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Max as a novel co-activator of myeloid transcription factor C/EBPα and the critical role of PIN1 in Acute Myeloid Leukemia with C/EBPα mutation

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Submitted by

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Dedicated To My Beloved Father Antony Pulikkan and

Mother Treesa Antony

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Abbreviations:

AML	Acute Myeloid Leukemia
ALL	Acute Lymphoid Leukemia
APL	Acute Promyelocytic Leukemia
BR-LZ	Basic Region-Leucine Zipper
CLP	Common Lymphoid Progenitor
C/EBP	CCAAT Enhancer Binding Protein
CHCA	α-Cyano-4-Hydroxy Cinnamic Acid
CML	Chronic Myeloid Leukemia
DAPI	4, 6-Diamino-2-Phenylindole Dihydrochloride
DTE	Dithioerythritol
DHB	2,5-Dihydroxy-Benzoicacid
DMEM	Dulbecco´s Modified Eagle Medium
DMSO	Dimethylsulfoxide
FAB	French American British Classification
FBS	Foetal Bovine Serum
FACS	Fluorescence Activated Cell Sorting
GMP	Granulocyte/Macrophage Progenitor
GCSFR	Granulocyte Colony Stimulating Factor Receptor
HSC	Hematopoietic Stem Cell
IB	Immunoblot
IEF	Isoelectric Focussing
IP	Immunoprecipitation
Ivt	In-vitro Translated
LC	Liquid Chromatogrphy
MALDI	Matrix Assisted Laser Desorption Ionisation
MEP	Megakaryocyte/Erythroid Progenitor
MS	Mass Spectrometry
NK	Normal Karyotype
Nbm	Normal Bone Marrow
PBS	Phosphate Buffered Saline
PMF	Peptide Mass Fingerprinting
pI	Isoelectric Point
PIN1	Peptidyl-prolyl cis/trans isomerase
RA	Retinoic Acid
SDS	Sodium Dodecyl Sulphate
TE	Transactivation Element
TAD	Transactivation Domain
TOF	Time of Flight
USF	Upstream Stimulatory Factor

1. Introduction

1.1 Hematopoiesis

Hematopoiesis is the process by which mature blood cells of different lineages develop from pluripotent hematopoietic stem cells (HSCs) through a highly organized hierarchy of successive differentiation events (Figure 1). Hematopoiesis takes place in several distinct anatomical sites during mouse embryogenesis. Primitive blood cells are first identifiable in the blood islands of the embryonic yolk sac at embryonic day 7.5 of gestation. The aorta gonad mesonephros (AGM) and fetal liver represent the principal intraembryonic fetal hematopoietic organs (Houssaint, 1981). From birth and throughout adult life, all mature blood cells are produced in the bone marrow.

Hematopoietic stem cells (HSCs) constitute 0.05% of mouse bone marrow cells, and can be divided into three distinct populations: long-term self-renewing HSCs, short-term self-renewing HSCs, and multipotent progenitors without detectable self-renewable potential (Reya et al., 2001). The multipotent progenitors give rise to common lymphoid progenitors (CLPs; the precursors for lymphoid cells) and common myeloid progenitors (CMPs). The CMPs give rise to granulocyte/macrophage progenitors (GMPs) and megakaryocyte/erythroid progenitors (MEPs). The GMPs develop into granulocytes, monocytes and dendritic cells while MEPs develop into platelets and erythrocytes (Figure 1).

1.2 Acute Myeloid Leukemia

The main focus in cancer research has been identification of oncogenes as well as tumor suppressors and identification of the pathways coordinated by oncogenes and tumor suppressors. It is proposed that a block in the differentiation programme from precursor to mature cells can cause cancer. Recent evidence suggests that disruption of myeloid transcription factors as being important step in acute myeloid leukemia.

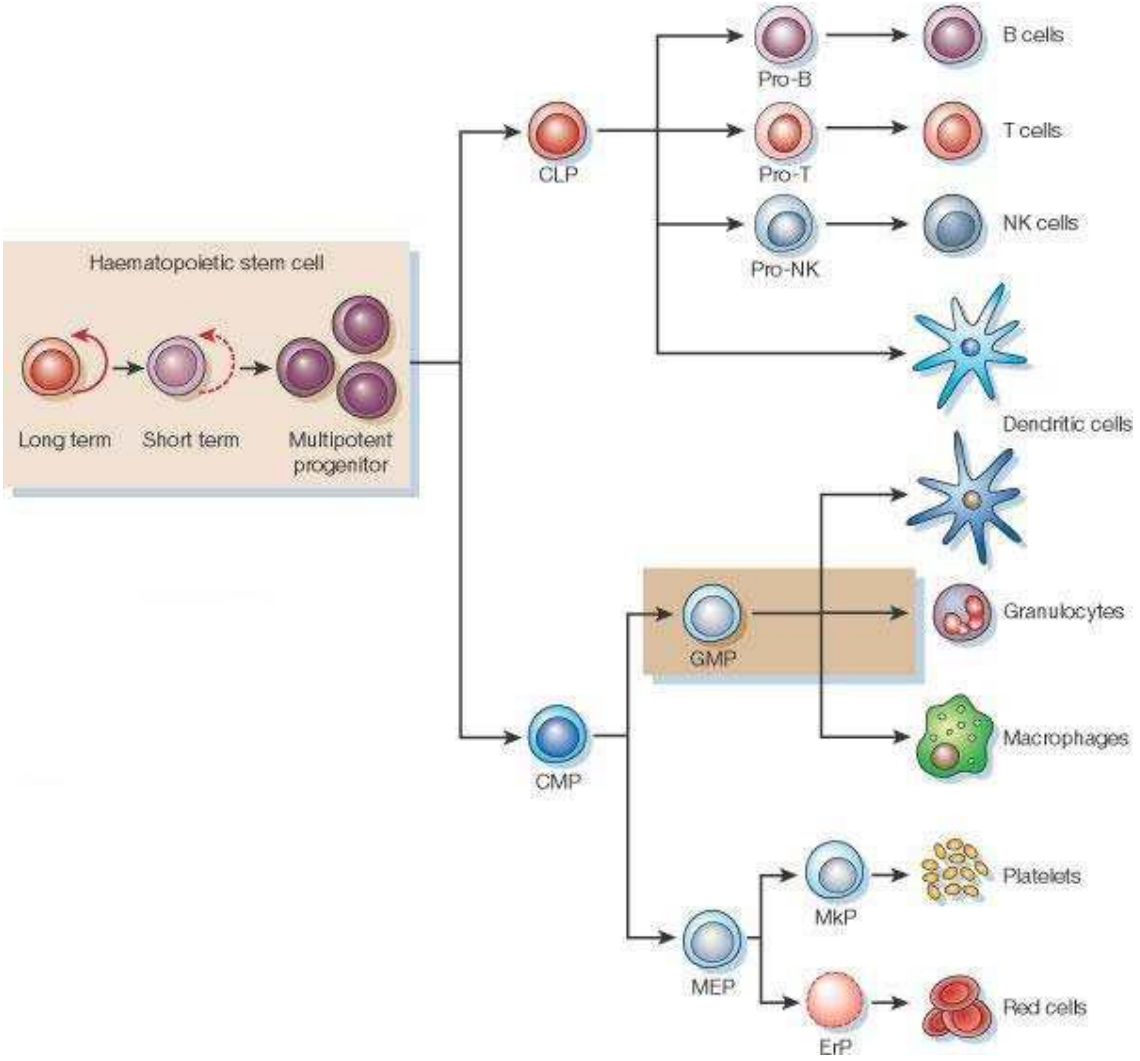


Figure 1. Hematopoietic lineage diversification: Development of hematopoietic stem cell to various lineages (Reya et al., 2001)

Leukemia is a common term used for a group of hematological malignancies characterized by accumulation of hematopoietic precursor cells, which fail to undergo terminal differentiation (Tenen, 2003). Leukemia is classified as acute and chronic based on how quickly it progresses. Acute leukemia is fast growing and can overrun the body within a few weeks or months, while chronic leukemia is slow growing and progresses in years. According to the type of lineage which is affected, leukemia is classified as myeloid and lymphoid. The four major types of leukemia are:

- Acute Myeloid Leukemia (AML)
- Chronic Myeloid Leukemia (CML)
- Acute Lymphocytic Leukemia (ALL)
- Chronic Lymphocytic Leukemia (CLL)

According to French-American-British (FAB) classification, AML is divided into 9 subtypes based on the morphological appearance of the blasts and their reactivity with biochemical stains (Bennett et al., 1976; Lowenberg et al., 1999). The subtypes of the FAB classification of AML are represented in Table 1.

Table 1. French-American-British (FAB) classification of AML

FAB subtype	**Description**	**Associated translocations and rearrangements**
M0	Acute myeloblastic leukemia with minimal differentiation	inv(3q26), t(3;3)
M1	Acute myeloblastic leukemia without maturation	
M2	Acute myeloblastic leukemia with maturation	t(8;21), t(6;9)
M3	Acute promyelocytic leukemia	t(15;17), t(11;17), t(5;17)
M4	Acute myelomonocytic leukemia	11q23, inv(3q26), t(3;3), t(6;9)
M4E0	Acute myelomonocytic leukemia with abnormal eosinophils	inv(16), t(16;16)
M5	Acute monocytic leukemia	11q23, t(8;16)
M6	Erythroleukemia	
M7	Acute megakaryocytic leukemia	t(1;22)

Adapted from Lowenberg B et al, New England Journal of Medicine 1999.

Recent findings show that cell cycle control and the regulation of differentiation programme are two closely related events which can have crucial role in tumorigenesis. More recently, the concept of the existence of a leukemic stem cell (LSCs) has been proposed (Huntly and Gilliland, 2005). The formation of LSCs, which exhibit little differentiation capacity but fail to differentiate into mature cells, is thought to be a result of somatic mutations in HSCs or in committed progenitors. The mutations vary from chromosomal translocations leading to fusion proteins (eg. AML1/ETO, PML/RARα) to point mutations in critical transcription factors (Rosenbauer et al., 2005); (Passegue et al., 2003; Zhang and Rosen, 2006). Figure 2 compares the cellular hierarchy of normal and malignant hematopoiesis (leukemia).

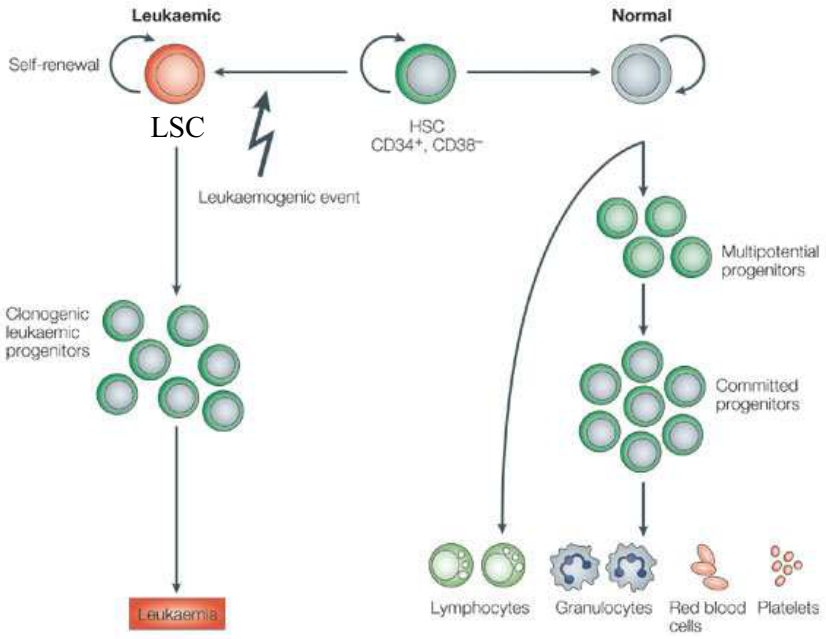


Figure 2. Cellular hierarchy of normal and malignant hematopoiesis (Huntly BJ and Gilliland DG, Nature Review Cancer, 2005)

1.3 Transcription Factors in Hematopoiesis

Transcription factors are specialized nuclear proteins that can bind specifically to their DNA binding site and activate transcription. A major factor which

determines the cell fate in hematopoiesis is the interplay between tissue specific transcription factors, which in turn, modulate a specific set of genes necessary for differentiation to a specific lineage (Cantor and Orkin, 2001; Tenen et al., 1997); (Lutterbach and Hiebert, 2000). Transcription factors play an important role in regulating major steps of hematopoiesis, such as differentiation, proliferation and survival. Several of those transcription factors have narrow expression patterns in that they are limited to a few hematopoietic lineages.

The major transcription factors involved in granulopoiesis are runt-related transcription factors (RUNX1; also known as AML1) (Okuda et al., 1996), stem-cell leukemia factor (SCL, also known as Tal-1) (Shivdasani et al., 1995), PU.1 (Klemsz et al., 1990), CCAAT/enhancer-binding proteins (in particular C/EBPα, C/EBPβ, C/EBPε) (Tanaka et al., 1995; Yamanaka et al., 1997; Zhang et al., 1997), interferon-regulatory factor 8 (IRF8) (Holtschke et al., 1996) and growth-factor independent 1 (GFI1) (Hock et al., 2003). The major transcription factors in granulopoiesis and the critical steps they regulate are depicted in figure 3.

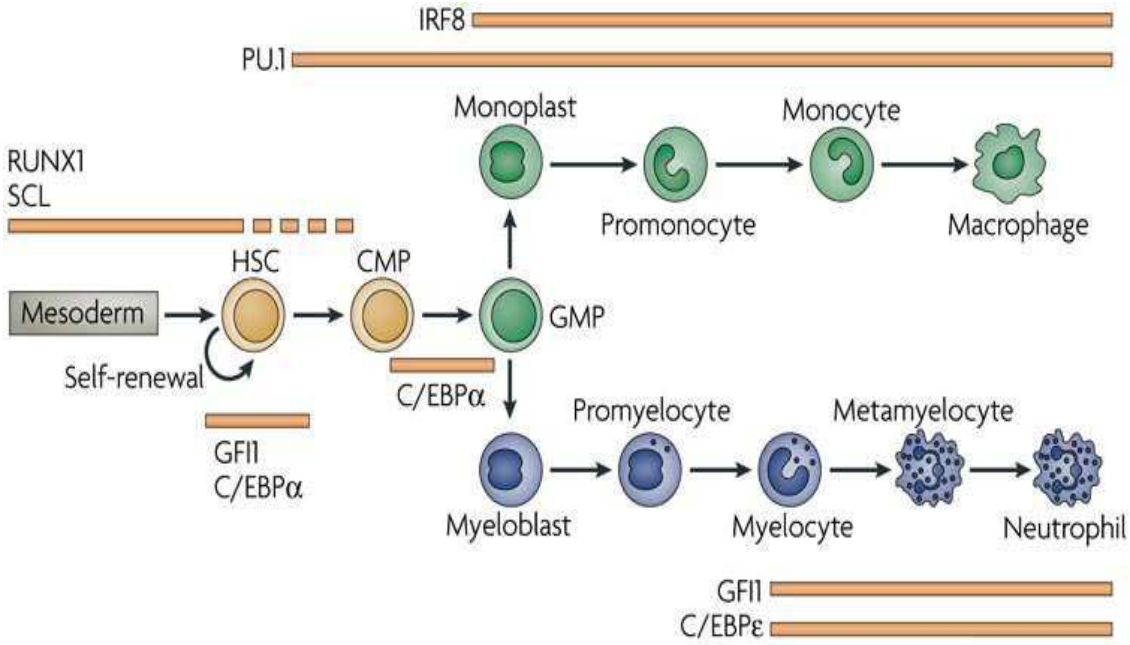


Figure 3. Role of transcription factors in granulopoiesis (Rosenbauer F and Tenen DG, Nature Reviews Immunology, 2007)

Transcription factors such as RUNX1 and SCL are the critical factors that orchestrate formation of hematopoietic stem cells from mesoderm during embryogenesis. Mice that are deficient for either RUNX1 or SCL die during embryogenesis and lack hematopoiesis (Okuda et al., 1996; Shivdasani et al., 1995). The function of PU.1 in granulopoiesis is the development of CMPs from HSCs (Dakic et al., 2005; Iwasaki et al., 2005; Scott et al., 1997). Mice that lack PU.1 show complete absence of macrophages (McKercher et al., 1996; Scott et al., 1997). Studies show that high PU.1 levels support the production of macrophages, while low PU.1 level support granulocyte development (Dahl et al., 2003; Rosenbauer et al., 2004).

The role of C/EBPα in granulopoiesis was underlined by the finding that nonconditional targeted disruption of C/EBPα results in a selective early block in granulocytic maturation, without affecting other hematopoietic lineages (Zhang et al., 1997). C/EBPα conditional knock-out mice show a selective block in the transition from the CMP to GMP stage of granulopoiesis and an increase in HSC self renewal (Zhang et al., 2004). This study points out that C/EBPα is necessary for CMP to GMP transition as well as in regulating the self renewal of HSC compartment of bone marrow during granulopoiesis. The concept of C/EBPα as granulocyte specific transcription factor is questioned by the finding that fetal liver from C/EBPα$^{-/-}$ mice also lack mature macrophages and macrophage progenitors, suggesting that C/EBPα can have crucial role in the development of macrophages (Heath et al., 2004).

The role of IRF8 (interferon-regulatory factor 8) in granulopoiesis is underlined by the finding that IRF8 knock-out mice had a reduced number of macrophages and increased number of granulocytes, suggesting that IRF8 favors macrophage development (Holtschke et al., 1996). After GMP development, transcription factors necessary for granulopoiesis are GFI1 and C/EBPε. Mice that lack GFI1 as well as C/EBPε exhibit abnormal granulopoiesis beyond the promyelocyte stage suggesting the importance of these factors during the final

stages of granulopoiesis (Yamanaka et al., 1997); (Hock et al., 2003). GFI1 has been shown to repress monocyte specific genes (Hock et al., 2003). The function of C/EBPε in the final stage of granulopoiesis is the regulation of genes necessary for the development of secondary and tertiary granule proteins such as lactoferrin and gelatinase (Yamanaka et al., 1997).

1.4 The C/EBP family

The CCAAT/enhancer-binding proteins (C/EBPs) are a family of transcription factors that include six members C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε and C/EBPζ (Ramji and Foka, 2002); (Akira et al., 1990); (Cao et al., 1991); (Roman et al., 1990). Except for C/EBPζ, which lacks the basic region, each protein contains similar basic region and leucine zipper sequences at its C-terminus, which mediate DNA binding and dimerization, respectively. The C/EBP proteins form leucine zipper mediated homodimers as well as heterodimers with other C/EBP members (Ramji and Foka, 2002); (Akira et al., 1990); (Cao et al., 1991); (Roman et al., 1990); (Ryden and Beemon, 1989). The dimer resembles an inverted Y shaped structure in which each arm of the Y is made of the basic region, which binds to palindromic DNA sequence in the DNA major groove. The predicted structure of a C/EBP bZIP dimer bound to its cognate DNA site is depicted in figure 4 (Johnson, 2005; Miller et al., 2003).

The N-terminal portion of each protein contains effector domains that mediate transcriptional activation, repression and autoregulatory functions. The expression pattern of each C/EBP varies in different tissues pointing out to the fact that each member could have specific roles in each cell type. In hematopoiesis, the C/EBP members shown to have specific function are C/EBPα, C/EBPβ and C/EBPε. C/EBPα is necessary for early granulocytic differentiation i.e., from hematopoietic stem cell to promyelocyte and C/EBPε is necessary for terminal granulocyte differentiation i.e., from promyelocyte to mature neutrophil (Theilgaard-Monch et al., 2005). C/EBPβ is necessary for

granulopoiesis in emergency conditions such as fungal infection or cytokine stimulation (Hirai et al., 2006).

1.5 C/EBPα

CCAAT/enhancer-binding protein alpha (C/EBPα) is the first leucine- zipper (bZIP) group of transcription factors discovered (Landschulz et al., 1988). C/EBPα was identified originally as a heat stable protein present in rat liver nuclei and having sequence specific DNA binding activity (Graves et al., 1986; Johnson et al., 1987). The DNA sequences to which C/EBPα binds are the "CCAAT homology" common to many promoters of genes that encode mRNA, and the "enhancer core homology" common to many viral enhancers. These findings point out the role of C/EBPα as a transcriptional regulatory protein (Johnson et al., 1987).

C/EBPα is highly expressed in a variety of tissues including liver, lung, placenta, adipose tissue, intestine, mammary gland, skin and peripheral blood mononuclear cells (Birkenmeier et al., 1989); (Antonson and Xanthopoulos, 1995). Human C/EBPα is encoded by an intronless gene and is located at chromosome band 19q13.1 (Hendricks-Taylor et al., 1992).

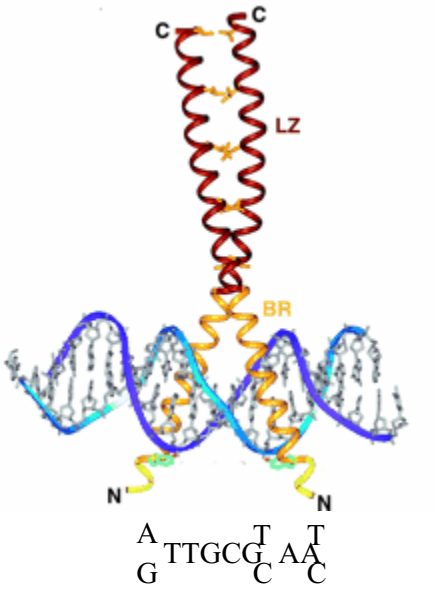


Figure 4. The predicted structure of a C/EBP bZIP dimer bound to its cognate DNA site

1.5.1. Domains of C/EBPα

C/EBPα contains a transactivation domain, a DNA binding basic region and a leucine zipper dimerization domain. The leucine zipper is a heptad of leucine repeats that intercalate with repeats of the dimer partner, forming a coiled coil of alpha-helices in parallel orientation (Agre et al., 1989; Landschulz et al., 1988; Vinson et al., 1993). The basic region, which contacts the DNA, is a stretch of approximately 20 amino acids, upstream of the leucine zipper (Johnson, 1993). The N-terminal domain which is responsible for transcriptional activation and/or repression consists of three transactivation elements - TE-I, TE-II and TE-III.

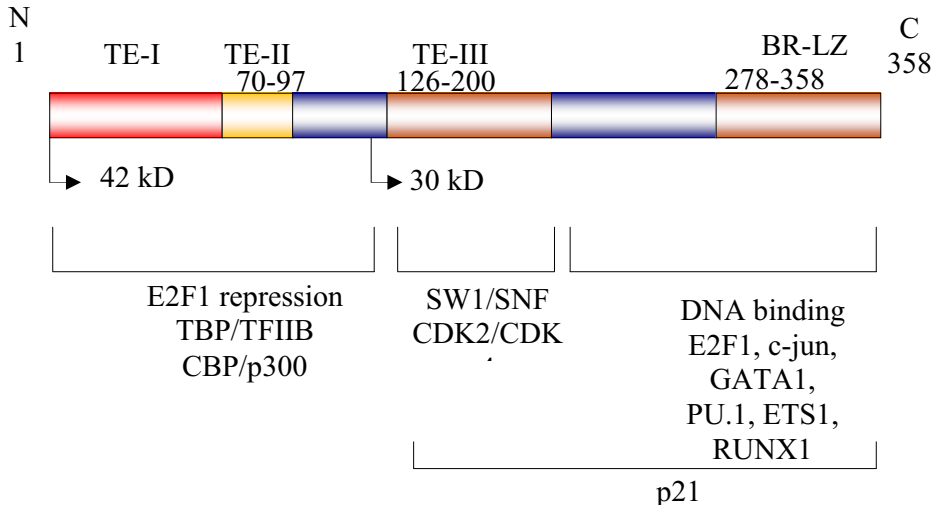


Figure 5. Different domains of C/EBPα and proteins regulated by C/EBPα (Claus Nerlov, Nature Reviews Cancer, 2004)

The C/EBPα mRNA gives rise to two different translational products by using two different AUGs within the same open reading frame (Lin F et al, 1993, Ossipow V et al, 1993): a full length 42 kD protein (p42) and a truncated 30 kD version (p30), which in contrast to p42 lacks transactivation elements TE-I and TE-II.

1.5.2 Distribution of C/EBPα functions

C/EBPα directly activates transcription from lineage-specific promoters. C/EBPα has been shown to regulate a number of genes mainly in

granulopoiesis, adipogenesis, and in tissues such as lung, liver, skin and mammary epithelial cells (Ramji and Foka, 2002). Beyond acting as a classical transcription factor, C/EBPα has been shown to interact with and/or regulate a number of proteins. The transactivation elements TE-I and TE-II of C/EBPα have been found to interact with the basal transcriptional apparatus, TBP/TFIIB (Nerlov and Ziff, 1995). It has been found that these elements are necessary for E2F repression during granulopoiesis and adipogenesis (Porse et al., 2001) as well as for interaction with histone acetyl transferases, CBP/p300 (Kovacs et al., 2003); (Schwartz et al., 2003). The transactivation element TE-III is responsible for recruitment of chromatin-remodelling complexes, SW1/SNF (Pedersen et al., 2001). It is also found that TE-III is responsible for binding and inhibition of CDK2/4 (Wang et al., 2001).

