Aus der Medizinischen Klinik und Poliklinik III, der Ludwig-Maximilians-Universitäts München,

Direktor: Prof. Dr. med. Wolfgang Hiddemann

Proteomic identification of C/EBPα multiprotein complex reveals that JNK1, an activator of C/EBPα is downregulated in patients with acute myeloid leukemia (AML)

Dissertation

zum Erwerb des Doktorgrades der Humanbiologie

an der Medizinischen Fakultät

der Ludwig-Maximilians-Universität zu München

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München 2006

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Thesis
Submitted for a Doctoral degree in Human Biology,
at the Faculty of Medicine
Ludwig-Maximilians-University, Munich

Submitted by
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München 2006

Mit Genehmigung der Medizinischen Fakultät

der Universität München

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Dekan:	Prof. Dr. med. D. Reinhardt
Tag der mündlichen Prüfung:	15.02.2006

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University of Munich

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Dean:	Prof. Dr. med. D. Reinhardt
Date of Submission:	28.09.2005
Date of Oral Exam:	15.02.2006

Dedicated to my esteemed father
Late Mr. Sita Ram Tiwari

Abbreviations:

AML	Acute Myeloid Leukemia
ALL	Acute Lymphoid Leukemia
APL	Acute Promyelocytic Leukemia
β-ME	β-Mercaptoethanol
BR-LZ	Basic Region-Leucine Zipper
CLP	Common Lymphoid Progenitor
CHAPS	3-[(3-Cholamidopropyl) Dimethylammonio]-1-Propanesulfonate
C/EBP	CCAAT Enhancer Binding Protein
CML	Chronic Myeloid Leukemia
CK	Complex Karyotype
CHX	Cycloheximide
2 DE	2 Dimensional Gel Electrophoresis
DTE	Dithioerythritol
DTT	Dithiothreitol
DHB	2,5-Dihydroxy-Benzoicacid
DMEM	Dulbecco´s Modified Eagle Medium
DMSO	Dimethylsulfoxide
EDTA	Ethylenediamine Tetra-Acetic Acid
EGTA	Ethylene Glycol bis (2-aminoethyl ether)-N,N,N'N'-Tetra Acetic Acid
FAB	French American British Classification
GMPs	Granulocyte/Macrophage Progenitors
GST	Glutathione-S-Transferase
GCSF	Granulocyte Colony Stimulating Factor
GCSFr	Granulocyte Colony Stimulating Factor Receptor
HSC	Haematopoietic Stem Cell
HA	Hemeagglutinin
IB	Immunoblot

IEF	Isoelectric Focussing
IPTG	Isopropyl-beta-D-Thiogalactopyranoside
Ip	Immunoprecipitation
Ivt	*In-vitro Translated*
JNK	c-Jun NH_2 -Terminal Kinase
JNK*	Kinase dead c-Jun NH_2-Terminal Kinase (mutant JNK)
MALDI	Matrix Assisted Laser Desorption Ionisation- Time of Flight
MEPs	Megakaryocyte/Erythroid Progenitors
MAPK	Mitogen Activated Protein Kinase
µl	Microlitres
µg	Micrograms
µCi	Micro Curie
MS	Mass Spectrometry
NP40	Nonidet P-40
ng	Nanogram
NK	Normal Karyotype
nBM	Normal Bone Marrow
PMF	Peptide Mass Fingerprinting
pI	Isoelectric Point
RIPA	Radioimmunoprecipitation
RT	Room Temperature
Rpm	Revolutions per Minute
SDS	Sodium Dodecyl Sulphate
TE	Transactivation Elements
TAD	Transactivation Domain
TBE	Tris-Borate EDTA
TOF	Time of Flight
UVB	Ultra Violet Radiation B
WHO	World Health Organization

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1. Introduction:

1.1 Haematopoiesis:

The haematopoietic system is a paradigm for the development of different specialized cell types from multipotent progenitors. Pluripotential haematopoietic stem cells — an extremely rare population (<0.1%) of nucleated bone-marrow cells — undergo a decision to either self-renew or remain pluripotent or to differentiate into immature but committed progenitor cells. These progenitor cells differentiate into either lymphoid, erythroid, megakaryocytic or myeloid precursors in the bone marrow (Tenen, 2003).

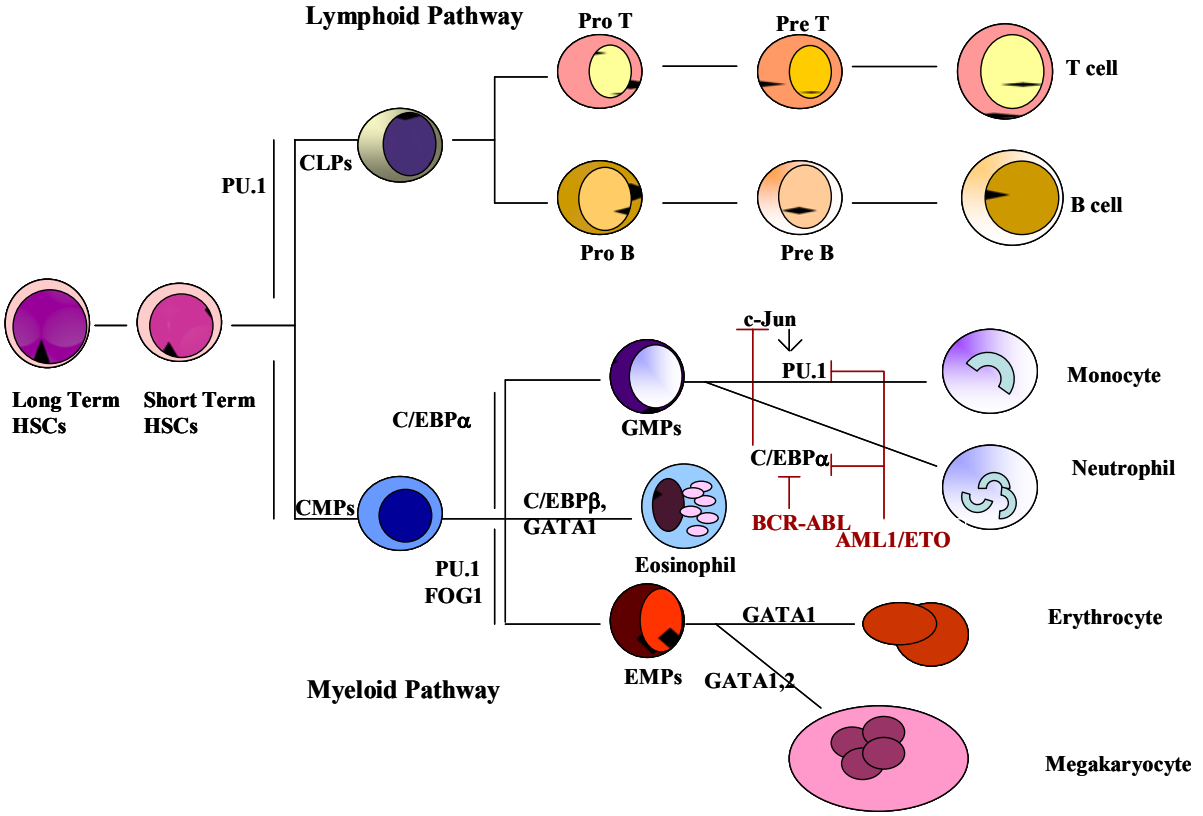

Fig 1. Role of transcription factors in haematopoietic development: Long-term and short-term haematopoietic stem cells (HSCs) provide long-term (more than 3 months) and short-term reconstitution in lethally irradiated mice. The common lymphoid progenitor (CLP) gives rise to T and B cells, whereas the common myeloid progenitor (CMP) gives rise to granulocyte/macrophage progenitors (GMPs) and megakaryocyte/erythroid progenitors (MEPs). Upregulation of the transcription factor PU.1 is essential for the transition from HSC to CLP, whereas downregulation of PU.1 is required for the differentiation of CMP to MEP. CCAAT/enhancer binding protein- (C/EBP)α upregulation initiates the transition from CMP to GMP. One hypothesis is that the 'default' pathway involves differentiation of GMPs to monocytes and macrophages, which depends on PU.1 activity. In this model, C/EBP subverts

this default monocytic pathway and promotes differentiation into granulocytes — perhaps through inhibition of PU.1 function. On contrary, C/EBPα inhibition by AML1/ETO and BCR-ABL has been implicated in patients with acute myeloid leukemia. Adapted from Nature reviews, Tenen DG,(Tenen, 2003)

The past two decades have witnessed significant advances in our understanding of the cellular physiology and molecular regulation of haematopoiesis and myelopoiesis. Several recent reviews and studies focus on the importance of lineage specific transcription factors for the specification of the myeloid, lymphoid and erythroid lineages (Friedman, 2002; Orkin, 1996; Sieweke and Graf, 1998; Tenen, 2000).

The central role of transcription factors in these processes has been highlighted by gene inactivation studies, promoter analysis, and ectopic expression of lineage restricted factors (Sieweke and Graf, 1998). Figure 1, depicts a schematic representation of transcription factors required at different lineage specification while haematopoiesis.

1.2 Transcription factors involved in normal myelopoiesis:

Rather than being controlled by single master regulators, lineage specific gene expression appears to depend on the combination of factors in overlapping expression domains (Ness and Engel, 1994). Important transcription factors involved in normal haematopoiesis are listed in Table 1.

Table 1: Transcription factors involved in normal haematopoiesis:

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

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

Adapted from Nature Reviews Cancer (Tenen, 2003).

1.3 Acute Myeloid Leukemia:

Acute myeloid leukemia (AML) is a malignant disease of the haematopoietic system in which cells of one of the myeloid lineages (erythrocytes, megakaryocytes, granulocytes, monocytes/macrophages) accumulate in an undifferentiated state due to accumulation of mutations that prevent their normal differentiation and allow undifferentiated cells to survive (Bob Löwenberg, 1999; Nerlov, 2004; Nerlov and Graf, 1998). The fundamental biological features of the malignant cells in AML are their ability to proliferate continuously with an aberrant or arrested differentiation (Scandura et al., 2002). The French American British classification, introduced approximately 25 years ago (Bennett et al., 1976; Harris et al., 1999) remains the foundation on which the morphologic diagnosis of AML and ALL is based (Table 2). However, the new World Health Organization (WHO) classification takes into account cytogenetics, molecular genetics, morphologic and immunophenoytic findings (Harris et al., 1999; Harris et al., 2000a; Harris et al., 2000b; Harris et al., 2000c).

Table 2: The French American British classification of AML:

FAB Subtype	Description	Comments
M0	Undifferentiated	Myeloperoxidase negative, Myeloid markers positive;
M1	Myeloblastic without maturation	Some evidence of granulocytic differentiation
M2	Myeloblastic with maturation	Maturation at or beyond the promyelocytic stage of differentiation; can be divided into those with t(8;21) AML1-ETO fusion and those without
M3	Promyelocytic	APL; most cases have t(15;17) PML-RARα or another translocation involving RARα
M4	Myelomonocytic	
M4e0	Myelomonocytic with bone marrow eosinophilia	Characterized by inversion of chromosome 16 involving CBFb, which normally forms a heterodimer with AML1
M5	Monocytic	
M6	Erythroleukemia	
M7	Megakaryoblastic	GATA mutations in those associated with Down´s syndrome.

Adapted from Nature Reviews Cancer (Harris et al., 1999; Tenen, 2003)

Much is known about the signals that control cell survival and proliferation, whereas signals required for differentiation are not well defined (Tenen, 2003). In the past few years, a number of studies have pointed to the dominant role of lineage specific transcription factors in normal hematopoietic differentiation (Nerlov and Graf, 1998; Rangatia et al., 2002; Zhang et al., 1997). These studies predicted that the function of these transcription factor pathways would be disrupted in AML. Recent studies have confirmed this hypothesis, showing that a number of cases of AML are not associated with a consistent translocation, but

with small mutations in the coding regions of these lineage specific transcription factors or their inhibition by other inhibitory proteins by direct protein-protein interactions (Nerlov, 2004; Nerlov, 2000; Pabst et al., 2001a; Pabst et al., 2001b; Vangala et al., 2003). In addition, in many of those cases the resulting translocation product disrupts the expression and/or function of lineage specific factors (Pabst et al., 2001a; Perrotti et al., 2002). In fact, the most common genetic mechanism that is associated with AML and ALL, is the aberrant expression of transcription factors or the production of an abnormal hybrid transcription factor (Jing, 2004; Pabst et al., 2001a; Perrotti et al., 2002). Hematopoietic transcription factors often associated with AML are listed in table 3.

Table 3: Transcription Factors associated with AML:

Factor	Subtype	Mutation
AML1	FAB M0	Often biallelic mutations
AML1	FAB M2	t(8;21) AML1-ETO and AML1/Evi1
GATA1	FAB M7 associated with Down´s syndrome	Amino terminal mutations similar to those seen with C/EBPα
PU.1	M0,M4,M5,M6	Not associated with common chromosomal translocations; mostly M0 and M4 without inv 16
C/EBPα	M1,M2 few M4	Mutation not associated with t(8;21); amino-terminal dominant negative
C/EBPα	M2 with t(8;21)	No mutation; downregulation at RNA level
C/EBPα	CML myeloid-blast crisis	No mutation; downregulation at protein level
C/EBPα	APL	No downregulation; loss of DNA binding in cell lines;

		C/EBPβ and C/EBPε mediate ATRA response in AML.
CBFβ	M4e	Inversion of chromosome 16 involving CBFβ
FLT3		Involved in translocation with ITD and is often mutated

Adapted from Nature Reviews, Cancer (Tenen, 2003).

1.4 Role of C/EBPα in myelopoiesis:

The CCAAT enhancer binding protein alpha (C/EBPα) is a key transcription factor involved in granulocytic differentiation and in general myelopoiesis (McKnight, 1991; Radomska et al., 1998; Zhang et al., 1997). C/EBPα belongs to the C/EBP family of proteins first identified from rat liver nuclear proteins (Landschulz et al., 1988; Landschulz et al., 1989). CCAAT enhancer binding proteins encompass a family of transcription factors with structural as well as functional homologies. C/EBP proteins consist of an activation domain at the N-terminal, a DNA binding basic region (BR) and a Leucine-rich dimerization domain aptly termed the "leucine zipper" (LZ) at the carboxyl terminus (Landschulz et al., 1989).

In the past few years, the study of lineage specific transcription factors such as C/EBPs has greatly increased our knowledge of mechanisms underlying the regulation of myelopoiesis and haematopoiesis in general (Friedman, 2002; Nerlov, 2004; Pabst et al., 2001a; Pabst et al., 2001b; Sieweke and Graf, 1998; Tenen, 2000; Tenen, 2001; Tenen, 2003). The genes of six C/EBP members have been cloned to date from several species and have been given a systematic nomenclature in which members are designated as C/EBP followed by a Greek letter indicating the chronological order of their discovery (C/EBPα-ζ) (Akira et al., 1990; Cao et al., 1991; Chang et al., 1990; Descombes et al., 1990; Ramji

and Foka, 2002; Roman et al., 1990; Ron and Habener, 1992; Williams et al., 1991). A brief description of C/EBP proteins involved in haematopoiesis, myelopoiesis and their association with AML is given in table 1and 3.

