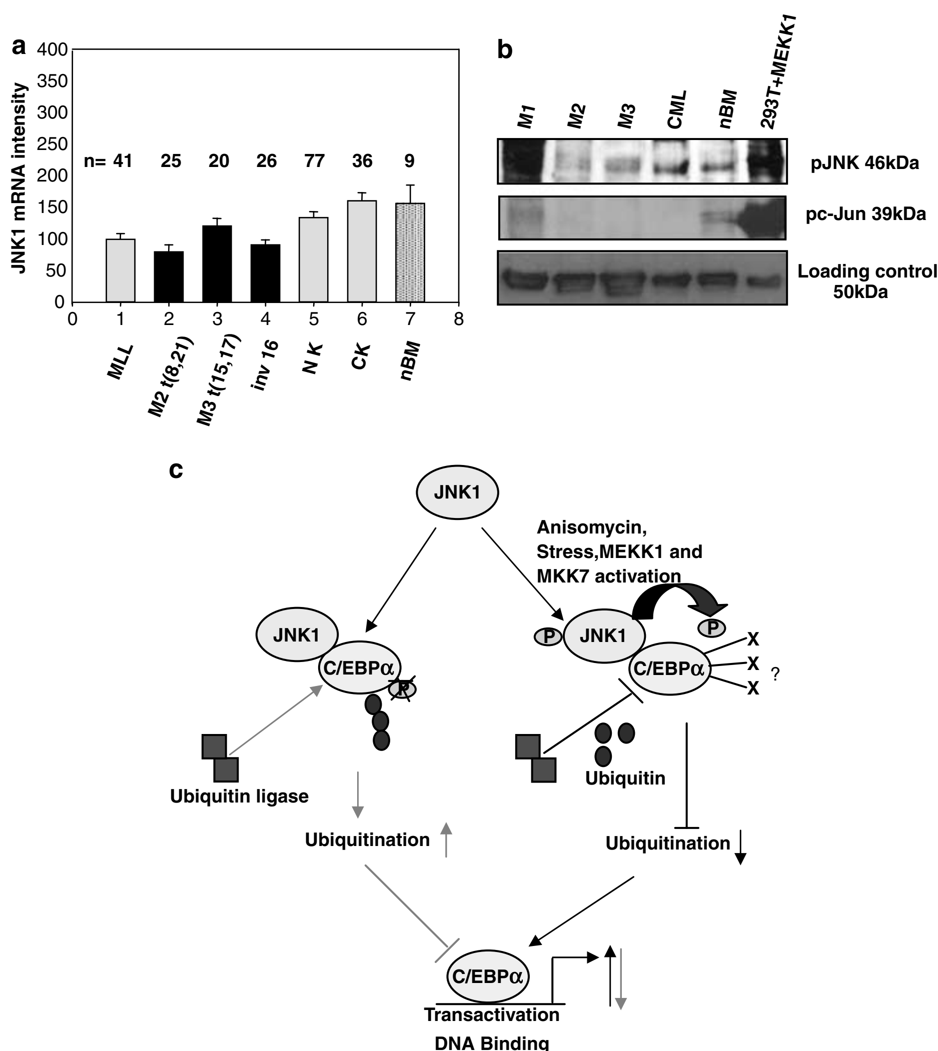



**Figure 6** JNK1 is inactive and downregulated in AML subtypes. (**a**) JNK1 mRNA expression is decreased in AML patient samples ($n=225$) in comparison with normal bone marrow (nBM) mononuclear cells ($n=9$). Expression signal intensities are given as absolute numbers. The error bars indicate the s.e.m., $n$ indicates the number of patient samples analysed in each subgroup. (**b**) pJNK expression is decreased in some AML subtypes. Patient samples from different AML subtypes M1, M2 with t(8,21); M3 with t(15,17); and CML were lysed in RIPA lysis buffer, equal amount of protein was separated on 10% SDS–PAGE; immunoblot against pJNK antibody shows that pJNK expression is decreased in M2 and M3 (upper panel) as compared to normal bone marrow; subsequently, the same membrane stripped and blotted for phospho-c-Jun shows that c-Jun phosphorylation is consistent with decreased JNK1 kinase activity in these patients. Lower panel is loading control. 293T co-transfected with c-Jun and MEKK1 was used as positive control. (**c**) Model for C/EBPα activation by inhibition of JNK targeted ubiquitination of C/EBPα: Diagrammatic representation of C/EBPα physical association with active and inactive JNK. Active JNK1 (pJNK) interaction prevents C/EBPα ubiquitination presumably by phosphorylating later and changing its conformation and thereby contributes to enhanced C/EBPα transcriptional activation.

SDS–PAGE. Immunoblot against pJNK antibody shows that pJNK expression (served as measure of JNK1 kinase activity Figure 6b; upper panel) is decreased in these AML patients as compared to normal bone marrow mononuclear cells. Also, phospho-c-Jun expression (Figure 6b; middle panel) was consistent with decreased JNK kinase activity as shown by re-blotting the same membrane. The same membrane was stripped and blotted with calreticulin as loading control. As expected, JNK kinase activity was increased in CML patients (Raitano *et al*., 1995). These findings led us to hypothesize that physical interaction of active JNK with C/EBPα may positively regulate later whereas down-regulation and/or inactivation of JNK may lead to C/EBPα inactivation as seen in AML.

## Discussion

Protein–protein interactions operative at every biological step are important to the formation of complexes and signal transduction through protein pathways. Perturbation in such multiprotein complexes often leads to improper functioning as seen in leukemia. The molecular mechanism by which C/EBPα is regulated at protein level in normal stem cell development and AML is not fully elucidated. Therefore, we applied a mass spectrometry-based proteomic approach to identify interacting proteins of C/EBPα. The significance of our approach stems from the fact that we could identify JNK1, PAK6, MADP-1, ZNF45 and other proteins (Figure 1f) interacting with the DBD of C/EBP transcription factors. Furthermore, we show that JNK1 indeed interacts with full-length C/EBPα and modulates its activity via ubiquitination.

JNK, a prompt stress-responsive kinase has been reported to regulate protein stability of its associated substrates. Several recent studies have shown the requirement of physical association of the stress-responsive factor JNK1 with other proteins in order to affect protein stability (Fuchs *et al*., 1996, 1997). Our finding shows, like with other proteins, JNK does physically associate with C/EBPα *in vivo* and *in vitro* in co-immunoprecipitation and GST pull down, respectively; Note that JNK and phospho-JNK both interact with C/EBPα (Figure 2b and 3i). We show that physical association of JNK with C/EBPα inhibits ubiquitination of C/EBPα. *In vivo* ubiquitination assay showed that, under normal conditions, JNK interaction with C/EBPα targets ubiquitination of C/EBPα presumably by attracting the enzymes of the ubiquitination machinery to C/EBPα, thereby marking it for proteasome-dependent degradation. However, in response to stress, that is, when phosphorylated active JNK interacts with C/EBPα, this ubiquitination is inhibited (Figure 3f and g). Our data are consistent with the findings that JNK regulates ubiquitination-dependent degradation of a different subset of substrates by acquiring a specific phosphorylation pattern that affects conformation, stability and transcriptional activation (Musti *et al*., 1997; Fuchs *et al*., 1998; Ronai, 2004). JNK regulates C/EBPα protein stability is even more evident from upregulation of C/EBPα protein expression in different myeloid cell lines upon JNK activation (Figure 3a–d). Additionally, amount of C/EBPα immunoprecipitated from anisomycin-induced U937RIPA lysates was more as compared to the uninduced cells (Figure 3g; lanes 7 and 8). Moreover, MEKK1-induced JNK activation leading to C/EBPα protein stability was abrogated when these cells were treated with JNK inhibitor, which strengthens the fact that JNK does regulate C/EBPα protein stability (Figure 3d). More recently, Yoon K *et al*. have shown that C/EBPα protein is upregulated up to 70-fold upon ultraviolet B (UVB) irradiation in keratinocytes which supports our finding, as UVB is a potent activator of JNK (Yoon and Smart, 2004).

In addition to its function as a transcription factor, C/EBPα acts as tumor suppressor which inhibits cell proliferation via transcription-independent mechanism in which C/EBPα forms a complex with cdk2 and cdk4 preventing cyclin/cdk complex formation, E2F inhibition and cell cycle progression. In case of signal-dependent transcription factors, it is apparent that changes in their half-life could have a significant impact on the activity of the corresponding target genes. Therefore, regulation of C/EBPα protein levels through an ubiquitin–proteasomal pathway would serve to control both the transcription-dependent and transcription-independent activities of C/EBPα (Shim and Smart, 2003). Active JNK-induced increase in C/EBPα protein was accompanied by a significant increase in C/EBPα transactivation activity, which dramatically reduced with JNK inhibitor treatment suggesting JNK-specific C/EBPα activation (Figure 5a). Additionally, we also observed an increase in DNA-binding capacity of C/EBPα in the presence of active JNK in EMSA (Figure 5b). Taken together, our findings suggest that JNK is required for C/EBPα stability which has impact on its activity.