The bZip domain is shown to interact with E2F (Johansen et al., 2001) and c-Jun (Rangatia et al., 2002) as well as PU.1, ETS1, GATA1, RUNX1 (McNagny et al., 1998; Reddy et al., 2002); (Zhang et al., 1996a); (Yamaguchi et al., 1999).

1.5.3 C/EBPα –mechanisms of action

One of the most important functions of C/EBPα is its role in cell cycle. C/EBPα induces cell cycle arrest in a variety of tissues (Johnson, 2005; McKnight, 2001). There are different mechanisms proposed for the C/EBPα mediated cell cycle arrest. The initial studies investigating the C/EBPα mediated cell cycle arrest revealed that C/EBPα interacts with the cyclin dependent kinase (CDK) inhibitor p21 in developing liver by protein-protein interaction (Timchenko et al., 1996). It is observed that p21 levels increase 20 fold during C/EBPα induction and p21 binds to and inhibits the kinase activity of CDK4, CDK6 and CDK2. Another mechanism proposed for C/EBPα mediated cell cycle arrest is its interaction with cyclin dependent kinase CDK2 and CDK4 (Wang et al., 2001).

The mechanism that has gained the most acceptance is the C/EBPα mediated repression of the E2F transcription factors. The E2F group of transcription factors regulates genes required for cell cycle progression. Experiments in murine fibroblast cell lines showed that C/EBPα inhibits proliferation of fibroblast cells and that C/EBPα is present in a complex that binds to E2F sites in genes such as dihydrofolate reductase (DHFR) and E2F-1 that are upregulated during the G1-S transition (Slomiany et al., 2000). C/EBPα also represses transcription from reporter constructs containing the DHFR or E2F1 promoters. Porse et al provided further proof for the E2F repression model, deciphering domains of C/EBPα required for repression of E2F driven transcription (Porse et al., 2001). They showed that transactivation element (TE-I) at the N-terminus as well as residues residing on the non-DNA binding face of C/EBPα basic region as the critical domains for the cell cycle inhibitory effect of C/EBPα. Later studies showed that the bZip domain of C/EBPα is able to interact with E2F (Johansen et al., 2001).

All the above studies show that the mechanism by which C/EBPα inhibits cell proliferation seems to differ from cell type to cell type. Findings from myeloid systems support the concept that C/EBPα mediated E2F repression is the major pathway that mediates cell cycle exit and differentiation in granulopoiesis (Porse et al., 2001); (D'Alo et al., 2003); (Porse et al., 2005); (Rosenbauer and Tenen, 2007).

1.5.4 C/EBPα in normal hematopoiesis.

Granulocytes play a major role in host defense, and patients with granulocyte deficiency are extremely vulnerable to bacterial infection. Unlike long-lived lymphocytes, granulocytes have a short lifespan, necessitating a tight relationship between supply and demand. After production and release by the bone marrow, the life span of neutrophils is only 8 hours. Mature neutrophils are

unable to undergo cell division. The factors that play critical role in granulopoiesis have been a major research focus in hematology.

Studies in the early 1990s focused on the role C/EBPα in the differentiation programmes in tissues such as adipocyte, liver, lung etc. Later, the role of C/EBPα in granulopoiesis was elucidated by the finding that a large number of myeloid genes contain C/EBP binding sites in their promoters (Tenen et al., 1997). The role of C/EBPα in granulopoiesis was underlined by the finding that targeted disruption of C/EBPα results in a selective early block in granulocytic maturation, without affecting other hematopoietic lineages (Zhang et al., 1997). Even though C/EBPα is expressed at low levels in hematopoietic stem cell (HSC), it is found that C/EBPα is specifically upregulated during granulocytic differentiation. The relative levels of C/EBPα during different stages of granulopoiesis are depicted in figure 6 (Akashi et al., 2000; Bjerregaard et al., 2003).

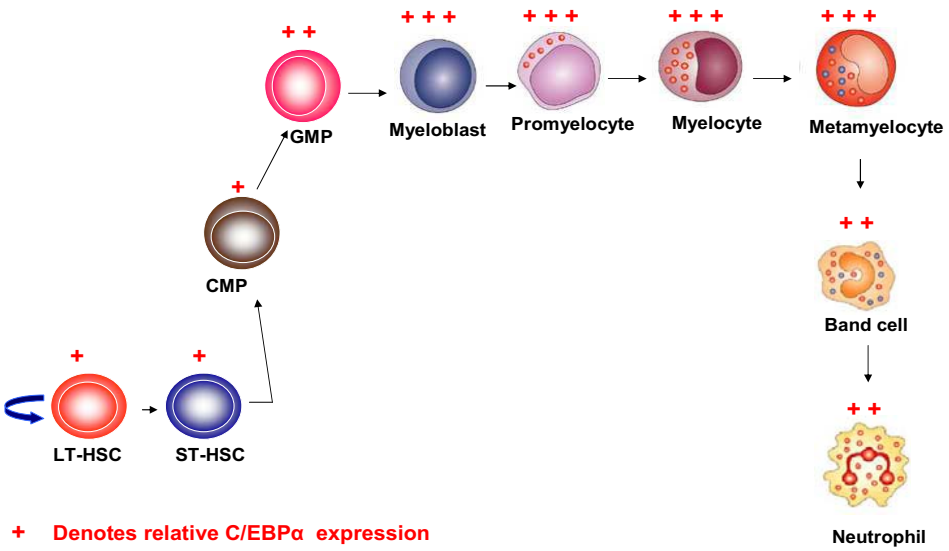


Figure 6. Relative levels of C/EBPα in different stages of granulopoiesis

Radomska et al., have shown that conditional expression of C/EBPα alone is sufficient to trigger neutrophilic differentiation (Radomska et al., 1998). Primary

$CD34^+$ cells isolated from human bone marrow differentiate into granulocytes when transduced with a retroviral vector expressing C/EBPα (Iwama et al., 2002). C/EBPα conditional knock-out mice show a selective block in the transition from the CMP to the GMP stage of granulopoiesis (Zhang et al., 2004). Loss of C/EBPα leads to an increase in HSC self renewal compared to that of wild type HSC.

In the hematopoietic system, a number of C/EBPα target genes have been found, including a number of primary granule protein genes (Oelgeschlager et al., 1996); (Iwama et al., 1998). C/EBPα was also described to regulate the genes encoding the receptors for the granulocytic growth factors- granulocyte colony-stimulating factor (G-CSF) and interleukin 6 (IL-6) (Smith et al., 1996; Zhang et al., 1997; Zhang et al., 1998). However, knock-out studies of these factors suggested that these were not the critical target genes, since disruption of one or more growth factors failed to show the complete granulocyte differentiation block observed in C/EBPα knock-out mice (Liu et al., 1996); (Liu et al., 1997). Recently it has been shown that C/EBPα regulates microRNA-223 during granulopoiesis (Fazi et al., 2005). Induction of C/EBPα in myeloid differentiation models results in the displacement of NFI-A from the miR-223 promoter by C/EBPα and concomitant upregulation of miR-223 and granulopoiesis. It was shown that downregulation of c-Jun expression by C/EBPα is important for granulocytic lineage commitment (Rangatia et al., 2002).

Recent studies suggest that inhibition of E2F pathways by C/EBPα is the most critical step in granulopoiesis (D'Alo et al., 2003; Porse et al., 2005; Porse et al., 2001; Rosenbauer and Tenen, 2007). Transactivation element-I (TE-I) at the N-terminus as well as residues residing on the non-DNA binding face of C/EBPα basic region are the critical domains for the E2F repression by C/EBPα (Porse et al., 2001). Mutation of either results in loss of C/EBPα inhibition of E2F, which results in block of granulocytic differentiation (D'Alo F et al, 2003). Another

study showed that inhibition of E2F leads to downregulation of E2F target gene c-Myc (Johansen et al., 2001). Knock-in mice with a targeted mutation in the C/EBPα-E2F interaction domain (the BRM2 mutation) displayed a block in granulocytic differentiation and expansion of myeloid progenitor population (Porse et al., 2005). All these studies suggest that C/EBPα mediated repression of E2F pathways is a major event in granulopoiesis.

1.6 C/EBPα and leukemia

Recent studies show that C/EBPα function is impaired by various mechanisms in leukemia. The first report of deregulation of C/EBPα was shown by AML1/ETO, the product of the t(8;21) translocation (Westendorf et al., 1998). C/EBPα mRNA was found suppressed by AML1/ETO fusion protein both *in vitro* and *in vivo* (Pabst et al., 2001a). AML-M2 patients with t(8;21) show up to six fold less C/EBPα mRNA than AML-M2 patients with normal karyotype (Pabst et al., 2001a). BCR-ABL, the product of the t(9;22) translocation in chronic myeloid leukemia (commonly known as Philadelphia chromosome) has been found to inhibit C/EBPα translation by interaction of the poly(rC)-binding protein hnRNP E2 with CEBPA mRNA (Perrotti et al., 2002).

Another fusion protein that was found to downregulate C/EBPα in AML was AML1-MDS-EVI1 (AME), the product of the t(3;21) translocation (Helbling et al., 2004). AME was shown to induce expression of the RNA binding protein, calreticulin. Calreticulin interacts with GCN repeats within the C/EBPα mRNA and inhibits the translation of C/EBPα protein (Helbling et al., 2004). Similarly, CBFB-SMMHC fusion protein found in AML patients with inv(16) has been shown to upregulate calreticulin expression and inhibit C/EBPα (Helbling et al., 2005).

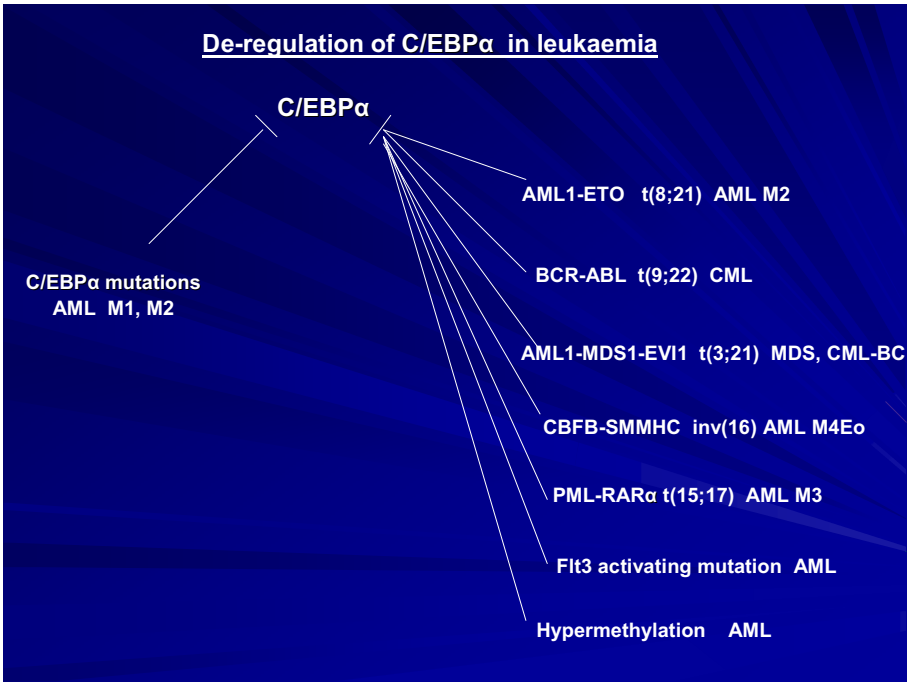


Figure 7. Deregulation of C/EBPα in leukemia: C/EBPα as tumor suppressor

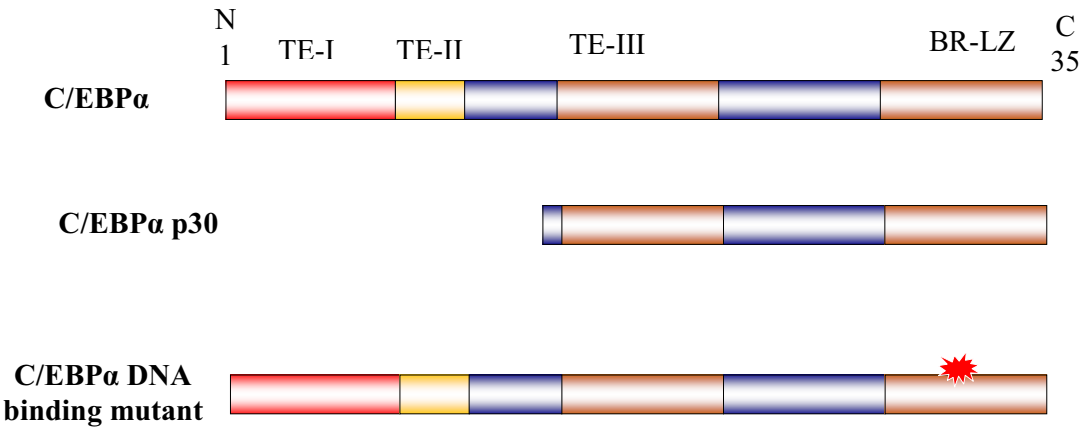
PMR-RARα, the leukemic fusion protein observed in acute promyelocytic leukemia deregulates C/EBPα by repressing C/EBPα promoter activity (Guibal et al., 2005). C/EBPα mRNA is repressed by FLT3/ITD signaling *in vitro* in 32D myeloid cells (Zheng et al., 2004). This repression can be overcome by treatment with CEP-701, a FLT3 inhibitor (Zheng et al., 2004). It is found that activation of FLT3 in AML inhibits C/EBPα function by ERK1/2-mediated phosphorylation of C/EBPα (Radomska et al., 2006). Also, hypermethylation was detected in the CEBPA promoter in AML patients (Chim et al., 2002). All these studies show that the myeloid master regulator C/EBPα is deregulated in acute myeloid leukemia in different ways.

1.6.1 C/EBPα mutations in AML

The role of C/EBPα as a tumor suppressor gene is underlined by the discovery that C/EBPα is mutated in acute myeloid leukemia (Pabst et al., 2001b); (Gombart et al., 2002). C/EBPα is mutated in around 9 % of AML patients (Nerlov, 2004; Rosenbauer and Tenen, 2007). The mutations reported in

C/EBPα are point mutations at basic region leucine zipper and frame shift mutation at N- terminus resulting in a 30 kD form of C/EBPα initiated from the second AUG (Pabst et al., 2001b). The mutant forms of C/EBPα observed in AML are depicted in figure 8.

Figure 8. Wild type and mutant forms of C/EBPα in AML



1.6.2 C/EBPα-p30

The C/EBPα mRNA gives rise to two different translation products based on two different AUGs in the same open reading frame. The 30 kD form of C/EBPα, C/EBPα-p30 lacks the N-terminal transactivation domain but retains the C-terminal DNA-binding domain. C/EBPα-p30 shows decreased DNA binding and is deficient in transactivation potential (Pabst et al., 2001b). It has been reported previously that C/EBPα-p30 lacks antimitotic activity (Lin et al., 1993). Conditional expression of the C/EBPα-p30 in myeloid precursor cells fails to induce granulocytic differentiation (Pabst et al., 2001b). Interestingly, C/EBPα-p30 inhibits DNA binding and transactivation of the wild type protein in a dominant negative manner (Pabst et al., 2001b). Expression of the dominant negative C/EBPα-p30 form in human hematopoietic progenitor $CD34^{+}$ cells inhibits granulocytic differentiation (Schwieger et al., 2004).

The mechanism by which C/EBPα-p30 exerts its dominant negative function is not understood. It was suggested initially that C/EBPα-p30 could be forming heterodimers with wild type C/EBPα. A recent report shows that C/EBPα-p30 modified at the leucine zipper, which is unable to form heterodimers with C/EBPα-p42, still exhibits dominant negative properties over the wild type protein (Cammenga et al., 2005). The first study that identified C/EBPα mutations failed to detect C/EBPα-p42/C/EBPα-p30 heterodimers (Pabst et al., 2001b). One recent study showed that C/EBPα-p30 plays transcriptional regulatory roles distinct from C/EBPα-p42 in a hepatocyte cell line (Wang et al., 2007). This study suggests that C/EBPα-p30 could regulate a unique set of genes distinct from wild type C/EBPα. All these findings suggest that the mechanism with which C/EBPα-p30 modulates its functions could be by upregulating a unique set of genes that are different from C/EBPα-p42 and not by forming heterodimers with C/EBPα-p42.

In order to identify the role of C/EBPα-p30 in leukemogenesis, a mouse line carrying a germline Cebpa mutation resulting in the specific ablation of the p42 isoform of C/EBPα was generated (Kirstetter et al., 2008). This was accomplished by introducing a nonsense codon between the two ATG codons functioning as tranlational start sites for the p42 and p30 forms. Mice heterozygous for C/EBPα-p30 developed normally and didn‘t display any hematopoietic abnormalities. Mice homozygous for C/EBPα-p30 survived until adulthood. These animals showed defects in differentiation of myeloid progenitors in the bone marrow at weaning. These animals developed AML in 12 months with massive invasion of liver and spleen. All these findings, the patient data and the genetic loss of function studies in mice show that loss of C/EBPα expression or function in leukemic blasts leads to a block in myeloid cell differentiation. This supports the concept that C/EBPα disruption is one of the central events in acute myeloid leukemia (Rosenbauer and Tenen, 2007).

1.7 Regulation of C/EBPα

1.7.1 C/EBPα and dimerization

The C/EBPα protein can form a homodimer with another C/EBPα protein as well as heterodimer with other members of the CEBP family (Ramji and Foka, 2002); (Akira et al., 1990); (Cao et al., 1991); (Roman et al., 1990); (Ryden and Beemon, 1989). It has been observed that heterodimerization could potentially alter several functional activities of C/EBPα protein, including DNA binding, transactivation potential, responsiveness to signaling pathways, and the ability to cooperate with other transcription factors. Heterodimers between C/EBP members possess regulatory activities that are distinct from homodimers. C/EBPα-C/EBPβ heterodimers have been observed in liver nuclear extracts and monocytic cells (Ossipow et al., 1993; Pan et al., 1999). Heterodimerization of C/EBPα with ATF-2 results in decreased activation of transcription driven from consensus C/EBP-binding sites (Shuman et al., 1997). One study shows that heterodimerization of C/EBPα with c-Jun blocks C/EBPα DNA binding (Rangatia et al., 2003). These studies show that dimerization of C/EBPα with different proteins can modulate its functions.

1.7.2 C/EBPα and post translational modifications

C/EBPα has been shown to be phosphorylated at serine 21 by extracellular signal-regulated kinases 1 or 2 (ERK1/2)(Ross et al., 2004). This phosphorylation has been shown to block granulopoiesis. It is shown that ras signaling enhances the activity of C/EBPα to induce granulocytic differentiation in mouse by phosphorylation of C/EBPα at serine 248 (Behre et al., 2002). PP2A mediated dephosphorylation at serine 193 mediated by PI3K/Akt pathway has been shown to block the growth inhibitory effect of C/EBPα (Wang et al., 2004). Also, glycogen synthase kinase 3 (GSK3), has been found to phosphorylate C/EBPα on T222, T226, and S230 in vivo (Ross et al., 1999).

Another post translational modification reported for C/EBPα is sumoylation (Subramanian et al., 2003).

1.7.3 Auto regulation of C/EBPα mRNA

C/EBPα mRNA is autoregulated in mouse as well as human via different mechanisms. Activation of the murine promoter by direct binding of C/EBPα was shown to increase the transactivation up to 3 fold (Christy et al., 1991; Legraverend et al., 1993). It is shown that USF (upstream regulatory factor) is also critical for the murine C/EBPα promoter activity (Legraverend et al., 1993). Human C/EBPα protein can activate its own promoter without direct binding, but by stimulating USF to bind to the consensus USF binding site in the C/EBPα promoter (Timchenko et al., 1995).

1.7.4 C/EBPα and protein-protein interaction

An important level of transcription factor regulation is through protein-protein interaction. Such interactions have been shown to be important in hematopoiesis (Stopka et al., 2005). C/EBPα has been shown to physically interact with E2F1 (Johansen et al., 2001). Knock-in mice with a targeted mutation in the C/EBPα-E2F interaction (the BRM2 mutation) displayed block in granulocytic differentiation and expansion of myeloid progenitor population (Porse et al., 2005). Direct physical interaction of C/EBPα with PU.1 is important for PU.1 inactivation by C/EBPα to drive granulocytic differentiation (Reddy et al., 2002). Protein-protein interaction of C/EBPα and c-Jun have been shown to be essential in granulopoiesis (Rangatia et al., 2002). C/EBPα has been also shown to interact with several other proteins including ETS1, GATA1, RUNX1 (McNagny et al., 1998; Yamaguchi et al., 1999; Zhang et al., 1996b). The importance of protein-protein interaction for C/EBPα mediated functions is underlined by the finding that C/EBPα interacts with different protein partners in

the liver of young versus old mice to execute its function (Iakova et al., 2003; Timchenko, 2003).

1.8 Max

Max is a basic region helix loop helix leucine zipper protein (Amati et al., 1993; Amati et al., 1992; Amati and Land, 1994; Blackwood and Eisenman, 1991). Max belongs to Myc-Max-Mad network of proteins which has been shown to have critical role in regulating transcription. Max can form homodimers as well as heterodimers with Myc, Mad and several members of the Myc-Max-Mad family including Mnt and Mga. These homodimers as well as heterodimers can bind specifically to E box DNA sequences with consensus CACGTG elements (Amati et al., 1993; Amati et al., 1992; Amati and Land, 1994; Blackwood and Eisenman, 1991).

The Myc-Max-Mad network proposed by Eisenman postulates a central role of Max (Ayer et al., 1993). According to this model, Max forms transactivating complexes when associated with Myc but repressive complexes when bound to Mad proteins (Luscher, 2001). The binding of Myc-Max to promoters results in binding of the SW1/SNF complex which mediates chromatin remodeling. Also, Myc interacts with the TRAAP complex which has histone acetyl transferase activity. So binding of Myc-Max to promoters results in chromatin remodeling and histone acetylation, which in turn, makes binding sites for transcription factors accessible, and results in transcriptional activation. Binding of the Mad-Max complex to promoters results in the recruitment of the mSin3 repressor complex, which has histon deacetylase activity and chromatin compaction. This results in decreased accessibility of transcription factors to their binding sites and finally transcriptional repression. The shift in equilibrium from Myc-Max complexes to Mad-Max complexes results in shift from proliferation to differentiation (Luscher, 2001). The model for transcriptional regulation by Myc-Max-Mad network of proteins is depicted in figure 9.

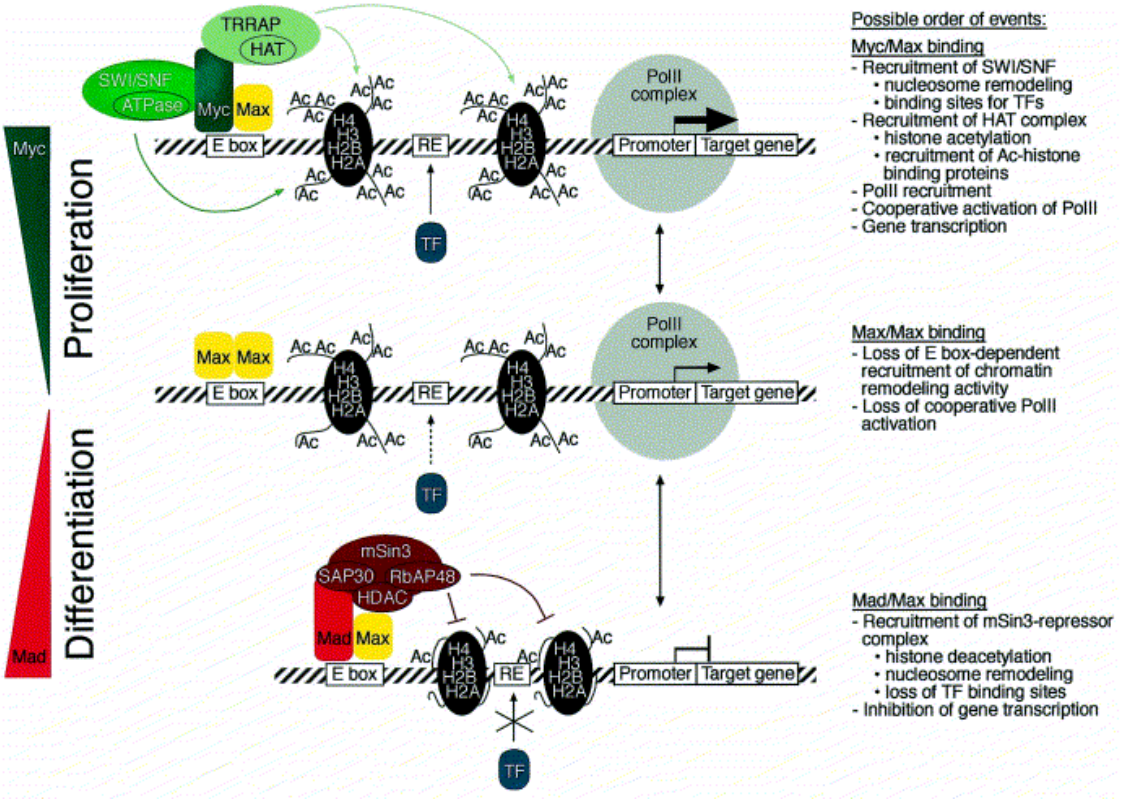


Figure 9. Transcriptional regulation by Myc-Max-Mad network of proteins (Luscher B, Gene, 2001)

Studies of a Max knock-out mouse model show that Max is essential during growth and development (Shen-Li et al., 2000). Max $^{-/-}$ mice die at days E5.5 to 6.5. Switching from Myc-Max to Mad-Max heterocomplexes accompanies monocyte/macrophage differentiation (Ayer and Eisenman, 1993). One important study which suggests Max could have profound significance in granulopoiesis is that a mouse line that carries transgene encoding Max, exhibits a 50 to 60 fold elevation of blood neutrophils (Metcalf et al., 1995). Overexpression of Max has been shown to attenuate Myc-induced lymphoproliferation and lymphomagenesis in transgenic mice. (Lindeman et al., 1995). This finding is very interesting since it is reported that downregulation of c-Myc by C/EBPα is critical for granulopoiesis (Johansen et al., 2001) and c-

Myc is found to inhibit C/EBPα dependent gene activation (Mink et al., 1996). These findings suggest that Max can have important functions in granulopoiesis.