C/EBPs are key regulators of growth and differentiation of many tissues and cell types *in-vivo*; The transcription factor C/EBPα in particular has been shown to play a pivotal role during differentiation in various cell types, including adipocytes, hepatocytes, keratinocytes, lung, prostrate and ovary cells (Cao et al., 1991; Flodby et al., 1996; Lin and Lane, 1994; Radomska et al., 1998; Umek et al., 1991; Zhang et al., 1997). Within hematopoiesis, full length C/EBPα, C/EBPβ and C/EBPδ are expressed in the monocyte, granulocyte and eosinophil lineage (Friedman, 2002). However, C/EBPα is the predominant isoform expressed in early myeloid cells. Figure 2, depicts the temporal expression of various C/EBP proteins during granulopoiesis.

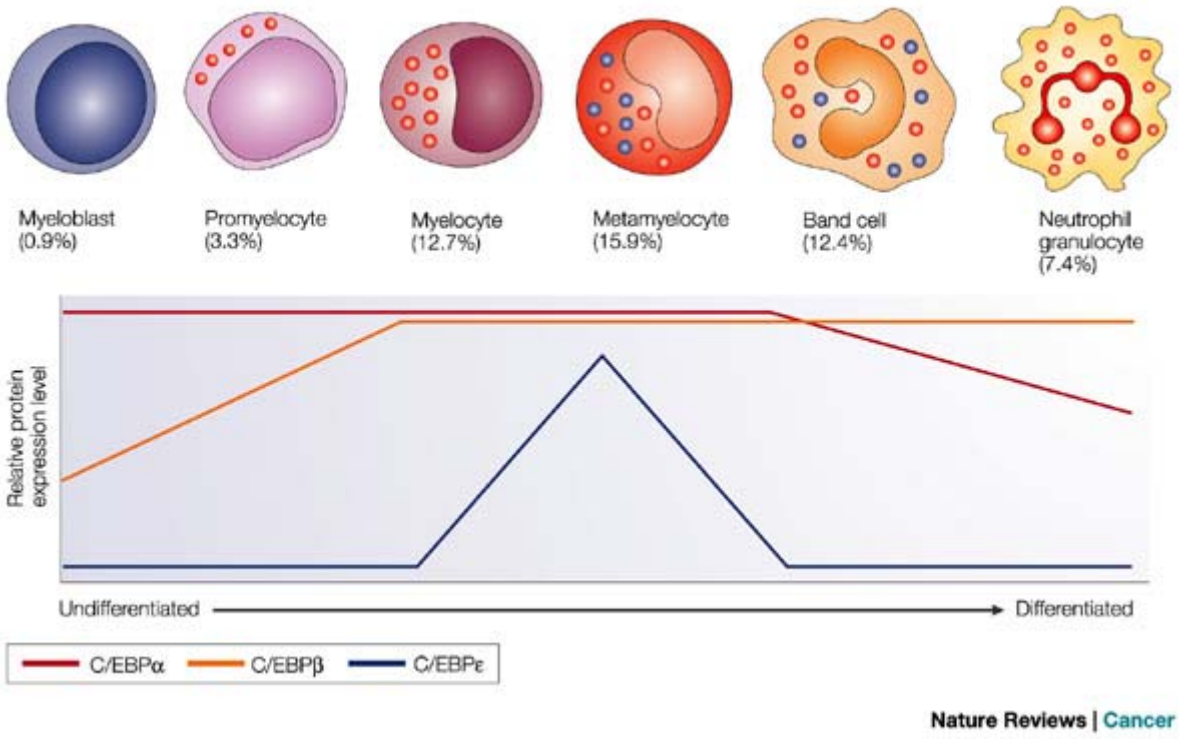

Fig 2. Expression profile of C/EBP proteins during Granulopoiesis: Granulopoiesis is the formation of mature neutrophil granulocytes from immature myeloblasts through a differentiation process that involves the cessation of cellular proliferation concomitant with the sequential synthesis of a number of enzymes and structural proteins that make up the different types of granules contained in the granulocyte. The figure shows their relative contribution to the granulocytic compartment (as percent of total nucleated bone-marrow cells (Duprez et al., 2003), as well as the expression pattern of the C/EBPα, C/EBPβ and

C/EBPε (Bjerregaard et al., 2003), (Weinstein, 1995) proteins during the process of granulocyte differentiation, Adapted from Nature Reviews, Cancer. (Nerlov, 2004).

Previous studies have demonstrated that the expression of C/EBPα correlates with the commitment of multipotential precursors to the myeloid lineage, and is specifically upregulated during neutrophil differentiation (Radomska et al., 1998; Scott et al., 1992). Moreover, conditional expression of C/EBPα is sufficient to induce neutrophil differentiation (Radomska et al., 1998; Wang et al., 1999) and can block the monocytic differentiation program in bipotential myeloid precursors (Radomska et al., 1998). In addition, studies with targeted disruption of the C/EBPα gene demonstrate a selective block in the differentiation of neutrophils while all the other blood cell types are present in normal proportions (Zhang et al., 1997). C/EBPα knockout mice do not express granulocyte colony-stimulating factor receptor (G-CSFr) which is a critical target gene of C/EBPα. As a result, multipotential myeloid progenitors from the mutant foetal liver are unable to respond to granulocyte colony-stimulating factor (G-CSF) signalling, although they are capable of forming granulocyte-macrophage and macrophage colonies in methylcellulose in response to other growth factors (Zhang et al., 1997). In fact, C/EBPα is not only essential for granulocyte development but, in addition, is a regulator of haematopoietic stem cell activity (Zhang et al., 2004).

Few critical target genes of C/EBPα have been identified and studied thoroughly. The C/EBPα protein activates the CD14 promoter and mediates TGF-β signalling during monocyte development. CD14 is a monocyte/macrophage differentiation marker which is strongly upregulated during monocytic differentiation. Myelomonoblastic U937 cells differentiate into monocytic cells upon treatment with vitamin D3 and TGF-β which is accompanied by a specific increase in the DNA binding and the expression of C/EBPα and C/EBPβ (Zhang et al., 1997). c-Myc is another target gene of C/EBPα, regulated negatively in granulopoiesis as shown by Johansen *et. al.* (Johansen et al., 2001). They

mapped an E2F binding site in the c-Myc promoter as the cis-acting element critical for C/EBPα negative regulation; they show that stable expression of c-Myc from an exogenous promoter not responsive to C/EBPα-mediated down-regulation, forces myeloblasts to remain in an undifferentiated state. Therefore, C/EBPα negative regulation of c-Myc is critical for allowing early myeloid precursors to enter a differentiation pathway. This was the first report to demonstrate that C/EBPα directly affects the level of c-Myc expression and; the decision of myeloid blasts to enter into the granulocytic differentiation pathway (Johansen et al., 2001). Moreover, C/EBPα directly activates transcription from lineage-specific promoters.

Recent studies have emphasized that the interaction of transcription factors with other nuclear proteins plays an important role in a combinatorial fashion in stem cell development, lineage commitment and differentiation in the haematopoietic system (Behre et al., 2002a; Behre et al., 1999b; Nerlov, 2000; Sieweke and Graf, 1998; Zhang et al., 2000). Indeed C/EBPα also functions via protein-protein interaction; Janki *et al.* have shown that downregulation of c-Jun by C/EBPα is critical for granulocytic differentiation. Their data demonstrate that C/EBPα interacts directly with c-Jun in the leucine zipper domain and thereby attenuates its DNA binding, which in turn inhibits c-Jun expression and its transactivation capacity (Rangatia et al., 2002). Direct physical interaction of C/EBPα with PU.1 in its β3, β4 domains has been reported to be essential for PU.1 inactivation by C/EBPα to drive granulocytic differentiation (Reddy et al., 2002). Besides, the importance of protein-protein interaction is also evidenced by the fact that C/EBPα interacts with different protein partners in young and old mice liver to execute its function (Polina Iakova, 2003; Timchenko, 2003). Direct contact with the basal transcription apparatus (TBP/TFIIB) (Nerlov and Ziff, 1995), interaction with histone acetyltransferases (CBP/p300) (Schwartz et al., 2003) and recruitment of chromatin-remodelling complexes (SWI/SNF) (Pedersen et al., 2001) have all been implicated in the activation of lineage-

specific genes by C/EBPα. An important function of the lineage specific factor C/EBPα is the inhibition of proliferation (Harris et al., 2001; Wang et al., 2001). Cells that are terminally differentiated and do not undergo proliferation have an inhibition in their cell cycle apparatus. This is particularly true for C/EBPα which works through multiple mechanisms to activate p21, inhibit E2F, and inhibit CDK function (Harris et al., 2001; Porse et al., 2001; Wang et al., 2001; Wang et al., 2003). In fact, several studies indicate that inhibition of E2F might be the most critical target of C/EBPα. Figure 3, depicts the characterized functional domains and interacting proteins of wild type C/EBPα (p42) and the N-terminal dominant negative mutant form C/EBPα(p30) which is often upregulated in patients with acute myeloid leukemia (AML).

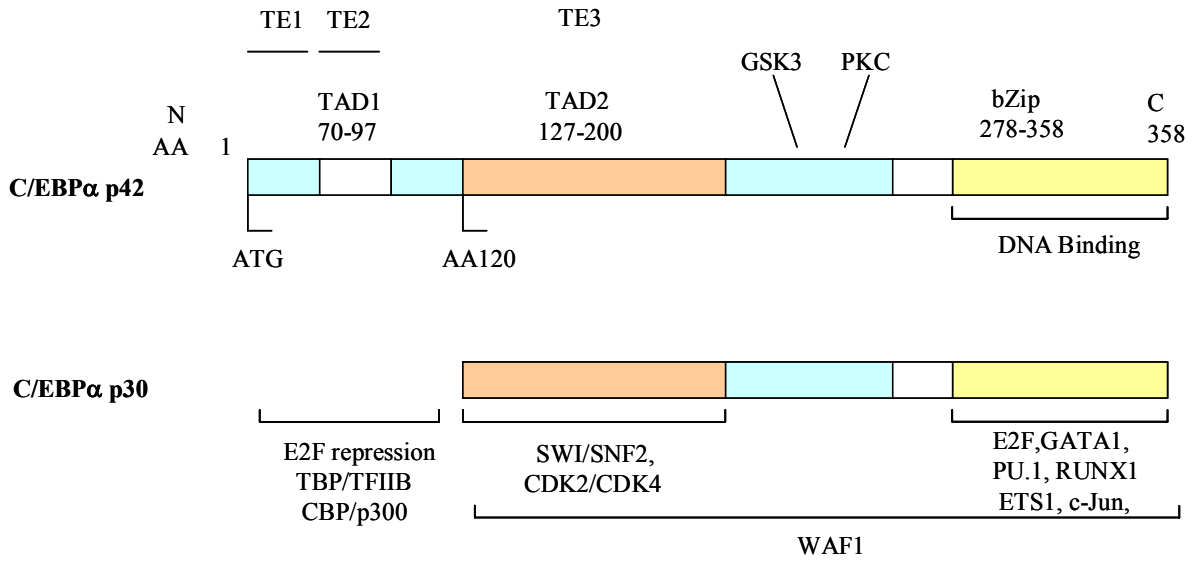

Fig 3. Distribution of C/EBPα domains and its known interacting proteins: The location of characterized functional domains within the CCAAT/enhancer-binding protein-α (C/EBPα) protein. The basic region leucine zipper (BR-LZ) domain has been shown to mediate interaction with other transcription factors involved in lineage-specific gene regulation (such as GATA1, PU.1, ETS1 and RUNX1) and growth control (such as c-JUN and E2F). The three transactivation elements; TE-I, TE-II and TE-III mediate interactions with the transcriptional machinery (TBP/TFIIB; CBP/p300; SWI/SNF). In addition, p30 has been proposed to interact with the cyclin-dependent kinase inhibitor WAF1 (also known as p21), and with the cyclin-dependent kinases CDK2 and CDK4. Finally, glycogen synthase kinase 3 (GSK3) and protein kinase C (PKC) have been reported to phosphorylate distinct sites (Charles W. Mahoney, 1992; Ross et al., 1999)

1.5 C/EBPα inactivation in acute myeloid leukemia:

Acute myeloid leukemia (AML) is characterized by an increase in the number of myeloid cells in the bone marrow and an arrest in their maturation, frequently resulting in hematopoietic insufficiency with or without leukocytosis (Bob Löwenberg, 1999). On the basis of morphology and cytogenetics AML has been classified in different FAB subtypes (Bennett et al., 1976). Although precise aetiology of AML is not clearly known, various somatic genetic alterations in hematopoietic stem or progenitor cells are known to cause malignant transformation which leads to leukemia (Gilliland et al., 2004). Recent studies have confirmed that function of lineage specific transcription factors is disrupted in AML which is either due to mutation or their functional inactivation by other inhibitory proteins (Nerlov, 2004). In addition, in many AML cases that are associated with a chromosome translocation, the resulting translocation product disrupts the expression and/or function of lineage specific transcription factors (Tenen, 2003).

We recently reported that dominant-negative mutations of C/EBPα are found in patients with acute myeloid leukemia of subtypes M1 and M2. Heterozygous mutations were found in the CEBPα gene in ten patients with acute myeloid leukemia (AML); five mutations in the amino terminus truncated the full-length protein, but did not affect a 30-kD protein initiated further downstream. The mutant proteins block wild-type C/EBPα DNA binding and transactivation of granulocyte target genes in a dominant-negative manner resulting in the impairment of differentiation (figure 3). Since then several reports of C/EBPα gene mutation have been observed and reported (Frohling and Dohner, 2004; Frohling et al., 2004; Perrotti et al., 2004). In fact, C/EBPα is mutated in approximately 9% of AML — most prominently in the M2 subtype, where C/EBPα mutations are observed in up to 20% of patients but also in other granulocyte-lineage leukaemias (Nerlov, 2004). Furthermore, Fröhling et al. have demonstrated that mutation of C/EBPα predicts a favourable prognosis in

AML with normal cytogenetics. Recently C/EBPα mutation has been implicated in inherited AML (Smith et al., 2004).

Besides mutations in CEBPα gene, C/EBPα protein function is also disrupted in AML by negative protein-protein interactions (Rangatia et al., 2003; Westendorf et al., 1998). AML1-ETO associates with C/EBPα, inhibits C/EBPα dependent transcription of the myeloid specific rat defensin NP-3 promoter, and blocks granulocytic differentiation (Westendorf et al., 1998). Moreover, we have also shown that the leukemic fusion protein AML1-ETO, found in patients with acute myeloid leukemia with translocation t(8;21), downregulates C/EBPα expression and inhibits its function in primary AML patients (Pabst et al., 2001a).