The fact that c-Jun is protected from ubiquitination after being phosphorylated by JNK (Fuchs *et al*., 1996; Musti *et al*., 1997) prompted us to investigate whether JNK apart from physically associating, also phosphorylates C/EBPα in order to inhibit its ubiquitination. In *in vitro* JNK kinase assay, we show that wild-type JNK does phosphorylate C/EBPα and GST-C/EBPα whereas kinase-dead JNK could partially phosphorylate (Figure 4). However, further study is required to more fully determine the contribution of direct CEBPα phosphorylation in its stabilization and the amino-acid residues of C/EBPα being targeted by JNK. In any case, physical interaction of JNK with C/EBPα and phosphorylation of later led us to hypothesize that JNK inhibits C/EBPα ubiquitination by phosphorylating and presumably changing its conformation making it inaccessible to the ubiquitin ligase and ubiquitination machinery as such to target C/EBPα for degradation.

Affymetrix analysis of JNK1 mRNA using an indirect measure for JNK activity in AML subtypes where C/EBPα function is impaired such as M2 with translocation t(8;21), M3 with t(15;17) and AML with inversion

16, shows reduced JNK1 mRNA expression in comparison with normal bone marrow mononuclear cells from healthy volunteers (Figure 6a). Additionally, phospho-JNK protein expression (kinase activity) in these subtypes is also decreased (Figure 6b). A function for JNK is implicated in cancer but the mechanism of JNK action is unclear. Although number of patient samples used for pJNK expression is very few to state if pJNK kinase activity in AML is downregulated in general. However, it gives a rough estimate that JNK kinase activity is decreased in certain AML patients. A follow-up study on the expression levels of pJNK and C/EBPα in large number of patients might give important insights into JNK-regulated C/EBPα expression.

In conclusion, we propose a hypothetical model (Figure 6c) for the importance of physical interaction of JNK with C/EBPα. Targeting ubiquitination of C/EBPα by JNK requires tight interaction with C/EBPα; whereas interaction with pJNK and subsequent C/EBPα phosphorylation at unidentified amino-acid residue by JNK inhibits its ubiquitination, probably owing to altered conformation of C/EBPα, which is likely to inhibit the ubiquitination machinery. This results in increased C/EBPα stability and availability which is reflected by enhanced DNA binding and transcriptional activity of C/EBPα. Moreover, downregulation of JNK mRNA and kinase activity in different AML subtypes implicates that JNK activity is required for C/EBPα activation in myeloid cells and that loss of JNK-regulated C/EBPα expression may render it inactive.

## Materials and methods

*GST pull down*

We used plasmid constructs in which full-length C/EBPα and region comprising amino acids 270–358 encoding the conserved DBD of C/EBPs are cloned in frame with the GST in pGEX bacterial expression vector. Fusion proteins were expressed in transformed DH5α *E. coli* bacterial strain after 0.5 mM IPTG induction at 37°C for 2 h and subsequently purified using immobilized glutathione sepharose 4B beads (Amersham Biosciences, Germany) by lysing in NETN buffer as described before (Rangatia *et al*., 2002). Following washing, purified fusion proteins were lysed in SDS sample buffer, separated on 12% SDS–PAGE and visualized by commassie blue staining. NE of U937 cells was prepared using lysis buffer A and C, respectively, as described before (Rangatia *et al*., 2002). Equal amount of fusion proteins were incubated with 1 mg of NE (in NETN buffer) for 3 h at 4°C on a rotating shaker. After GST pull down, protein-bound GST sepharose beads were washed three times in NETN buffer. In addition, $S^{35}$-methionine-labelled *ivt* JNK1 was pulled down with GST fusion proteins as described before (Reddy *et al*., 2002).

*2D-gel electrophoresis and MALDI–TOF mass spectrometry*

GST and GST-DBD were incubated with NE of myelomonocytic U937 cells. Beads with their associated proteins from NE were lysed in urea lysis buffer (66% urea plus one, 1% DTE, 4% CHAPS, 2.5 mM ethylenediaminetetraacetic acid (EDTA) and 2.5 mM ethyleneglycoltetraacetate) for 1 h at room temperature. Lysed beads were passed through RNA quiashredder (Quiagen, Germany), and resulting supernatant was ultracentrifuged for 1 h at 50 000 r.p.m. at 22°C to eliminate DNA and other cellular debris. In the first dimension, 350 μl of dissolved proteins were separated on 18 cm long immobiline dry strip in the pH range 3–10 (Amersham Biosciences, Germany) by IEF. The reduction and alkylation of separated proteins were carried out in urea buffer containing 2% DTE and 2.5% iodoacetamide. Proteins were then separated in the second dimension using 12% SDS–PAGE. 2D gels were silver stained to visualize the protein spots. After comparison of the two silver-stained gels, differentially appearing protein spots from GST-DBD gel were excised and digested with 200 ng Trypsin (Promega, Mannheim, Germany) in ammonium bicarbonate solution for 16 h. Digested peptides were eluted in 70% acetonitrile, lyophilized and resuspended in 5 μl of 0.1% trifluoacetic acid in 10% acetonitrile. The dissolved peptides were mixed in 1:1 ratio with 2,5-dihydroxybenzoic acid matrix solution and loaded on anchorChip target plate (Bruker Daltonics, Leipzig, Germany). Peptide mass fingerprint and peptide sequencing was performed by MALDI–TOF (Reflex III, Bruker) and confirmed by MALDI–TOF–TOF (AB4700, Applied Biosystems, Darmstadt, Germany) mass spectrometer, corresponding proteins were identified by MASCOT database search.

*Cell culture, plasmids*

293T, U937 and HL 60 cells were cultured as described previously (Reddy *et al*., 2002). NB4 cells were maintained in Rosewell Park Memorial Institute medium (RPMI) supplemented with 20% fetal bovine serum (FBS), 2.5% pen-strep and 2.5% glutamine. K562C/EBPα-Er and K562Er cells were maintained in RPMI (without phenol red) supplemented with 10% charcoal treated FBS (Hyclone, Nürtingen, Germany) and 2 μg/ml puromycin. HA-JNK and kinase-dead HA-JNK mutant were provided by Dr A Keiser, GST-c-Jun corresponding to 35 kDa (1–79 amino acids fused with GST) was bought from Cell Signalling technology (Beverly, MA, USA), whereas GST-c-Jun with 40 kDa molecular size was bacterially purified. pCDNA3-human C/EBPα is described previously (Pabst *et al*., 2001b). BR3 harbors mutations in the basic region that prevent DNA binding (amino acids: 297R, 298K, 300R and 302K of C/EBPα mutated to glycine, threonine, glycine and aspargine, respectively); Leu1, 2-Val cannot dimerize or bind DNA because of mutation of two leucines to valine within the leucine zipper; and GZ is a variant in which the C/EBPα leucine zipper is replaced with the leucine zipper from a yeast protein, GCN4. GZ retains the ability to homodimerize, bind DNA, and activate transcription, but is not expected to interact with endogenous basic region leucine zipper (bZIP) proteins via the leucine zipper. C/EBP-DBD plasmid constructs were kindly provided by Dr Alan Friedman and is described elsewhere (Landschulz *et al*., 1989). Plasmid construct HA-ubiquitin (pMT123HA-Ubi) was kind gift from Dr Dirk Bohmann.

*Co-immunoprecipitation and* in vitro *kinase assay*

Co-immunoprecipitation assay was performed form RIPA lysate of U937 cells (RIPA: 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 5 mM EDTA and 50 mM Tris pH8.0) as described previously (Rangatia *et al*., 2002). Immunoprecipitated proteins were heated at 56°C for 90 min in 2 × SDS loading buffer and then boiled at 95°C for 5 min before separation on 8% SDS–PAGE. Proteins were immunoblotted with JNK1 and C/EBPα antibody (Santa Cruz Biotechnology Inc., Heidelberg, Germany), other antibodies used are HA (Roche Diagnostics, Manheim, Germany) and Phospho-JNK antibody (Cell Signalling Technologies, USA).

*In vitro* kinase assay was performed as described before (Zada *et al*., 2003).

*EMSA and reporter assays*
For EMSA analysis, a double-stranded G-CSF receptor promoter oligonucleotide extending from bp −57 to −38 was used as a probe; OligoA (AAG GTG TTG CAA TCC CCA GC) and OligoB (GCT GGG GAT TGC AAC ACC TT) were annealed and labelled with γ-[$^{32}$P]dATP (GE Health Care, Munich, Germany) using T4 polynucleotide kinase (Invitrogen, Karlsruhe, Germany). EMSA was performed by incubating 10 μg of NE with 1 ng of the radiolabelled probe in binding buffer (10 mM HEPES pH 7.9, 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM dithiothreitol and 10% glycerol) and 0.5 μg of poly (dI–dC) (Amersham Biosciences, Munich, Germany) for 30 min as described before (Behre *et al*., 2002b). For reporter assays, 293T cells were transfected using LipofectAMINE Plus as described by the manufacturer (Invitrogen, Karlsruhe, Germany). Firefly luciferase activities from the constructs phC/EBPα, pTK, pCDNA3-MEKK1, p(C/EBP)2TK and *Renilla* luciferase activity from the internal control plasmid pRL-null were determined 24 h after the initiation of the transfection protocols as described previously (Behre *et al*., 2002b), The reporter construct p(C/EBP)2TK contains two adjacent ATTGCGCAAT consensus C/EBPα-binding sites cloned into pTK81 luciferase, was kindly provided by Dr Alan Friedman and is described previously (Nordeen, 1988; Friedman, 1996). Results are given as means ±s.e.m. of three independent experiments.