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. Our data reveal Max as a novel co-activator of C/EBPα, thereby suggesting a possible link between C/EBPα and the Myc–Max–Mad network.

1.9 The Peptidyl-prolyl *cis/trans* isomerase, PIN1

Phosphorylation of proteins at serine or threonine has been shown to play an essential role in signal transduction and cell cycle progression. It has been shown recently that phosphorylation of proteins on serine or threonine residues that immediately precede a proline (pSer/Thr-Pro), known as Pro-directed phosphorylation, is a central signaling mechanism controlling normal cell proliferation and malignant transformation (Blume-Jensen and Hunter, 2001; Lu et al., 2002). Interestingly, many oncogenes as well as tumor suppressors are strictly regulated by Pro-directed phosphorylation. The identification and characterization of the peptidyl-prolyl *cis/trans* isomerase, PIN1, which regulates the conformation of specific Pro-directed phosphorylation sites in certain proteins, has led to the discovery of a new post phosphorylation regulatory mechanism (Lu et al., 1996; Ranganathan et al., 1997; Yaffe et al., 1997).

PIN1 belongs to the evolutionarily conserved peptidyl-prolyl isomerase (PPIase) family of proteins (Lu, 2003). PIN1 contains two functional domains-

an amino-terminal WW domain and a carboxy-terminal PPIase domain (Lu et al., 1999). The WW domain of PIN1 binds only to specific pSer/Thr-Pro motifs via protein-protein interaction and the PPIase domain catalyses a *cis-trans* isomerization of the pSer/Thr-Pro motifs.

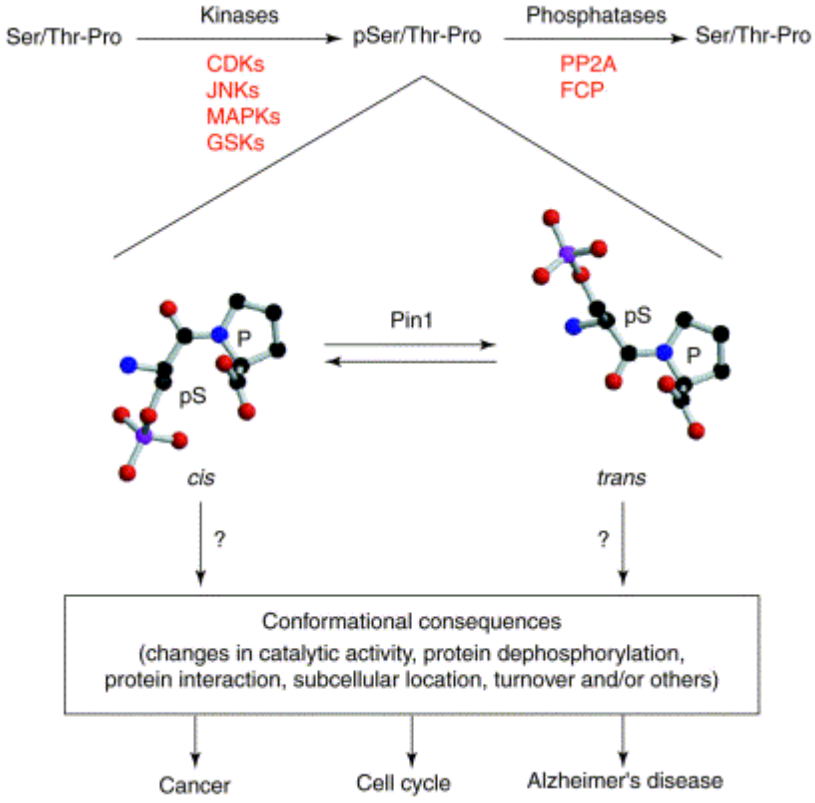


Figure 10. Model for phosphorylation specific prolyl isomerization (Lu KP et al, Trends in Cell Biology, 2002)

1.9.1 PIN1 as a molecular timer

PIN1 was originally discovered as a cell cycle protein essential for the regulation of mitosis (Lu et al., 1996). Later studies showed that overexpression of PIN1 prevents entry into mitosis (Shen et al., 1998). *Pin1* depletion results in tumor cell survival and entry into mitosis. (Rippmann et al., 2000). Studies from *Pin1*$^{-/-}$ mouse embryo fibroblast cells display slower asynchronus growth than wild type cells (Fujimori et al., 1999). *Pin1*- null mice had a marked increase in cell cycle duration in the primordial germ cells due to prolonged G1-S transition (Atchison et al., 2003). Also, Pin1 has been shown to be positively regulated by

E2F and PIN1 expression is increased as cells progress from G0 to S phase of the cell cycle (Ryo et al., 2002). Collectively, all these data suggest that PIN1 can have an important role in different phases of cell cycle by functioning as a molecular timer (Yeh and Means, 2007).

1.9.2 Targets of PIN1

Recent works show that PIN1 mediated conformational changes following phosphorylation can have profound effects on catalytic activity, phosphorylation status, protein-protein interaction, subcellular localization, and/or protein stability of its substrates (Lu, 2003; Lu et al., 1999; Yeh and Means, 2007). PIN1 regulates the stability of many proteins including p53, Cyclin D1, ß-catenin etc. PIN1 increases the protein half life of p53 by inhibiting its binding to the Mdm2 ubiquitin ligase, which regulates the degradation of p53 (Wulf et al., 2002). PIN1 binds and isomerizes cyclin D1 and thereby prevents its nuclear export and ubiquitin-mediated degradation, resulting in cyclin D1 stabilization (Liou et al., 2002).

PIN1 has been shown to control cell cycle progression through regulating some key proteins such as c-Myc, c-Jun, Fos, Cyclin E and Cyclin D1. PIN1 binds to c-Myc which is phosphorylated on Ser62 and Thr58. The binding of PIN1 has been shown to be necessary for the ubiquitination and degradation of c-Myc. Depletion of PIN1 results in stabilization of c-Myc (Yeh et al., 2004). Another important function PIN1 has is to regulate RNA polymerase II. PIN1 increases C-terminal domain (CTD) phosphorylation of human RNA polymerase II by inhibiting the CTD phosphatase FCP1 and stimulating CTD phosphorylation by cdc2 / cyclin B (Xu et al., 2003). It was shown that PIN1 binds to c-Jun and increases the transcriptional activity of c-Jun in breast cancer development (Wulf et al., 2001). Some of the targets of PIN1 are depicted in figure 11.

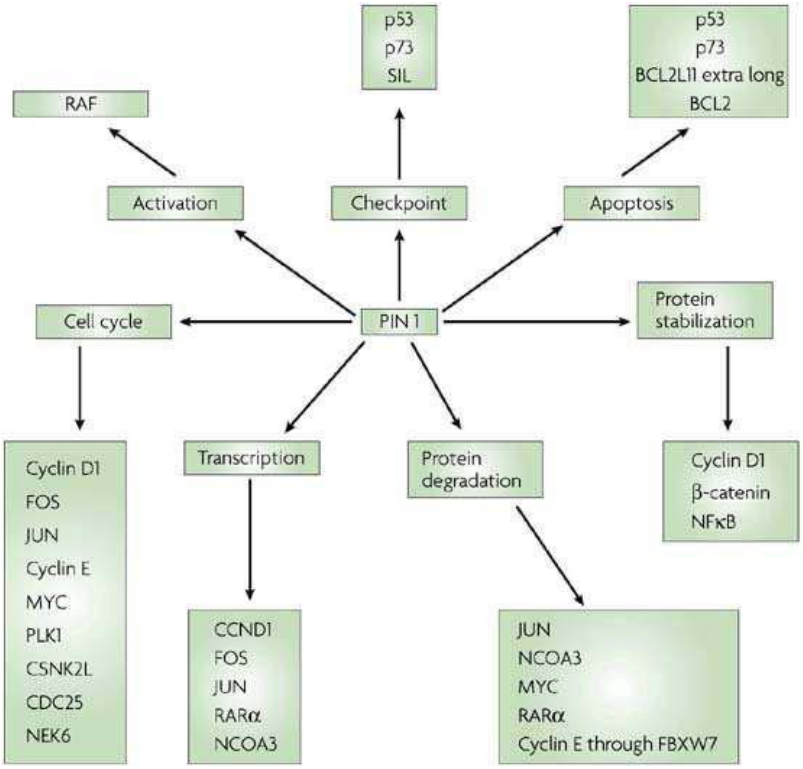


Figure 11. Functional targets of PIN1 (Yeh ES and Means AR, Nature Reviews Cancer, 2007)

1.9.3 PIN1 and tumorigenesis

A role of PIN1 in tumorigenesis is suggested by the fact that it is overexpressed in many cancers including prostate, lung, ovary, cervical, breast, brain and skin (Bao et al., 2004; Wulf et al., 2001). Initial reports show that *Pin1* knock-out mice develop normally (Fujimori et al., 1999). Even though the *Pin1* null animals display age dependent defects such as retinal atrophy, decreased body weight and testicular atrophy, no other phenotypic characteristics related to cancer were detected (Liou et al., 2002). Mice lacking *Pin1* are resistant to tumorigenesis induced by oncogenic Neu or Ras (Wulf et al., 2004). Inhibition of PIN1 in cancer cells via multiple approaches triggers apoptosis or suppresses the transformed phenotype (Lu et al., 1996; Rippmann et al., 2000). These studies show that PIN1 is essential for tumorigenesis. PIN1 is overexpressed in breast cancer and has been found to cooperate with Ras signaling in increasing

c-Jun transcriptional activity towards cyclin D1 (Wulf et al., 2001). Overexpression of cyclin D1 has been reported in many cancers (Hunter and Pines, 1994), including around 50% human breast tumors (Bartkova et al., 1994). These results indicate that PIN1 overexpression could be a prevalent event in human cancers (Lu, 2003).

It is suggested that PIN1 overexpression can function as a critical catalyst that amplifies multiple oncogenic signaling pathways during oncogenesis (Ryo et al., 2003). Even though PIN1 is known to have important role in the development of many cancers, no data are published so far about any possible role of PIN1 in any type of leukemia.

A recent study proposes that C/EBPα could be a PIN1 target (Miller, 2006). This study points out that S/T-P motifs in C/EBPα could be regulated by PIN1 mediated isomerization. PIN1 has been shown to be positively regulated by E2F and PIN1 expression is increased as cells progress from G0 to S phase of cell cycle (Ryo et al., 2002). It is important to note that C/EBPα-p30 doesn't repress E2F. E2F repression by C/EBPα is required for granulopoiesis (Porse et al., 2001). This demonstrates that lack of E2F repression by the C/EBPα-p30 may leads to upregulation of PIN1. Rangatia et al has shown that downregulation of the proto-oncogene c-Jun by C/EBPα is critical for granulocytic lineage commitment (Rangatia et al., 2002). Overexpression of c-Jun blocks granulopoiesis and c-Jun expression is high in AML patients with C/EBPα mutations (Rangatia et al., 2003). Moreover, PIN1 increases the transcriptional activity of c-Jun (Wulf et al., 2001) and PIN1 is upregulated in response to c-Jun overexpression (Rinehart-Kim et al., 2000). It is proposed that PIN1 can increase the stability of c-Jun (Wulf et al., 2005). Taken together, these studies suggest that PIN1 may have profound effects in AML patients in which C/EBPα is mutated.

In this study we show that C/EBPα-p30 induces PIN1 mRNA levels and it is upregulated in patients with acute myeloid leukemia. Inhibition of Pin1 leads to

myeloid differentiation suggesting that inhibition of Pin1 as a novel strategy in treating AML patients with C/EBPα mutation.

1.10 Aims of the study

Recent findings show protein-protein interactions coordinated by transcription factors have profound effect in hematopoiesis. Protein-protein interactions of C/EBPα have been shown to be essential in granulopoiesis. In the present study we aimed to identify the interacting proteins of C/EBPα. Here we demonstrate the role of Max as an interacting partner of C/EBPα and how this interaction is essential for the myeloid differentiation programme. These findings give new insights to the molecular mechanisms in granulopoiesis orchestrated by C/EBPα.

Experimental data from animal models as well as AML patient samples suggest that loss of function or expression of C/EBPα is critical in AML development. C/EBPα is mutated in around 9% of acute myeloid leukemia. The mutant form of C/EBPα i.e., C/EBPα-p30 exhibits dominant negative function over the wild type protein and blocks myeloid differentiation. The mechanism with which C/EBPα-p30 mediates this differentiation block is poorly understood. An increasing number of studies suggest that the regulatory network around C/EBPα-p30 could have a critical role in the development of AML. In the present study we also sought to demonstrate the role of PIN1 in AML with C/EBPα mutation and how the regulatory network coordinated by PIN1 can have an important role in the dominant negative function of C/EBPα-p30. These findings might lead to novel strategies for treating AML with C/EBPα mutation.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals

Acetonitrile	Sigma, Germany
Acetic Acid	Merck, Germany
Acrylamide-Bisacrylamide	Biorad, Germany
Agar	Life Technologies, Scotland
Agarose	Life Technologies, Scotland
APS	Fluka, Switzerland
Bromphenolblue	Sigma, Germany
CHCA	Sigma, Germany
DAPI	Sigma, Germany
DTT	Sigma, Germany
Dimethyl Sulfoxide	Sigma, Germany
Dithioerythritol	Merck, Germany
Deoxycholate	Merck, Germany
β-Estradiol	Sigma, Germany
Ethanol	Merck, Germany
Ethidium bromide	Sigma, Germany
Ethylenediamine Tetra-Acetic Acid	Merck, Germany
Formaldehyde	Sigma, Germany
Formamide	Sigma, Germany
Glycine	Sigma, Germany
Glycerol	Merck, Germany
Isopropanol	Merck, Germany
Methanol	Merck, Germany
PiB	Calbiochem, USA
Puromycin	Sigma, Germany
Silver Nitrate	Merck, Germany

Sodium Carbonate	Merck, Germany
Sodium Chloride	Sigma, Germany
Sodium Thiosulfate	Merck, Germany
Sodium Dodecyl Sulphate (SDS)	Sigma, Germany
Trifluoroacetic Acid (TFA)	Merck, Germany
Triton X-100	Sigma, Germany
Trizol	Invitrogen, Germany
Tween-20	Sigma, Germany
Urea Plus	Merck, Germany

2.1.2 Cell culture reagents

Charcoal treated FBS	Hyclone, Germany
DMEM	PAN, Germany
Foetal bovine serum	GIBCO, Germany
IMDM	Cambrex, United States
Lymphocyte Separation Medium	PAA, Austria
PBS	PAN, Germany
Penicillin/Streptomycin	GIBCO, Germany
RPMI 1640	PAA, Austria
RPMI 1640	ATCC, Germany
Trypsin EDTA	GIBCO, Germany

2.1.3 Cell lines, AML blast cells, Primary Cells

293T cells (human embyronic kidney fibroblast cells)

U937 (human myeloid cell line, monoblastic)

K562-C/EBPα-p42-ER and K562-C/EBPα-p30-ER (Erythroleukemic cells K562 cells stably transfected with C/EBPα-p42-ER and C/EBPα-p30-ER resepectively) (D'Alo et al., 2003).

Kasumi-6 (Myeloid leukemia cell line eastablished from the bone marrow cells of a patient with C/EBPα mutation (Asou et al., 2003).

Blast cells from different AML patients were kind gift from the laboratory for leukemia diagnostic, Med III, Klinikum Großhadern, University of Munich, Germany.

Human cord blood samples were collected after full term delivery with informed consent of the mothers from Klinikum Kröllwitz, Halle, Germany. Hematopoietic CD34+ cells were isolated from cord blood samples using CD34+ selection kit (Miltenyi Biotech, Germany).

2.1.4 Plasmids

Max expression plasmid (kind gift from Dr. Dirk Eick)

In vitro translatable Max (kind gift from Dr. Robert Eisenman)

BR3/C/EBPα (basic region mutant) and LZ/C/EBPα (leucine zipper domain mutant) (kind gift from Dr. Alan Friedman)

Wild type C/EBPα and mutant C/EBPα (C/EBPα-p30) (Pabst et al., 2001b)

Reporter construct p(C/EBP)2TK having two consensus C/EBPα binding sites

PIN1 promoter luciferase construct (Ryo et al., 2002)

PIN1 pcDNA (Zacchi et al., 2002)

E2F1 expression vector (Johansen et al., 2001)

c-Jun expression vector (Rangatia et al., 2002)

2.1.5 shRNA / siRNA

Max shRNA	Cat. No. RHS1764-9690535; Open Biosystems, Germany
Control shRNA	Cat. No. RHS1707; Open Biosystems, Germany
PIN1 siRNA	Cat. No. SI02662128; Qiagen, Germany
Control siRNA	Cat. No 10277280; Qiagen, Germany

2.1.6 Antibody

Anti-C/EBPα	Cat. No. sc-61, sc-9315, Santa Cruz Biotechnology, Germany
Anti-Max	Cat. No. sc-765, Santa Cruz Biotechnology, Germany
Anti-c-Myc	Cat. No. sc-42, Santa Cruz Biotechnology, Germany
Anti-PIN1	Cat. No. sc-15340, Santa Cruz Biotechnology, Germany
Anti-β-Tubulin	Cat. No. sc-9104, Santa Cruz Biotechnology, Germany
CD15	Cat. No. 555401, BD Pharmingen, Germany
CD11b	Cat. No. 555388, BD Pharmingen, Germany
Anti-HA	Cat. No. 1867423, Roche Applied Science, Germany

2.1.7 Reagent Kits

Biorad-protein estimation kit	Biorad, Germany
$CD34^+$ selection kit	Miltenyi Biotech, Germany
Dual Luciferase assay kit	Promega, Germany
ECL detection kit	Amersham Biosciences, Germany
ImProm-II Reverse Transcription system	Promega, Germany
LipofectAMINE plus	Invitrogen, Germany
Nucleofector kit	AMAXA, Cologne, Germany
Plasmid DNA Isolation kit	Qiagen, Germany
SYBR Green kit	Qiagen, Germany
TNT-Reticulocyte lysate system	Promega, Germany
TRizol reagent	Invitrogen, Germany

2.1.8 Mass Spectrometry:

Reflex III MALDI-TOF	Bruker Daltonics, Germany
AB4700 MALDI-TOF/TOF	Applied Biosystems, Germany
Nano LC	LC Packing, Dionex, United States
Mascot database search software	Matrix Science
AnchorChip plate	Bruker Daltonics, Germany

2.1.9 Miscellaneous

ECL hyperfilm	Amersham Biosciences, Germany
Immobiline dry strip (pH 3-10)	Amersham Biosciences, Germany
Protein agarose beads	Roche Molecular Diagnostics, Germany
Phosphatase inhibitor Cocktail	Sigma, Germany
Protease inhibitors	Sigma, Germany
PVDF membrane	Schleicher and Schüll, Germany
RNA quiashredder columns	Quiagen, Germany

2.2 Methods

2.2.1 Cell culture

U937 cells were cultured in RPMI 1640 supplemented with 10% heat inactivated foetal bovine serum and 1% Penicillin-Streptomycin; human embryonic kidney 293T cells were maintained in Dulbecco´s modified Eagle medium supplemented with 10% foetal bovine serum, 1% glutamine and 1% Penicillin-Streptomycin; K562-C/EBPα-p42-ER and K562-C/EBPα-p30-ER cells were maintained in RPMI 1640 without phenol red supplemented with 10% charcoal treated foetal bovine serum, 1% Penicillin-Streptomycin and 2 μg/ml Puromycin; Kasumi-6 cells were cultured in RPMI 1640 supplemented with 20% foetal bovine serum, 1% Penicillin-Streptomycin and 2 ng/ml GM-CSF; human CD34+ cells were 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), 50 ng/ml of interleukin-3 (IL-3) and 100 U/ml penicillin/streptomycin.

2.2.2 Transfection

2.2.2.1 Transient transfection by LipofectAMINE

The day before transfection, 293T cells were trypsinized, counted and plated so that they were 50-80% confluent the day of transfection. Antibiotics were avoided during plating to help cell growth and increased transfection efficiency.

DNA was diluted in serum free medium and precomplexed with PLUS reagent (Invitorgen, Germany). The complete mixture was incubated at room temperature for 15 min. LipofectAMINE reagent was diluted and mixed in a serum free medium in a second tube. Pre-complexed DNA and diluted LipofectAMINE reagent (Invitorgen, Germany) were mixed and incubated for 15 min at room temperature. During this incubation period, cells were rinsed with serum free medium to enable higher transfection activity. DNA-PLUS LipofectAMINE reagent complexes were added to each well containing fresh medium. The complexes were gently mixed with the medium; incubated at 37C at 5% CO2 for 3 hrs. After 3 hrs of incubation medium volume was increased to normal volume by adding medium containing 20% fetal bovine serum.

2.2.2.1 Transient transfection by AMAXA

The Nucleofector kit (AMAXA, Cologne, Germany) was used essentially as described by the manufacturer. 2 μg portion of plasmid DNA constructs were used for each transfection and the transfection efficiency was analyzed using a plasmid with eGFP marker. $CD34^+$ cells, U937 and Kasumi-6 cells were transfected with nucleofection programmes U-08, V-01 and T-03, respectively.

2.2.3 Immunoprecipitation

The immunoprecipitation (IP) was performed from 500–1000 μg nuclear extracts of U937 cells, 50 μl slurry of protein A agarose beads and 2 μg of respective antibodies and the corresponding IgGs as controls in an IP buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.5% NP-40, 0.25% sodium deoxycholate). Immunoprecipitated proteins were heated at 56°C for 30 minutes in 2X SDS loading buffer and then boiled at 95°C for 5 minutes. Denatured proteins were subsequently separated on 8% SDS PAGE and immunoblotted against respective antibodies.

2.2.4 Western blotting

For Western blotting, 50 μg total protein was denatured in the SDS sample loading buffer, separated on 8-10% SDS-polyacrylamide gels and transferred to an immobilon-P membrane (Millipore, USA). The membrane was incubated with respective primary antibody overnight and with horseradish peroxidase conjugated secondary antibodies for one hour. Signals were detected with the ECL Western blotting detection reagents. In all immunoblotting experiments a 1:1000 dilution for primary and 1:2000 dilutions for secondary antibody was used.

2.2.5 Proteomics

Nuclear extracts of U937 cells were used for immunoprecipitation with C/EBPα antibody using protein A Agarose beads. After immunoprecipitation, beads with their associated proteins were lysed in urea lysis buffer for 1hr at RT on a rotating shaker. Lysed beads were passed through RNA quiashredder columns, and resulting supernatant containing dissolved proteins was ultracentrifuged for 50 minutes at 50,000 rpm at 22°C. In the first dimension, 350 μl of dissolved proteins after ultracentrifugation were separated on an immobiline dry strip pH 3-10 by isoelectric focussing (IEF) where proteins are separated on the basis of their isoelectric point (pI). The reduction and alkylation of separated proteins was carried out in urea buffer containing 2% DTE and 2.5% iodoacetamide, respectively. Proteins were then 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.