The PML-RARα fusion protein, which is the signature of acute promyelocytic leukemia (APL) results from the translocation t(15;17) and blocks promyelocytic differentiation (Zelent et al., 2001). In primary human APL cells PML-RARα inhibits C/EBPα function by downregulating its expression. AML specific Flt3 mutations also downregulate C/EBPα expression and hence contribute to the leukemic phenotype (Zheng et al., 2004). C/EBPα is not only inhibited at the protein level, but also at the mRNA level. Perrotti *et al.,* have demonstrated that in growth factor-independent cell lines expressing BCR-ABL and in primary bone marrow cells from individuals with CML blast crisis, C/EBPα expression is suppressed at the translational level by a mechanism that depends on the specific interaction of the RNA-binding protein hnRNP E2 with the cytosine-rich intercistronic region of CEBPα mRNA. In fact, ectopic expression of C/EBPα in cells expressing BCR-ABL allows G-CSF-induced differentiation; by contrast, overexpression of hnRNP E2 in myeloid precursor cells downregulates expression of C/EBPα and G-CSFr and promotes rapid cell death upon culture of these cells in G-CSF containing medium (Perrotti et al., 2002). In addition, Timchenko et al. have shown that calreticulin downregulates C/EBPα by inhibiting CEBPα mRNA (Timchenko et al., 2002). In some AML cases CEBPα hypermethylation leading to its gene inactivation has also been

reported. Several studies demonstrate that C/EBPα protein activity is modulated by post translational modifications (Behre et al., 2002b). SUMOylation of C/EBPα in its synergy control motif provides a means to rapidly control higher order interactions among transcription factors and suggests that SUMOylation may be a general mechanism to limit transcriptional synergy (Subramanian et al., 2003). Phospho modification of C/EBPα at Ser21 in the transactivation domain inhibits granulopoiesis (Ross et al., 2004) while phosphorylation at Ser299 in the DNA binding domain attenuates its DNA binding activity leading to its inactivation and inhibition of granulopoiesis (Charles W. Mahoney, 1992).

These studies point to a crucial role of C/EBPα in both normal myeloid differentiation and leukemogenesis. However, how the transcriptional activity of C/EBPα is regulated both in normal myelopoiesis as well as in leukemogenesis is not fully understood. Recent reviews focusing on disruption of differentiation in AML have set the perspective for the future research in this field. Many questions remain unanswered regarding the transcriptional regulation of granulocyte and monocyte development. What are the relative contributions of different lineage specific factors in a particular lineage commitment, how do they operate within regulatory networks to orchestrate lineage selection and haematopoietic development?, What is the role of lineage specific transcription factors on stem cells, and how does this relate to AML. Do some granulocyte or monocyte progenitors develop directly from pluripotent stem cells; how irreversible are commitment decisions? Are there additional transcription factors yet to be revealed; are there lineage-restricted co-activators or co-repressors which participate in lineage specification? Detailed studies for further clarification of the regulatory network are needed. Another important area for the investigation is the further identification of protein-protein interactions to find out if there is a switch between different interacting partners of C/EBPα in normal haematopoiesis and AML as seen in case of young and old mice livers (Iakova et al., 2003). The answers to these questions will provide general

lessons in developmental biology and insights into leukemogenesis and will enable applications in clinical haematology, oncology, and gene therapy (Friedman, 2002; Frohling and Dohner, 2004; Tenen, 2003).

1.6 c-Jun NH_2-terminal kinase (JNK):

The transmission of extracellular signals into intracellular responses is a complex process which often involves the activity of mitogen-activated protein kinases. Mitogen-activated protein (MAP) kinase signalling pathways relay, amplify and integrate signals from a diverse range of extracellular stimuli; thereby the genomic and physiological response of a cell to changes in the respective environment (Weston and Davis, 2002). Each MAPK is activated by dual phosphorylation on a Thr- Xaa-Tyr motif by upstream kinases referred to as MAPK kinase or MKKs. Biochemical studies led to the identification and purification of JNK as a "p54 microtubule-associated protein kinase" that was activated by cycloheximide (Kyriakis and Avruch, 1990).

JNK was found to bind the NH_2-terminal activation domain of c-Jun (Hibi et al., 1993) and to phosphorylate c-Jun on Ser-63 and Ser-73 (Woodgett et al., 1993) leading to increased transcriptional activity. JNK is activated by the treatment of cells with cytokines (e.g., TNF and IL-1) and by exposure of cells to many forms of environmental stress e.g., osmotic stress, redox stress, and radiation (figure 4). A major target of the JNK signalling pathway is the activation of the AP-1 (Activator protein-1) transcription factor that is mediated, in part, by the phosphorylation of c-Jun and related molecules implicated in regulating altered gene expression, cellular survival and proliferation in response to cytokines, growth factors, to noxious stimuli and to oncogenic transformation (Davis, 2000; Dong et al., 2001; Manning and Davis, 2003); (Fuchs et al., 1998d). Figure 4 schematically depicts JNK kinase signalling pathway.

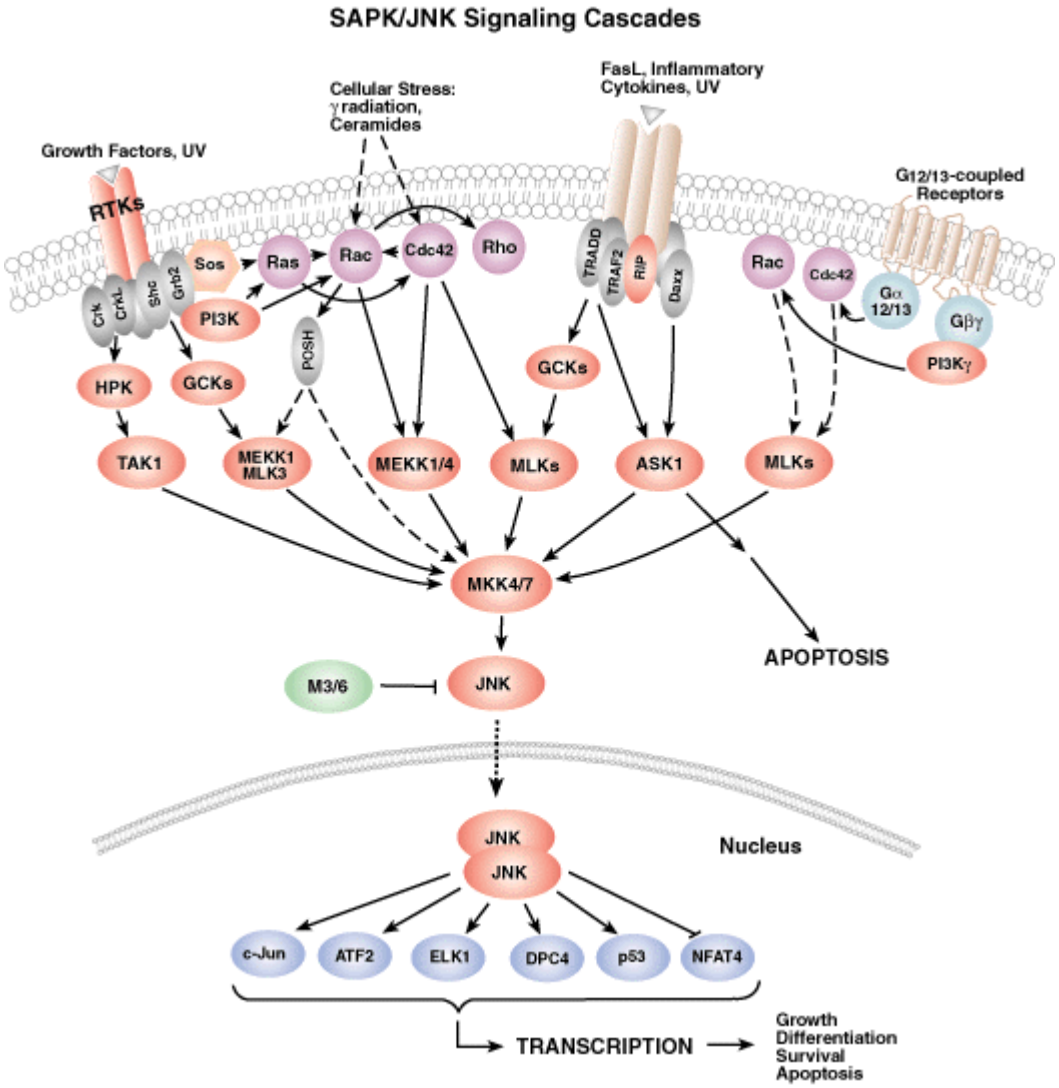

Fig 4. SAPK/JNK signalling cascades: Stress-activated protein kinases (SAPK)/Jun N-terminal kinases (JNK) are members of the MAPK family and are activated by a variety of environmental stresses, inflammatory cytokines, growth factors and GPCR agonists. Stress signals are delivered to this cascade by members of small GTPases of the Rho family (Rac, Rho, cdc42). As with the other MAPKs, the membrane proximal kinase is a MAPKKK, typically MEKK1-4, or a member of the mixed lineage kinases (MLK) that phosphorylates and activates MKK4 (SEK) or MKK7, the SAPK/JNK kinases. Alternatively, MKK4/7 can be activated by a member of the germinal center kinase (GCK) family in a GTPase-independent manner. SAPK/JNK translocates to the nucleus where it regulates the activity of several transcription factors such as c-Jun, ATF-2 and p53 (Leppa et al., 1998) Adapted from cell signalling technology.

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1.6.1 JNK signalling in human leukemia and tumour development:

Dramatic advances have occurred over the last few years in the research of MAP kinases in haematological malignancies. The realization that map kinase – dependent signalling cascades play important roles in the regulation of apoptosis

and growth of malignant hematopoietic cells has led to extensive studies, aimed to characterize the precise mechanisms that are responsible for such effects (Milella et al., 2001). It is now clear that the Raf/MEK/ERK pathway participates in the generation of mitogenic responses in essentially all hematopoietic malignancies, including acute and chronic leukemias, lymphomas and multiple myeloma (Davis, 2000; Kennedy and Davis, 2003; Manning and Davis, 2003; Milella et al., 2001; Weston et al., 2002). On the other hand, the regulatory effects of the JNK and p38 MAP kinase pathway in acute myeloid leukemias are not well understood. A recent report demonstrated a relationship between the constitutive activity of JNK in leukemic blasts and treatment failure in acute myelogenous leukemia (Cripe et al., 2002). However owing to its important biological function in activation of the gene transcription and inducing apoptosis, the JNK signalling pathway is often involved in pathogenesis of cancer. In a recent study, inducible activation of JNK in APL cells harbouring PML-RARα induced apoptosis of NB4 cells. This suggests a possible role of JNK in myeloid leukemia (Davison et al., 2004).

JNK also seems to play a significant role in tumour development (Johnson et al., 1996). In fact, several tumour cell lines have been reported to possess constitutively active JNK (Ip and Davis, 1998). The transforming potential of several oncogenes is reduced after introduction of antisense JNK oligonucleotides or dominant-negative versions of proteins belonging to the JNK-pathway. This indicates that JNK activity is necessary for efficient transformation and tumourigenesis by oncogenes (Kennedy and Davis, 2003; Manning and Davis, 2003; Potapova et al., 2000; Potapova et al., 1997). However, in a recent study, fibroblasts isolated from mice that lack expression of JNK due to compound mutations of *Jnk* genes were efficiently transformed by Ras, and actually formed increased numbers of tumour nodules and the size of individual tumours in mice injected with these cells (Kennedy et al., 2003). Taken together these studies suggest that JNK could play more than one role in

tumour development, and that in certain cases this role might be to promote or inhibit tumour development. Understanding the genetic and mechanistic basis for these different roles of JNK in tumours may pave the way for potential therapeutic approaches (Davis, 2000; Dong et al., 2001; Manning and Davis, 2003).

1.6.2 Stress activated kinases regulate protein stability:

In addition to their function in signal transduction, SAPK kinases have been implicated in the regulation of protein stability (Musti et al., 1997). The mechanisms that modulate ubiquitination by stress signals are: association with auxiliary proteins, phosphorylation by specific kinases, and a combination of both. Therefore, changes in ubiquitination would depend on the conformation of a specific substrate and its associated proteins (Fuchs et al., 1998d). Ubiquitination dependent protein degradation has been shown to participate in regulating several stress activated transcription factors (Alarcon-Vargas and Ronai, 2004; Fuchs et al., 1998a; Fuchs et al., 1998b; Fuchs et al., 1998c; Fuchs et al., 1997; Isaksson et al., 1996). Cellular responses to stress and damage, as well as mitogenic stimuli, initiate signal transduction cascades that modify the duration and magnitude of transcriptional activity of stress-responsive and mitogen-regulated transcription factors. The resulting changes in gene expression are implicated in differentiation or proliferation and apoptosis (Alarcon-Vargas and Ronai, 2004; Fuchs et al., 1998b; Fuchs et al., 1998c; Fuchs et al., 1998d).

Among stress responsive kinases, JNK1 regulates a different subset of substrates by acquiring a specific phosphorylation pattern that affects conformation, stability, subcellular localization and transcriptional activation (Fuchs et al., 1998c; Fuchs et al., 1996; Fuchs et al., 2000; Musti et al., 1997). Phosphorylation of JNK associated proteins prevent their targeting by ubiquitination through possible changes in their conformation which is expected to abrogate the association of the respective ubiquitin ligase (Fuchs et al., 1997;

Sabapathy et al., 2004). The emerging model from recent observations suggests that JNK, by virtue of its targeting ubiquitination, is the key regulator of associated proteins, c-jun, ATF2, c-Myc, p53, and Jun B in non-stressed normal growing cells. After stress, JNK´s activation by upstream kinases, followed by phosphorylation of its associated substrates, regulates their stability and activity (Alarcon-Vargas and Ronai, 2004; Fuchs et al., 1998b; Fuchs et al., 1998c; Fuchs et al., 1996; Fuchs et al., 2000; Fuchs et al., 1997; Musti et al., 1997; Musti et al., 1996).

1.7 Proteomics:

The array of proteins found within the cell, their interactions and modifications hold the key to understanding biologic systems. This is encapsulated in the term "proteome." It can be defined as the protein population of a cell, characterized in terms of localization, post translational modification, interactions, and turnover, at any given time. The proteome is fundamentally dynamic and has an inherent complexity that surpasses that of the genome or the mRNA complement found within a cell (Cristea et al., 2004). Analysis of the whole proteome of a cell by isolating individual proteins on 2-Dimensional gel or by liquid chromatography subsequently followed by their identification by mass spectrometry comprises proteomics. Recent success illustrates that mass spectrometry based proteomics is an indispensable tool for molecular and cellular biology and for the emerging field of systems biology (Aebersold and Mann, 2003). Proteomics-based approaches, which enable the quantitative investigation of both cellular protein expression levels and protein-protein interactions involved in signalling networks, promise to define the molecules controlling the processes involved in cancer (Simpson RJ, 2001). Owing to its high throughput, MS-based proteomics has been extensively applied for probing molecular mechanisms in signal transduction, identifying cancer biomarkers and profiling various diseases (Celis

and Gromov, 2003; Celis et al., 2004; Jain, 2002; Michaud and Snyder, 2002; Wang et al., 2004).