In vivo *HA ubiquitination assay, pulse chases labelling and Western blotting*
For *in vivo* HA ubiquitination assay, $1 \times 10^6$ 293T cells were transiently transfected with different constructs as described. Twenty-four hours post-transfection, cells were lysed in RIPA buffer and C/EBPα was immunoprecipitated from 500 μg protein. For pulse chase labelling, $3 \times 10^5$/ml HL60 cells in 50 ml were plated in normal RPMI medium 1 day before the experiment. Next day cells were washed twice with phosphate-buffered saline and grown in 3 ml labelling medium (RPMI without methionine and cysteine, supplemented with 0.2% dialysed FBS) for 15 min with 200 μCi/ml methionine with constant shaking at 37°C in water bath. The cells were then washed and chased for various time points with normal RPMI medium in the absence or presence of anisomycin. Cells were lysed and C/EBPα was immunoprecipitated from equal amount of lysates. The resulting precipitates were subjected to 10% SDS–PAGE, autoradiography and densitometry analysis. Western blotting was performed as described before (Rangatia *et al*., 2002).

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. The percentage of blast cells within the bone marrow carrying the respective fusion genes as detected by fluorescence *in situ* hybridization ranged from 52 to 99% (median 90%). For Western blot analysis, the healthy bone marrow cells were purchased from stem cell technologies (Cell Systems biotechnology, Vertrieb Gmbh, St Katharinen, Germany). There was no correlation between the percentage of blast cells and the fusion gene transcript levels. Microarray analyses were performed as reported previously (Schoch *et al*., 2002) by use of the GeneChip System (Affymetrix U95Av2 and U133A microarrays, Santa Clara, CA, USA).

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ORIGINAL ARTICLE

# Proteomic discovery of Max as a novel interacting partner of C/EBPα: a Myc/Max/Mad link

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**The transcription factor CCAAT/enhancer binding protein a (C/EBPα) is important in the regulation of granulopoiesis and is disrupted in human acute myeloid leukemia. In the present study, we sought to identify novel C/EBPα interacting proteins *in vivo* through immunoprecipitation using mass spectrometry-based proteomic techniques. We identified Max, a heterodimeric partner of Myc, as one of the interacting proteins of C/EBPα in our screen. We confirmed the *in vivo* interaction of C/EBPα with Max and showed that this interaction involves the basic region of C/EBPα. Endogenous C/EBPα and Max, but not Myc and Max, colocalize in intranuclear structures during granulocytic differentiation of myeloid U937 cells. Max enhanced the transactivation capacity of C/EBPα on a minimal promoter. A chromatin immunoprecipitation assay revealed occupancy of the human C/EBPα promoter *in vivo* by Max and Myc under cellular settings and by C/EBPα and Max under retinoic acid induced granulocytic differentiation. Interestingly, enforced expression of Max and C/EBPα results in granulocytic differentiation of the human hematopoietic CD34+ cells, as evidenced by CD11b, CD15 and granulocyte colony-stimulating factor receptor expression. Silencing of Max by short hairpin RNA in CD34+ and U937 cells strongly reduced the differentiation-inducing potential of C/EBPα, indicating the importance of C/EBPα–Max in myeloid progenitor differentiation. Taken together, our data reveal Max as a novel co-activator of C/EBPα functions, thereby suggesting a possible link between C/EBPα and Myc–Max–Mad network.**
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**Keywords:** C/EBPα; proteomics; Myc–Max–Mad network; mass spectrometry; differentiation

## Introduction

Hematopoietic differentiation proceeds in a largely irreversible fashion and the role of transcription factors in regulating hematopoiesis has been well documented. This is particularly true for CCAAT/enhancer binding protein a (C/EBPα), one of the lineage-specific transcription factors that is essential for commitment to and development of the granulocytic lineage.[1,2] Recent data have indicated that C/EBPα may also regulate hematopoietic stem cell activity[3] and act as a tumor suppressor gene in acute myeloid leukemias (AMLs), indicating an important role for C/EBPα in the control of cellular proliferation *in vivo*.[4] Inactivation of C/EBPα is an important event in AML, and ectopic overexpression of C/EBPα leads to differentiation and growth arrest in AML.[5] It is therefore suggested that C/EBPα has a crucial role in regulating the balance between cell proliferation and differentiation, which is crucial for lineage commitment of any cell type. These findings and data from our laboratory indicate that for AML to develop, the activity of C/EBPα must be curbed by either mutations or antagonistic protein–protein interactions.

C/EBPα can form protein–protein interactions with other bZIP and non-bZIP factors. Among them, c-Jun and PU.1,[6,7] E2F, p21, and cyclin-dependent kinases CDK2 and CDK4 have been well characterized.[8–10] Thus, it has become increasingly clear that like most proteins, C/EBPα might not work alone, but in association with other factors regulates gene transcription. However, studies involving protein–protein interactions of C/EBPα at the global proteomic level are lacking. We therefore took advantage of high-throughput proteomics by mass spectrometry (LC-MS/MS) to identify proteins that specifically associate with C/EBPα *in vivo*. In our screen, Max was identified as a novel interacting partner of C/EBPα in addition to other new and known partners of C/EBPα.

Max is a member of the basic region-helix–loop–helix-leucine zipper protein that belongs to a network of transcription factors, which includes the Myc and Mad families of protein (commonly referred to as a Myc–Max–Mad network).[11] The Myc–Max–Mad proteins can affect different aspects of cell behavior, including cell cycle, proliferation and differentiation, by modulating distinct target genes.[12–15] Max can form a homo- or a heterodimer and bind specifically to E-box DNA elements in target promoters (consensus CACGTG).[16,17] To function as transcriptional regulators, the members of the Myc and Mad families must heterodimerize with Max. Whereas Myc–Max activates transcription, Mad–Max and Mnt–Max repress transcription.[18–20] Indirect evidences to the fact that C/EBPα could be a part of the Myc–Max–Mad network do exist in the literature.[21,22] However, no direct evidence has been reported so far.

In this study, we have characterized the role of Max as an interacting partner of C/EBPα. We show that Max is an important co-activator of C/EBPα and the stable silencing of Max inhibits the differentiation-inducing potential of C/EBPα. C/EBPα and Max not only colocalize but also the heterocomplex is preferentially formed on the human C/EBPα (hC/EBPα) promoter *in vivo* during granulocytic differentiation, thereby contributing to increased transactivation and differentiation capacity of C/EBPα.

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## Materials and methods

### Transfection of human hematopoietic CD34+ progenitors

Human CD34+ hematopoietic cells were selected, using a magnetic CD34 selection kit system (Milteny Biotec, Bergisch, Gladbach, Germany), from small aliquots of leukapheresis products collected from either healthy donor or a patient undergoing stem/progenitor cell collection after granulocyte-colony stimulating factor treatment for non-hematologic malignancy at Klinikum Krollwitz Hospital Halle, Germany, following their informed consent. After magnetic selection, more than 85% of the cells expressed the CD34 antigen. An aliquot containing $5\times10^5$ CD34+ cells was cultured in Iscove's modified Dulbecco's medium with 20% heat-inactivated fetal calf serum, 100 ng/ml Flt3-ligand, 100 ng/ml of stem cell factor, 100 ng/ml thrombopoietin, 100 ng/ml of interleukin-6 (IL-6) and 50 ng/ml of IL-3, 100 U/ml penicillin/streptomycin and 2 mM L-glutamine. The cells were transfected with various expression constructs using AMAXA nucleofection technology essentially as described by the manufacturer and analyzed for CD11b and CD15 expression by flow cytometry.

### Cell lines, antibodies and treatments

Human myeloid cell lines U937 and K562-ER-C/EBPα were cultured under standard conditions. β-Estradiol and retinoic acid (RA) (Sigma-Aldrich, Munich, Germany) were used at a concentration of 1–5 μM and $10^{-6}$ M, respectively. The antibodies used in this study were purchased from Santa Cruz (Heidelberg, Germany); for C/EBPα, SC-61 (14AA), SC-9315 (N-19) Max, SC-765 (C-124) and c-Myc, SC-42 (C-33) and Molecular Probes, Gmbh, Karlsruhe, Germany).

### Immunoprecipitation and immunoblotting

The immunoprecipitation (IP) was performed from 500–1000 μg nuclear extracts of U937 cells in an IP buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.5% NP-40, 0.25% sodium deoxycholate), followed by washing in the buffer (50 mM Tris pH 7.5, 0.1% NP-40, 0.05% sodium deoxycholate) with respective antibodies (Santa Cruz) and the corresponding IgGs as controls. A Western blot analysis was used to confirm the identity of immunoprecipitated and/or co-precipitated proteins as described previously.[23] Alternatively, the immunocomplexes were incubated with urea lysis buffer for further proteomic analysis.