The protein spots were excised from gels, destained, and in gel digestion was performed with 200 ng trypsin in ammonium bicarbonate solution for 16 hours. The digested peptides were eluted in 70% acetonitrile, lyophilised and resuspended in 5 μl of 0.1% TFA in 10% acetonitrile. The dissolved peptides were mixed in 1:1 ratio with DHB (2, 5-dihydroxybenzoic acid) matrix solution and loaded on an anchorChip target plate (Bruker Daltonics, Germany). Peptide

mass fingerprint (PMF) was generated by Matrix Assisted Laser Deionization-Time of Flight (MALDI TOF; REFLEX III, Bruker Daltonics) mass spectrometry and corresponding proteins were identified by MASCOT database searches.

For LC-MS/MS analysis of interacting proteins of C/EBPα, the beads after immunoprecipitation were denatured with SDS sample loading buffer, separated on 10% SDS-polyacrylamide gel and silver stained. The individual bands were excised and processed for trypsin digestion. The digested protein samples were fractionated by Nano LC (LC Packing, Dionex) using 500 µm i.d. x 5mm, C18 reverse phase column with a flow rate of 200 nl/min and loaded on an 4700 sample plate. Peptide mass fingerprint (PMF) was generated by Matrix Assisted Laser Deionization-Time of Flight (MALDI TOF/TOF, Applied Biosystem) mass spectrometry and corresponding proteins were identified by MASCOT database searches.

2.2.6 Immunofluorescence

U937 cells (3 X 10^5), under uninduced condition or induced with RA, 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/EBPa, anti-Max and anti-Myc 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 DAPI (1 mg/ml) for 15 min. The cells were mounted in aqueous mounting medium and the images were acquired and analyzed using Leica fluorescence microscope (X100, X60).

2.2.7 Promoter assay

293T cells were transiently transfected using LipofectAMINE (Invitorgen, Germany) as described by the manufacturer. Firefly luciferase activities from

the constructs pTK, p(C/EBP)2TK and Renilla luciferase activity from the internal control plasmid pRL-null were determined 24 h after transfection using the Dual-Luciferase Reporter Assay System (Promega, Germany). Firefly luciferase activities were normalized to the Renilla luciferase values of pRL-null. Results are given as means and standard errors of the means of three independent experiments. The following DNA concentrations of the reporter constructs and expression plasmids were used for LipofectAMINE Plus transfections: 0.1 μg of pCDNA3-human C/EBPα (wild type), 0.1 μg of C/EBPα-p30, 0.1 μg of Max, 0.1 μg of pTK, 0.1 μg of pCDNA3 PIN1, 0.1 μg of c-Jun, 0.05 μg of E2F1, 0.1 μg of p(C/EBP) 2TK and 0.01 μg of the internal control plasmid pRL-null.

2.2.8 FACS analysis

For flow cytometry analysis, 10^6cells were washed twice with PBS and resuspended in 50 μl of PBS with 2 μl of the respective antibody. Incubation was performed for 20 minutes in ice. After the incubation, cells were washed with PBS, resuspended in PBS and analyzed by flow cytometry in FACScan (Becton Dickinson).

2.2.9 Chromatin immunoprecipitation assay

Crosslinking of proteins to DNA was done by the direct addition of formaldehyde (final concentration 1% (v/v) to cultured cells for 10 min at 37^0C. Glycine was added to a final concentration of 0.125M to stop cross-linking. Fixed cells were pelleted by centrifugation and equentially 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/EBPa, Max and c-Myc were used overnight at 4^0C. After serial elution, washing and reverse cross linking, the samples were extracted twice with phenol/chloroform and precipitated with ethanol overnight in the presence of 20 mg glycogen as a carrier. DNA fragments were recovered

by centrifugation, resuspended in distilled water, and used for PCR amplification. For detection of immunoprecipitated C/EBPα promoter region, two primers, forward 5′-CCGCTACCGACCACGTGGGCG-3′(which corresponds to -280 to -260 bases) and reverse 5′-GCACCTCCGGGTCGCGAATGG-3′(which corresponds to -2 to +19 bases), were used for Q-RT-PCR amplification. The amplified product (299 bases) encompasses the CACGTG site in the C/EBPα promoter.

2.2.10 mRNA expression analysis

Total RNA was isolated from leukemic patient samples, processed and analyzed on the Affymetrix HG-U133A and HG-U133B chips as described before (Schoch et al., 2002). The data from Affymetrix analysis were normalised together according to the procedure described before (Huber et al., 2002). Normalized expression data were then analyzed with the R software package and the "boxplot" function (www.r-project.org). Expression signal intensities are expressed on a logarithmic scale.

2.2.11 Quantitative Real-Time PCR

Total RNA was isolated from cells with Trizol reagent (Invitorgen, Germany). 750 µg of RNA was used to synthesize cDNA by Reverse Transcription. Equal amounts of cDNA were taken for a subsequent quantitative real-time PCR (Q-RT-PCR) using the SYBR Green PCR kit (Qiagen, Germany) in a Rotor-Gene RG-3000 (Corbett Research, Australia). The delta delta C_T value ($\Delta\Delta C_T$) was then calculated from the given C_T value by the formula $\Delta\Delta C_T = [C_T \text{ (gene of interest)} – C_T \text{ (GAPDH)}]_{\text{Test}} - [C_T \text{ (gene of interest)} – C_T \text{ (GAPDH)}]_{\text{Control}}$. The fold change was calculated as fold change = $2^{-\Delta\Delta C_T}$ (Livak and Schmittgen, 2001).

The primers used in the analysis are shown in table 2:

Table 2.

Gene	Acc. No.	Sequence	Amplicon (bp)	Position at the cDNA sequence
PIN1	NM_006221	For. 5′AAG ATG GCG GAC GAG GAG 3′ Rev. 5′CAC TCA GTG CGG AGG ATG AT 3′	494	1-491
c-Jun	NM_002228	For.5′GCA TGA GGA AAC GCA TCG CTG CCT CCA AGT 3′ Rev. 5′GCG ACC AAG TCC TTC CCA CTC GTG CAC ACT 3′	400	776-1175
G-CSFR	BC053585	For. 5′AAG AGC CCC CTT ACC CAC TAC ACC ATC TT 3′ Rev. 5′TGC TGT GAG CTG GGT CTG GGA CAC TT 3′	340	1666-2005
GAPDH	NM_002046	For. 5′ACC ACA GTC CAT GCC ATC AC 3′ Rev. 5′TCC ACC ACC CTG TTG CTG TA 3′	452	526-977

2.2.12 Ubiquitination Assay

293T cells were transiently transfected with different constructs as described (Figure 27), 24 hours after transfection cells were lysed in RIPA buffer followed by c-Jun immunoprecipitation from 500µg total protein. The protein samples after immunoprecipitation were analysed in a 10% SDS-PAGE gel and probed for HA antibody.

3. Results

3.1 Max as a novel co-activator of myeloid transcription factor C/EBPα

3.1.1 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 cellular level, we applied proteomics technique coupled with mass spectrometry using the immunoprecipitation 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 12A) and co-immunoprecipitate other endogenous proteins (as positive controls) such as c-Jun (Figure 12B) which was not present in the isotype IgG control. Immunocomplexes were further processed for proteomic analysis. The protein spots excised from the 2D gels (Figure 12C, spots are numbered) were identified by MALDI-TOF MS. Additionally, the individual bands were excised from Coomassie/Silver-stained SDS-PAGE gels (Figure 12D) and processed for LC-MS/MS. From both screens, we were able to identify 10 proteins by MS, which potentially interact with C/EBPα (Table 3). Among these proteins, was Max. C/EBPα was also identified by MS analysis of the corresponding band (Figure 12D), thereby serving as a control for our experimental setup. The discovery of Max as a novel C/EBPα partner is intriguing because of the role Max plays in switching from Myc-Max to Mad-Max heterocomplexes during myeloid differentiation (Ayer and Eisenman, 1993). We therefore selected Max for further functional and biological characterization.

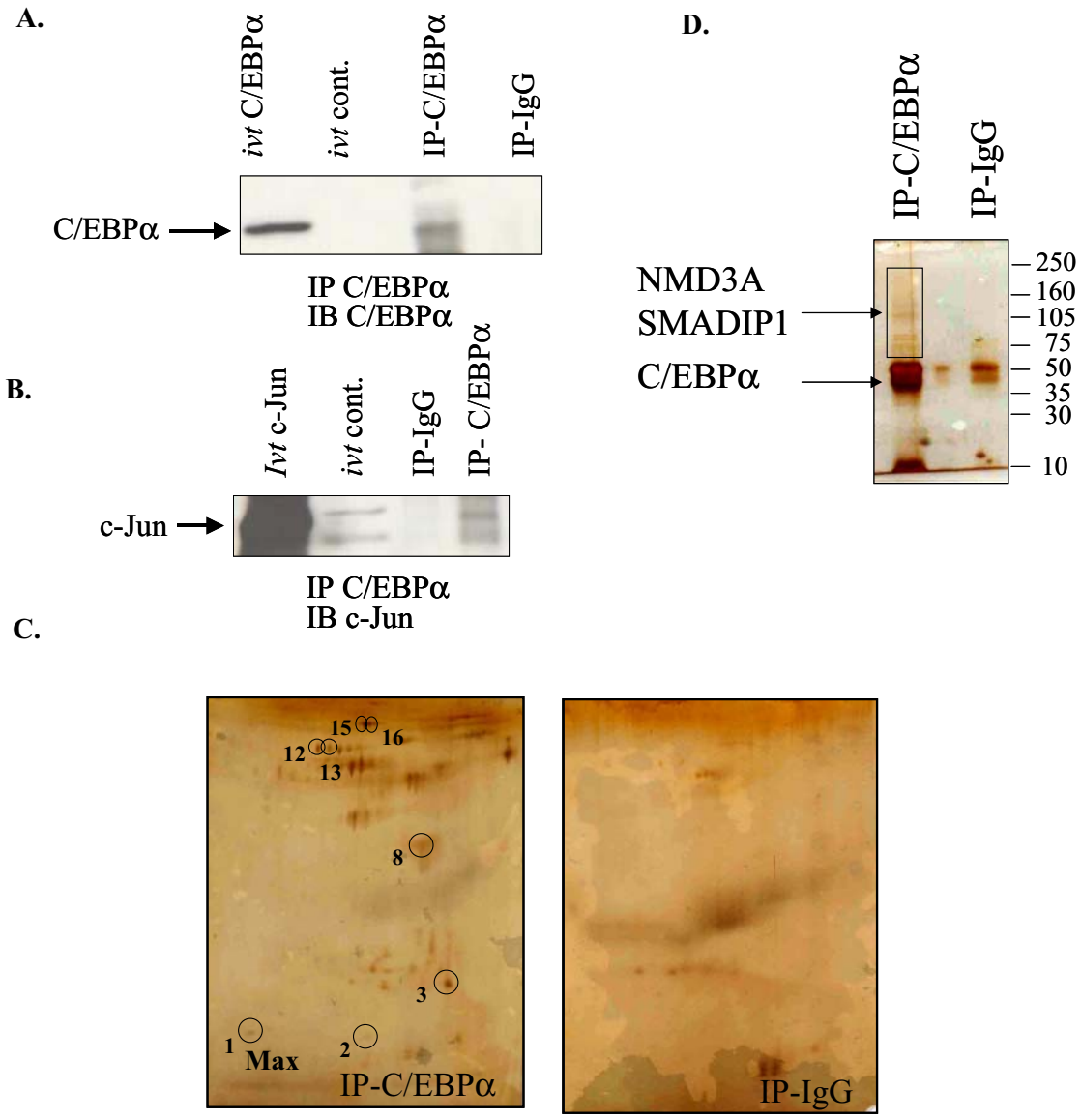


Figure 12. Mass spectrometry based proteomics identification of C/EBPα interacting proteins. A) C/EBPα IP from nuclear extracts of U937 cells and a corresponding immunoblotting (IB) with anti-C/EBPα antibody to confirm the presence of C/EBPα protein in the IP complex. *In vitro*-translated C/EBPα (ivt, 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. *In vitro* translated 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 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 liquid chromatography and the peptides identified by MALDI-TOF-TOF

Table 3. Mass spectrometry based identification of interacting proteins of C/EBPα

Spot no.	Acc.no.	Protein name	Mol.wt.
1	A42611	Max	21.029
2	Q9UP93	Macrophin1 fragment	620.00
3	M2OM_Hum	Mitochondrial 2-oxoglutarate/malate carrier protein	34.08
8	A47213	Beta fodrin	146.55
12	Q96QA8	RPGR interacting protein 1	147.33
13	Q9P1U9	ZNF45	80.44
15	FAHUAA	Actinin 1	103.48
16	Q9UKD2	60 S ribosomal protein	127.60
*	NMD3A	N-methyl-D-aspartate receptor 3A	126.67
*	SMADIP1	Smad interacting protein 1	137.84

* represents proteins identified by liquid chromatography coupled tandem mass spectrometry

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

To confirm the observed interaction of Max with C/EBPα, we performed reciprocal immunoprecipitation. Our results demonstrate that C/EBPα interacts with Max and vice versa (Figure 13) *in vivo*, and thereby confirm the results from the interaction screen. It is important to note that for the same amounts of nuclear extracts used (5 and 10 mg) 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.

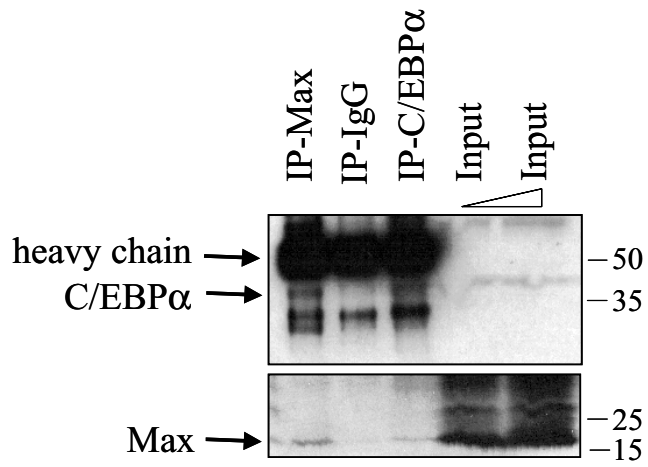


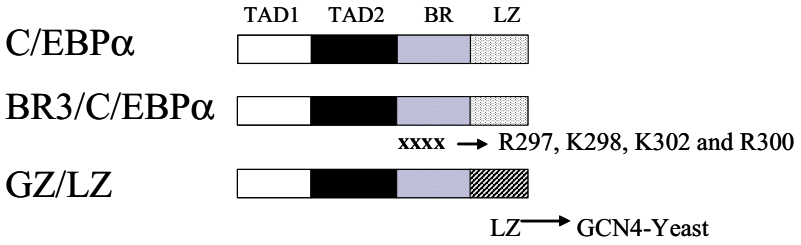
Figure 13. Confirmation of C/EBPα-MAX interaction. Reciprocal IPs: C/EBPα and Max were immunoprecipitated (IP C/EBPα, IP Max) from nuclear extracts of U937 cells by incubation with anti-C/EBPα and anti-Max, respectively, and respective IgG as controls. The blot was first probed with anti-C/EBPα antibody, stripped and reprobed with anti-Max antibody.

3.1.3 The BR3 domain of C/EBPα is involved in its interaction with Max

To investigate the protein domains that might be involved in the C/EBPα–Max interaction, we performed co-immunoprecipitation studies using different mutants of C/EBPα. The different C/EBPα mutants used in this study were BR3/C/EBPα (arginine 297, lysine 298, aginine 300 and lysine 302 in the basic region mutated), GZ/LZ (the leucine zipper of C/EBPα is replaced with GCN4 leucine zipper) and L1-2V (leucine 1 and 2 in the leucine zipper mutated to valine) (Liu et al., 2003). C/EBPα and its various mutants were transiently transfected into 293T cells, and cotransfected with an expression plasmid for Max containing a carboxy-terminal HA tag. Max was then immunoprecipitated from nuclear extracts using Max antibody. The associated complexes were assayed by immunoblotting for C/EBPα using C/EBPα antibody. Our results demonstrate that C/EBPα could be co-immunoprecipitated when IP was performed using 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 co-expressed (Figure 15B, 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

14B, lane 2). We also show that Max could be specifically immunoprecipitated (as controls) with immunoblot for Max using HA antibody (Figure 14B, lower panel). These data show that the basic region of C/EBPα is involved in its interaction with Max in a cellular setting.

A.



B.

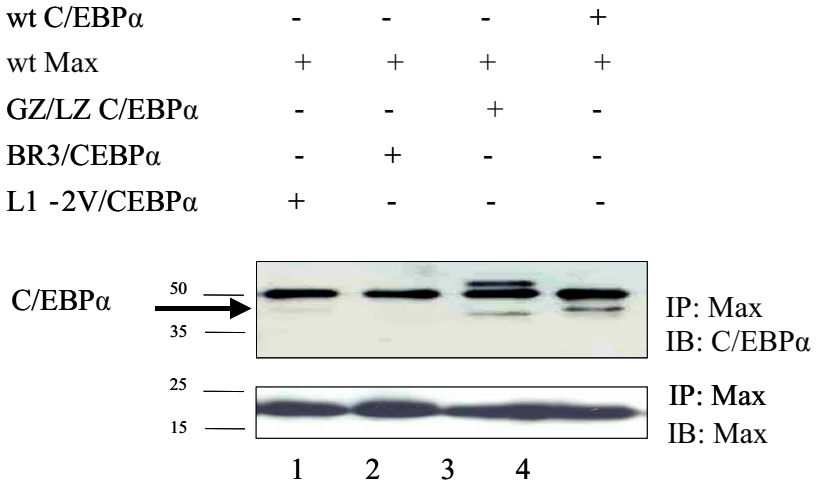


Figure 14. Interaction of C/EBPα with Max involves the DNA-binding domain of C/EBPα: A) The basic region of C/EBPα is involved in its interaction with Max. Schematic representation of wild-type hC/EBPα and different mutants used in this study. TAD, transactivation domains 1 and 2; BR, basic region; LZ, leucine zipper domain; HLH, helix–loop–helix. **B)** 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.

3.1.4 C/EBPα–Max but not Myc–Max colocalize during granulocytic differentiation of myeloid U937 cells

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 15A) and the overlay of the two images shows that both proteins colocalize in these intranuclear structures (Figure 15A, panel 4; yellow signal).

We next investigated the effect on C/EBPα–Max colocalization when the cells were triggered for granulocytic differentiation by RA for 24 hr. 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 15B, 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 15A, lower panels). On the other hand, no intranuclear c-Myc signal could be detected after RA treatment (Figure 15C, lower right 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 (nuclear fraction (NF) and cytoplasmic fraction (CF) by Western blotting, using specific antibodies (Figure 16D). Our results revealed that the c-Myc protein level was drastically decreased in both fractions (Figure 15D) by RA. However, C/EBPα was undetectable in the CF and slightly increased in the NF by RA when analyzed by immunoblotting. Max, on the other hand, was relatively unchanged under induced and uninduced conditions. These data suggest 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.

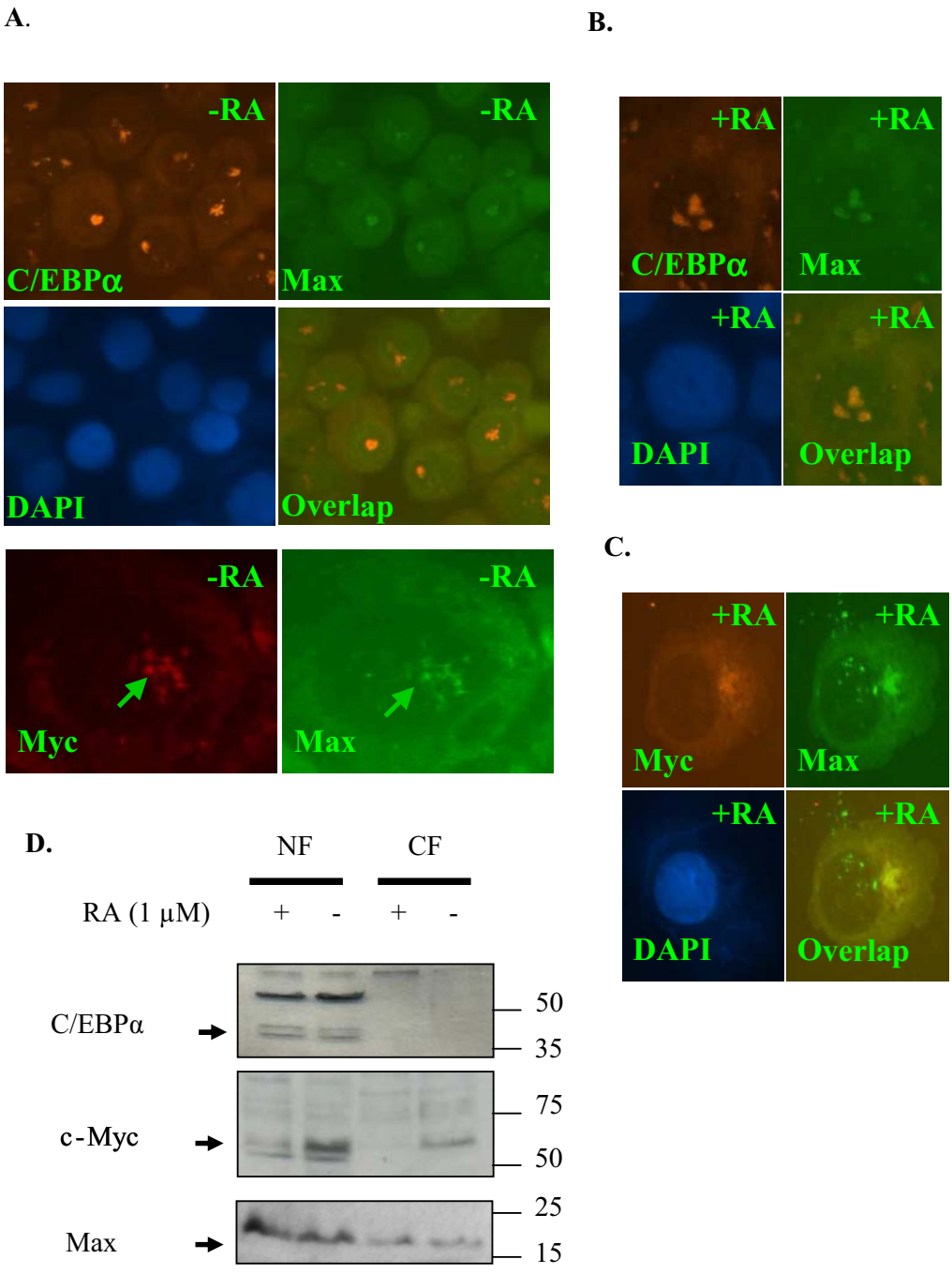


Figure 15. Endogenous C/EBPα-Max but not Myc–Max remains colocalized during granulocytic differentiation of U937 cells. A) Indirect immunofluorescence staining for C/EBPα (anti-goat; Santa Cruz), Max (anti-rabbit; Santa Cruz) and Myc (anti-mouse, Santa Cruz) using respective conjugated secondary antibodies (Molecular Probes). 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 nuclear and cytoplasmic fractions. Blots were stripped and reprobed with specific antibody. NF: nuclear fraction; CF: cytoplasmic fraction.

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

To investigate the functional importance of the C/EBPα –Max interaction and the colocalization of these proteins, we performed transient transfection assays in the human embryonal kidney cell line 293T and the myeloid cell line U937 using as a reporter a minimal TK promoter containing two CCAAT binding sites cloned upstream of the luciferase reporter gene. Transfection of a Max expression construct significantly enhanced the ability of C/EBPα to transactivate a minimal TK promoter containing two CCAAT binding site in a dose dependent manner (Figure 16A). In control experiments, no effect of Max on C/EBPα activity was observed when promoter with no CCAAT binding sites was used. Similar results were obtained with myeloid U937 cells (Figure 16B). Interestingly, co-transfection studies with the human 2200 bp C/EBPα promoter construct having bases -1 to -2200 with respect to the transcription start site (which has an 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 weak but significant increase in the promoter activity (Figure 16C). It is important to point out that Max itself does show some activation.