Proteins are important targets for drug discovery and may also be of prognostic importance which can be treated as diagnostic and prognostic factor (Jain, 2002). Moreover, aberrant regulation and function of transcription factors has been reported to be involved in the pathogenesis of AML (Chava Parry, 2002; McKnight, 2001; Nerlov, 2000; Pabst et al., 2001b; Pedersen et al., 2001; Perrotti et al., 2004; Rangatia et al., 2002; Timchenko, 2003; Vangala et al., 2003; Westendorf et al., 1998; Zheng et al., 2004). Mapping of protein interactions of crucial transcription factors can highlight new functions of a known protein or can even define the function of novel proteins (Figeys, 2002; Michaud and Snyder, 2002). Few years back yeast two-hybrid was the method of choice to screen protein-protein interactions but, nowadays this has been replaced by high throughput mass spectrometry (MS) based proteomics which is extensively being used for screening targets as well as interacting proteins. Proteomics, a qualitative and quantitative method has been the technique of choice to identify target and interacting proteins (Mann et al., 2001; Pandey et al., 2000). Proteomics encompasses many platform technologies (figure 5) for protein separation and identification, for determining their biomolecular interactions, function, and regulation, and for annotating, storing, and distributing protein information (Aebersold and Goodlett, 2001; Celis and Gromov, 2003; Celis et al., 2004; Figeys, 2002; Figeys, 2003; Gavin et al., 2002; Panisko et al., 2002).

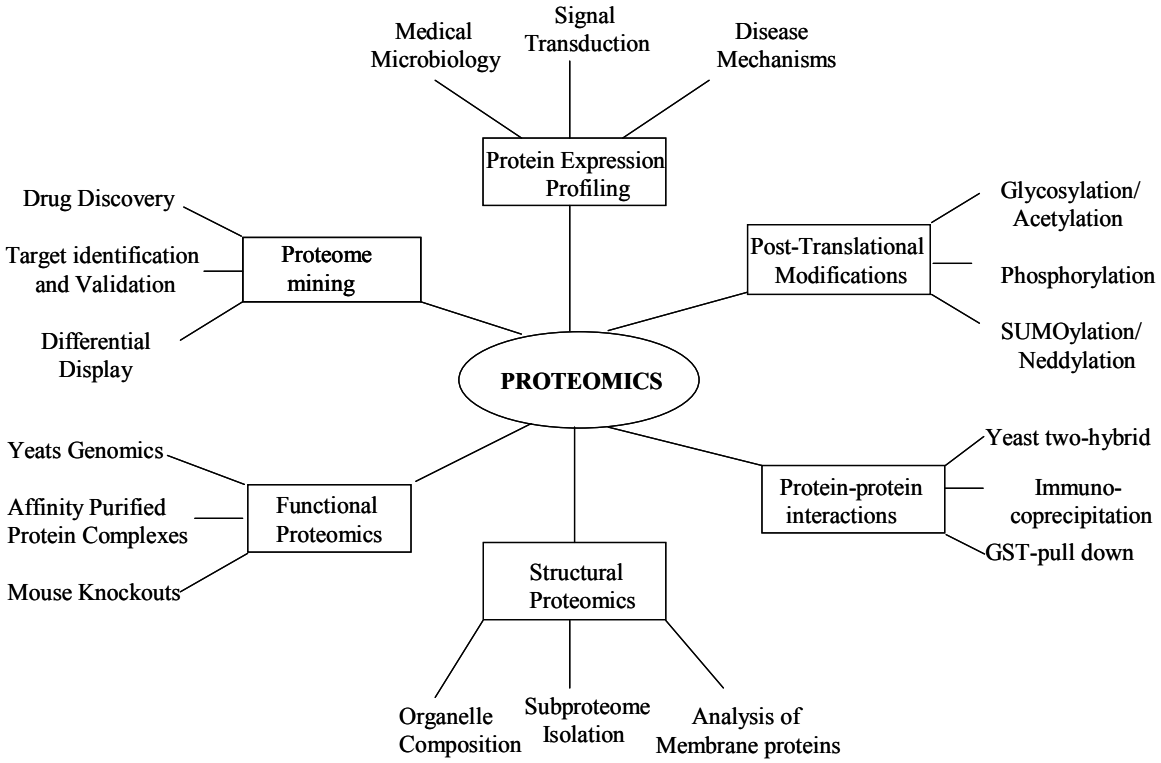

Fig 5. Technology and resources in proteomics: Technologies and resources in proteomics encompasses many platform technologies for protein separation and identification, for determining their biomolecular interactions, structure, function, and regulation, and for annotating, storing, and distributing protein information. In the context of translational cancer research, proteomics requires well-established tissue banks as well as tools from genomics and transcriptomics Adapted from molecular´s biologists guide for proteomics (Graves and Haystead, 2002).

1.8 Aim of the study:

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

2. Materials and Methods:

2.1 Materials:

2.1.1 Mammalian Cell Lines:

293T cells (human embyronic kidney fibroblast cells)

U937 (human myeloid cell line, monoblastic)

Myelomonocytic HL60 cells

K562 ER-C/EBPα (Erythroleukemic cells stably transfected with C/EBPα-ER fusion vector)

NB4 cells (Acute promyeloblastic cell line carrying PML-RARα fusion Gene)

Cells from different AML patients for western blot analysis were kindly provided by the laboratory for leukemia diagnostic, Med III Klinikum Großhadern, University of Munich, Germany.

2.1.2 Cell Culture:

RPMI (PAA, Cölbe, Germany)

RPMI without Phenol red (PAA, Cölbe, Germany)

Foetal bovine serum (Invitrogen/GIBCO, Germany)

Penicillin/Streptomycin (GIBCO, Germany)

Charcoal treated FBS (Hyclone, Greiner, Nürtingen, Germany)

Dialysed FBS (SIGMA, Germany)

DPBS (PAN, Germany)

DMEM (PAN, Germany)

Trypsin EDTA (GIBCO, Germany)

β-Estradiol (SIGMA, Germany)

2.1.3 Immunoblots:

GST sepharose beads 4B (Amersham Biosciences, USA)

Protein agarose beads (Roche Molecular Diagnostics, Germany)

Phosphatase inhibitor Cocktail I and II (Sigma, Germany)

Protease inhibitors (Sigma, Germany)

8 and 10% SDS PAGE (Acryl amide; Roth, Germany)

Bradford assay buffer (Biorad Laboratories, Germany)

Nitrocellulose membrane (Millipore, Germany)

ECL detection kit (Amersham Biosciences, Germany)

ECL hyperfilm (Amersham Biosciences, Germany)

2.1.4 Antibodies:

Anti-C/EBPα (cat# sc-61 and sc-61X for Gelshift; Santa Cruz Biotechnology, CA, USA)

Anti-β-tubulin (cat# sc-9104 Santa Cruz Biotechnology, CA, USA)

Anti-JNK1 (cat# sc-474 Santa Cruz Biotechnology, CA, USA)

Anti-pJNK1 (cat# 9251S Cell Signalling Technology, USA)

Anti-pc-Jun (cat# 9261S Cell Signalling Technology, USA)

Anti-GST (cat# sc-131 Santa Cruz Biotechnology, CA, USA)

Anti-PU.1 (cat# sc-5972 Santa Cruz Biotechnology, CA, USA)

Anti-HA (cat# 3F10, Roche Molecular Diagnostics, Germany)

2.1.5 2D-gel Electrophoresis:

Urea plus (Amersham biosciences, Germany)

Resolyte buffer (Rehydration buffer; Amersham Biosciences, Germany)

Bromophenol blue (Sigma, Germany)

Immobilin dry strips (IPG strips, pH 3-10, Amersham Bioscience, Germany)

Colloidal Coomassie blue G-250 (Sigma, Germany)

Bromophenol Blue (Sigma, Germany)

2.1.6 Peptide Extraction:

50% and 70% Acetonitrile and 50mM Ammonium Bicarbonate (NH_4HCO_3) for washing and fixing the gel pieces

Trypsin enzyme reconstituted with 50mM Ammonium Bicarbonate (NH_4HCO_3).

Lyophilized peptides dissolved in solution containing 20% Acetonitrile and 0.1% Trifluoroacetic acid (TFA).

DHB matrix solution of 9:1 ratio of DHB: HMB

2.1.7 Mass Spectrometry:

Reflex III MALDI-TOF for Peptide mass fingerprints i.e PMF (Bruker Daltonics, Leipzig, Germany)

AB4700 MALDI-TOF/TOF for MS/MS analysis and GPS explorer software for spectral annotation (Applied Biosystems, Darmstadt, Germany)

Mascot database search (Matrix Science)

AnchorChip plate (Bruker Daltonics, Leipzig, Germany)

2.1.8 Chemicals:

2,5-Dihydroxy-Benzoic Acid (Sigma Aldirch, USA)

2-Hydroxy-5-Methoxy-Benzoic Acid (Sigma Aldirch, USA)

Acetonitrile (Sigma Aldirch, USA)

Acetic Acid (Merck, Darmstadt, Germany)

Ammonium Bicarbonate (Sigma, USA)

Anisomycin (Calbiochem, Germany)

Colloidal Coomassie Blue G-250 (Sigma, Germany)

Dimethyl Sulfoxide (Sigma, USA)

Dithioerythritol (Merck, Darmstadt, Germany)

Dithiothreitol (Merck, Darmstadt, Germany)

Deoxycholate (Merck, Darmstadt, Germany)

Ethanol (Merck, Darmstadt, Germany)

Formaldehyde (Merck, Darmstadt, Germany)

Ethylenediamine Tetra-Acetic Acid (Merck, Darmstadt, Germany)

Ethylene Glycol bis (2-aminoethyl ether)-N,N,N'N'-Tetra acetic Acid

(Merck, Darmstadt, Germany)

Formaldehyde (Merck, Darmstadt, Germany)

Glycine (ICN Biomedicals)

Glycerol (Merck, Darmstadt, Germany)

Isopropanol (Merck, Darmstadt, Germany)

JNK inhibitor SP600125 (Calbiochem, Germany)

Methanol (Merck, Darmstadt, Germany)

Silver Nitrate (Merck, Darmstadt, Germany)

Sodium Carbonate (Merck, Darmstadt, Germany)

Sodium Chloride (ICN Biomedicals)

Sodium Thiosulfate (Merck, Darmstadt, Germany)

Sodium Dodecyl Sulphate (SDS) (Sigma, USA)

Trifluoroacetic Acid (TFA) (Merck)

Triton X-100 (Sigma, USA)

Tween-20 (Sigma, Germany)

Urea Plus (Merck, Darmstadt, Germany)

2.2 Methods:

2.2.1 GST-Purification:

We used plasmid constructs in which full length C/EBPα and regions comprising amino acids 270 to 358 encoding the DNA binding domain of C/EBP (conserved in all C/EBP family proteins) are cloned in frame with the Glutathione-S-Transferase (GST) in a pGEX bacterial expression vector (Amersham Biosciences, Germany). Fusion proteins were expressed in transformed DH5α *E. coli* bacterial strains after 0.5mM IPTG induction for 2 hours. Induced bacterial pellet was lysed in NETN buffer {150mM NaCl, 20mM Tris pH 8.0, 1mM EDTA pH 8.0, 0.1% NP40 and protease inhibitors (Sigma)} and sonicated, followed by subsequent protein purification using immobilised Glutathione Sepharose 4B beads (Amersham Biosciences, USA). Sepharose beads bound with GST proteins were washed twice with NETN buffer on a rotating shaker at 4°C for 10 minutes each and then lysed in 2X SDS sample loading buffer (125mM Tris pH 6.8, 4% SDS, 20% Glycerol, 10% 2-Mercapto-

ethanol and pinch of bromophenol blue). Subsequently proteins were separated on 12% SDS PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis) and visualised by Coomassie blue staining.

2.2.2 GST-Pull Down: In the present study, we used the myelomonocytic U937 cell line as our model system. Nuclear extract from U937 cells was prepared using lysis buffer A (20mM Tris pH 8.0, 10mM NaCl, 3mM $MgCl_2$, 0.1% NP40, 10% Glycerol, 0.2mM EDTA and Protease inhibitors 1µg/ml) and lysis buffer C (20mM Tris pH 8.0, 400mM NaCl, 0.2mM EDTA, 20% Glycerol, 1mM DTT and 1µg/ml Protease inhibitors). Equal amounts of bacterially purified proteins were incubated with 500µg of nuclear extract (volume made up to 1ml with NETN buffer) for 3h at 4°C. After pull down, protein bound beads were washed 3 times with NETN buffer at 10 rpm on a rotating shaker for 5 minutes at 4°C. Beads were lysed in urea lysis buffer (66% Urea plus one, 1% DTE, 4% CHAPS, 2.5mM EDTA, 2.5mM EGTA) to completely denature the interacting proteins which were then applied for 1D and 2D separation in a pH range of 3-10. In addition, S^{35}-Methionine labelled *in-vitro* translated JNK1 was pulled down with GST-fusion proteins by incubating for 3 hours, separated on 10% SDS PAGE and detected by autoradiogram using Kodak films.

2.2.3 2D-Gel Electrophoresis: GST and GST-DBD were incubated with nuclear extracts of the myelomonocytic cell line U937. Beads with their associated proteins from nuclear extract were lysed in urea lysis buffer for 1h at RT on a rotating shaker. Lysed beads were passed through an RNA quiashredder (Quiagen, Germany), and resulting supernatant containing dissolved proteins was ultracetrifuged for 50 minutes at 50,000 rpm at 22°C to get rid of DNA and other cellular debris. In the first dimension, 350µl of dissolved proteins after ultracentrifugation were separated on an immobiline^{TM} dry strip pH 3-10 (Amersham Biosciences, Germany) 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.

2.2.4 MALDI-TOF Mass Spectrometry: On comparison of the two silver stained gels, differentially appearing protein spots from GST-DBD gel were carefully excised, destained and in gel digestion was performed with 200ng trypsin (Promega) in ammonium bicarbonate solution for 16 hours. The digested peptides were eluted in 70% acetonitrile, lyophilised and resuspended in 5µl 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 DALONICS) mass spectrometry and corresponding proteins were identified by a MASCOT database search (Matrix Sciences).

2.2.5 Plasmid Constructs: In this article we used pCDNA3-MKK7, pCDNA3-MEKK1-flag (kind gift from Dr. Roger Davis); GST, GST-DBD, GST-C/EBPα (kind gift from Dr. Claus Nerlov); C/EBPα-GZ, (Leucine zipper replaced with leucine zipper of yeast GCN4), BR3 basic region (Amino acids: 297R, 298K, 300R, and 302K of C/EBPα mutated to Glycine, Threonine, Glycine and Aspargine respectively); L1,2 (Leucine 1 and 2 form leucine zipper mutated to alanine) was kindly provided by Dr. Alan Friedman and is described elsewhere (Landschulz et al., 1989); pCDNA3-humanC/EBPα was previously described (Pabst et al., 2001b), GST-c-Jun corresponding to 35 kDa (1-79 amino acids

fused with GST) was bought from Cell Signalling technology while bacterially purified GST-c-Jun (40kDa) was a kind gift from Dr. Arndt Keiser.

2.2.6 Cell Culture: U937 and HL 60 cells were cultured in RPMI supplemented with 10% heat inactivated foetal bovine serum (GIBCO, Aidenbach, Germany) and 1% Penicillin-Streptomycin (GIBCO, Aidenbach, Germany); human embryonic kidney 293T cells were maintained in Dulbecco´s modified Eagle medium (DMEM; GIBCO) supplemented with 10% foetal bovine serum (FBS), 1% glutamine, and 1% Pen/strep (GIBCO); NB4 cells were maintained in RPMI supplemented with 20% FBS, 2.5% Penicillin-Streptomycin and 2.5% glutamine. K562 Er-C/EBPα and K562Er cells were maintained in RPMI without phenol red supplemented with 10% charcoal treated FBS (Hyclone, USA) and 2µg/ml Puromycin (ICN Biochemicals).