### Proteomic analysis: two-dimensional gel electrophoresis and protein identification by mass spectrometry

The proteomics methodology was used essentially as described recently by our group.[34]

### Transient transfections using AMAXA and effectene

Effectene transfection reagent (Qiagen, Gmbh, Hilden, Germany) and lipofectamine (Invitrogen, Gmbh, Karlsruhe, Germany) were used for transient transfections according to the manufacturer's instructions. Transient transfections were carried out with minimal promoter/luciferase construct, which has been derived from an oligo 5′-GATCCAGATTGCGCAATCG-3′ by self-annealing, followed by ligation into a *Bam*HI site of the thymidine kinase (TK) promoter and co-transfected with expression plasmids for hC/EBPα, *Renilla* Luciferase-null and/or Max as described.[23] The Nucleofector kit (AMAXA, Gmbh, Cologne, Germany) was used essentially as described by the manufacturer. A 5 μg portion of plasmid DNA constructs was used for each transfection and the transfection efficiency was analyzed using a plasmid with eGFP marker (2 μg). For CD34+ and U937 cells, nucleofector solution kits used were VPA-1003 and VCA-1003 with nucleofection programs U-08 and V-01, respectively. The voltages are automatically adjusted according to the program and are essentially 110 V AC with a frequency of 50–60 Hz and a power consumption of 16 VA/fuse.

### Immunofluorescence and flow cytometry

U937 cells ($3\times10^5$), under uninduced condition or induced with RA (Sigma-Aldrich), were cytocentrifuged on glass slides with coverslips, fixed using 1:1 methanol/acetone and permeabilized using 0.3% Triton X. After blocking in PBG (0.5% BSA, 0.045% Fish–gelatin in phosphate-buffered saline) containing 5% FBS, the fixed cells were incubated with anti-C/EBPα (anti-goat; Santa Cruz), anti-Max (anti-rabbit; Santa Cruz) and anti-Myc (anti-mouse; Santa Cruz) antibodies, followed by incubation with corresponding Alexa Fluor 488 chicken anti-goat, Alexa Fluor 594 chicken anti-rabbit and anti-mouse IgG secondary antibodies (Molecular Probes) and 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI, 1 μg/ml) for 15 min. The cells were mounted in aqueous mounting medium and the images were acquired and analyzed using a fluorescence microscope (×100, ×60). Flow cytometry was performed for CD11b, CD14 and CD15 expression on Bectin Dikinson flow cytometer, using the supplied analysis software.

### Quantitative real-time PCR

RNA isolation from CD34+ and U937 cells, transfected with different expression constructs, by TRIZOL (Invitrogen, Germany) was followed by cDNA synthesis using standard conditions. Equal amount of cDNA was taken for a subsequent quantitative real-time PCR (Q-RT-PCR) using the Quantitech SyBR Green PCR kit (Qiagen, Germany) in a Rotor-Gene RG-3000 (Corbett Research, Sydney, Australia). The delta ct value (Δct) was then calculated from the given ct value by the formula $\Delta ct = ct_{sample} - ct_{control}$). The fold change was calculated as fold change $= 2^{-\Delta ct}$. The following primer sequences were used: myeloperoxidase (MPO), 5′-TCG GTA CCC AGT TCA GGA AG-3′ (forward) and 5′-CCA GGT TCA ATG CAG GAA GT-3′ (reverse); neutrophilelastase (NE), 5′-TGC TCA ACG ACA TCG TGA TT-3′ (forward) and 5′-CTC ACG AGA GTG CAG ACG TT-3′ (reverse); GCSFR, 5′-AAG AGC CCC CTT ACC CAC TAC ACC ATC TT-3′ (forward) and 5′-TGC TGT GAG CTG GGT CTG GGA CAC TT-3′ (reverse); CD14, 5′-CAA CTT CTC CGA ACC TCA GC-3′ (forward) and 5′-CCA GTA GCT GAG CAG GAA CC-3′ (reverse).

### Chromatin immunoprecipitation assay

Logarithmically growing and differentiating U937 cells (~$1\times10^8$ cells) were fixed with formaldehyde (final concentration 1% (v/v)) in serum free RPMI-1640 medium, at 4°C for 1 h. Glycine was added to a final concentration of 0.125 M to stop cross-linking. Fixed cells were pelleted by centrifugation and sequentially washed and sonicated (five times for 20 s each) to make soluble chromatin. Samples of total chromatin were taken at this point to use as a positive control in the PCRs (input chromatin). Antibodies against C/EBPα, Max and c-Myc were used overnight at 4°C. After serial elution, washing and cross-

link reverse, the samples were extracted twice with phenol/chloroform and precipitated with ethanol overnight in the presence of 20 μg glycogen as a carrier. DNA fragments were recovered by centrifugation, resuspended in $ddH_2O$, and used for PCR amplification. For detection of immunoprecipitated C/EBPα promoter region, two primers, forward (5′-ACCGCTACCGACCACGTGGGCG-3′) and reverse (5′-AGCACCTCCGGGTCGCGAATGG-3′), specific for a 280 bp region in the cellular C/EBPα promoter that encompasses the C/EBP site were used for Q-RT-PCR amplification.

## Results

### *Identification of Max, a heterodimeric partner of Myc, as a novel interacting protein of C/EBPα*

To identify interacting proteins of C/EBPα *in vivo* under physiological conditions on a global level, we applied proteomics technique coupled with mass spectrometry using the IP conditions of endogenous C/EBPα from myeloid U937 cells as a model system.

Under our experimental conditions, we could specifically immunoprecipitate endogenous C/EBPα from the nuclear extracts of U937 cells (Figure 1a) and co-immunoprecipitate other endogenous proteins (as positive controls) such as c-Jun and CDK4 (Figure 1b and data not shown) that were not present in the isotype IgG control. Immunocomplexes were further processed for proteomic analysis. The protein spots excised from the 2D gels (Figure 1c, spots are numbered) were identified by MALDI-TOF MS. Additionally, the individual bands were excised from Coomassie/silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (Figure 1d) and processed for LC-MS/MS. From both screens, we were able to reveal the identity of 10 proteins by MS, which specifically interact with C/EBPα (Table 1). Among these proteins, we identified Max as one interacting partner of C/EBPα. C/EBPα was also identified by MS analysis of the corresponding band (Figure 1d), thereby serving as a control for our experimental setup. Proteins in other bands could not be determined because of the poor quality of the spectrum. The discovery of Max as a novel C/EBPα partner is intriguing because of the role Max plays in switching of the complexes during myeloid differentiation.[24] We therefore

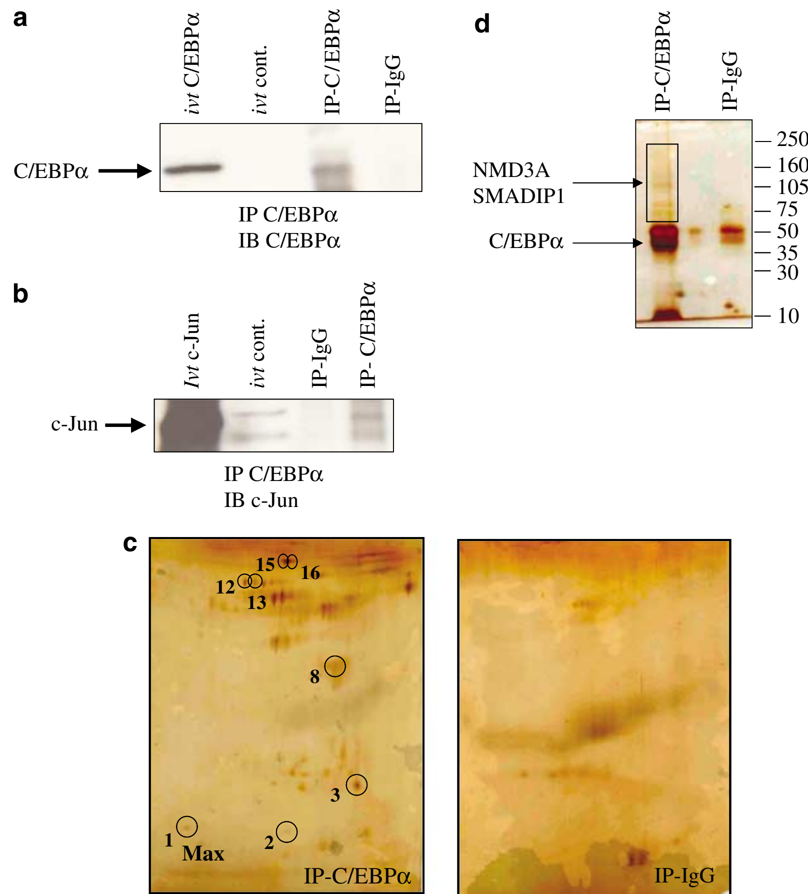



**Figure 1** MS-based proteomics identifies proteins specifically interacting with C/EBPα *in vivo* after its immunoprecipitation from myeloid U937 cells. (**a**) C/EBPα IP from nuclear extracts of U937 cells and a corresponding immunoblotting (IB) with anti-C/EBPα antibody to confirm the presence of C/EBPα protein in the IP complex. *In vitro*-translated C/EBPα (*ivt*, lane 1) was used as a positive control in the Western blot. (**b**) C/EBPα IP and corresponding IB with anti-c-Jun antibody to show endogenous proteins co-precipitated with C/EBPα. *ivt* c-Jun was used as a positive control for c-Jun. (**c**) Silver-stained 2D gels showing proteins specifically interacting with C/EBPα. C/EBPα was immunoprecipitated from nuclear extracts using anti-C/EBPα antibody (anti-rabbit; Santa Cruz) and the immunocomplex separated in the first dimension by pH 4–7 IPGphor strips followed by their separation in the second dimension using 12% SDS-PAGE. As a specificity control, we used immunoprecipitation with IgG under similar conditions. (**d**) Silver-stained SDS-PAGE gels after IP with anti-C/EBPα and anti-IgG. The bands were excised and peptide mixture after trypsin digestion was run on a reverse-phase high-pressure liquid chromatography and the peptides identified by MALDI-TOF-TOF (Applied Biosystems, Darmstadt, Germany).