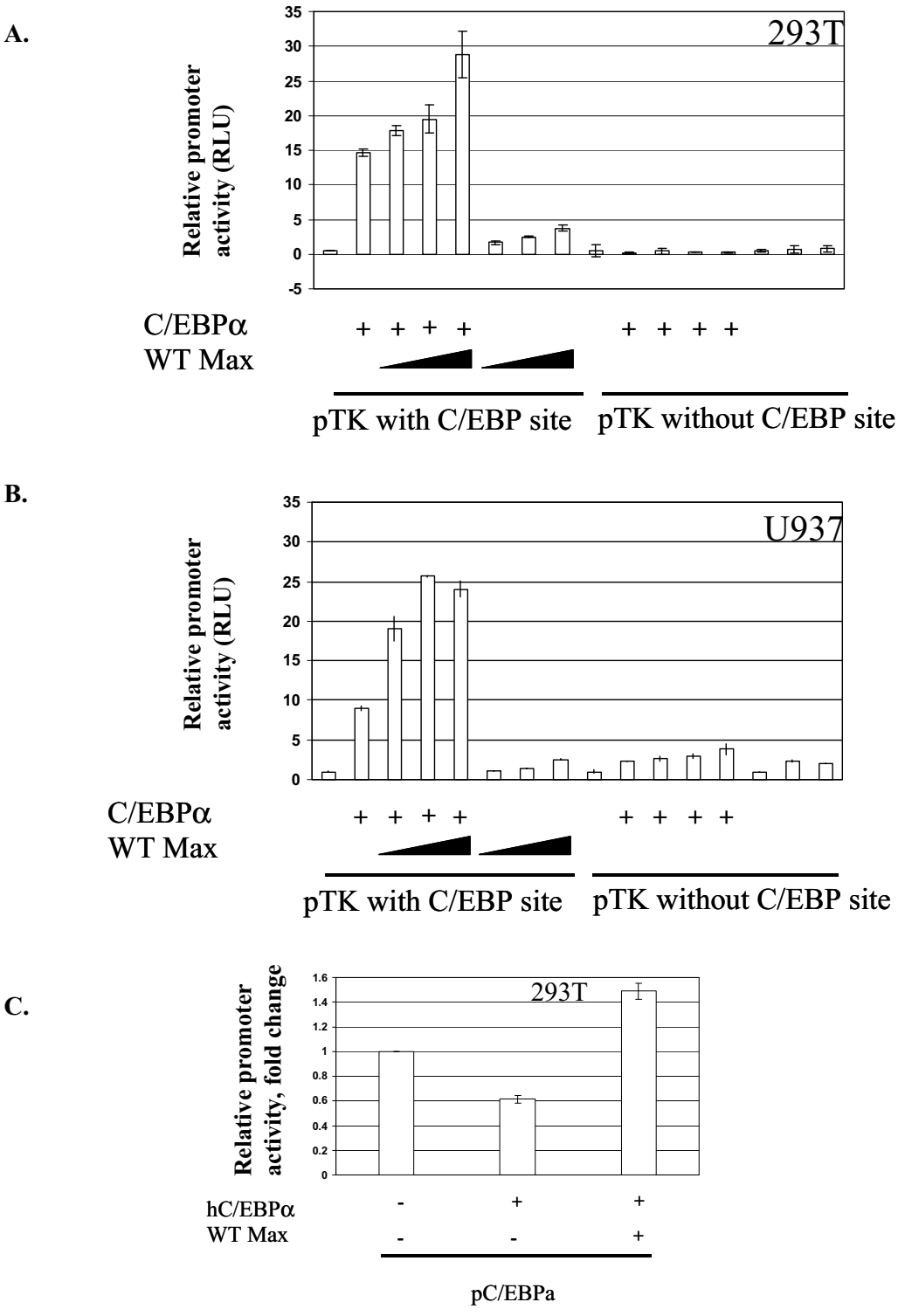


Figure 16. Max enhances the transactivation capacity of C/EBPα in transient transfection assays. **A, B)** Transient transfection in 293T and U937 cells with a reporter construct of a minimal TK promoter with CEBP binding sites only p(CEBP)2TK and expression plasmids for hC/EBPα and Max. pTK (without 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 coexpressed.

3.1.6 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 17). Chromatin was subjected to IP by using antibodies directed against C/EBPα, c-Myc and Max. The presence of Max in the C/EBPα promoter was detected by amplifying a promoter region using primers specific for a 280 bp region of the C/EBPα promoter that encompasses the CACGTG site (commonly referred to as E-box; Figure 17A). The E-box is conserved in the human and mouse C/EBPα promoter (Figure 17A). We observed that under normal physiological conditions (uninduced), endogenous c-Myc and Max were present on the C/EBPα promoter and there was undetectable endogenous C/EBPα occupancy on the hC/EBPα promoter (Figure 17B). IP using an isotype-matched IgG served as a negative control.

We next investigated the effect on heterocomplex formation of Max and C/EBPα at the hC/EBPα promoter upon differentiation by RA. We observed that both Max and C/EBPα appeared on C/EBPα promoter and in fact, more C/EBPα was associated with the promoter in the context of chromatin upon differentiation induction (Figure 17C, 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). A promoter without the CACGTG site, such as GAPDH promoter (Figure 17D), was used as a negative control for C/EBPα and Max occupancy and hTERT promoter (Figure 17E) as a positive control for Myc and Max interaction on the CACGTG site (E-box). 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.

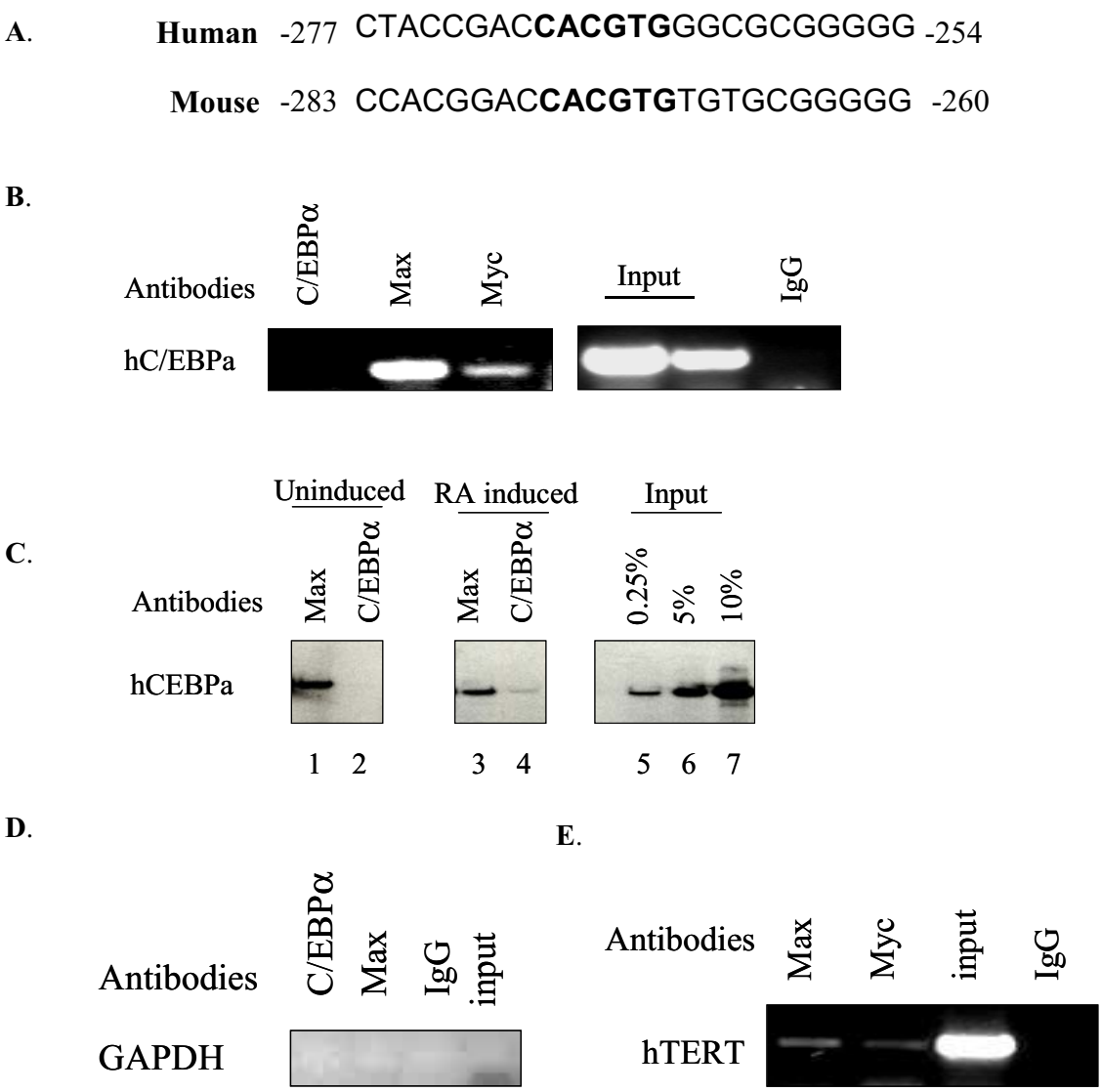


Figure 17. Max is associated at the hC/EBPα promoter in vivo and Max–C/EBPα associate strongly during granulocytic differentiation. ChIP assay was performed with untreated and RA-treated U937 cells, and the precipitated chromatin was PCR-amplified using specific primers.**A)** Comparison of the human and mouse C/EBP promoters encompassing a consensus CACGTG 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. **D, E)** ChIP assay using GAPDH promoter (negative control) and human TERT promoter (positive control with a non-radioactive RT-PCR.

3.1.7 Overexpression of Max and C/EBPα promotes differentiation along the granulocytic pathway in human hematopoietic CD34$^+$ cells

We next asked whether the interaction of Max with C/EBPα is biologically important for C/EBPα functions. For this, we performed overexpression studies using human hematopoietic CD34$^+$ 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 18, upper panel) and CD15+ (Figure 18, lower panel) cells compared to the mock-transfected control, respectively.

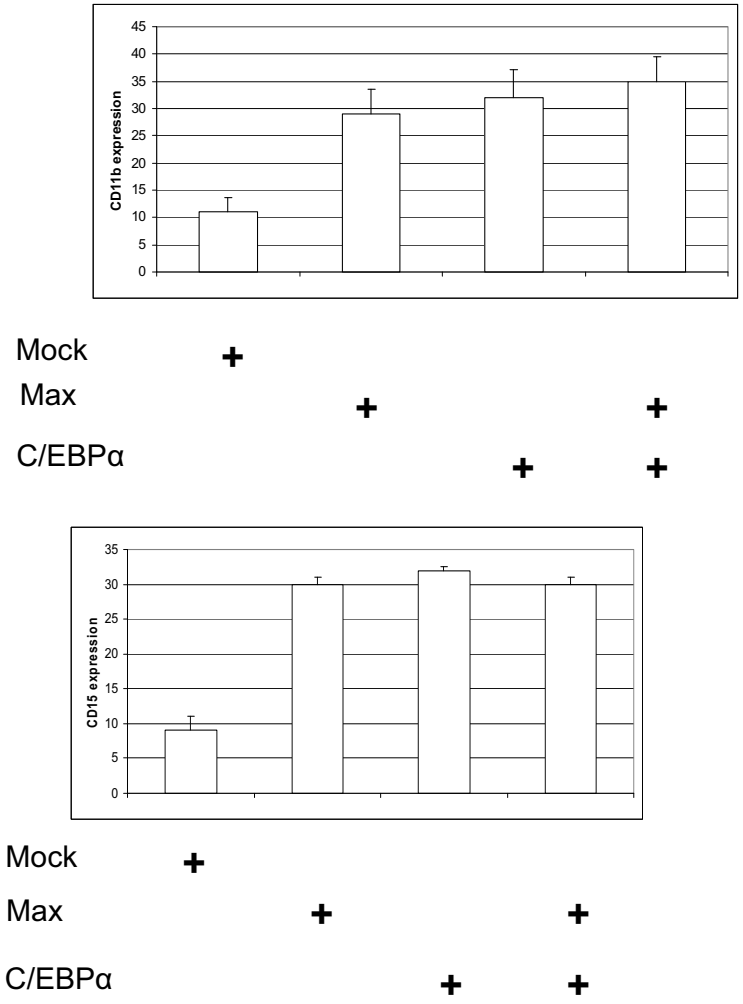


Figure 18. Overexpression of Max induces gramulocyte differentiation. The expression plasmids for human C/EBPα and Max were transfected into human hematopoietic CD34+ cells by using AMAXA. The surface expression of CD11b and CD15 was analyzed by flow cytometry at day 4. The histograms represent data from three different experiments.

3.1.8 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 by using short hairpin RNA (shRNA) against Max and control shRNA. 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 for two days. 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 ($CD15^+$) as compared with the mock-transfected $CD34^+$ (Figure 19A). Coexpression of Max shRNA led to a significant decrease in $CD15^+$ population (about two fold), whereas control shRNA did not lead to any significant reduction in $CD15^+$ population (Figure 19A, 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 19B). Thus, Max is important for C/EBPα-mediated effects on granulocytic differentiation and might have an important role in stem cell development.

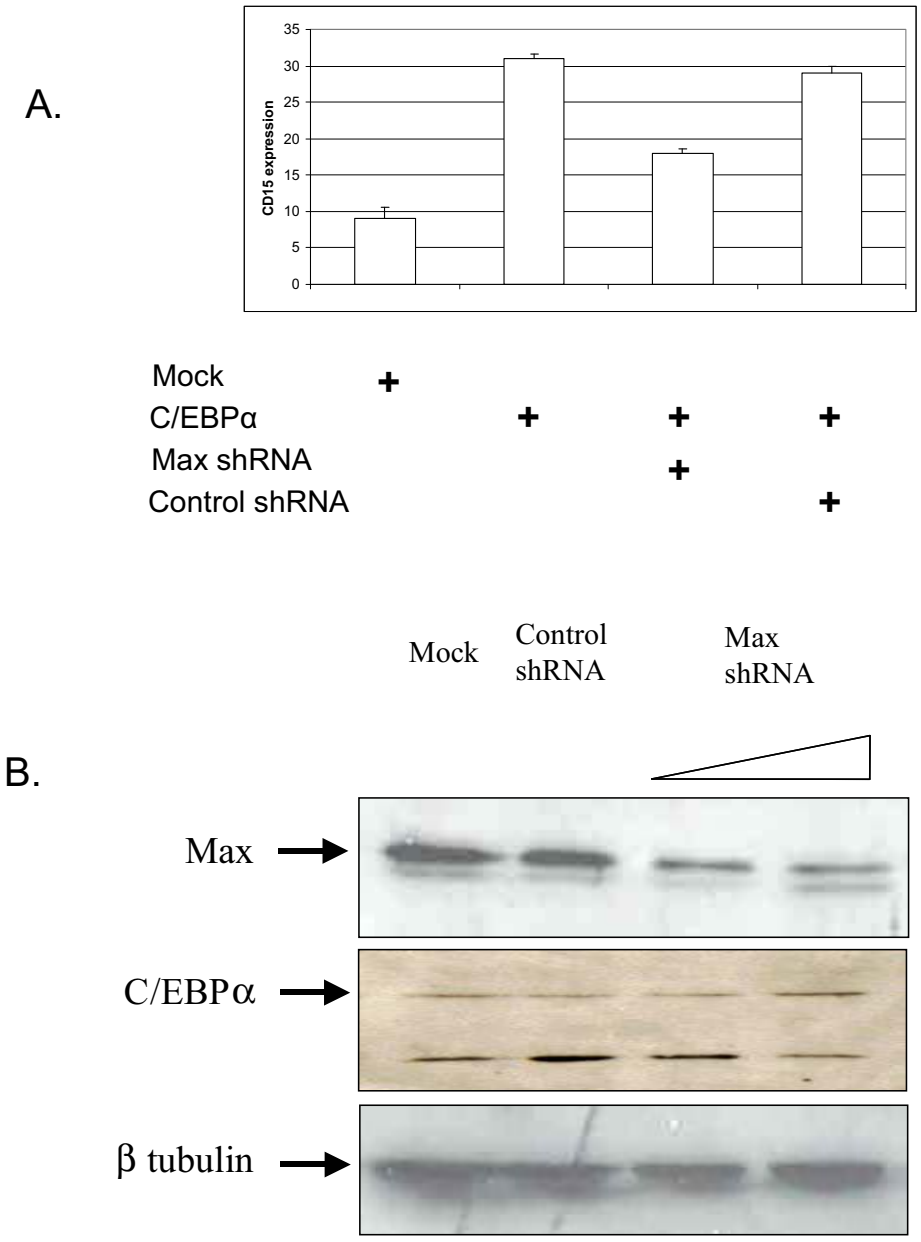


Figure 19. Stable silencing of Max by shRNA inhibits C/EBPα induced differentiation in human hematopoietic CD34$^+$ cells. A) The expression plasmid for human C/EBPα and/or expression Arrest shRNA Max plasmid (Open Biosystems, Cat. No. RHS1764-9690535) were transfected into human hematopoietic CD34$^+$ cells by AMAXA transfection method. After their selection in puromycine, the cells were analyzed for the surface expression of CD15 by flow cytometry. Control shRNA was also used in all the experiments and is shown. The histograms represent the data from three different experiments. **B)** 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.

3.2 The critical role of PIN1 upregulation in Acute Myeloid Leukemia with C/EBPα mutation

3.2.1 C/EBPα-p30 induces PIN1 mRNA expression in myeloid cells

It has been shown that mutant C/EBPα (C/EBPα-p30) blocks the wild type C/EBPα in a dominant negative manner and has been shown to block the differentiation of myeloid precursor cells (Pabst et al., 2001b). Recent findings suggest that C/EBPα mediated E2F repression as critical step in myeloid differentiation programme and lack of this repression provides a platform on which AML like syndrome evolve (Schuster and Porse, 2006). Based on this, we asked how E2F target genes could have a role in the differentiation block observed in AML with C/EBPα mutation. Growing number of studies support the oncogenic potential of PIN1, which is an E2F1 target gene. We investigated how C/EBPα-p30 regulates PIN1 levels. We chose K562-ER cell lines (D'Alo et al., 2003) as well as Kasumi-6 cells (Asou et al., 2003) for the initial experiments. K562-ER cells are an early multipotential line derived from the K562 cell line, which was obtained from a patient with chronic myeloid leukemia. Kasumi-6 is a myeloid leukemia cell line established from the bone marrow cells of an individual with acute myeloid leukemia, subtype M2 having C/EBPα mutation and expressing both the p42 and p30 isoforms of the C/EBPα protein endogenously. We induced C/EBPα-p30 by ß-Estradiol treatment in the K562-C/EBPα-p30-ER cell line. We observed that C/EBPα-p30 induces PIN1 mRNA expression (Figure 20A). Next we overexpressed C/EBPα-p30 by AMAXA transfection in Kasumi-6 cells. Similar results were obtained with myeloid Kasumi-6 cells also (Figure 20B).

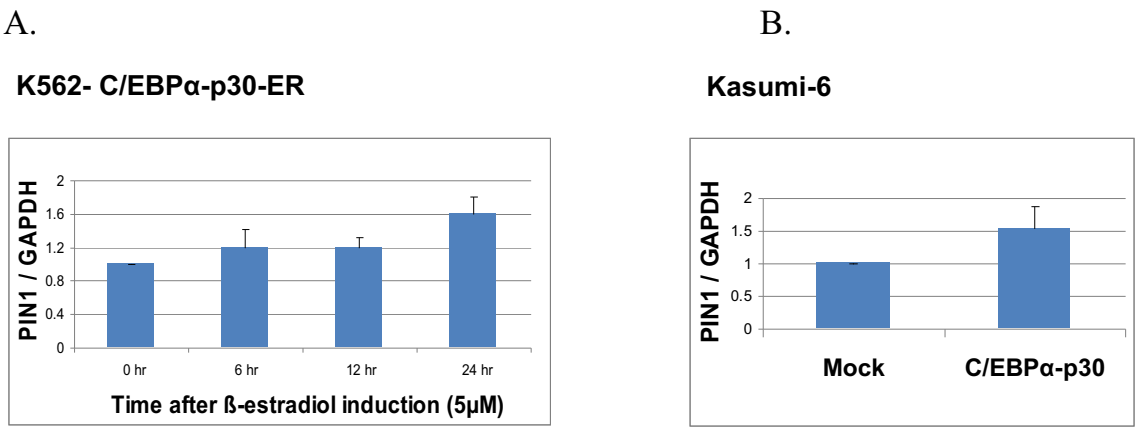


Figure 20. C/EBPα-p30 induces PIN1 mRNA expression. **A)** K562- C/EBPα-p30-ER cells were induced with ß Estradiol for respective time points to induce C/EBPα-p30 and total RNA was isolated at respective time points, proceeded for reverse transcription followed by quantitative real time PCR for PIN1 **B)** Kasumi-6 cells were transfected with mock vector or C/EBPα-p30 vector by AMAXA and isolated total RNA after 24 hr and proceeded for Real time PCR for PIN1. Values were normalized with GAPDH mRNA. The results are the means from three independent experiments, and error bars represent the standard errors of mean.

3.2.2 PIN1 is upregulated in different AML subypes including AML with C/EBPα mutations.

It is suggested that PIN1 overexpression can function as a critical catalyst that amplifies multiple oncogenic signaling pathways during oncogenesis (Ryo et al., 2003). Recent studies show that PIN1 is overexpressed in many cancers including prostate, breast, lung, ovary and colon cancer (Bao et al., 2004). PIN1 is transcriptionally regulated by E2F (Ryo et al., 2002). Given the fact that C/EBPα mediated E2F repression is a critical step in granulopoiesis (Porse et al., 2001) and that C/EBPα is inactivated by various mechanisms in AML including mutations of C/EBPα itself, we hypothesized that PIN1 expression in AML patient samples could be high in comparison to normal control samples. mRNA expression analysis and Western blot analysis show that PIN1 mRNA and protein are upregulated in various AML subtypes including AML with C/EBPα mutation (Figure 21).

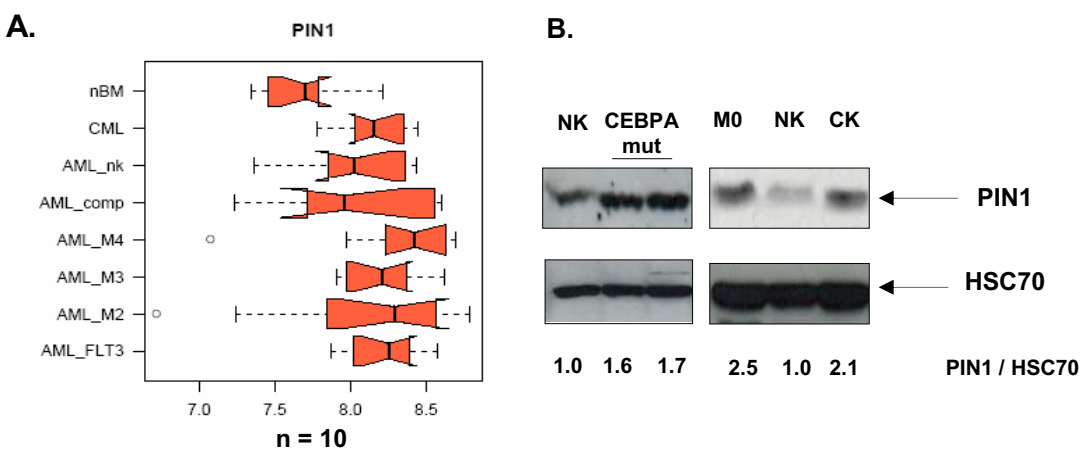


Figure 21. PIN1 mRNA and protein expression is high in different subtypes of leukemia. **A)** Affymetrix analysis was done with AML blast cells using total RNA isolated from leukemic patient samples, processed and analyzed on the Affymetrix HG-U133A and HG-U133B chips. Expression signal intensities are expressed in logarithmic scale. **B)** Western blot analyis for Pin1 using lysates prepared from AML blast cells. nBM: normal bone marrow, CML: chronic myeloid leukemia, AML NK: Normal karyotype, AML Comp (CK): Complex karyotype, AML M4: AML with the CBF/MYH11 fusion gene, AML M3: AML with PML/RARA fusion gene, AML M2: AML with AML1/ETO fusion gene, AML FLT3: AML with FLT3 activating mutation and CEBPA Mut : AML with CEBPA mutation.

3.2.3 Silencing PIN1 overcomes the dominant negative action of the mutant C/EBPα over the wild type protein in a promoter assay

To investigate the functional importance of PIN1 upregulation by C/EBPα-p30 and how this can be important for the dominant negative function of C/EBPα-p30, we performed reporter gene assays in 293T cells. Cells were transfected with a minimal TK promoter containing two CCAAT binding sites cloned upstream of the luciferase reporter gene (pTK CEBPA). Transfection of wild type C/EBPα enhanced the promoter activity and C/EBPα-p30 was able to block the promoter activity of the wild type protein (Figure 22). Transfection of PIN1 siRNA (Cat. No. SI02662128; Qiagen, Germany) was shown to overcome the dominant negative effect of C/EBPα-p30, while control siRNA (Cat. No 10277280; Qiagen, Germany) didn't make any effect. No effect was observed when cells were transfected with PIN1 siRNA / control siRNA alone.