2.2.7 Co-Immunoprecipitation: Co-immunoprecipitation is an *in-vitro* biochemical assay which we used to detect *in-vivo* interaction of C/EBPα with JNK1. Here we used 25µl slurry of protein A agarose beads (Roche Molecular Diagnostics, Germany) and 2µg of the anti JNK1 antibody (Santa Cruz Biotechnology) to immunoprecipitate JNK1 from whole cell extracts of the myeloid cell line U937 using co-immunoprecipitation buffer (1X TBS, 0.5% NP40 and protease inhibitors). Immunoprecipitated proteins were heated at 56°C for 90 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 rabbit polyclonal anti-JNK1 and anti C/EBPα antibody (Santa Cruz Biotechnology) as required after transferring to a PVDF immobilon-P membrane.

2.2.8 *In-vitro* Kinase Assay: To analyse whether JNK1, a substrate specific kinase, phosphorylates C/EBPα, an *in-vitro* kinase assay was performed using

GST-DBD as substrate and JNK1 as kinase which was previously immuno-precipitated using an anti-HA antibody (Roche Molecular Biochemicals, Germany) from a whole cell extract of 293T cells co-transfected with C/EBPα and MEKK1; proteins bound to beads after immunoprecipitation were equilibrated with kinase buffer. GST c-Jun (1-79 amino acids of c-Jun containing S63 and S73; Cell Signalling Technology) a known substrate of JNK1 was used as positive control. Kinase reaction was performed for 30 minutes at 30°C using 1X kinase buffer (Cell Signalling Technology), 4μCi [γP^{32}]ATP (3000Ci/mMol), 50μM ATP, 10μl GST-DBD and immuno-precipitated HA-JNK1. After 30 minutes the reaction was terminated using 2X SDS loading buffer. The proteins were separated on 10% SDS PAGE and visualised by autoradiography.

2.2.9 Identification of Phosphopeptides by Mass Spectrometry: In order to identify phosphopeptides from tryptic peptides of C/EBPα, immunoprecipitated C/EBPα from 293T alone and co-transfection of C/EBPα and MKK7 was separated on 10% SDS PAGE and excised bands were sent to Toplab (Munich, Germany) for further analysis. Both protein bands were digested with the protease trypsin and MALDI MS was done to make identification. For database search, the program MS Fit (Protein Prospector) was used with the NCBI database (release 210604). Two protein bands, control (Ip C/EBPα from 293T) and protein of interest (Ip C/EBP from 293T cotransfected with C/EBPα+ MKK7) were clearly identified as the CCAAT/enhancer binding protein alpha (NCBI Accession # 28872794, 37.6 kDa, pI 7.3) from *Homo sapiens.* In a further process the digested sample of the possibly phosphorylated C/EBPα was incubated for 2h with bovine alkaline phosphatase (0.1u/μl /2h /30°C) to remove phosphorylation of peptides. A direct comparison of the three MALDI spectra CCAAT (control), CCAAT (phosphorylated) and CCAAT (phosphorylated + alkaline phosphatase) confirmed the presence of a phosphopeptide. The mass of

the non-phosphorylated peptide is 1466.74Da with the sequence (K)SVDKNSN EYRVR(R) (Start: 277 / End: 288).

2.2.10 Electrophoretic Mobility Shift Assay (EMSA): To assess C/EBPα DNA binding activity, a double-stranded G-CSF receptor promoter oligo-nucleotide extending from bp -57 to -38 was used as a probe (Smith et al., 1996). EMSA was performed by incubating 5µg of nuclear extract with 1ng of the radiolabelled probe in binding buffer [10 mM HEPES pH 7.9, 50 mM KCl, 2.5 mM $MgCl_2$, 1mM dithiothreitol (DTT)], 10% glycerol and 0.5µg of poly dI-dC (Amersham Biosciences, Germany) for 30 minutes at room temperature. For competition experiments, a 50-fold excess of unlabeled competitor oligo-nucleotide was added before addition of the labelled oligonucleotide. For supershift experiments, 1.0µl of specific C/EBPα antisera (Santa Cruz Biotechnology, CA USA) were added before addition of the radiolabelled probe. Complexes were resolved in 5.2 % non-denaturing polyacrylamide gels in 1X TBE buffer. Oligos used were OligoA (AAG GTG TTG CAA TCC CCA GC) and OligoB (GCT GGG GAT TGC AAC ACC TT; Metabion, Germany). Nuclear extracts used for EMSA were prepared from transiently transfected 293T cells with expression plasmids of hC/EBPα (human C/EBPα), JNK1 and MEKK1.

2.2.11 Transient Transfections using LipofectAMINE Plus and Reporter Assays Firefly and Renilla Luciferase: 293T cells were transiently transfected using LipofectAMINE Plus (Invitrogen Life Technologies, Germany) as described by the manufacturer. Firefly luciferase activities from the constructs phC/EBPα, pCDNA3-MEKK1, pTK, p(C/EBP)2TK and *Renilla* luciferase activity from the internal control plasmid pRL-null were determined 24 h after the initiation of the transfection protocols using the Dual-Luciferase Reporter Assay System (Promega, Germany). Firefly luciferase activities were normalized

to the *Renilla* luciferase values of pRL-null. Results are given as means ± S.E.M. 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-hC/EBPα, pCDNA3-MEKK1, pTK and p(C/EBP) 2TK each; 0.01 µg of the internal control plasmid pRL-null; and the same concentrations of the empty expression vectors were used as controls, respectively. In the transfections without cotransfection of MEKK1, the empty vector pCDNA3 was included to equalize the amount of DNA in each transfection set. pRL-null was chosen as internal control plasmid, because it was not transactivated by Ras in 293T cells which in general activates the pTKα promoter in the presence of C/EBPα (Behre et al., 2002b; Behre et al., 1999a)

2.2.12 *In-vivo HA* Ubiquitination Assay: $1*10^6$ 293T cells were transiently transfected with different constructs as described (figure 12A), 24 h post transfection cells were lysed in RIPA buffer, and C/EBPα was immuno-precipitated from 500µg protein, as described before in this article. HA-Ubiquitin was a kind gift from Dr. Bohmann (Musti et al., 1997) .

2.2.13 Pulse Chase Labelling: $3*10^5$/ml HL60 cells were plated in 50ml normal RPMI medium (RPMI supplemented with 10% FBS and 1 % Pen/Strep) one day before the experiment. In the next day cells were washed twice with PBS and grown in 3ml labelling medium (RPMI without methionine and cysteine, supplemented with 0.2% dialysed FBS; Sigma USA) together with 200µCi [S-35]-methionine/ml for 15 minutes with constant shaking at 37°C in a water bath. The cells were then washed to remove labelling medium and were chased for various time points as indicated (figure 12B) with normal RPMI medium in the absence or presence of 50ng anisomycin. The cells were lysed in RIPA buffer supplemented with protease inhibitors, and then lysates were subjected to immunoprecipitation with a C/EBPα antibody. The resulting

precipitates were separated on 10% SDS PAGE followed by autoradiography and densitometry analysis.

2.2.14 Western Blotting: 24 h post-transfection, whole cell extract was prepared with RIPA buffer (1% NP40, 0.5% Sodium Deoxycholate (DOC), 0.1% SDS, 150mM NaCl, 5mM EDTA, 50mM Tris pH 8.0 and 1μg/ml Protease Inhibitors). Equal amount of total protein was denatured in the SDS sample loading buffer, separated on 10% SDS-polyacrylamide gels and transferred to an immobilon-P membrane (Millipore, USA). The membranes were incubated with anti-C/EBPα antibody (dilutions; Santa Cruz Biotechnology) or anti-β–tubulin antibody as an internal control (Roche Molecular Biochemicals) for 1-3h and horseradish peroxidase conjugated to secondary antibodies (Amersham Biosciences, Germany) for 1h. Signals were detected with the ECL Western blotting detection reagents (Amersham Biosciences, Germany). In parallel, Anisomycin (Calbiochem, Germany) treated (25ng/ml) myeloid HL60 cells for 30, 60 and 90 minutes were lysed in RIPA buffer and immunoblotted against the C/EBPα antibody. In the experiment with cycloheximide treatment, U937 cells were treated with 50μg/ml cycloheximide prior to treatment with 25ng/ml anisomycin, whole cell extract was prepared and equal amount of protein was separated. In all immunoblotting experiments a 1:1000 dilution for primary and 1:2000 dilutions for secondary antibody was used.

3. Results:

3.1 Purification of GST fusion constructs: In order to understand the molecular mechanism underlying C/EBPα inactivation in patients with acute myeloid leukemia (AML), we applied proteomics based mass spectrometry to identify the interacting proteins of C/EBPα which might be important in AML pathogenesis. Figure 6A depicts the location of characterized functional domains within the CCAAT/enhancer-binding protein (C/EBP); BR-LZ DNA-binding

domain (DBD) is required for dimerization and DNA binding. The three transactivation elements TE-I, TE-II and TE-III mediate interactions with the transcriptional machinery; GST fused to wild type C/EBPα and the DNA binding domain of C/EBP was used in this study.

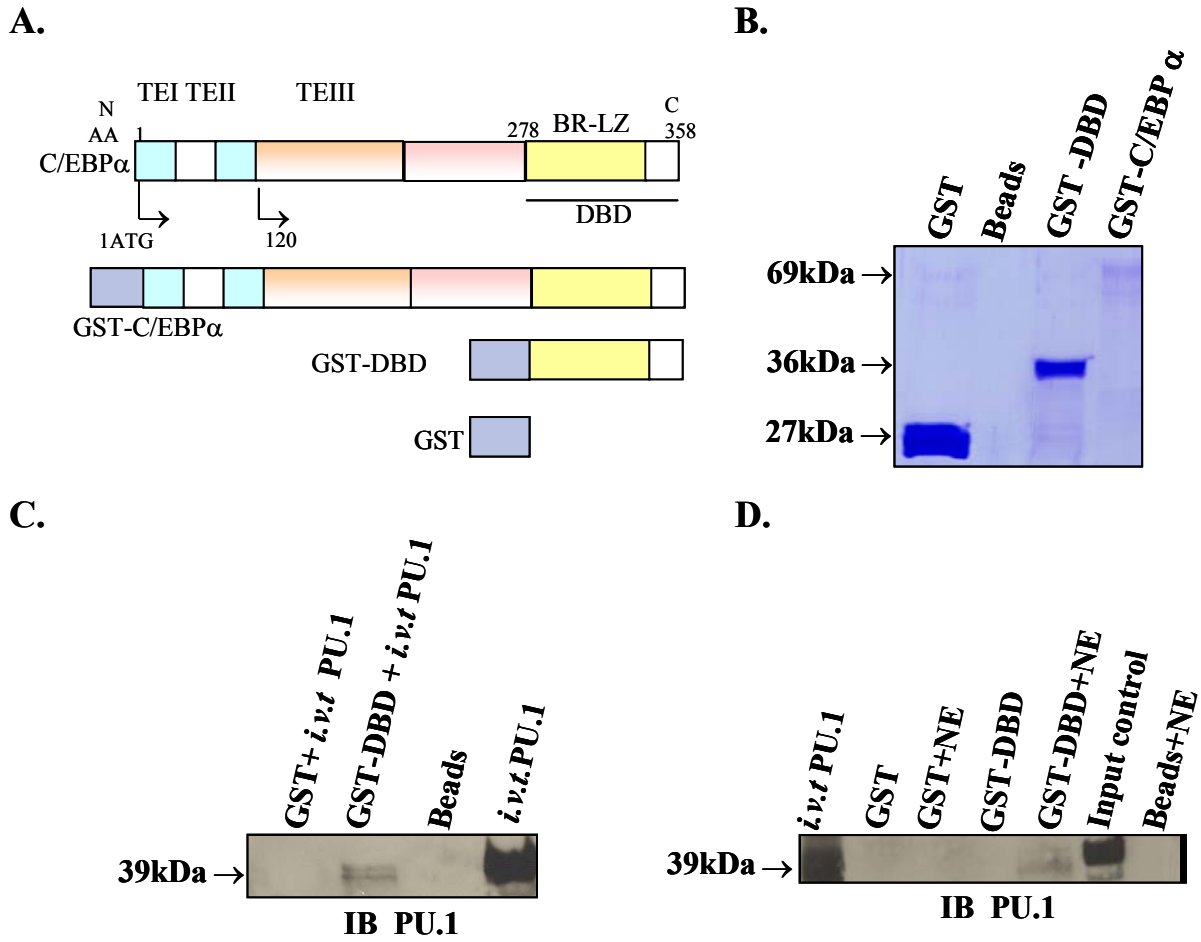

Fig 6. Bacterial purification of GST fusion proteins: A) The location of characterized functional domains within the CCAAT/enhancer-binding protein (C/EBP) protein. The basic region leucine zipper (BR-LZ) has been shown to mediate protein interaction with other transcription factors involved in lineage-specific gene regulation. The three transactivation elements TE-I, TE-II and TE-III mediate interactions with the transcriptional machinery (TBP/TFIIB; CBP/p300; SWI/SNF), GST fused with full length C/EBPα and DBD region of C/EBP is schematically depicted. **B)** Coomassie stained gel showing purified GST-C/EBPα, GST DBD, and GST proteins from *E.coli* after induction with IPTG. **C,D)** Physical activity of bacterially purified proteins was confirmed by immunoblotting against an anti PU.1 antibody after incubating them with *ivt* PU.1 (c) and nuclear extract (NE) of U937(d); PU.1 is known to bind DBD of C/EBPα .

The Coomassie stained gel picture shows the purification of GST fused with the DNA binding domain of C/EBP (GST-DBD) and GST alone from bacterial culture after induction with IPTG (figure 6B). Physical activity of the bacterially

purified GST fusion constructs was confirmed by assessing their interaction with PU.1 which is known to bind with C/EBPα in its DNA binding domain (figure 6C,D).

3.2 2D Gel Electrophoresis of differentially interacting proteins:

We next performed GST pull down assays using GST-DBD and GST from a U937 nuclear extract and separated the differentially interacting proteins in 2D gel (2-dimensional gel electrophoresis) after lysing the protein bound beads in the sample buffer described in *materials and methods*.