**Table 1** MS results of the proteins interacting with C/EBPα: MALDI-TOF *Reflex III (Bruker Daltonics) and LC-MS/MS*

| *Spot no.* | *Acc. no.* | *Protein name* | *Score* | *Mol. wt.* | *pI* | *Sequence coverage* |
|---|---|---|---|---|---|---|
| 1 | A42611 | Max | 85 | 21.029 | 5.64 | 53 |
| 2 | Q9UP93 | Macrophin1 fragment | 95 | 620 (full) | 5.27 | 35 |
| 3 | M2OM_Hum | Mitochondrial2-oxoglutarate/malate carrier protein | 65 | 34.08 | 9.92 | 37 |
| 8 | A47213 | Beta fodrin | 68 | 146.55 | 5.18 | 14 |
| 12 | Q96QA8 | RPGR interacting protein 1 | 88 | 147.33 | 5.47 | 14 |
| 13 | Q9P1U9 | ZNF45 | 71 | 80.44 | 9.0 | 16 |
| 15 | FAHUAA | Actinin 1 | 238 | 103.48 | 5.22 | 31 |
| 16 | Q9UKD2 | 60 S ribosomal protein | 74 | 127.60 | 7.68 | 29 |
| * | NMD3A | *N*-methyl-D-aspartate receptor 3A | * | 126.67 | * | * |
| * | SMADIP1 | Smad interacting protein 1 | * | 137.84 | * | * |

Abbreviations: C/EBPα, CCAAT/enhancer binding protein a; LC-MS/MS, liquid chromatography-coupled tandem mass spectrometry.
Proteins identified by MALDI-TOF mass spectrometry and LC-MS/MS from 2D gels and normal SDS-PAGE gels (represented as *), respectively.
Acc. no: SwissProt. protein accession numbers; Mol. wt: apparent molecular weight; pI: isoelectric point of the protein.

selected Max for further functional and biological characterization.

### *C/EBPα and Max interact in a cellular setting: confirmation of proteomics data*

To confirm the observed interaction of Max with CEBPα by an alternative technique, we performed reciprocal immunoprecipitation. Our results demonstrate that C/EBPα interacts with Max and vice versa (Figure 2a) *in vivo*, and thereby confirm proteomic results. It is important to note that for the same amounts of nuclear extracts used (5 and 10 $\mu$g) as input controls, the levels of the two transcription factors are dramatically different, which is likely due to Max being more stable than C/EBPα.

### *BR3 region of C/EBPα is involved in its interaction with Max*

To investigate the protein domains that might be involved in C/EBPα–Max interaction, we performed co-immunoprecipitation studies using different mutants of C/EBPα as shown. C/EBPα and its various mutants (kind gift from Dr Alan Friedman; Figure 2b) were transiently transfected into 293 cells, and co-transfected with an expression plasmid for Max (a kind gift from Dr Dirk Eick) containing a carboxy-terminal HA tag.[25] Max was then immunoprecipitated from nuclear extracts using anti-Max antibody. The associated complexes were assayed by immunoblotting for C/EBPα using anti-C/EBPα antibody. Our results demonstrate that C/EBPα could be co-immunoprecipitated when IP was performed using anti-Max antibody in samples in which wild-type C/EBPα: wild-type Max, GZ/LZ C/EBPα: wild-type Max and L1-2V C/EBPα: wild-type Max were coexpressed (Figure 2c, lanes 4, 3, 1, respectively). However, C/EBPα could not be co-immunoprecipitated in immunoprecipitated samples in which basic region mutant BR3-C/EBPα: wild-type Max was co-expressed (Figure 2c, lane 2). We also show that Max could be specifically immunoprecipitated (as controls) with immunoblot for Max using anti-HA antibody (Figure 2c, lower panel). The relative expression of C/EBPα mutants was the same (data not shown). These data show that the basic region of C/EBPα is involved in its interaction with Max in a cellular setting. Furthermore, we observed that wild-type Max and its basic region mutants have the same ability to interact with C/EBPα (Supplementary Figure S1a and b).

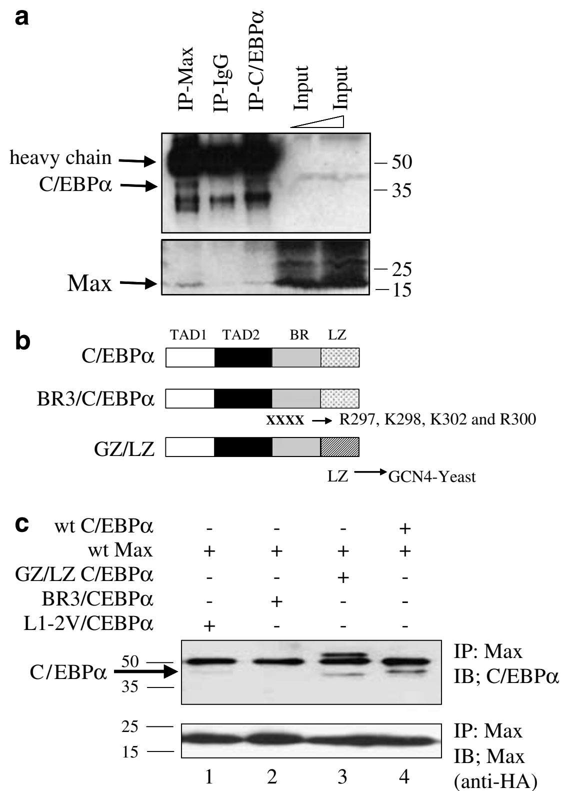



**Figure 2** *In vivo* interaction of C/EBPα with Max confirmed by reciprocal IP involves the DNA-binding domain of C/EBPα. (**a**) Reciprocal IPs: C/EBPα and Max were immunoprecipitated (IP C/EBPα, IP Max) from nuclear extracts of U937 cells by incubation with anti-C/EBPα and anti-Max, respectively, and respective IgG as controls. The blot was first probed with anti-C/EBPα antibody, stripped and reprobed with anti-Max antibody. (**b**) Basic region of C/EBPα is involved in its interaction with Max. Schematic representation of wild-type hC/EBPα and different mutants used in this study. TAD, transactivation domains 1 and 2; BR, basic region; LZ, leucine zipper domain; HLH, helix–loop–helix. (**c**) hC/EBPα wild type and its mutants were transfected in 293T cells and co-transfected with wild-type Max expression plasmid. At 24 h post-transfection, the nuclear extracts were prepared and IP of Max performed for the samples followed by immunoblot for C/EBPα or Max using anti-C/EBPα and HA antibodies, respectively.

### *C/EBPα and Max colocalize*

Given the fact that C/EBPα and Max are nuclear transcription factors and the observation that they interact *in vivo*, we next investigated the localization of these proteins by indirect immunofluorescence in myeloid U937 cells. We observed both endogenous C/EBPα and Max to be localized in intranuclear structures (Figure 3a) and the overlay of the two images shows