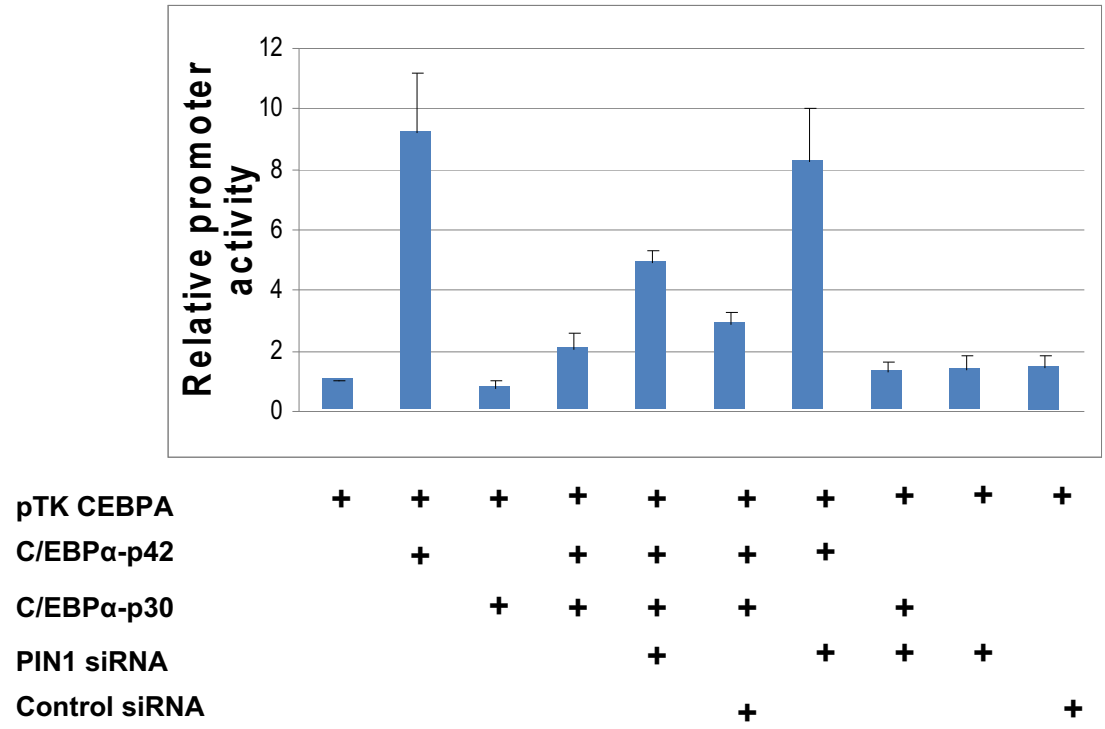


Figure 22. Silencing of PIN1 overcomes the dominant negative action of C/EBPα-p30 over the wild type protein in promoter assay. Transient transfection in 293T cells with a reporter construct having minimal TK promoter with CEBP binding sites and expression plasmids for C/EBPα–p42, C/EBPα–p30, PIN1 siRNA and control siRNA. Luciferase activities were measured 24 h after transfection and the values were normalized by using Renilla luciferase PRL0. The results are the means from three independent experiments, and error bars represent the standard errors of mean.

3.2.4 PIN1 inhibition by PiB can overcome the differentiation block observed in human myeloid cells.

To further understand how silencing of PIN1 activity is biologically significant in the context of C/EBPα mutation, we carried out myeloid differentiation experiments in AML blast cells with C/EBPα mutation and Kasumi-6 cells. For silencing PIN1, we used the PIN1 inhibitor, PiB (Uchida et al., 2003). PiB has been shown to inhibit PIN1 activity by binding to the PPIase domain of PIN1. Myeloid cell differentiation was assessed by CD11b and CD15 expression by FACS analysis as well as G-CSFR expression by Real Time RT-PCR. As shown in figure 23, treatment of cells with PiB was able to induce the differentiation.

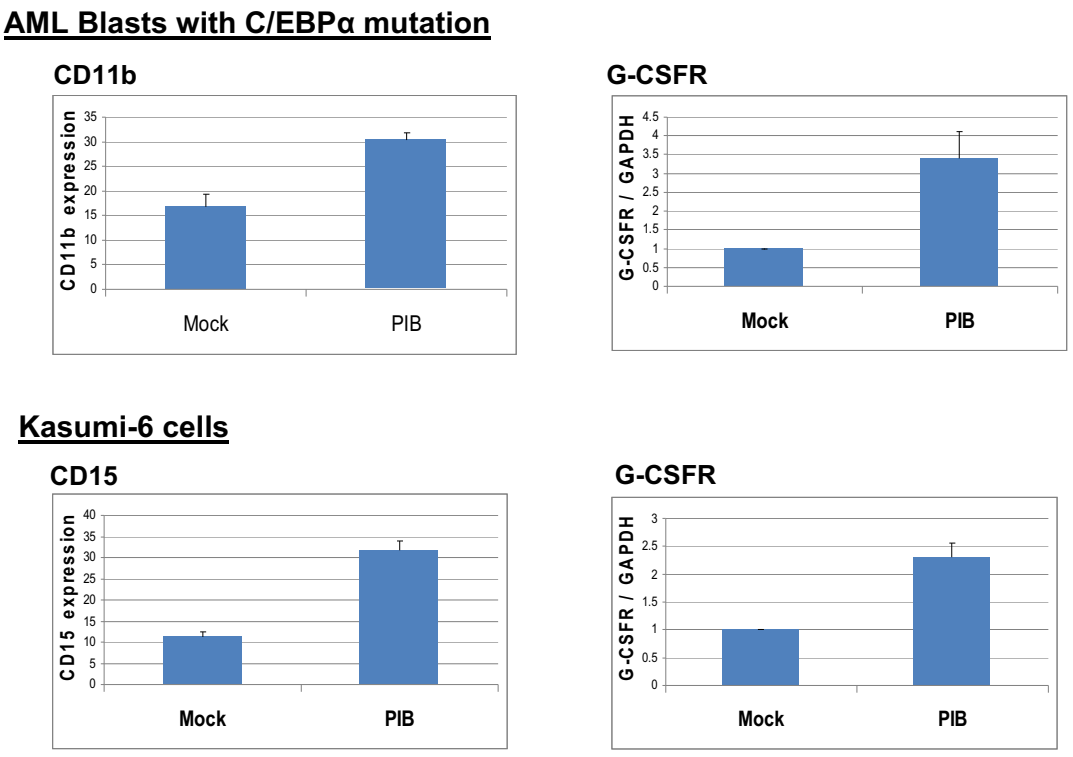


Figure 23. PIN1 inhibition using the PiB can overcome the differentiation block observed in human myeloid cells. AML blast cells with C/EBPα mutation and Kasumi-6 cells were treated with PiB (5μM) for 6 days and myeloid cell differentiation was assessed by FACS analysis using CD11b and CD15 expression as well G-CSFRexpression by Real Time RT-PCR analysis. The results are the means from three independent experiments, and error bars represent the standard errors of mean.

3.2.5 PIN1 inhibition can upregulate C/EBPα-p42 protein level.

C/EBPα mutations are characterized by low C/EBPα wild type protein level (Pabst et al., 2001b). We have shown that inhibition of PIN1 can induce differentiation in myeloid cells (Figure 23). We hypothesized that inhibition of PIN1 could upregulate the C/EBPα wild type protein level. We induced Kasumi-6 cells with PIN1 inhibitor, PiB for different time points followed by RIPA lysis and Western blot with C/EBPα specific antibody. As shown in figure 24, C/EBPα protein level increased during inhibition of PIN1.

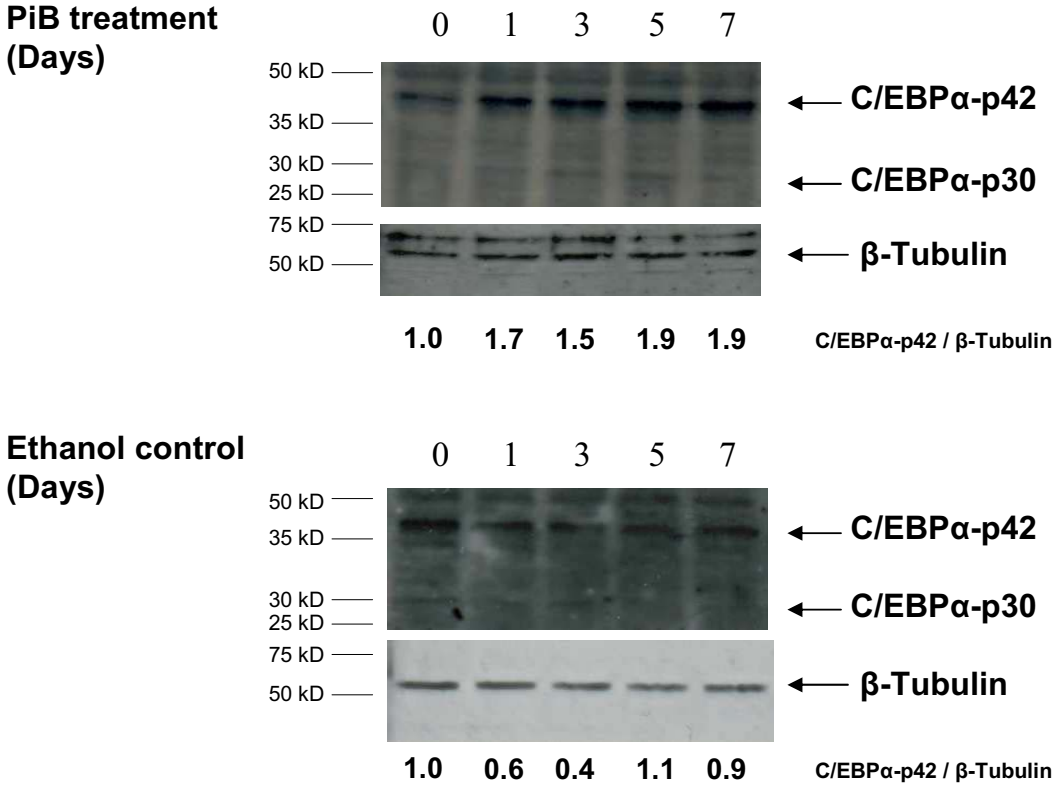


Figure 24. PIN1 inhibition by PiB can upregulate C/EBPα-p42 protein level. Kasumi-6 cells were treated PiB or ethanol for different time points. Cells were lysed and subjected to Western blot analysis using C/EBPα antibody. Values below the gel image indicate the upregulation (fold) of C/EBPα wild type protein level normalised to ß-Tubulin.

3.2.6 C/EBPα-p30 induces PIN1 promoter activity in association with E2F1 and C/EBPα-p42 interferes with the transactivation of the PIN1 promoter

Since we could observe C/EBPα-p30 induces PIN1 expression, we next investigated how C/EBPα proteins regulate PIN1 mRNA level. PIN1 is transcriptionally regulated by E2F (Ryo et al., 2002). E2F repression by C/EBPα is necessary for granulopoiesis and loss of E2F inhibition by C/EBPα has been shown to be sufficient to initiate AML like transformation of the granulocytic lineage (Porse et al., 2005). C/EBPα-p30 lacks the domain necessary for E2F inhibition. We hypothesized that in the presence of C/EBPα-p30, E2F is able to transactivate PIN1 promoter activity and/or C/EBPα-p30 can increase the transactivation in cooperation with E2F. We performed luciferase promoter assay for PIN1 promoter (Ryo et al., 2002) with E2F, C/EBPα-p30 and C/EBPα-

p42. We observed that C/EBPα-p30 could increase the transactivation of PIN1 promoter activity in association with E2F1 (Figure 25). However C/EBPα-p30 alone did not increase PIN1 promoter activity. C/EBPα-p42 was able to interfere with endogeneous as well as E2F1 mediated transactivation of PIN1 promoter activity.

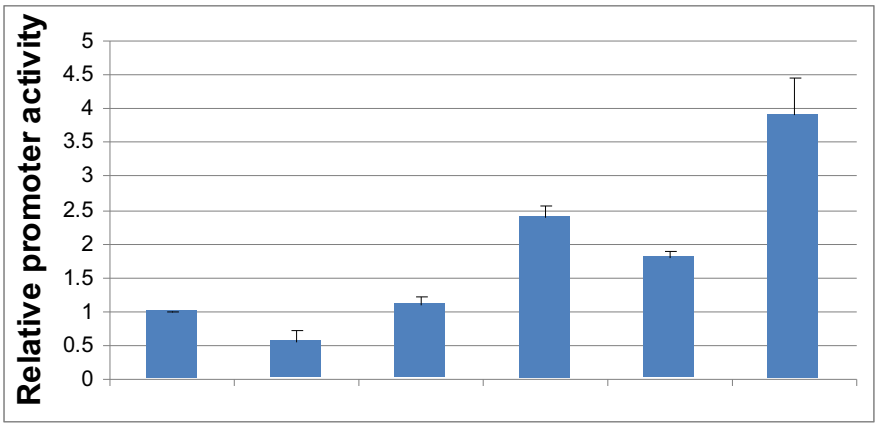


Figure 25. C/EBPα-p42 interferes with transactivation of the PIN1 promoter; C/EBPα-p30 induces PIN1 promoter activity in association with E2F1. Transient transfection in 293T cells with a reporter construct of 2.3 kb of the human PIN1 promoter cloned in pGL3 basic vector and expression plasmids for C/EBPα–p42, C/EBPα–p30 and E2F1. Luciferase activities were measured 24 h after transfection and values were normalized by using Renilla luciferase PRL0. The results are the means from three independent experiments, and error bars represent the standard errors of mean.

3.2.7 C/EBPα-p42 downregulates PIN1 expression

Based on our finding that C/EBPα-p42 interferes with transactivation of the PIN1 promoter (Figure 25), we next asked how C/EBPα-p42 regulates PIN1 mRNA level. We induced the C/EBPα-p42 by ß-Estradiol treatment in K562-C/EBPα-p42-ER cell lines for various time intervals. As shown in figure 26, wild type C/EBPα is able to down regulate PIN1 expression which is opposite to the upregulation of PIN1 expression by the p30 form of C/EBPα, as shown in

figure 20. This demonstrates that C/EBPα wild type and its mutant p30 form can regulate genes differently. A recent study has shown that C/EBPα-p30 displays transcriptional regulatory roles distinct from the wild type C/EBPα protein (Wang et al., 2007). This study shows that C/EBPα-p30 binds to a unique set of genes more strongly than C/EBPα-p42. Our finding that PIN1 is regulated differently by p42 and p30 reflects the strikingly different biological properties of the two protein isoforms.

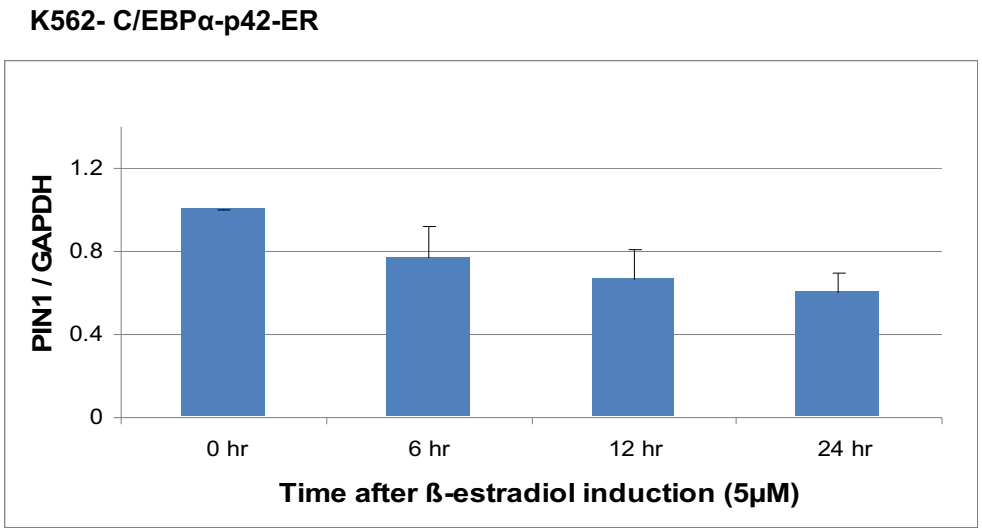


Figure 26. C/EBPα-p42 downregulates PIN1 mRNA expression. K562- C/EBPα-p42-ER cells were induced with ß-Estradiol for respective time points to induce C/EBPα-p42 and total RNA was isolated at respective time points, proceeded for reverse transcription followed by quantitative real time PCR for PIN1. Values were normalized with GAPDH mRNA. The results are the means from three independent experiments, and error bars represent the standard errors of mean.

3.2.8 PIN1 protects c-Jun from ubiquitination mediated protein degradation

PIN1 has shown to regulate the protein degradation of many targets with which it interacts (Wulf et al., 2005). Upon JNK activation, PIN1 binds c-Jun that is phosphorylated on ser63/73-Pro motifs (Wulf et al., 2001). c-Jun has been shown to be be subjected to ubiquitin mediated degradation in a JNK dependent manner (Gao et al., 2004). It has been shown previously that ubiquitination plays a central role in c-Jun degradation (Fang and Kerppola, 2004). Hence, we

hypothesized that PIN1 could play a role in regulating degradation of c-Jun. We perfirmed ubiquitination assay in 293T cells by overexpressing c-Jun, PIN1 and HA-Ubiquitin constructs. When c-Jun was co-transfected with HA.tagged ubiquitin, higher molecular weight ladders were detected in the immunoprecipitation with HA antibody that reflects c-Jun ubiqutination (Figure 27, lane 4). Overexpression of PIN1 prevents the ubiquitination of c-Jun (lane 5).

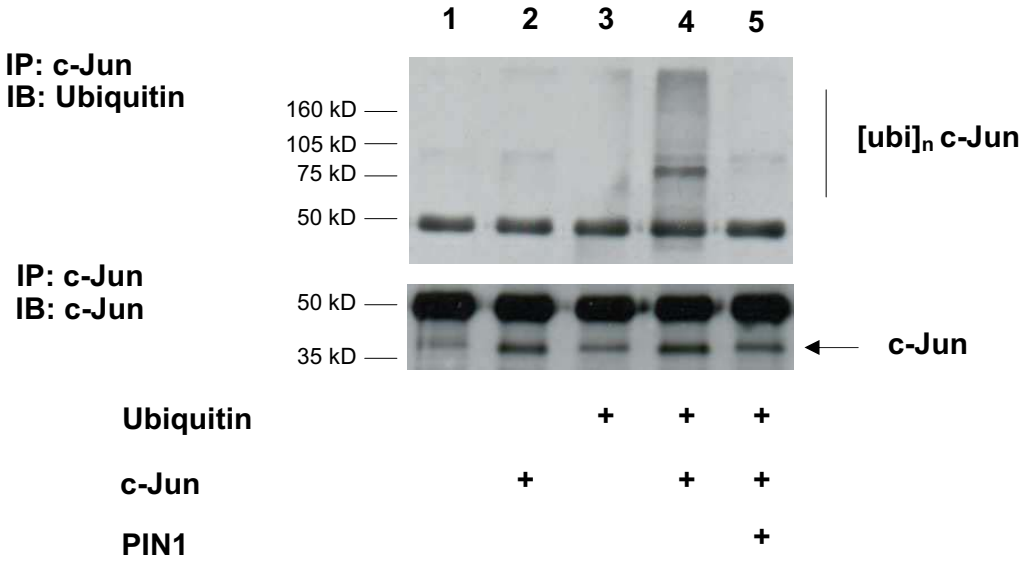


Figure 27. PIN1 protects c-Jun from ubiquitination mediated protein degradation. Ubiquitination assay was performed by transfecting 293T cells with the expression plasmids for HA-biquitin, c-Jun and PIN1 as indicated, 24 post transfection cells were lysed and c-Jun was immunoprecipitated and immunoblot against HA antibody 8upper panel). Membrane was stripped and reprobed for c-Jun (lower panel) as control for c-Jun immunoprecipitation

3.2.9 PIN1 regulates c-Jun mRNA level: overexpression of PIN1 upregulates c-Jun mRNA, while inhibition of PIN1 downregulates c-Jun

PIN1 increases the transcriptional activity of c-Jun towards cyclin D1 (Wulf et al., 2001). Interestingly, PIN1 is upregulated in response to c-Jun overexpression (Rinehart-Kim et al., 2000). c-Jun has been shown to bind to its own promoter at AP-1 sites and this has been shown to regulate c-Jun mRNA level in a positive manner (Angel et al., 1988). Our data suggests that PIN1

prevents the degradation of c-Jun (Figure 27). Based on these findings, we hypothesized that PIN1 could have a major role in regulating c-Jun mRNA level. We overexpressed PIN1 in Kasumi-6 cells and analysed the c-Jun mRNA level by quantitative Real Time PCR. We observed that overexpression of PIN1 leads to an increase in c-Jun mRNA level (Figure 28A). We next investigated the effect of silencing PIN1 on c-Jun mRNA level. We found that silencing PIN1 with the inhibitor PiB could downregulate c-Jun levels as observed by quantitative Real Time PCR (Figure 28B).

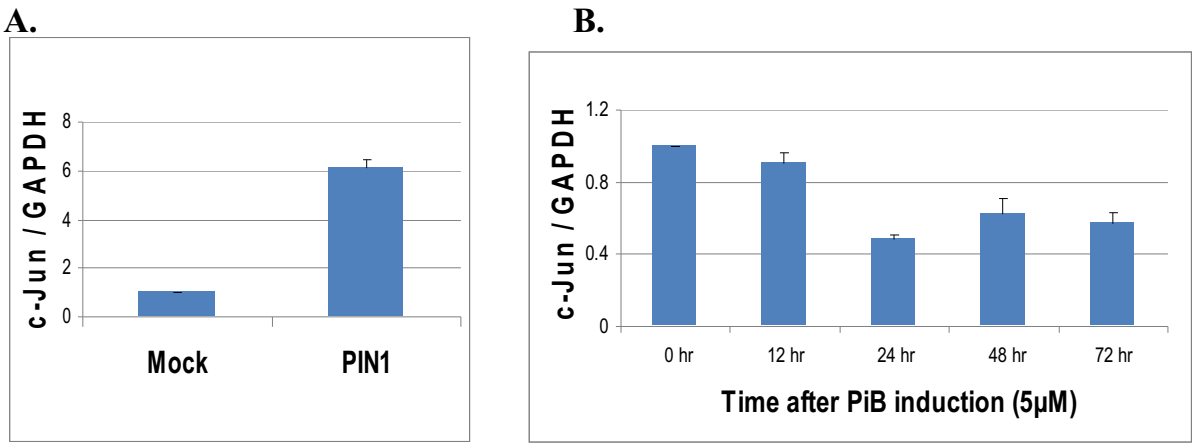


Figure 28. Overexpression of PIN1 upregulates c-Jun mRNA, while inhibition of PIN1 downregulates c-Jun. A) Kasumi-6 cells were transfected with PIN1 expression vector. Total RNA was isolated after 24 hr with TRIzol reagent and proceeded for reverse transcription followed by quantitative real time PCR for c-Jun. Values were normalized with GAPDH mRNA. **B)** Kasumi-6 cells were treated with PIN1 inhibitor, PiB for various time points and proceeded for quantitative real time PCR for c-Jun as described. The results are the means from three independent experiments, and error bars represent the standard errors of mean.

3.2.10 c-Jun blocks the transactivation capacity of C/EBPα-p42.

It was observed that livers from neonatal C/EBPα knock-out mice contain increased levels of Jun transcripts (Flodby et al., 1996). Another study showed that C/EBPα could prevent c-Jun from binding to AP-1 site through leucine zipper domain interaction (Rangatia et al., 2002). Overexpression of c-Jun was shown to block granulopoiesis (Rangatia et al., 2002). These observations show how C/EBPα and c-Jun are negatively regulating each other. Given the fact that c-Jun expression is high in different subtypes of AML including C/EBPα

mutation and c-Jun is able to block DNA binding of C/EBPα (Rangatia et al., 2003), we hypothesised that c-Jun is able to block the transactivation capacity of C/EBPα. As shown in figure 28, c-Jun is able to inhibit the transactivation capacity of C/EBPα. This finding shows how overexpressed c-Jun could block C/EBPα functions.