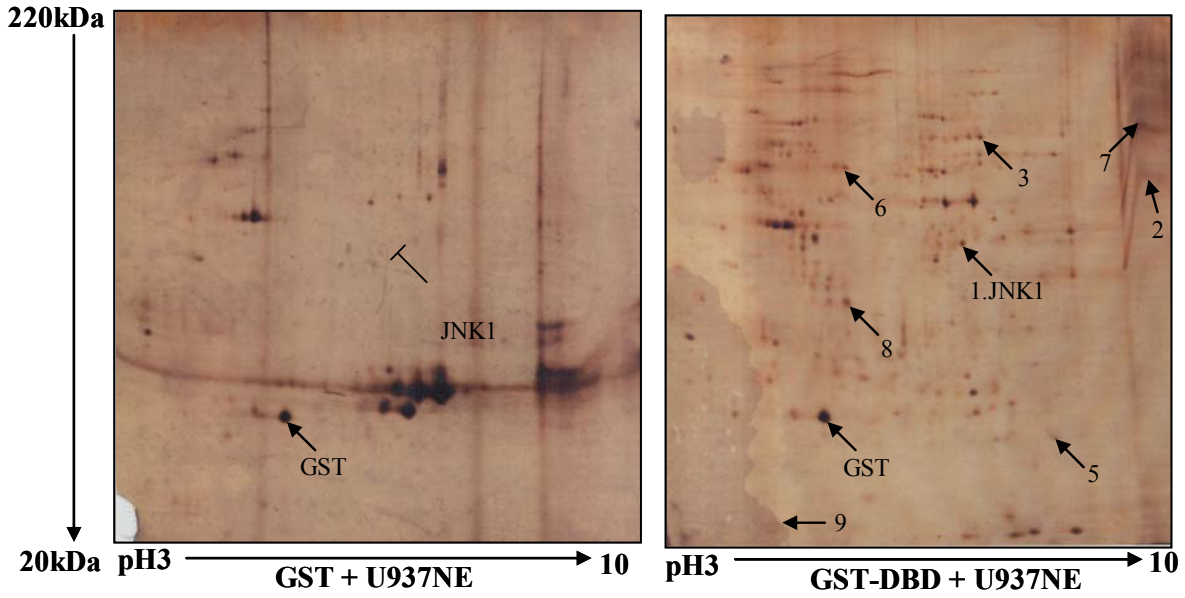

Fig 7. 2D-Gel electrophoresis of differentially interacting proteins: GST and GST-DBD were incubated with nuclear extract of myelomonocytic cell line U937. Beads with their associated proteins from nuclear extract were lysed in urea lysis buffer (described in materials and methods). In the first dimension, 350µl of soluble proteins after ultracentrifugation were separated on immobiline™ dry strip pH 3-10 (Amersham Biosciences) by isoelectric focussing (IEF) where proteins are separated on the basis of their isoelectric point (pI). 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.

Silver staining was done to visualise differentially interacting proteins in the GST-DBD gel (figure 7); protein spots present only in GST-DBD gel were excised and analysed by MALDI TOF mass spectrometry for identification.

Identified proteins are listed in table 4. We were not able to identify all proteins owing to their low amount and the sensitivity of mass spectrometer.

Table 4: Interacting Proteins of C/EBPα identified by MALDI TOF Mass Spectrometry from GST-DBD gel.

Accession No.	Theoritcal Mol Mass (kDa)	Description
1. JNK1	40.36	c-Jun N-Termnial Kinase rsidues
2. Q9NQU5	75.00	p21 Activated Protein Kinase
3. Q9UQ36	137.50	RNA Binding Protein Fragment human
4. O78168	23.00	HLA-A 24* (var)
5. Q96TA6	24.80	MADP-1
6. Q8TD91	72.43	Hepatocellular Carcinoma Associated protein HCA2
7. Q9P1U9	80.44	Zinc Finger Protein 45
8. Q9UPN3	532.40	Macrophin 1 isoform fragment (homo sapiens)
9. Q9NZT1	15.91	Calmodulin like skin protein

3.3 JNK1 physically interacts with C/EBPα *in-vitro*:

Among the identified interacting proteins of C/EBPα (table 4), we chose JNK for further characterization since JNK is known to regulate the activity of its physically associated substrates (Fuchs et al., 1997). To examine whether there is a direct protein-protein interaction between GST-DBD and JNK, we used GST-purified GST-DBD and incubated it with [35S]-methionine-labelled *in-vitro* translated JNK1 for 90 minutes. After washing with stringent buffer, an interaction between JNK1 and GST-DBD was observed by autoradiography (figure 8A). As our major focus is C/EBPα, we next asked if JNK1 interacts with full length C/EBPα. We performed a GST-Pull down assay using GST-DBD; GST-C/EBPα; and GST alone from 25ng/ml anisomycin induced (RIPA*) and uninduced U937 RIPA lysates, The immunoblot against JNK1 shows a direct interaction of JNK1 and pJNK1 with GST-DBD and GST-C/EBPα; GST-c-Jun served as a positive control for this interaction (figure 8B).

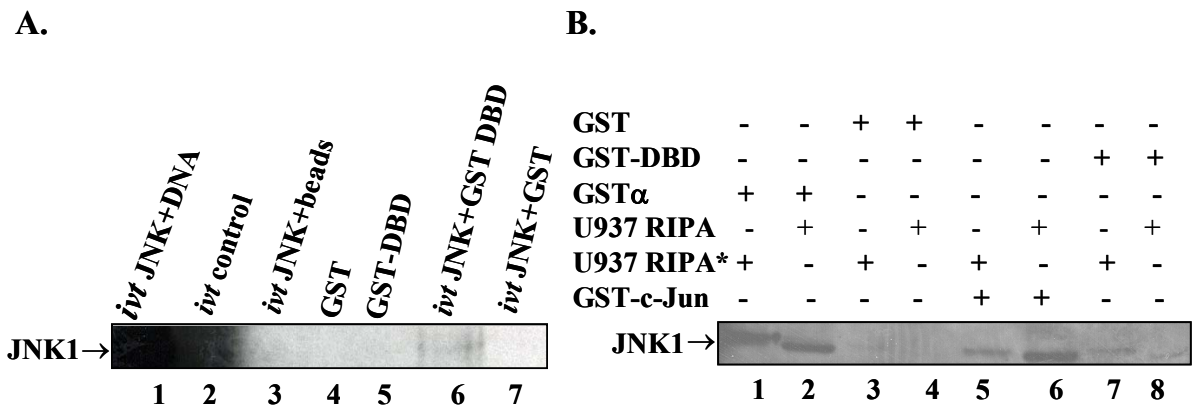

Fig 8. C/EBPα physically binds to JNK1 *in-vitro*. A) GST pull-down assay was performed from [35S]-methionine-labelled *in-vitro*-translated JNK1 incubated with equal amounts of bacterially expressed GST-DBD (lane 6), GST (lanes 7) and sepharose beads (lane 3) GST-DBD or GST was washed stringently and separated on SDS-PAGE prior to autoradiography. **B)** Immunoblot against JNK1 after GST pull down of JNK1 from a whole cell extract of anisomycin induced (RIPA*) and uninduced U937 cells shows an *in-vitro* interaction of JNK1 with GST-C/EBPα, GST-DBD and positive control c-Jun while there is unspecific or no interaction with GST.

3.4 JNK1 physically interacts with C/EBPα *in-vivo*: Next we asked if C/EBPα and JNK1 also interact in *in-vivo,* for this we performed JNK1 co-immunoprecipitation using an anti JNK1 rabbit polyclonal antibody from whole cell extract of U937 cells. An immunoblot against JNK1 (upper panel) followed by C/EBPα (lower panel) after stripping the same membrane confirms an *in-vivo* interaction of JNK and C/EBPα in myeloid cells (figure 9A). Since JNK interacts with DBD region of C/EBPα, We tried to narrow down DBD region interacting with JNK1 using already published DBD point mutants of C/EBPα (Liu H et al., 2003). Note that among these, two are point mutants of DBD region while other is C/EBPα with its leucine zipper replaced with that of yeast GCN4 luecine zipper. Therefore, all the mutants have almost equal molecular weight (MW) of 42kDa like wild type C/EBPα. DBD mutants of C/EBPα used are described in *materials and methods*. 293T cells were separately cotransfected with various mutants and HA-JNK; 24h posttransfection, cells were lysed and JNK was immunoprecipitated with an Anti-HA antibody from 500μg protein. After separation on 10% SDS PAGE,

immunoblots with an anti HA antibody (upper panel) and then with C/EBPα antibody after stripping the same membrane

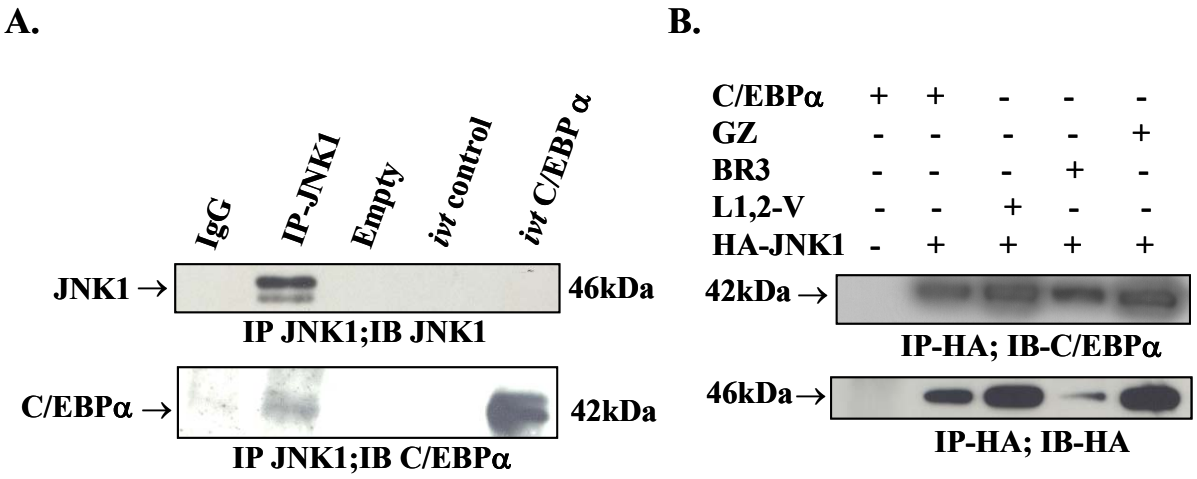

Fig 9. C/EBPα and JNK1 physically interact *in-vivo*: A) C/EBPα and JNK1 interaction was confirmed *in-vivo* in myeloid cell line U937 by co-immunoprecipitation of JNK1 from U937 nuclear extract using an anti JNK1 rabbit polyclonal antibody and immunoblotted with JNK1 (upper panel) same membrane stripped and reblotted with C/EBPα antibody (lower panel). **B)** JNK1 interacts with different C/EBPα DNA binding mutants; GZ, (Leucine zipper replaced with leucine zipper of yeast GCN4 protein), point mutants BR3 (basic region mutant) and L1,and 2 form leucine zipper mutated to valine. Although phosphopeptide region 271-288 identified later in this article is still intact in these mutants. 293T transfected with above mutants together with HA-JNK, cells were harvested and JNK1 was immunoprecipitated using an anti HA antibody (upper panel), same membrane stripped and reblotted with C/EBPα (lower panel).

shows an interaction of JNK1 with all of these mutants (Lower panel), though with varying extent. However, later in this study we identified one phosphopeptide from tryptic digests of phosphorylated C/EBPα belonging to the DBD region which spans from amino acid 277-288. This region is still intact in the above used C/EBPα mutants (figure 9B) and could be the putative region of interaction with JNK1.

3.5 JNK1 phosphorylates C/EBPα *in-vitro*:

Because JNK1 is a potent kinase from the MAPK family (Manning and Davis, 2003) and C/EBPα is known to be post translationaly modified by SUMO, ubiquitin and phosphorylation (Behre et al., 2002b; Mahoney et al., 1992; Ross et al., 1999; Ross et al., 2004; Subramanian et al., 2003) we asked if JNK1 also

modifies C/EBPα. For this, an *in-vitro* kinase assay using GST purified GST-C/EBPα as substrate and immunoprecipitated HA-JNK from transiently co-transefected 293T together with MEKK1 as kinase shows that GST- C/EBPα can be phosphorylated by JNK1 (figure 10A; lane 1). After confirming the phospho modification of GST-C/EBPα as expressed and purified from bacteria, we assessed the phosphorylation of C/EBPα purified from transfected 293T cells. We performed an *in-vitro* kinase assay using immunoprecipitated C/EBPα from transiently transfected 293T as substrate and immunoprecipitated HA-JNK

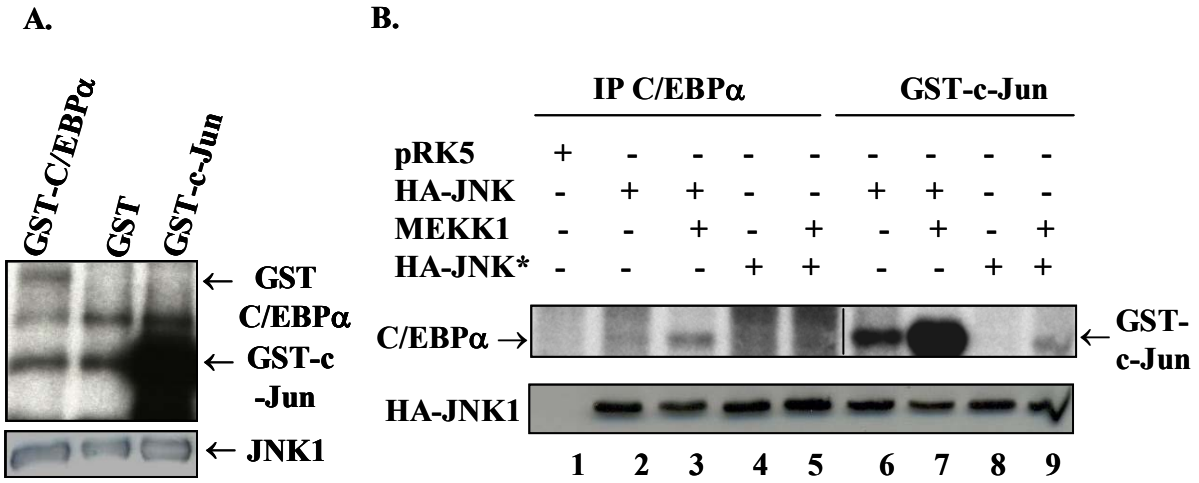

Fig 10. JNK1 phosphorylates C/EBPα in *in-vitro* kinase assay: A) JNK1 phosphorylates bacterially purified GST-C/EBPα in an *in-vitro* kinase assay performed using GST-C/EBPα as substrate and HA-JNK1 as kinase (lane 1), GST-c-Jun (lane 3) served as positive control (upper panel); autoradiogram developed after 4 hours. Membrane immunoblotted with an anti HA antibody confirms the presence of kinase in each lane (lower panel) **B)** An *in-vitro* kinase reaction using immunoprecipitated C/EBPα from transiently transfected 293T as substrate and immunoprecipitated HA-JNK1 from transiently transfected 293T as kinase also confirms phosphorylation of C/EBPα (lane 3) while there is much less phosphorylation of C/EBPα with HA-JNK* (kinase dead) in autoradiogram (upper panel) developed after 4hrs, membrane immunoblotted with anti JNK1 shows kinase in all lanes (lower panel). GST-c-Jun, a known substrate of JNK1 used as positive control in the above assays was bacterially purified (kind gift from Dr.A Keiser); bacterially purified GST-c-Jun has mol. weight 40kDa.

and HA-JNK* (from transiently cotransfected 293T together with MEEK1) as kinase. Wild type JNK phosphorylates C/EBPα (figure 10B; lane 3) while kinase dead HA-JNK could not (figure 10B; lane 5). GST-c-Jun (bacterially purified; Mol wt. 40kDa) was used as a positive control while the pRK5 empty vector transfected cells were used as control for JNK immunoprecipitation

(figure 10B; lane 1). Thus, the *in-vitro* kinase assay using wild type HA-JNK1 and kinase dead HA-JNK indicates that C/EBPα is a substrate of JNK1.