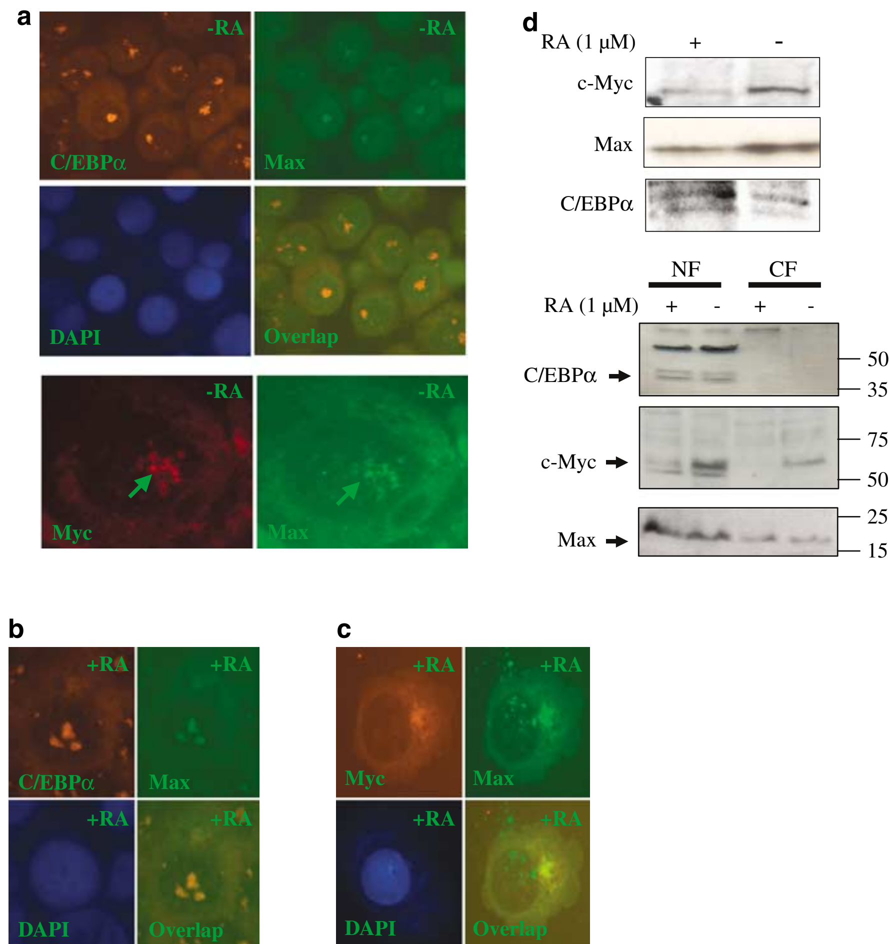



**Figure 3** Endogenous C/EBPα-Max but not Myc–Max remains colocalized during granulocytic differentiation of U937 cells. (**a**) Indirect immunofluorescence staining for C/EBPα (anti-goat; Santa Cruz), Max (anti-rabbit; Santa Cruz) and Myc (anti-mouse, Santa Cruz) using respective conjugated secondary antibodies (Molecular Probes). U937 cells were cytocentrifuged on glass slide cover slips, fixed with methanol/acetone, permeabilized with 0.3% Triton X stained with respective antibodies (Alexa Fluor, Molecular Probes) and DAPI. The morphology of the cells was visualized under fluorescence microscope (X60–100). Colocalization is demonstrated by the yellow signals. Indirect immunofluorescence staining for (**b**) C/EBPα-Max and (**c**) Myc–Max using conjugated antibodies (Molecular Pobes) in U937 cells after RA treatment. (**d**) Immunoblot analysis showing expression of c-Myc, Max and C/EBPα under RA-induced and uninduced conditions from various fractions. Blots were stripped and reprobed with specific antibody. Upper panel: whole-cell lysates; NF: nuclear fraction; CF: cytoplasmic fraction.

that both proteins colocalize in these intranuclear structures (Figure 3a,panel 4; yellow signal).

### *C/EBPα–Max but not Myc–Max remains colocalized during granulocytic differentiation of myeloid U937 cells*

We next investigated the effect on C/EBPα–Max colocalization when the cells were triggered for granulocytic differentiation by RA for 24 h. We observed intranuclear staining with C/EBPα and Max antibodies, and the overlay of the two images shows that both proteins remain colocalized even after RA treatment of the cells (Figure 3b, panel 4; yellow signal). As Max is associated with Myc, we also analyzed their localization in U937 cells. We observed that endogenous Myc–Max colocalize in the nucleus under uninduced condition (Figure 3a, panels 5 and 6). On the other hand, no intranuclear c-Myc signal could be detected after RA treatment (Figure 3c, panel 4; only green signal from Max). We next investigated the expression of c-Myc, Max and C/EBPα before and after RA treatment from various fractions (whole-cell lysates, nuclear fraction (NF) and cytoplasmic fraction (CF)) by Western blotting, using specific antibodies (Figure 3d). Our results revealed that the c-Myc protein level was drastically decreased in all the three fractions (Figure 3d, upper and lower panels) by RA. However, C/EBPα was undetectable in the CF and slightly increased in the NF by RA when analyzed by immunoblotting. Dot blot analysis revealed the presence of CEBPα in the CF as well. This indicates that the concentration of C/EBPα in the CF is quite low, so as not to be detected by immunoblotting (data not shown). Max, on the other hand, was relatively unchanged under induced and uninduced conditions. These data demonstrate that retention/colocalization of C/EBPα–Max, and not Myc–Max heterocomplexes, in the nucleus might be important events during granulocytic differentiation of U937 cells.

### *Max enhances the ability of C/EBPα to transactivate a minimal thymidine kinase promoter*

To investigate the functional importance of C/EBPα–Max interaction and their colocalization, we performed transient transfection assays in the fibroblast 293T and the myeloid U937 cells using a minimal TK promoter containing two CCAAT binding sites cloned upstream of the luciferase reporter gene. Transfection of a Max expression construct significantly en-

hanced the ability of C/EBPα to transactivate a minimal TK promoter containing two CCAAT binding site in a dose-dependent manner (Figure 4a). In control experiments, no effect of Max on C/EBPα activity was observed when promoter with no CCAAT binding sites was used, whereas C/EBPα alone was able to transactivate the minimal promoter construct ninefold. Similar results were obtained with myeloid U937 cells (Figure 4b). Interestingly, co-transfection studies with the human ~2200 bp C/EBPa promoter (which has intact E-box site and no CCAAT site) revealed that C/EBPα alone was unable to transactivate the promoter, whereas, co-transfection of Max led to a significant increase in the promoter activity (Figure 4c). It is important to point out that Max itself does show some activation.

### *C/EBPα and Max associate* in vivo*: a Myc–Max–Mad link*

To further elucidate the mechanism by which Max augments the transcriptional activity of C/EBPα, we hypothesized that Max might associate with the hC/EBPα promoter *in vivo* because similar to C/EBPα, Max also possesses a DNA binding basic region. To test this possibility, we performed quantitative radioactive and non-radioactive chromatin immunoprecipitation (ChIP) in U937 cells (Figure 5). Chromatin was subjected to IP by using antibodies directed against C/EBPα, c-Myc and Max. The presence of C/EBPα promoter was detected by amplifying a promoter region using primers specific for a 280 bp region in the C/EBPα promoter that encompasses the CACGTG site (commonly referred to as E-box; Figure 5a). The E-box is conserved in the human and mouse C/EBPα promoter (Figure 5a). We observed that under normal physiological conditions (uninduced), endogenous c-Myc and Max appeared on C/EBPα promoter and there was undetectable endogenous C/EBPα occupancy on the hC/EBPα promoter (Figure 5b). IP using an isotype-matched IgG served as a negative control.

We next investigated the affect on heterocomplex formation at the hC/EBPα promoter upon differentiation by RA. We observed that both Max and C/EBPα appeared on C/EBPα

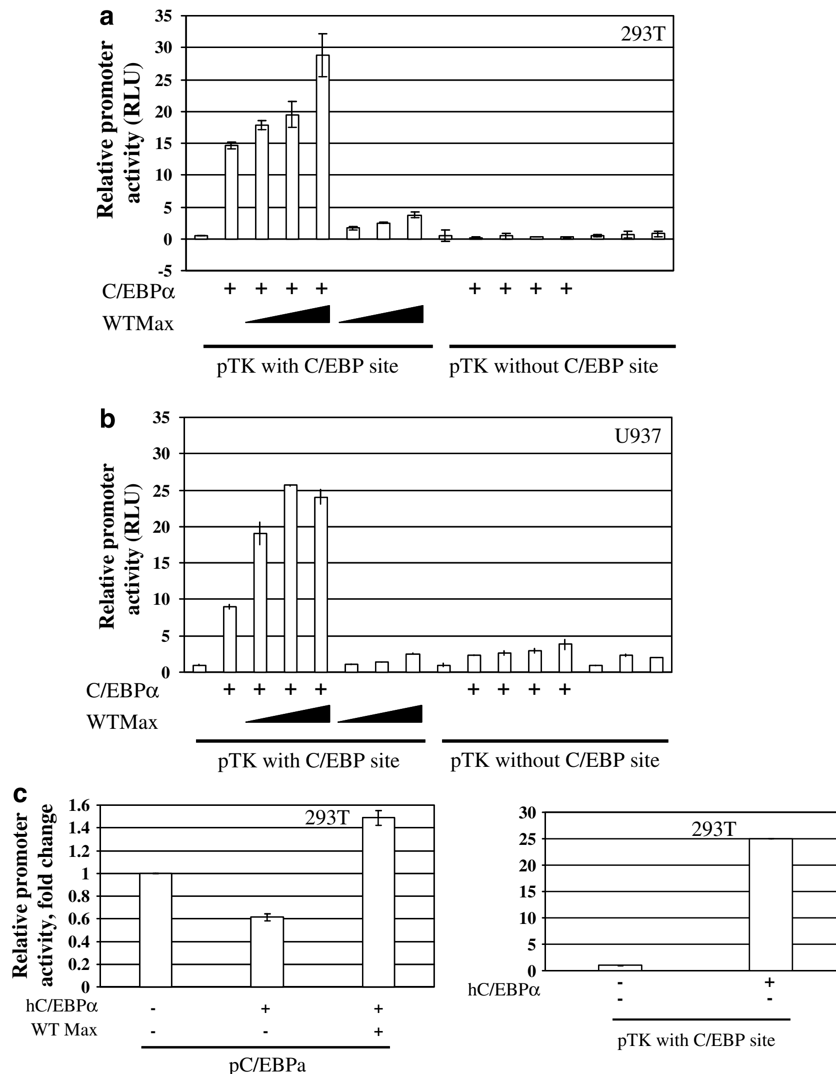



**Figure 4** Max enhances the transactivation capacity of C/EBPα in transient transfection assays. (**a, b**) Transient transfection in 293T and U937 cells with a reporter construct of a minimal TK promoter with CEBP binding sites only p(CEBP)2TK and expression plasmids for hC/EBPα and Max. pTK (without CEBP sites) was used as control. Luciferase activities were measured 24 h after transfection and the values normalized by using *Renilla* luciferase PRL0. (**c**) Transient transfection in 293T cells with a 2200 bp hC/EBPα promoter showing increased promoter activation when Max is co-expressed. Histogram on the right shows promoter activation by hC/EBPα on a minimal promoter, used as a positive control in this experiment.