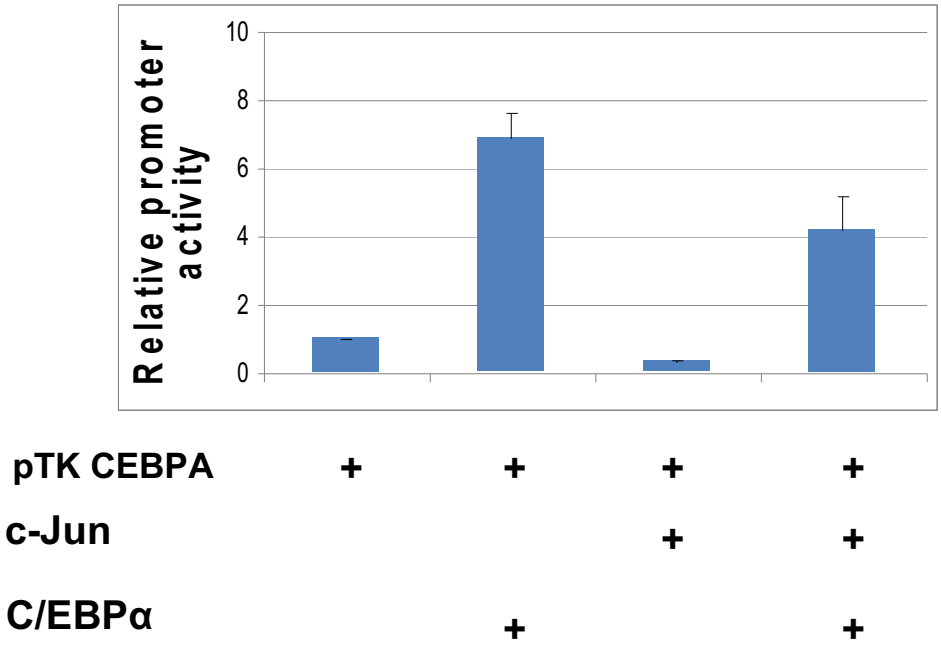


Figure 29. c-Jun can block C/EBPα-p42 transactivation capacity. Transient Transfections were carried out in 293T cells with a reporter construct having minimal TK promoter with C/EBPα binding sites and expression plasmids for C/EBPα–p42, and c-Jun. Luciferase activities were measured 24 hr after transfection and the values were normalized by using Renilla luciferase PRL0. The results are the means from three independent experiments, and error bars represent the standard errors of mean.

3.2.11 Overexpression of c-Jun blocks C/EBPα-p42 induced granulocytic differentiation.

It was shown that downregulation of the proto-oncogene c-Jun by C/EBPα is critical for granulocytic lineage commitment (Rangatia et al., 2002). Based on our findings that c-Jun is able to block transactivation capacity of C/EBPα (Figure 29) and DNA binding of C/EBPα (Rangatia et al., 2003), we hypothesized that c-Jun is able to block differentiation induced by C/EBPα. We

overexpressed C/EBPα and c-Jun in U937 cells by AMAXA transfection method. Four days after transfection, granulocyte differentiation was assessed by FACS analysis for CD15 expression. The increase in CD15 expression by C/EBPα was blocked in the presence of c-Jun (Figure 30). c-Jun transfected cells were also investigated for CD15 expression; however, no increase in its expression was observed (data not shown).

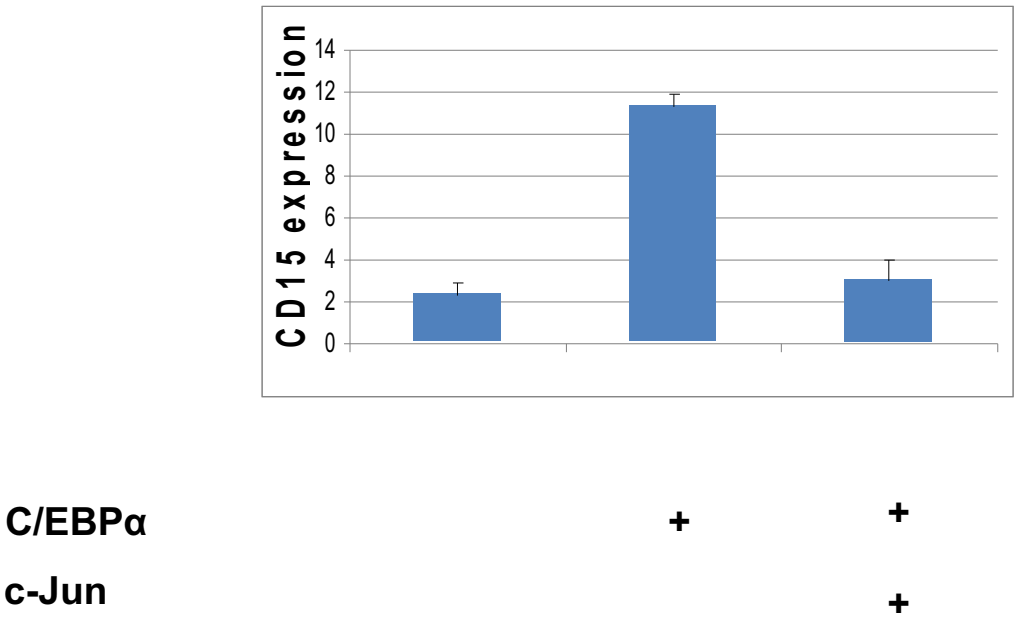


Figure 30. Overexpression of c-Jun blocks C/EBPα-p42 induced granulocytic differentiation. U937 cells were transfected with c-Jun and C/EBPα as indicated and granulocytic differentiation was investigated by FACS analysis for CD15 expression. Error bars indicate the standard errors of means from three independent experiments.

4. Discussion

4.1 Max as a novel co-activator of myeloid transcription factor C/EBPα

It has become increasingly clear that the 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 (Rangatia et al., 2002; Reddy et al., 2002) relatively little is known about how C/EBPα interacts with other nuclear proteins to regulate gene transcription. The results presented in this work provide evidence that Max, a heterodimerization partner of Myc, is a novel, functionally and biologically important co-activator of C/EBPα. C/EBPα and Max not only colocalize but also the heterocomplex of C/EBPα and Max is preferentially formed on the hC/EBPα promoter during granulocytic differentiation, thereby contributing to increased transactivation and differentiation capacity of C/EBPα.

We used mass spectrometry based proteomic analysis as a means of identifying the interacting partners of C/EBPα, utilizing immunoprecipitation of C/EBPα from myeloid U937 cells. U937 cells are a good model system for studying myeloid differentiation in general, as they are bipotential and can be differentiated into the granulocytic lineage by RA. In particular, a threefold increase in C/EBPα protein (above the level of endogenous C/EBPα) in U937 cells is sufficient for their granulocytic differentiation (Radomska et al., 1998). In addition to nine other proteins (see Table 2), we identified Max, an essential heterodimerization partner of Myc (Blackwood et al., 1991), as a novel interacting partner of C/EBPα in our screen (Figure 12). The discovery of Max as a novel C/EBPα partner is intriguing because of the role Max plays in switching from Myc-Max to Mad-Max heterocomplexes during myeloid

differentiation (Ayer and Eisenman, 1993). 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 (Metcalf et al., 1995). Additionally, Max is an essential heterodimerization partner of Myc family members to regulate transcription (Grandori et al., 2000) and c-Myc is an important target of C/EBPα (Johansen et al., 2001). We confirmed the *in vivo* interaction of C/EBPα with Max by immunoprecipitation and showed that the basic DNA-binding region of C/EBPα is involved in this interaction, as the mutant of C/EBPα (C/EBPα BR3), which lacks the DNA-binding region, could not be co-precipitated with Max (Figures 13, 14). C/EBPα BR3 carries mutations in four amino acids, residues Arg297, Lys298, Arg300 and Lys302 (Liu et al., 2003). 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 (Porse et al., 2001). Further study is required to pin point the exact amino acids involved in the C/EBPα and Max interaction.

The endogenous C/EBPα and Max proteins are not distributed evenly throughout the nucleoplasm (Figure 15), but are localized in intranuclear structures within the nucleus. In other cell systems, such as pituitary progenitor GHFT1-5 cells, C/EBPα has been shown to concentrate at chromatin surrounding the centromeres (Schaufele et al., 2001). The observation that C/EBPα–Max but not Myc–Max remain colocalized during granulocytic differentiation (Figure 15) indicates that these intranuclear structures 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. To our knowledge,

this is a first report showing occupancy of the hC/EBPα promoter by Max *in vivo*.

The occupancy of the hC/EBPα promoter by Max 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 (Legraverend et al., 1993). 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 (Timchenko et al., 1995). Our colocalization and ChIP data (Figures 15 and 17) and the data that C/EBPα is co-precipitated with Myc (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. Given that the C/EBPα–Max heterocomplex is formed on the 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 induce granulocytic differentiation (Figure 18). The role of C/EBPα in the transition from CMPs to GMPs in myeloid progenitors has been recently characterized (Zhang et al., 2004). The role of Max in inducing granulocytic differentiation indicates that Max can activate the myeloid differentiation programme either independent of C/EBPα or in association with it. *In vivo* interaction and retention of C/EBPα–Max heterocomplex in myeloid cells (Figures 13, 14 and 15) and inhibition of differentiation-inducing capacity of C/EBPα by silencing of Max using shRNA against MAX in $CD34^+$ cells

(Figure 19) suggest that the C/EBPα–Max association likely plays an important role in this process of myeloid progenitor differentiation. A very recent study has shown the role of C/EBPα in monopoiesis (Wang et al., 2006). 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 C/EBPα and has important co-activator functions for C/EBPα induced granulocytic differentiation in myeloid progenitors.

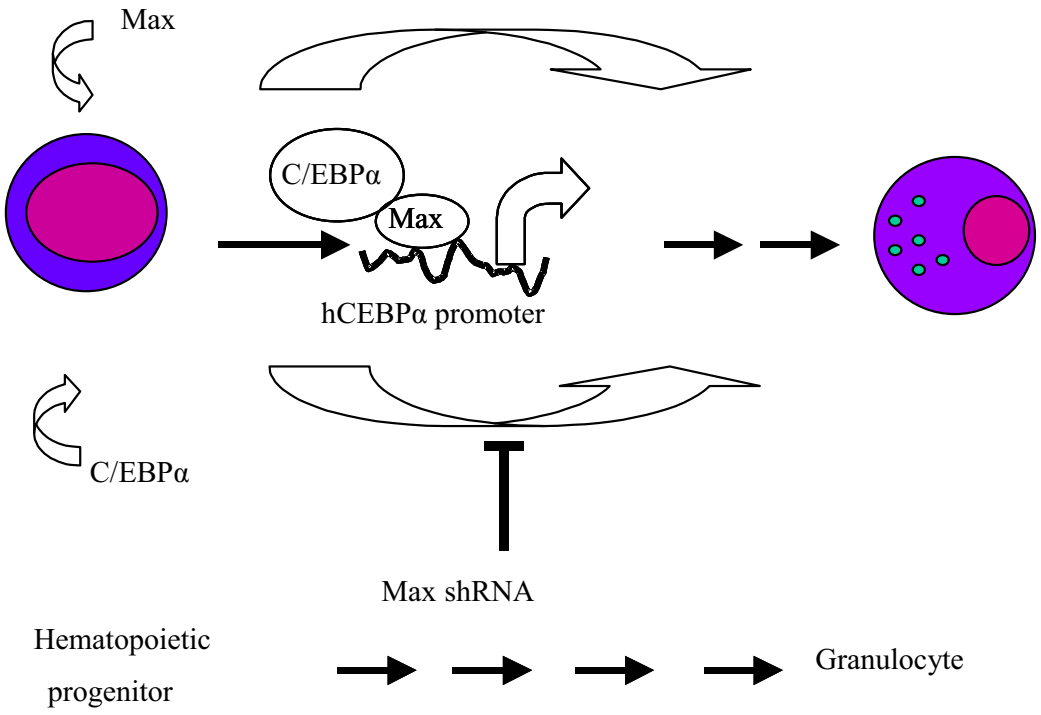


Figure 31. Model summarizing the importance of Max as a co-activator of C/EBPα in the differentiation of myeloid progenitors. During granulocyte differentiation Max-C/EBPα complex occupies human C/EBPα promoter. Max acts as a co-activator for C/EBPα and induces differentiation along the granulocytic pathway. Meanwhile, silencing of Max inhibits C/EBPα induced differentiation.

4.2 The critical role of PIN1 in AML with C/EBPα mutation

It is widely accepted that C/EBPα functions as a tumor suppressor in multiple tissues (Schuster and Porse, 2006). Experimental data from animal models as well as AML patient samples suggest that the loss of function or expression of C/EBPα provides a platform on which AML develops. Even though a number of different laboratories have pointed out that C/EBPα is mutated in around 9% of AML, the mechanism with which the mutated dominant negative form of C/EBPα i.e., C/EBPα-p30 mediates a differentiation block is poorly understood. Our findings suggest that C/EBPα-p30 might induce PIN1 expression and increase the stability of c-Jun, which in turn inhibits the function of wild type C/EBPα.

Initially, it was suggested that the dominant negative effect of C/EBPα-p30 is the result of heterodimer formation with wild type C/EBPα. A recent report shows that a C/EBPα-p30 with modification in the leucine zipper, which can not form heterodimers with C/EBPα-p42, still exhibits dominant negative properties over the wild type protein (Cammenga et al., 2005). This suggests that rather than heterodimerization, regulatory networks activated by C/EBPα-p30 could play a critical role in its dominant negative function over the wild type protein. One recent study showed that C/EBPα-p30 has quite distinct functional properties compared to the wild type protein (Wang et al., 2007). This study showed that C/EBPα-p30 binds to a unique set of genes more strongly than C/EBPα-p42. Mice with targeted disruption of C/EBPα-p42 while expressing C/EBPα-p30 develop leukemia (Kirstetter et al., 2008). This study is of particular interest since C/EBPα knock-out mice did not develop leukemia even though they exhibited a block of granulocytic differentiation (Zhang et al., 2004). These findings suggest that disruption of wild type C/EBPα alone is not sufficient to initiate leukemogenesis, but that pathways modulated by C/EBPα-p30 might be critical role in the development of AML.

Our finding that PIN1 is upregulated upon C/EBPα-p30 induction is intriguing since PIN1 overexpression was shown to act as a critical catalyst that amplifies multiple signaling pathways during oncogenesis (Ryo et al., 2003). Also, lack of E2F inhibition by C/EBPα has been shown to be a key event in AML (Porse et al., 2005) and PIN1 has been shown to be regulated by E2F (Ryo et al., 2002). Microarray analysis and Western blot analysis identified elevated PIN1 expression in different subtypes of AML (Figure 21). One possible explanation for the upregulation of PIN1 in different AML subtypes could be the fact that C/EBPα is shown to be downregulated by diverse mechanisms in different subtypes of leukemia (Schuster and Porse, 2006) and wild type C/EBPα downregulates PIN1 expression (Figures 25, 26). Also, loss of cell cycle regulatory mechanisms in leukemic basts could amplify oncogenic signaling pathways and could result in positive regulation of PIN1. Ours is the first report showing PIN1 upregulation in human AML.

Previous studies showed that PIN1 knock-out mice develop normally and do not exhibit any significant phenotype at a young age although they display age dependant defects such as retinal atrophy, decreased body weight and testicular atrophy (Fujimori et al., 1999; Liou et al., 2002). However, there is no report about any phenotypic characters related to hematopoiesis in the PIN1 null animals. The important role of PIN1 in tumorigenesis is underlined by the finding that mice lacking PIN1 are resistant to tumorigenesis induced by oncogenic Neu or Ras (Wulf et al., 2004). This study showed that these oncogenes need certain pathways in which PIN1 has a key regulatory role. All these findings point out that PIN1 is important for oncogenesis in AML as well.

The transcriptional activation function of C/EBPα is required for the induction of granulocytic differentiation (Keeshan et al., 2003). C/EBPα-p30 blocks the transactivation ability of the wild type C/EBPα in a dominant negative manner. Our finding that silencing of PIN1 could overcome C/EBPα-p30 mediated inhibition of C/EBPα transactivation (Figure 22) suggests that PIN1 might have

an important role in regulating the transcriptional activity of C/EBPα. It has been previously shown that PIN1 can regulate the transcriptional activity of transcription factors such as p53 and p73 (Mantovani et al., 2004; Zheng et al., 2002). A recent study proposes that C/EBPα could be a PIN1 target (Miller, 2006). This study point out that several S/T-P motifs in wild type C/EBPα could be regulated by PIN1 mediated isomerization. Most of the S/T-P motifs in wild type C/EBPα are present in C/EBPα-p30 also. Even though C/EBPα has been shown to be phosphorylated, there is no report about PIN1 mediated post phosphorylation mechanisms regulating C/EBPα function. Further studies are needed to explain how PIN1 regulates C/EBPα mediated transactivation.

It is known that C/EBPα-p30 not only fails to induce differentiation but also blocks the C/EBPα mediated granulocytic differentiation. Based on our findings that PIN1 is upregulated in AML (Figure 21) and silencing PIN1 could overcome the dominant negative action of C/EBPα-p30 over the wild type protein in promoter assay (Figure 22), we assessed how silencing of PIN1 affects myeloid differentiation. Differentiation experiments show that silencing PIN1 can overcome the differentiation block of AML blast cells with C/EBPα mutation and Kasumi-6 cells during granulopoiesis (Figure 23).

Human AML with C/EBPα mutation have a decreased levels of wild type C/EBPα-p42 and increased levels of C/EBPα-p30 (Leroy et al., 2005). Another study has shown that C/EBPα-p30 dominantly inhibits the wild type protein function when the C/EBPα-p42 to C/EBPα-p30 ratio is less than one (Calkhoven et al., 2000). The concentration of certain transcription factors is critical in hematopoiesis (Rosenbauer et al., 2004). For example, knocking down PU.1 below a threshold level has been shown to lead to a preleukemic phase which is susceptible to progress to leukemia when additional mutations are present. Even though such studies have not been carried out for C/EBPα, it seems that a similar mechanism might be relevant for C/EBPα. In AML with C/EBPα mutation, the wild type C/EBPα protein is expressed at lower levels than

normal. However, at this level, C/EBPα fails to promote differentiation. Whether this accounts for haploinsufficency awaits further studies. Our findings that silencing PIN1 can upregulate C/EBPα-p42 protein levels (Figure 24) and lead to myeloid differentiation (Figure 23) suggest that PIN1 might have a major role in contributing the differentiation block observed during C/EBPα mutation.

The mechanisms by which C/EBPα inhibits cell proliferation seem to differ from cell type to cell type. In granulopoiesis, inhibition of E2F activity has been proved as the unique mechanism for the anti-mitotic activity of C/EBPα (Porse et al., 2005; Porse et al., 2001). E2F1 activates transcription of the c-Myc oncogene, which had been shown to block granulopoeisis. C/EBPα inhibition of E2F1 has been shown to result in the downregulation of c-Myc, leading to granulopoeisis (Johansen et al., 2001). Even though the exact mechanism through which C/EBPα mediates E2F1 repression is not well understood, an increasing number of studies suggest that inhibition of E2F1 activity is one of the key events orchestrated by C/EBPα during the myeloid differentiation programme (Rosenbauer and Tenen, 2007). This raises the possibility that other E2F1 targets could be also regulated by C/EBPα in a similar mechanism as observed for c-Myc. Our observation that C/EBPα negatively regulates the PIN1 promoter (Figure 25) as well as PIN1 mRNA (Figure 26) suggests that PIN1 downregulation by C/EBPα is an important event in granulopoiesis. Loss or disruption of domains of C/EBPα necessary for E2F1 inhibition has been shown to result in block of granulocyte differentiation (D'Alo et al., 2003). Mice homozygous for a C/EBPα knock-in mutation that impairs E2F repression develop AML like transformation of the granulocytic lineage (Porse et al., 2005). At the same time, C/EBPα-p30 has been shown to be unable to inhibit E2F1 (Porse et al., 2001). Our observation that C/EBPα-p30 fails to inhibit PIN1, an E2F1 target gene, suggests intrinsic differences between C/EBPα wild type and C/EBPα-p30 in regulating PIN1 expression. Our finding that C/EBPα-p30 can increase the PIN1 promoter activity in association with E2F1 (Figure

25) suggests how regulatory networks coordinated by C/EBPα-p30 and E2F1 could play an important role in AML with C/EBPα mutation.

Previously it has been reported that PIN1 is overexpressed in breast cancer and increases the transcriptional activity of c-Jun (Wulf et al., 2001). PIN1 has been shown to bind to c-Jun and has been proposed to increase protein stability of c-Jun (Wulf et al., 2005). Rinehart-Kim et al has shown that c-Jun overexpression can result in increased expression of PIN1 (Rinehart-Kim et al., 2000). These studies demonstrate how PIN1 and c-Jun act in a positive feed back loop to control each others expression. PIN1 has shown to regulate the protein degradation of many targets with which it interacts (Wulf et al., 2005). Our finding that PIN1 could prevent c-Jun protein degradation (Figure 27) suggests that PIN1 might have a critical role in regulating c-Jun protein turn over. The stabilized c-Jun might bind to its own promoter and increase its expression in a positive feed back loop. c-Jun has been shown to be located at the end of signal cascades and to have crucial function in diverse mechanisms of oncogenesis (Vogt, 2001). The oncogenic role of c-Jun in AML is verified by the finding that c-Jun is overexpressed in different subtypes of AML (Elsasser et al., 2003; Rangatia et al., 2003). Leukemic fusion proteins such as BCR-ABL and AML1-ETO have been shown to induce c-Jun expression through JNK signaling pathway (Burgess et al., 1998; Elsasser et al., 2003). Neonatal livers of C/EBPα$^{-/-}$ mice contain increased levels of c-Jun transcript (Flodby et al., 1996). Rangatia et al demonstrated that downregulation of c-Jun expression by C/EBPα is critical for granulopoiesis (Rangatia et al., 2002). Their study showed that C/EBPα inactivates c-Jun via leucine zipper domain interaction. Interestingly, c-Jun expression is high in AML patient samples with C/EBPα mutation (Rangatia et al., 2003). One mechanism for the upregulation of c-Jun expression in AML might be the downregulation of C/EBPα by different mechanisms. The ability of c-Jun to block DNA binding of C/EBPα (Rangatia et al., 2003) as well as our finding that c-Jun is able to inhibit the transactivation capacity and

differentiation induced by C/EBPα (Figure 29,30) suggests a model in which c-Jun promotes proliferation and leads to differentiation block by inactivating C/EBPα. In other words, how c-Jun as well as C/EBPα regulate granulopoiesis depends upon their relative protein concentration - more C/EBPα favors differentiation while more c-Jun favors proliferation.

In summary, our study identifies PIN1 as an important player that might contribute to AML development through the inhibition of C/EBPα function. Here we demonstrate that PIN1 is upregulated by C/EBPα-p30 and that silencing of PIN1 was able to overcome the differentiation block mediated by C/EBPα-p30. Our observations suggest that C/EBPα-p30 exerts its dominant negative function through regulating PIN1 and c-Jun (Figure 32). Inhibiting PIN1 function could provide a novel strategy in the treatment of AML patients.

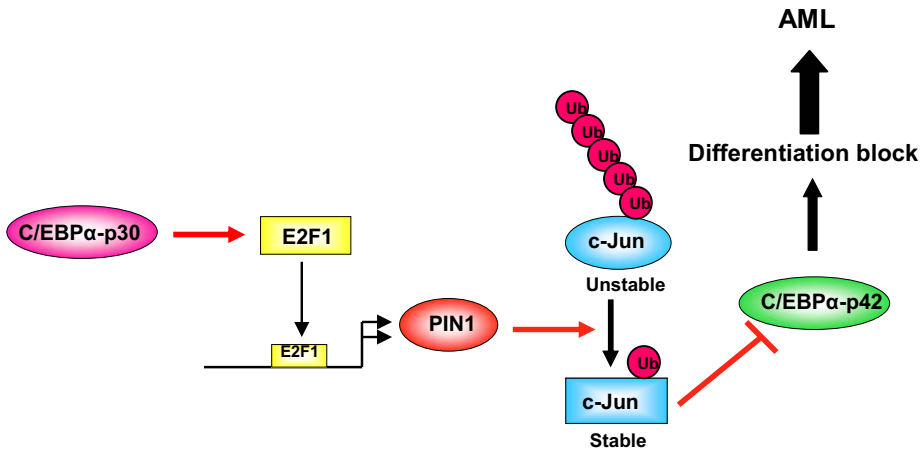


Figure 32. Model depicting the PIN1 mediated dominant negative role of C/EBPα-p30 in AML with C/EBPα mutation. C/EBPα–p30 cooperates with E2F1 and increase the PIN1 mRNA levels. PIN1 prevents the protein degradation of c-Jun, which in turn blocks C/EBPα functions and leads to differentiation block.

5. Summary

Transcription factor CCAAT enhancer binding protein α (C/EBPα) is crucial for the differentiation of granulocytes. In the present study, we sought to identify novel C/EBPα interacting proteins *in vivo* through immunoprecipitation and subsequent mass spectrometry based identification. We identified Max, a heterodimeric partner of Myc, as one of the interacting proteins of C/EBPα in our screen. We demonstrate that 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 as shown by promoter assay. Chromatin immunoprecipitation assays reveal occupancy of the human C/EBPα promoter by C/EBPα and Max under retinoic acid induced granulocytic differentiation. FACS analyis using granulocytic markers shows that enforced expression of Max and C/EBPα result in granulocytic differentiation of human hematopoietic CD34+ cells. Silencing of Max reduced the differentiation inducing potential of C/EBPα, indicating the importance of the C/EBPα–Max interaction in myeloid progenitor differentiation. Taken together, our findings demonstrate that Max as a novel co-activator of C/EBPα.