3.6 Phosphopeptide analysis of immunoprecipitated C/EBPα from transiently cotransfected 293T with MKK7 confirms presence of one phosphopeptide:

To identify the putative phosphopetides and localize the amino acid residue of C/EBPα targeted by JNK1, mass spectrometry analysis was done. C/EBPα from transiently transfected 293T and cotransfected together with MKK7 was immunoprecipitated and separated on 8% SDS PAGE. Further, the gel was colloidal Coomassie stained to visualise the C/EBPα band. The 42kD band corresponding to C/EBPα from lane 2 and 3 (figure 11A) was carefully excised and trypsin digested. After phosphopeptide enrichment, mass spectrometry analysis shows the presence of one phosphopeptide corresponding to a spectral peak at 1546.74 (1466.74Da+ ~80Da Phosphate group) in tryptic peptides of C/EBPα from cotransfection of MKK7 and C/EBPα (figure 11B, middle panel), whereas in the control tryptic peptides (figure 11B, upper panel, C/EBPα transfected alone) a spectral peak for the non phosphorylated peptide was observed.

In many cases, the final fraction of phosphorylated molecules is lower than 10%, and the percentage is usually even lower when the material stems from *in-vivo* phosphorylation (James, 2001). Thus, we can not rule out the possibility of more than one phosphopetide. Alkaline phosphatase (an enzyme which removes phosphate groups) treatment of phosphopetides shifts the spectral peak 1546.47 to a non-phosphorylated position at 1466.74 (figure 11B, bottom panel) which confirms the presence of a phospho peptide.

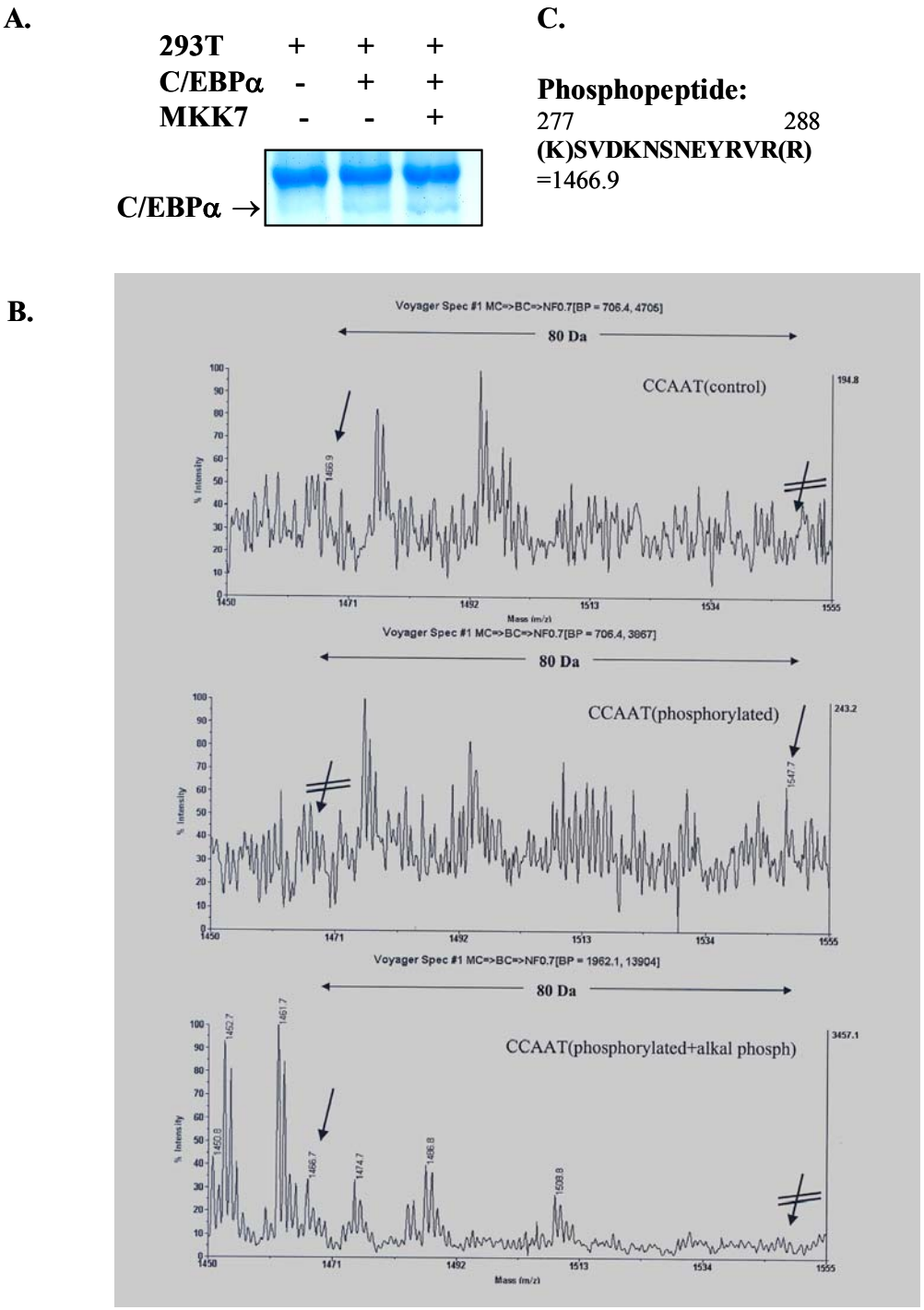

Fig 11. Phosphopeptide analysis of phosphorylated C/EBPα: A) Whole cell lysates from 293T transiently transfected with C/EBPα alone and together with MKK7 separated on 1D SDS PAGE, gel was Coomassie stained and C/EBPα band was excised for mass spectrometric analysis. B) After trypsin digestion and phosphopeptide enrichment, one phosphopeptide corresponding to C/EBPα tryptic peptide spanning from amino acid 277-286, having spectral peak at mass 1547Da (1466.9 + Additional 80Da corresponding to phospho group) was observed from C/EBPα band (middle panel spectra) of C/EBPα and MKK7 cotransfection, while in control (C/EBPα alone transfected; upper panel spectra) this spectral peak is seen at unmodified tryptic peptide at mass 1466.9. This was confirmed by alkaline phosphatase treatment (lower panel) which removes phospho group and hence peak at 1547

shifted back to original position at 1466.79. **C)** Amino acid sequence of identified phosphopeptide.

The spectral peak at 1466.74 corresponds to peptide (K)SVDKNSNEYRVR(R) which starts from amino acid 277 and ends at 288. This region contains two serine residues though they are not conventional MAPK sites for phosphorylation. However, there might be more than one amino acid residue getting phospho modified.

Since this region is also present in GST-DBD, we next asked if JNK1 can phosphorylate GST-DBD. An *in-vitro* kinase assay using GST-DBD as substrate and immunoprecipitated HA-JNK and HA-JNK* (kinase dead JNK mutant)

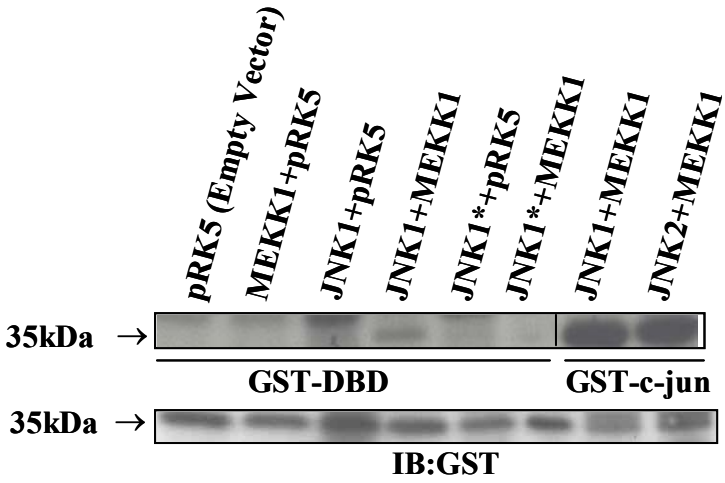

Fig 11. D) JNK1 phosphorylates GST-DBD in an *in-vitro* kinase assay : *In-vitro* kinase reaction using GST-DBD, GST-c-Jun as substrate and immunoprecipitated HA-JNK1 and HA-JNK1* from the transiently transfected 293T together with its activator MEKK1 as kinase shows that GST-DBD could be phosphorylated (lane 4) while much less with HA-JNK1* (lane 5) in autoradiogram (upper panel). Same membrane was stripped and blotted with anti GST to show presence of substrate, GST-c-Jun (GST fused with 1-79 amino acids of c-Jun; cell signalling technology, Germany) has size of 35 kDa.

respectively as kinase from transiently cotransfected 293T together with MEEK1 shows that GST-DBD can be phosphorylated by wild type JNK while there is little or no phosphorylation with mutant HA-JNK. GST-c-Jun was used as control (figure 11D).

3.7 JNK targets ubiquitination of C/EBPα:

JNK is known to regulate ubiquitination and hence protein stability of its physically associated substrates (Fuchs et al., 1997). Besides, C/EBPα is also known to be regulated by ubiquitination (Subramanian et al., 2003). Hence, we asked whether JNK can also target C/EBPα ubiquitination. We performed an *in-vivo* ubiquitination assay by transiently transfecting 293T cells with HA tagged ubiquitin (HA-Ubi) alone, together with C/EBPα, and MKK7 respectively. 24h post transfection cells were harvested and a whole extract was prepared.

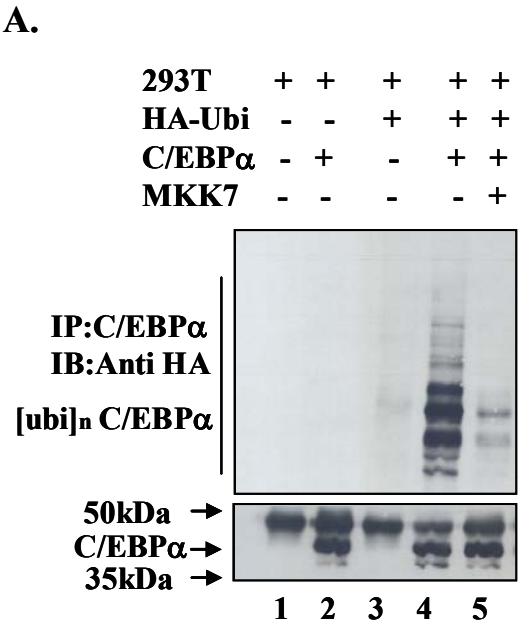

Fig 12. A) Inactive JNK targets C/EBPα ubiquitination: *In-vivo* HA-ubiquitination assay was performed by transfecting 293T cells with the expression plasmids for HA-Ubiquitin, hC/EBPα, and MKK7 as indicated, 24 post transfection cells were lysed and C/EBPα was immunoprecipitated. Immunoblot against HA antibody shows that C/EBPα is heavily ubiquitinated (lane 4), while this ubiquitination was inhibited upon JNK activation by co-transfection of MKK7 (upper panel). Membrane was stripped and reprobed for C/EBPα (lower panel) as control for C/EBPα immunoprecipitation.

An immunoblot against anti HA after immunoprecipitation of C/EBPα shows that C/EBPα is highly ubiquitinated when co-transfected with HA-Ubi (figure 12A, lane 4). However this ubiquitination was inhibited in HA-Ubi, C/EBPα and MKK7 co-transfected cells where JNK1 was activated by MKK7 (figure 12A, lane 5); MKK7 is known to directly interact and activate JNK1 (Moriguchi

et al., 1997). Owing to the fact that C/EBPα is overexpressed in this experiment, the steady state C/EBPα protein level in lane 5 does not seem to change upon inhibition of ubiquitination. However, the increase in the steady state C/EBPα protein level is expected after extended time points. Activation of JNK1 upon induction with a low amount of TNFα (20ng) also resulted in inhibition of C/EBPα ubiquitination, though it was not as efficient as with MKK7 induced JNK activation (data not shown). To our knowledge this is first report of the modulation of C/EBPα ubiquitination by JNK1.

3.8 Anisomycin induced activation of JNK1 enhances C/EBPα protein half life as compared to uninduced cells in pulse chase labelling assay: Next we asked when active JNK1 can inhibit C/EBPα ubiquitination; it should enhance the C/EBPα protein half life in a pulse chase assay. For this, HL 60 cells were grown in RPMI medium (lacking methionine) supplemented with

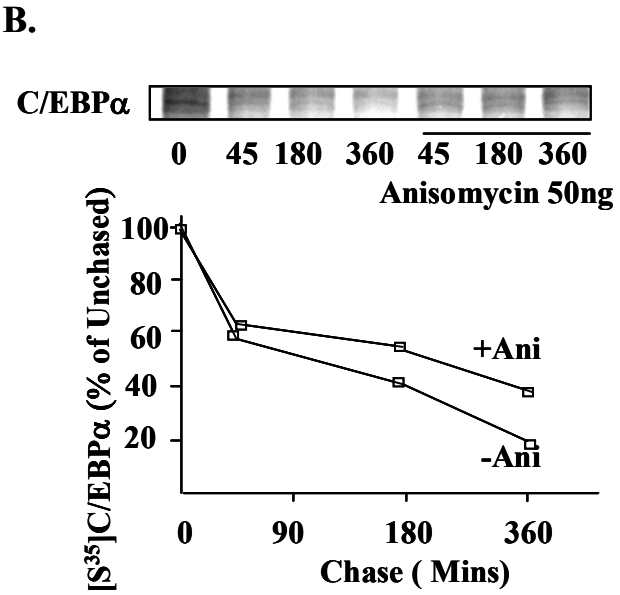

Fig 12. B) Anisomycin induced JNK activation prolongs C/EBPα half life: Myelo-monocytic HL 60 cells were labelled with [35S]-methionine and chased with the cold methionine for 0-6h with or without anisomycin treatment, as described in *materials and methods*. C/EBPα was immunoprecipitated from equal amount of protein (250µg), and analysed by autoradiography after separating on 10% SDS PAGE. Result of pulse chase was quantified using phosphor imager with AIDA software (Raytest, Germany), depicted in graph, which represents values with error of <15%).

[35S] - methionine and dialysed FBS for 10 minutes with continuous shaking at 37°C in a water bath, and then chased in normal medium (RPMI with methionine and non dialysed FBS) for different time points as indicated (figure 12B). Cells were harvested after indicated time points and C/EBPα was immunoprecipitated. The autoradiogram developed after 2 days shows that C/EBPα half life is prolonged when induced with anisomycin as compared to uninduced cells.