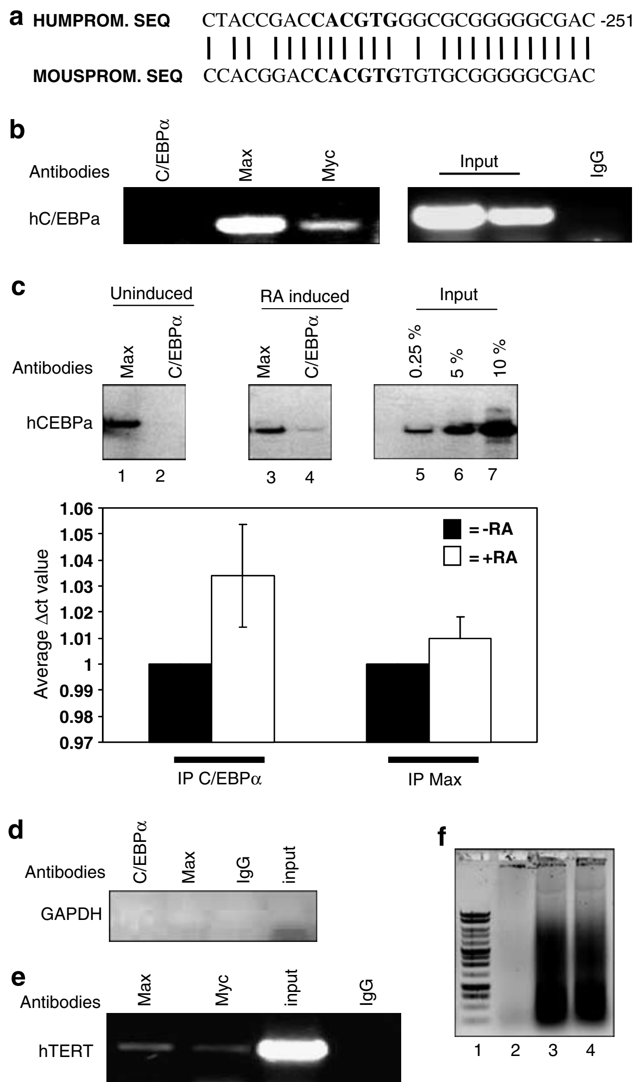



**Figure 5** Max is associated at the hC/EBPα promoter *in vivo* and Max–C/EBPα associate strongly during granulocytic differentiation. A ChIP assay was performed on logarithmically growing and RA-treated U937 cells, and the precipitated chromatin was PCR-amplified using specific primers. (**a**) Comparison of the human and mouse C/EBP promoters encompassing a consensus CACGT sequence, commonly referred to as E-box and known to be occupied by Myc + Max heterodimers. (**b**) *In vivo* occupancy by Myc and Max at the hC/EBPα promoter in logarithmically growing and (**c**) by Max-C/EBPα in RA-treated U937 cells. Input: Radioactive and Q-RT-PCR performed on total chromatin. The histograms beneath show the Q-RT-PCR average Δct values from two independent experiments normalized with the control sample. (**d, e**) ChIP assay using GAPDH promoter and human TERT promoter as controls with a non-radioactive RT-PCR. (**f**) Sheared DNA from U937 cells following 10 sonication pulses shows the optimal size range for IP (200–1000 bp). Lane: 1, unsheared; lanes: 2 and 3, sheared DNA.

promoter and in fact, more C/EBPα was associated with the promoter in the context of chromatin upon differentiation induction (Figure 5c, lane 4). The amount of Max bound to the promoter was fairly constant. DNA recovery was quantified as a percentage of the total input chromatin (lanes 5–7). Q-RT-PCR confirmed this observation and the histograms shown represent the average values from two independent experiments (Figure 5c, lower panel). A promoter without the CACGTG site, such as GAPDH promoter (Figure 5d), was used as a negative control for C/EBPα and Max occupancy and hTERT promoter (Figure 5e) as a positive control for Myc and Max interaction on the CACGTG site (E-box). The size of the DNA fragments before and after sonication is also shown (Figure 5f). Thus, C/EBPα and Max associate *in vivo* in the context of chromatin and are associated together more strongly on the hC/EBPα promoter when the cells are induced towards granulocytic differentiation.

### *Overexpression of Max and C/EBPα promotes differentiation along the granulocytic pathway in human hematopoietic CD34+ cells*

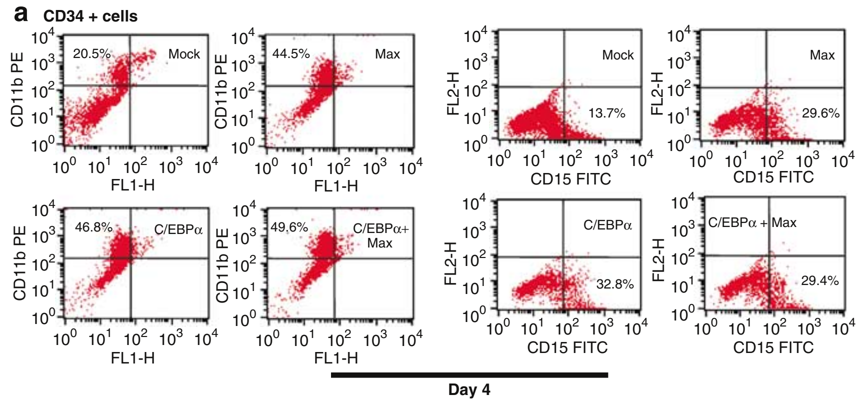
We next asked whether interaction of Max with C/EBPα is biologically important for C/EBPα functions. Hence, we performed overexpression studies using three different experimental systems: human hematopoietic CD34+ cells, estradiol-inducible K562-C/EBPα-ER cells and U937 cells. Our results revealed that overexpression of Max or C/EBPα alone in CD34+ cells leads to a significant increase in the proportion of CD11b+ (Figure 6a, dot plot 44 vs 20%) and CD15+ (Figure 6a, dot plot 29 vs 13%) cells compared with the mock-transfected control, respectively. The histograms represent the average values from three different experiments, and the viable cell count data (Trypan blue staining) under different conditions are also shown for days 1 and 4 (Figure 6b). Q-RT-PCR in these cells revealed increased GCSF receptor expression (Figure 6c). Similar results were observed with U937 and K562-C/EBPα-ER cells (Supplementary Figure S2a and data not shown). The morphology of the cells was observed to correlate with the surface marker expression (Supplementary Figure S2b). Q-RT-PCR in U937 cells for various granulocytic/ monocytic markers was also performed to complement the fluorescence-activated cell sorting results (Supplementary Figure S2c).

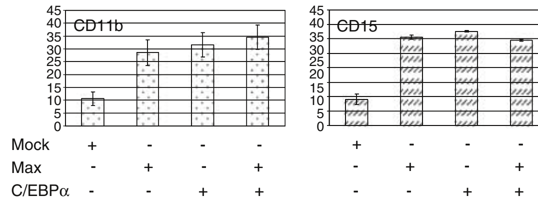
### *Stable silencing of Max by short hairpin RNA reduces the differentiation-inducing capacity of C/EBPα in human hematopoietic CD34+ cells*

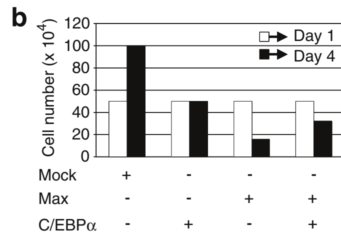
If Max is a biologically important co-activator of C/EBPα, silencing of Max should inhibit differentiation induction by C/EBPα. To address this, we performed RNA interference experiments in human hematopoietic CD34+ cells and myeloid U937 cells (Supplementary Figure S2d) by using short hairpin RNA (shRNA) against Max (cat. no. RHS1764-9690535; Open Biosystems, Heidelberg, Germany) and control shRNA (cat. no. RHS1707; Open Biosystems). Cells were transfected with expression plasmids for C/EBPα alone and/or co-expressed with shRNA against Max, control shRNA, and the cells cultured in media containing puromycine. After selection, the cells were analyzed for granulocytic differentiation, using CD15 expression as a marker. Our results revealed that C/EBPα alone induces granulocytic differentiation (CD15+) five- to six-fold as compared with the mock-transfected CD34+ (Figure 6d). Coexpression of Max shRNA led to a significant decrease in CD15+ population (about twofold), whereas control shRNA did not lead to any significant reduction in CD15+ population (Figure 6d, compare histograms). The reduction of Max protein level with shRNA was confirmed by Western blotting and Max shRNA did not affect the expression of C/EBPα (Figure 6e). In conclusion, we propose a model shown as Figure 6f. Thus, Max is important for C/EBPα-mediated effects on granulocytic differentiation and might have an important role in stem cell development.