Loss of C/EBPα expression or function in leukemic blasts contributes to a block in myeloid cell differentiation. C/EBPα is mutated in acute myeloid leukemia (AML). The mutant form of C/EBPα, C/EBPα-p30 exhibits dominant negative properties over the wild type protein. The Peptidyl-prolyl cis/trans isomerase, PIN1 is overexpressed in many cancers and has been shown to be critical in multiple oncogenic signaling pathways. Here we report that C/EBPα-p30 induces PIN1 mRNA expression as assessed by Real Time RT-PCR in K562-C/EBPα-p30ER cells. Affymetrix mRNA expression analysis and Western blot analysis show that PIN1 is upregulated in patients with AML. Silencing of PIN1 by PIN1 inhibitor (PiB) could overcome the dominant negative action of the C/EBPα-p30 over the C/EBPα-p42 transactivation

capacity as revealed by promoter assay in 293T cells. C/EBPα-p30 induces PIN1 promoter activity in association with E2F1. At the same time, wild type C/EBPα interferes with transactivation of the PIN1 promoter. Also, C/EBPα-p42 downregulates PIN1 mRNA expression as assessed by Real Time RT-PCR in K562-C/EBPα-p42ER cells. Furthermore, PIN1 inhibition was found to upregulate wild type C/EBPα protein level and lead to granulocytic differentiation in AML blast cells with C/EBPα mutation and Kasumi-6 cells. PIN1 increases the stability of c-Jun protein via inhibiting its ubiquitination. We further demonstrate that overexpression of PIN1 induces c-Jun mRNA expression, while inhibition of PIN1 downregulates c-Jun mRNA expression in Kasumi-6 cells as analysed by Real Time RT-PCR. Overexpression of c-Jun blocks the transactivation and differentiation induced by C/EBPα protein as shown by promoter assay and FACS anaylsis resepectively. In conclusion, inhibition of PIN1 leads to granulocytic differentiation and suggest PIN1 as a novel target in treating AML patients with C/EBPα mutation.

6. Zusammenfassung

Der Transkriptionsfaktor CCAAT Enhancer Binding Protein α (C/EBPα) spielt eine entscheidende Rolle bei der Differenzierung neutrophiler Granulozyten. In der vorliegenden Arbeit versuchten wir, durch *in vivo* Immunopräzipitation mittels auf Massenspektrometrie basierenden Proteomforschungs Techniken neue, mit C/EBPα interagierende Proteine zu identifizieren. In unseren Untersuchungen konnten wir Max, ein mit Myc Hetero-Dimere bildendes Protein, als eines dieser mit C/EBPα interagierenden Proteine identifizieren. Hierbei konnten wir zeigen, dass während der granulozytären Differenzierung myeloischer Zellen der U937 Zelllinie endogenes C/EBPα und Max, jedoch nicht Myc und Max, in intranukleären Strukturen co-lokalisiert vorzufinden sind. Max verstärkt dabei die Transaktivierungskapazität von C/EBPα wie durch einen Promoterassay gezeigt werden konnte. Der Chromatin Immunopräzipitations Assay zeigte eine Besetzung des C/EBPα Promoters durch C/EBPα und Max während der All-trans-Retinsäure induzierten granulozytären Differenzierung. FACS Analysen für granulozytische Marker zeigen, das eine verstärkte Expression von Max und C/EBPα in der granulozytären Differenzierung CD34-positiver humaner hämatopoetischer Stammzellen resultieren. Hingegen führt eine verminderte Expression von Max zu einer Reduktion des Potentials von C/EBPα eine Differenzierung zu induzieren und deutet damit auf eine wichtige Rolle der Interaktion von C/EBPα und Max während der Differenzierung myeloischer Vorläuferzellen hin. Zusammenfassend konnten wir Max, infolge unsere Ergebnisse, als einen neuen Co-Aktivator des Transkriptionsfaktors C/EBPα identifizieren.

Ein Verlust der C/EBPα Expression oder seiner Funktion in leukämischen Blasten führt zu einer Blockade in der myeloischen Differenzierung. C/EBPα liegt in Akut Myeloischen Leukämien (AML) in mutierter Form vor. Diese Mutation, C/EBPα-p30, zeigt eine dominant- negative Aktivität gegenüber dem

wild typ Protein auf. Die Peptidyl-prolyl cis/trans Isomerase, PIN1, ist in vielen verschiedenen Tumoren überexprimiert und ist als ein kritischer Faktor in multiplen onkogenen Prozessen bekannt. In der vorliegenden Arbeit zeigen wir durch Real Time RT-PCR in K562-C/EBPα-p30ER Zellen, dass C/EBPα-p30 die PIN1 mRNA Expression induziert. Affymetrix mRNA Expressions Analysen und Western Blot Analysen zeigten, dass bei Patienten mit AML eine verstärkte PIN1 Expression vorliegt. Durch einen Promoterassay in 293T Zellen konnte gezeigt werden, dass es bei der Inhibierung von PIN1 durch PIN1-Inhibitor (PiB) eine Überwindung der besagten dominant-negativen Wirkung von C/EBPα-p30 auf die Transaktivierungskapazität des C/EBPα-p42 Proteins erreicht wird. C/EBPα-p30 induziert gemeinsam mit E2F1 die PIN1 Promoteraktivität. Wohingegen das C/EBPα-p42 wild typ Protein die Promotertransaktivierung beeinträchtigt. Weiterhin reduziert C/EBPα-p42 die PIN1 mRNA Expression wie durch RT-PCR in K562-C/EBPα-p42ER Zellen gezeigt werden konnte. Desweiteren konnte gezeigt werden, dass eine Inhibition von PIN1 zu einer verstärkten C/EBPα Expression und zu granulozytärer Differenzierung in AML Blasten mit C/EBPα Mutationen und in Kasumi-6 Zellen führt. PIN1 erhöht die Stabilität des c-Jun Proteins durch Inhibierung seiner Ubiquitinierung. Außerdem konnten wir in Kasumi-6 Zellen durch Real Time RT-PCR zeigen, dass eine Überexprimierung von PIN1 die mRNA Expression von c-Jun hochreguliert, wohingegen die c-Jun mRNA Expression durch eine Inhibition von PIN1 vermindert wird. In einem Promoterassay und durch FACS Analysen konnte gezeigt werden, dass die Überexprimierung von c-Jun die, durch das C/EBPα Protein induzierte, Transaktivierung und Differenzierung, blockiert. Zusammenfassend kann gesagt werden, dass die Inhibition von PIN1 zur Differenzierung von Granulozyten führt und deutet daher auf eine mögliche Rolle von PIN1 als neues Zielprotein in der Therapie von AML Patienten mit C/EBPα Mutationen hin.

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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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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 5×10^5 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 (3×10^5), 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 ($\sim 1 \times 10^8$ 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′-ACCGC TACCGACCACGTGGGCG-3′) and reverse (5′-AGCACCTC CGGGTCGCGAATGG-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

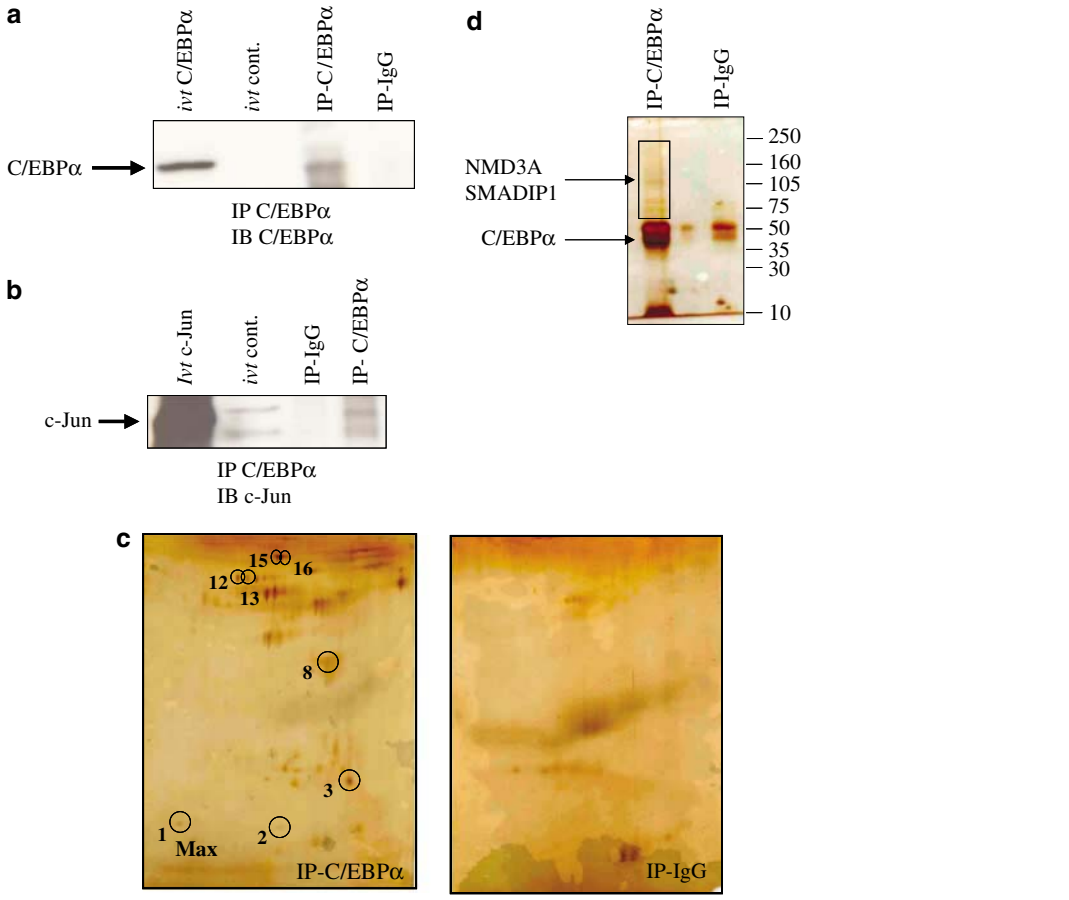


Figure 1 MS-based proteomics identifies proteins specifically interacting with C/EBPα *in vivo* after its immunoprecipitation from myeloid U937 cells. (**a**) C/EBPα IP from nuclear extracts of U937 cells and a corresponding immunoblotting (IB) with anti-C/EBPα antibody to confirm the presence of C/EBPα 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 μg) 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).

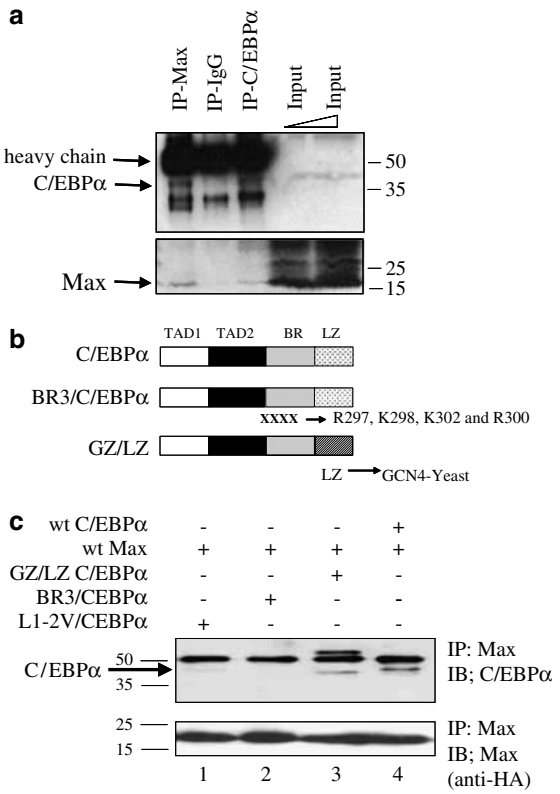


Figure 2 *In vivo* interaction of C/EBPα with Max confirmed by reciprocal IP involves the DNA-binding domain of C/EBPα. (**a**) Reciprocal IPs: C/EBPα and Max were immunoprecipitated (IP C/EBPα, IP Max) from nuclear extracts of U937 cells by incubation with anti-C/EBPα and anti-Max, respectively, and 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

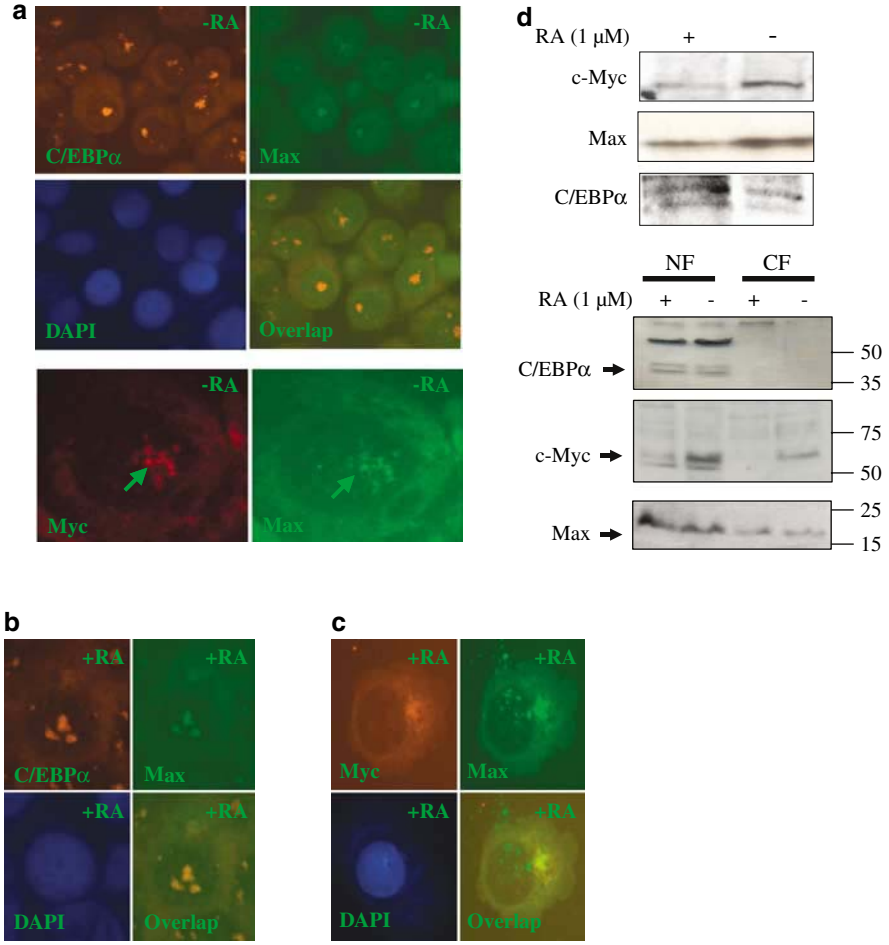


Figure 3 Endogenous C/EBPα-Max but not Myc–Max remains colocalized during granulocytic differentiation of U937 cells. (**a**) Indirect immunofluorescence staining for C/EBPα (anti-goat; Santa Cruz), Max (anti-rabbit; Santa Cruz) and Myc (anti-mouse, Santa Cruz) using respective conjugated secondary antibodies (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

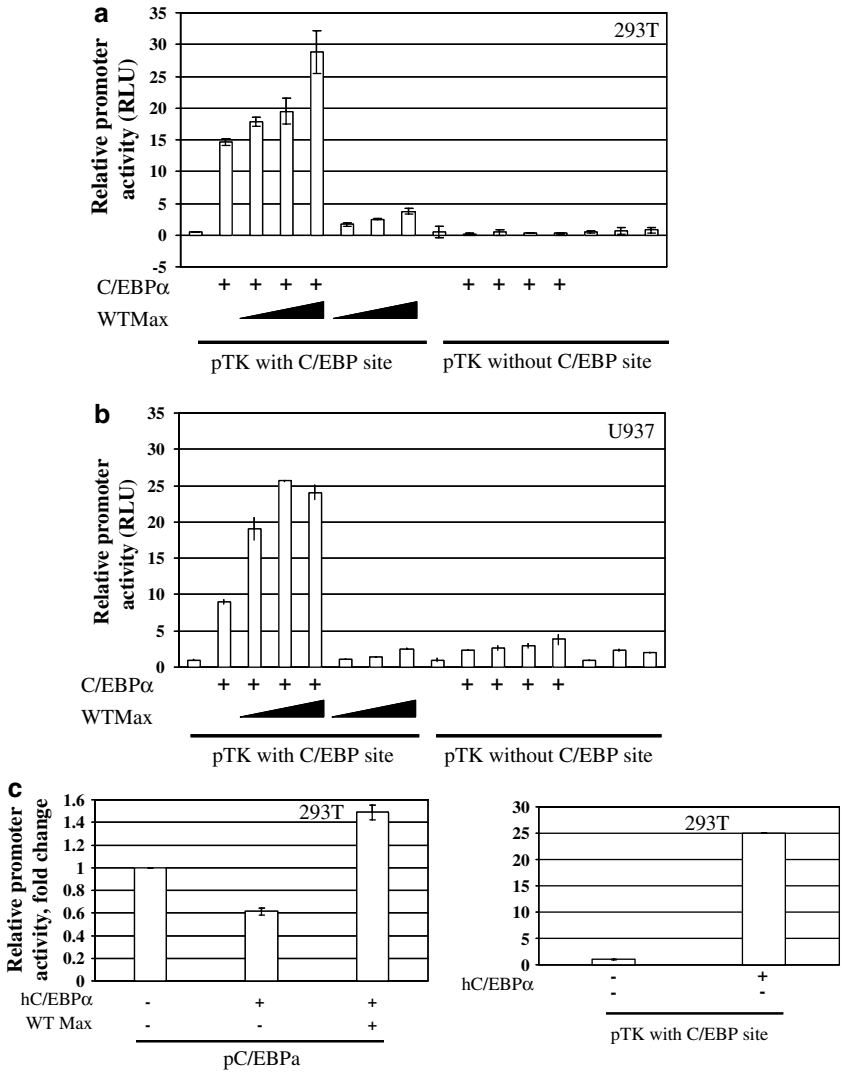


Figure 4 Max enhances the transactivation capacity of C/EBPα in transient transfection assays. (**a, b**) Transient transfection in 293T and U937 cells with a reporter construct of a minimal TK promoter with CEBP binding sites only p(CEBP)2TK and expression plasmids for hC/EBPα and Max. pTK (without 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.

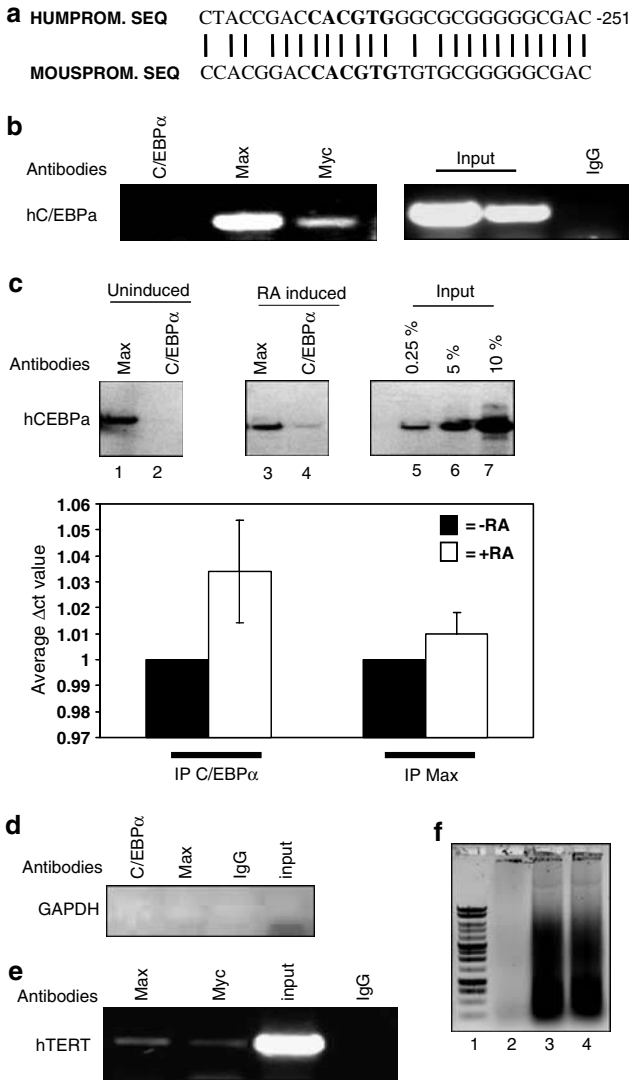


Figure 5 Max is associated at the hC/EBPα promoter *in vivo* and Max–C/EBPα associate strongly during granulocytic differentiation. A ChIP assay was performed on logarithmically growing and RA-treated U937 cells, and the precipitated chromatin was PCR-amplified using specific primers. (**a**) Comparison 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

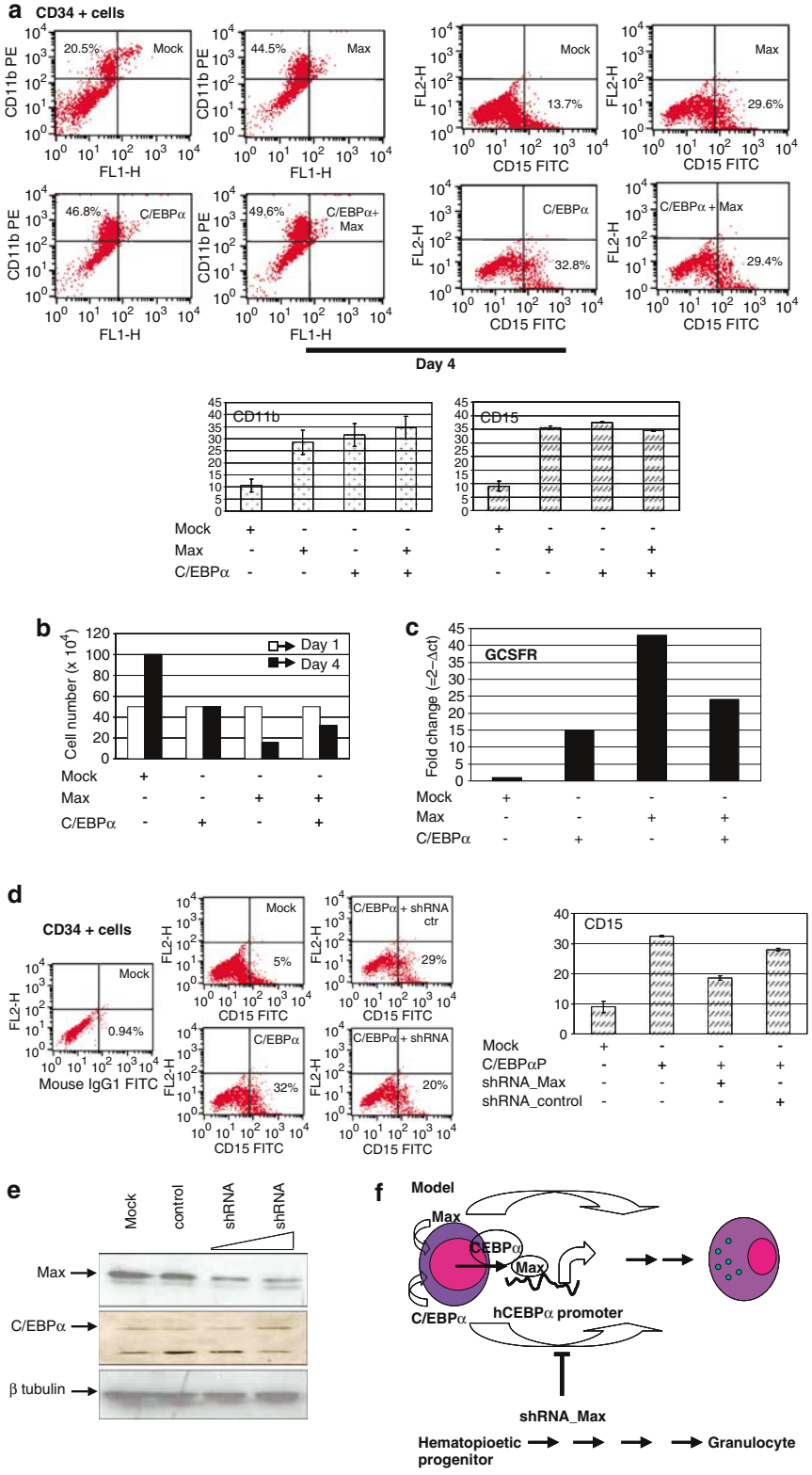
We next asked whether interaction of Max with C/EBPα is biologically important for C/EBPα functions. Hence, we performed overexpression studies using three different experimental systems: human hematopoietic CD34+ cells, estradiol-inducible K562-C/EBPα-ER cells and U937 cells. Our results revealed that 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)