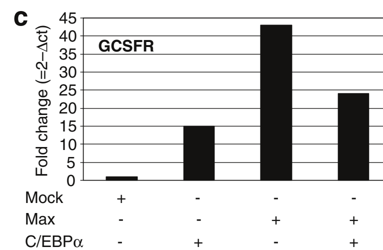
## Discussion

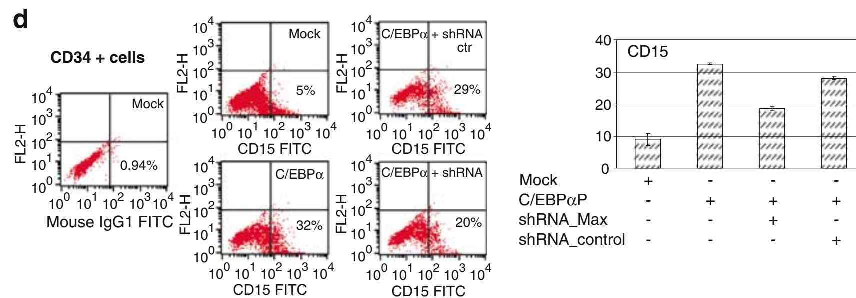
It has become increasingly clear that interaction of C/EBPα with other nuclear proteins plays an important role not only in lineage commitment and differentiation in the hematopoietic system but also in the pathogenesis of AML. Although the lineage commitment decision by C/EBPα was proposed by our laboratory to involve the functional inactivation of the myeloid master regulator PU.1 and/or its co-activator c-Jun through protein–protein interactions,[6,7] relatively little is known about how C/EBPα interacts with other nuclear proteins to activate gene transcription. The results presented in this article provide evidence that Max, a heterodimerization partner of Myc, is a novel, functionally and biologically important co-activator of

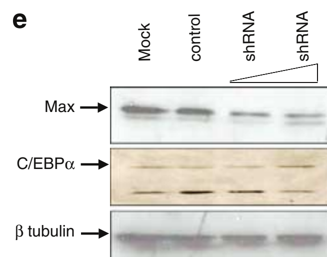


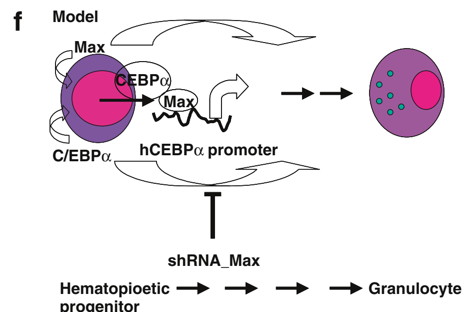



CEBPα. C/EBPα and Max not only colocalize but also the heterocomplex is preferentially formed on the hC/EBPα promoter during granulocytic differentiation, thereby contributing to increased transactivation and differentiation capacity of C/EBPα.

We used MS-based proteomic analysis as a means of identifying the interacting partners of C/EBPα, utilizing IP of C/EBPα from myeloid U937 cells as a model system. U937 cells are a good model system for studying myeloid differentiation in general, as they are bipotential and can be differentiated into granulocytic lineage by RA and in particular, with respect to the functions of C/EBPα, as a threefold level of C/EBPα protein (above the level of endogenous C/EBPα) in U937 cells is sufficient for their granulocytic differentiation.[26] In addition to nine other proteins (see Table), we identified Max, an essential heterodimerization partner of Myc,[16] as a novel interacting partner of C/EBPα in our screen (Figure 1). The discovery of Max as a novel C/EBPα partner is intriguing because of the role Max plays in switching of the complexes during myeloid differentiation.[24] Of particular importance is the fact that transgenic mice carrying an inserted transgene encoding Max have been shown to exhibit a 50- to 60-fold elevation of blood neutrophils.[27] Additionally, Max is an essential heterodimerization partner of Myc family members to regulate transcription[11] and c-Myc is an important target of C/EBPα.[26] We confirmed the *in vivo* interaction of C/EBPα with Max by IP technique and showed that the basic DNA-binding region of C/EBPα is involved in this interaction, as the mutant of CEBPα (C/EBPα BR3), which lacks DNA-binding region, could not be co-precipitated with Max (Figure 2). C/EBPα BR3 carries mutations in four amino acids, residues Arg297, Lys298, Arg300 and Lys302.[28] Of these, only Arg300 is expected to contact DNA. Neither the BR3 nor the Leu12Val variants bind DNA, suggesting that interaction with Max is likely via Arg297, Lys298 and/or Lys302. Arg297 is known to participate in the interaction between C/EBPα and E2F.[8] Further study is required to pin point the exact amino acid involved in the C/EBPα and Max interaction.

The endogenous C/EBPα and Max proteins are not distributed evenly throughout the nucleoplasm (Figure 3), but are localized in intranuclear structures within the nucleus. These structures represent, presumably, centromeres, which are chromosomal structures associated with intranuclear chromosome positioning and cell cycle regulation. Interestingly, C/EBPα is associated with cell cycle regulation.[29,30] In other cell systems, such as pituitary progenitor GHFT1-5 cells, C/EBPα has been shown to concentrate at chromatin surrounding the centromeres.[31] The observation that C/EBPα–Max but not Myc–Max remain colocalized during granulocytic differentiation (Figure 3) indicates that these intranuclear structures (centromeres) are selectively targeted by C/EBPα–Max during granulocytic differentiation. We observed the occupancy of the hC/EBPα promoter by Max *in vivo* under physiological conditions, and recruitment of more C/EBPα whereas Max is retained on the promoter during granulocytic differentiation. It is possible that the C/EBPα–Max heterocomplex regulates the balance of acetylated histones to modify chromatin structure at the hC/EBPα promoter and lead to transcriptional activation, as was shown by our results. In fact, TIP60, a histone acetyl transferase, was identified as an interacting partner of C/EBP to regulate histone acetylation at the hC/EBPα promoter α in an alternative approach (Bararia *et al.*, manuscript submitted for publication). To our knowledge, this is a first report showing occupancy of the hC/EBPα promoter by Max *in vivo*.

The occupancy by Max of the hC/EBPα promoter raises a possibility that Myc could also form a part of the complex under physiological conditions, as Max requires dimerization with Myc for efficient DNA binding. In fact, it was shown that purified Myc + Max heterodimers form stable complexes on the mouse C/EBPα promoter that includes the USF binding site.[21] The USF DNA recognition site CACGTG (which is the same as the E-box, occupied by Myc–Max) is found in both the human and the mouse C/EBPα promoter, and the USF binding site (for HLH-bZIP) is crucial for activation of the hC/EBPα promoter by C/EBPα.[32] Our colocalization and ChIP data (Figures 4 and 5) and the data that C/EBPα is co-precipitated with Myc IP (unpublished observation) support this Myc–Max link. Thus, it is tempting to speculate that C/EBPα exists in association with the Myc–Max–Mad network to regulate differentiation under cellular settings. Given that the C/EBPα–Max heterocomplex is formed on hC/EBPα promoter, specifically during granulocytic differentiation, this would mean that the balance between such complexes, under the influence of growth and differentiation signals, could be an important part of a molecular switch that is regulating genes important for growth and differentiation.

By using overexpression studies, we have demonstrated that enforced expression of C/EBPα and Max in human hematopoietic CD34 + cells induces granulocytic differentiation. The role of C/EBPα in the transition from CMPs to GMPs in myeloid progenitors has been recently characterized.[3] The role of Max in inducing granulocytic differentiation indicates that Max can activate myeloid differentiation program either independent of C/EBPα or in association with it. *In vivo* interaction and retention of C/EBPα–Max heterocomplex in myeloid cells (Figures 2, 4 and 5) and inhibition of differentiation-inducing capacity of C/EBPα by stable silencing of Max using shRNA against MAX in CD34 + cells (Figure 6) suggest CEBPα–Max association likely plays an important role in this process of myeloid progenitor differentiation. A very recent data from Alan Friedman's group has shown the role of C/EBPα in monopoiesis.[33] This means that the commitment decisions do not necessarily depend upon a single transcription factor but, in fact, on a number of cooperating factors.

In summary, we conclude that Max is a biologically and functionally important and relevant interacting partner of

**Figure 6** Overexpression of Max induces differentiation along granulocytic pathway in human hematopoietic CD34 + cells. (**a**) The expression plasmids for human C/EBPα and Max were transfected into human hematopoietic CD34 + cells by using AMAXA technology. The surface expression of CD11b and CD15 was analyzed by flow cytometry at day 4. The histograms underneath represent data from three different experiments. (**b**) Trypan blue staining, showing the number of viable cells under different conditions. (**c**) Q-RT-PCR for GCSF receptor expression under the conditions shown from two experiments. (**d**) Stable silencing of Max by shRNA inhibits C/EBPα-induced differentiation in human hematopoietic CD34 + cells. The expression plasmid for human C/EBPα and/or expression Arrest shRNA_Max plasmid (Open Biosystems) were transfected into human hematopoietic CD34 + cells or U937 cells by using the AMAXA technology. After their selection in puromycine, the cells were analyzed for the surface expression of CD15 by flow cytometry and the data shown as dot plot with percentage of positive cells representative of one experiment. shRNA control was also used in all the experiments and is shown. The histograms represent the data from three different experiments. (**e**) A Western blot for Max using anti-Max antibody showing silencing of Max at the protein level by shRNA_MAX. The blot was stripped and reprobed with C/EBPα antibody. (**f**) Model, a summary of our data showing the importance of Max as a co-activator of C/EBPα in the differentiation of myeloid progenitors. Enforced expression of Max and CEBPα induces differentiation along the granulocytic pathway, and stable silencing of Max inhibits CEBPα-induced differentiation.

C/EBPα and has important co-activator functions for C/EBPα-induced granulocytic differentiation in myeloid progenitors.

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)