3.9 Induced activation of JNK enhances C/EBPα protein expression in Western blot analysis: Because active JNK1 inhibits C/EBPα ubiquitination and prolongs its half life, we next asked whether activation of JNK1 can enhance the C/EBPα protein expression. For this, we induced acute promyelocytic NB4 cells with 25ng/ml anisomycin, which is a chemical activator of JNK (Bogoyevitch et al., 1995). Cell lysates prepared after different time points as indicated were separated on 8% SDS PAGE. The immunoblot against C/EBPα shows that C/EBPα protein expression is significantly enhanced upon JNK activation (figure 13A i; *in-vitro* translated C/EBPα -His, 47kDa used as positive control for C/EBPα). The same lysates were separated in a different gel and were immunoblotted against phospho-c-Jun as a measure of JNK kinase activity (figure 13Aii) upon anisomycin induction; note that a casual relationship between JNK activation and active JNK induced C/EBPα expression was established using SP600125 JNK inhibitor (figure 13D). The increase in C/EBPα protein expression was consistent with JNK activation. Similar effects were observed with U937 (data not shown) and acute myelomonocytic HL60 cells, when treated with 25ng/ml of anisomycin (figure 13B). That induction of HL60 cells with 25ng/ml anisomycin significantly activates JNK1 is published elsewhere (Terrance A. Stadheim, 2002). C/EBPα protein expression upon JNK1 activation was also investigated in the C/EBPα inducible cell line K562Er-C/EBPα, where Er-C/EBPα is activated when

induced with 1μM β-estradiol (Cleaves et al., 2004) but with accelerated degradation, The immunoblot against C/EBPα shows that anisomycin induction together with β-estradiol can reduce the degradation of Er-C/EBPα (figure 13C). This strengthens our data that inactive JNK targets C/EBPα for ubiquitination.

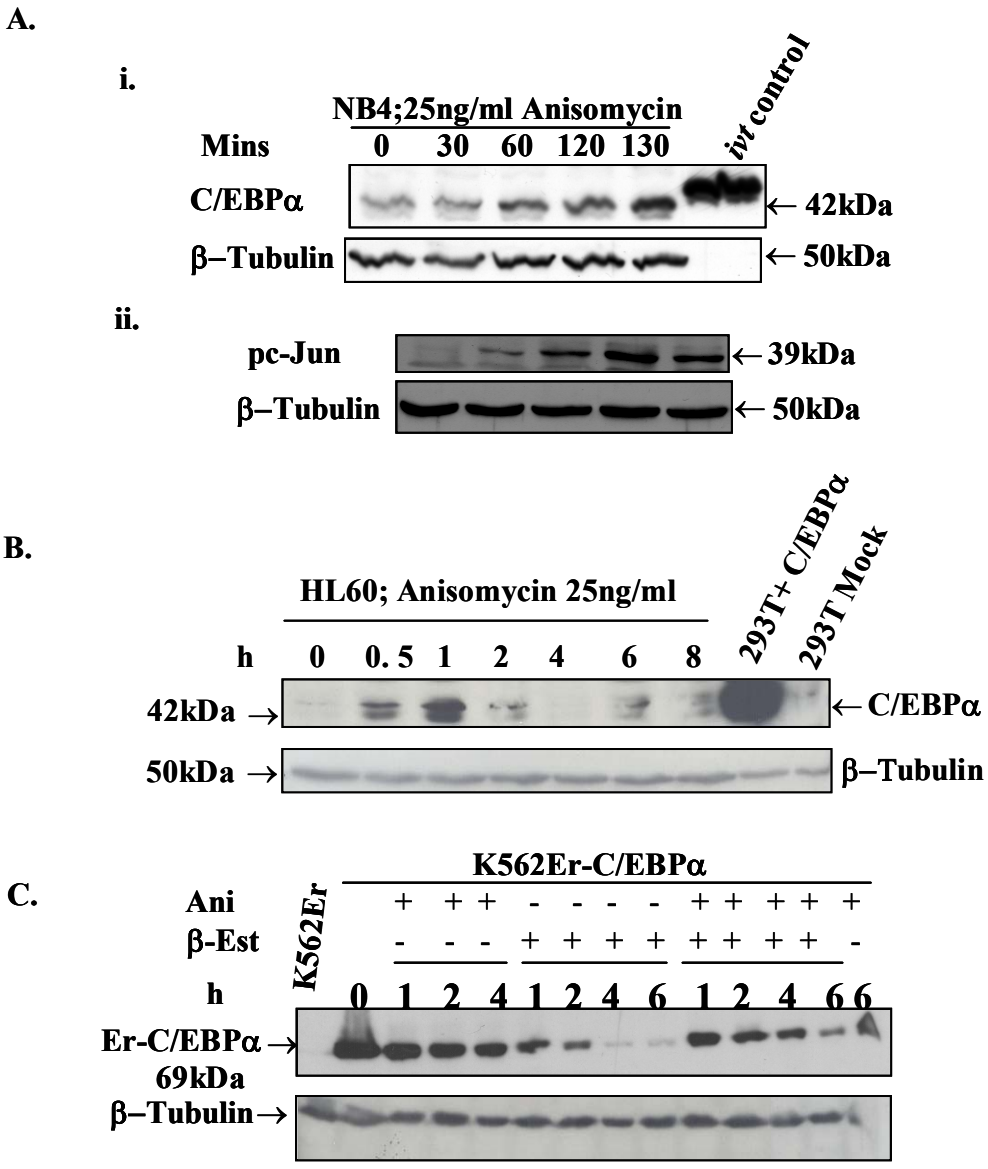

Fig 13. Anisomycin induced activation of JNK1 enhances C/EBPα protein expression: A) i. Immunoblot showing enhanced expression of C/EBPα in NB4 (PML/RAR harbouring) cells after induction with 25ng/ml anisomycin. *In-vitro* C/EBPα-His used as positive control (last lane), while lower panel is loading control. **ii.** Same lysates were separated on different gel and probed for phosphor c-Jun to measure anisomycin induced activation of JNK kinase activity. Lower panel is loading control probed with β-tubulin. **B).** HL 60 cells induced with 25ng/ml of anisomycin for different time points as indicated were lysed in RIPA buffer and separated on 10% SDS PAGE, immunoblot against C/EBPα antibody shows a significant increase in C/EBPα protein after 30 min as compared to uninduced cells, lower panel is loading control. **C) Anisomycin stabilizes C/EBPα in β-estradiol inducible C/EBPα cell line K562Er-C/EBPα:** Estradiol Receptor fused to C/EBPα stably transfected in K562 cells

was treated with 25ng/ml anisomycin for different time points as indicated and whole cell lysates were prepared in RIPA buffer. Immunoblot against an anti C/EBPα antibody shows the rate of degradation of C/EBPα is slower in anisomycin induced cell lysates as compared to cells treated with β-estradiol (Est) alone, lane one is control K562Er empty vector. Lower panel is loading control probed with β-Tubulin.

3.10 MEKK1 cotransfection with C/EBPα leads to accumulation of C/EBPα: To explore further if JNK1 activation contributes to C/EBPα elevated expression, we cotransfected 293T cells with JNK1 and MEKK1. It is well established that MEKK1 is an upstream activator of JNK (Fuchs et al., 1997). 24h post transfection cells were harvested by lysing in RIPA buffer. Equal proteins were separated on 8% SDS PAGE. The immunoblot against anti C/EBPα antibody shows that MEKK1 significantly enhances the C/EBPα protein expression while this effect was abrogated when cotransfected cells were treated with 20μM JNK inhibitor SP600125 (Han et al., 2001) 3h post transfection (figure 13D). This suggests that JNK1 activation is indeed required for stabilization of the C/EBPα protein. To investigate whether JNK affects C/EBPα at the protein level and not at mRNA, we treated U937 cells with 50μg/ml cycloheximide, a potent inhibitor of protein synthesis prior to the addition of 50ng/ml anisomycin for different time points as indicated.

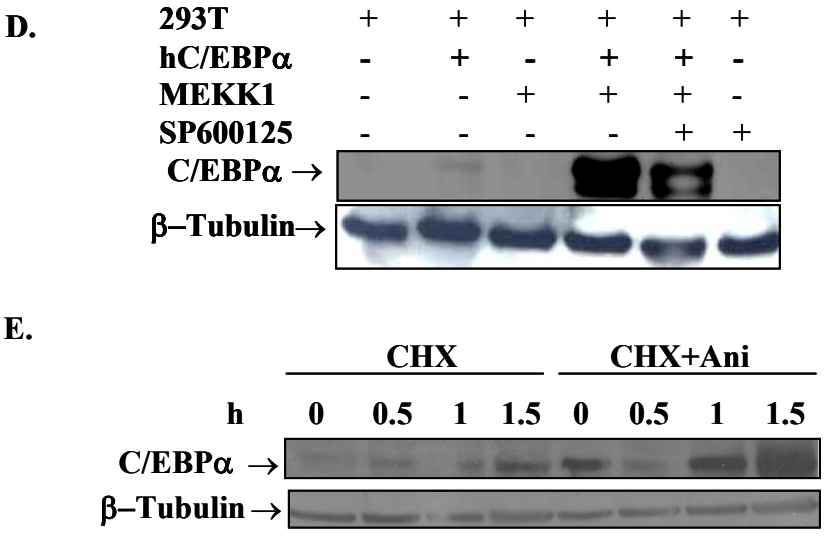

Fig 13. D) MEKK1 enhances the C/EBPα protein expression: 293T cells were transiently transfected with C/EBPα and MEKK1 expression plasmids, 24h post transfection cells were

lysed and equal amount of protein was separated on 10% SDS PAGE, Immunoblot against a C/EBPα antibody shows a significant increase in C/EBPα (lane 4) while, MEKK1 induced increase in C/EBPα protein was drastically decreased when cells were treated with 20μM JNK inhibitor SP600125, lower panel is loading control immunoblotted with β-tubulin. **E) Anisomycin induced activation of JNK affects C/EBPα at the protein level:** U937 cells were treated with cycloheximide (CHX) prior to treatment with anisomycin as described in materials and methods. A whole cell extract was separated on 10% SDS PAGE and immunoblotted against a C/EBPα (upper panel) antibody, Immunoblot against β- tubulin (lower panel) is loading control for equal amount of protein.

The immunoblot against C/EBPα after separating whole cell extracts on 8% SDS PAGE shows that C/EBPα protein degradation is reduced when treated together with anisomycin (figure 13E). The increase in protein amount after 0h may be attributed to JNK activation by cycloheximide itself (Kyriakis and Avruch, 1990).

3.11 Active JNK1 enhances the ability of C/EBPα to transactivate the minimal TK promoter driven by C/EBP DNA binding sites p(C/EBP) 2TK:

To ascertain if the active JNK induced increase in C/EBPα protein expression is also accompanied by an increase in C/EBPα transactivation activity, we transfected 293T cells with a minimal TK promoter containing 2C/EBP sites cloned upstream of the luciferase reporter gene along with expression plasmids for hC/EBPα (human C/EBPα) and MEKK1. The reporter gene expression of the luciferase reporter gene was determined 24h post transfection. Transfection of the MEKK1 expression construct significantly enhanced the ability of C/EBPα to transactivate the minimal C/EBP promoter upto 5fold, whereas this effect was drastically reduced to 1.5fold when C/EBPα and MEKK1 cotransfected cells were treated with a JNK inhibitor 3h post transfection. No significant change was observed in the pTK luc empty vector (figure 14). This confirms that the active JNK1 induced increase in C/EBPα protein level contributes to C/EBPα transactivation activity.

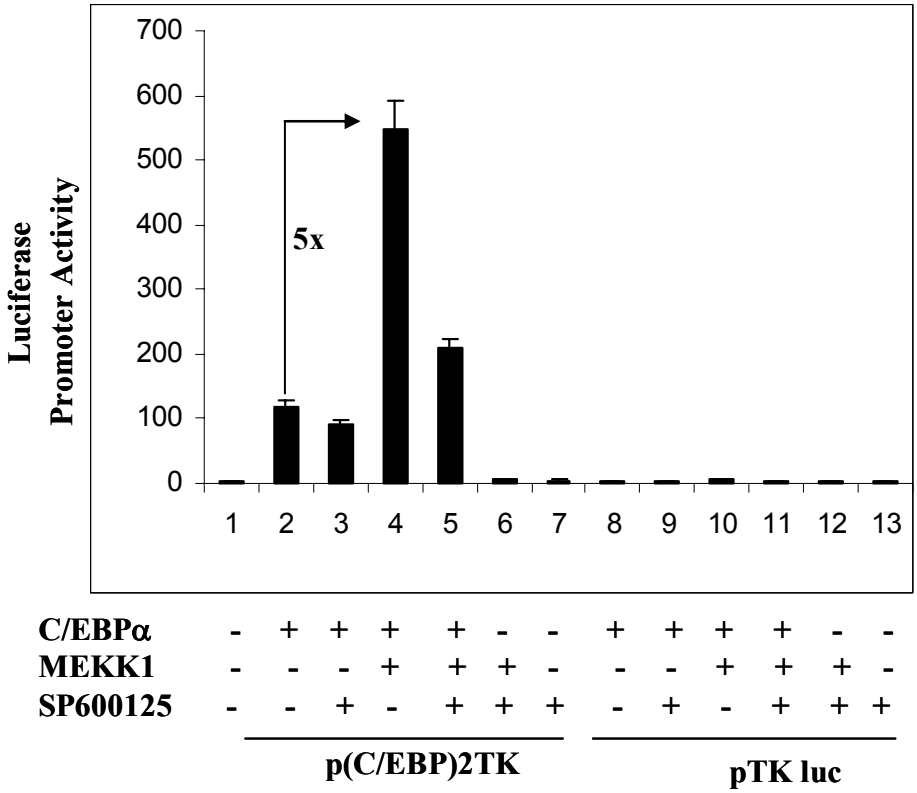

Fig 14. JNK1 enhances the C/EBPα transactivation capacity: MEKK1 induced activation of JNK enhances C/EBPα transactivation capacity; 293T cells were transiently transfected with expression plasmids pCDNA3-hC/EBPα, pCDNA3-MEKK1 and a minimal TK promoter driven by C/EBP DNA binding sites p(C/EBP)2TK, minimal promoter without any C/EBP binding site was used as control. Luciferase activities were determined 24h after transient transfection with LipofectAMINE Plus and normalized to the activities of the internal control plasmid pRL-null. MEKK1 enhances C/EBPα transactivity almost 5 fold while MEKK1 induced increase in C/EBPα transactivation was reduced to 1.5 fold when treated with 10μM JNK inhibitor SP600125 (Calbiochem, Germany). There was no major change in Luciferase activity in control transfections.

3.12 JNK1 enhances C/EBPα DNA binding activity in Electrophoretic Mobility Shift Assay (EMSA): Because JNK1 enhances C/EBPα protein expression and its transactivation activity we next asked, if JNK1 also increases the DNA binding capacity of C/EBPα. To investigate this, 293T cells were transfected with expression plasmids of hC/EBPα, JNK1 and MEKK1 as indicated (figure 15). A nuclear extract was prepared 24h post transfection and 5μg of nuclear extract was used in an EMSA reaction together with a labelled probe which is a 30 nucleotide oligomer corresponding to the C/EBPα binding region on the G-CSF receptor promoter (Behre et al., 2002b). A significant

increase in C/EBPα DNA binding with the probe was observed when the nuclear extract from cotransfection of JNK1, C/EBPα and MEKK1 (lane 10) was used as compared to a nuclear extract from C/EBPα transfected alone (lane 2). A supershift with a C/EBPα (lane 4,8 and 12) antibody was observed while no binding occurred when a 50-fold excess of the unlabelled probe was used as competitor (lane 3,5,7,9,11 and 13). However, antibody supershift like band seen in lane 6 and 10 could be unresolved protein-probe complex as this band is not seen in lane 2 while equal amount of protein was loaded in each well. These results indicate that binding of C/EBPα to the G-CSF receptor promoter DNA is enhanced upon C/EBPα stabilization by activated JNK1.

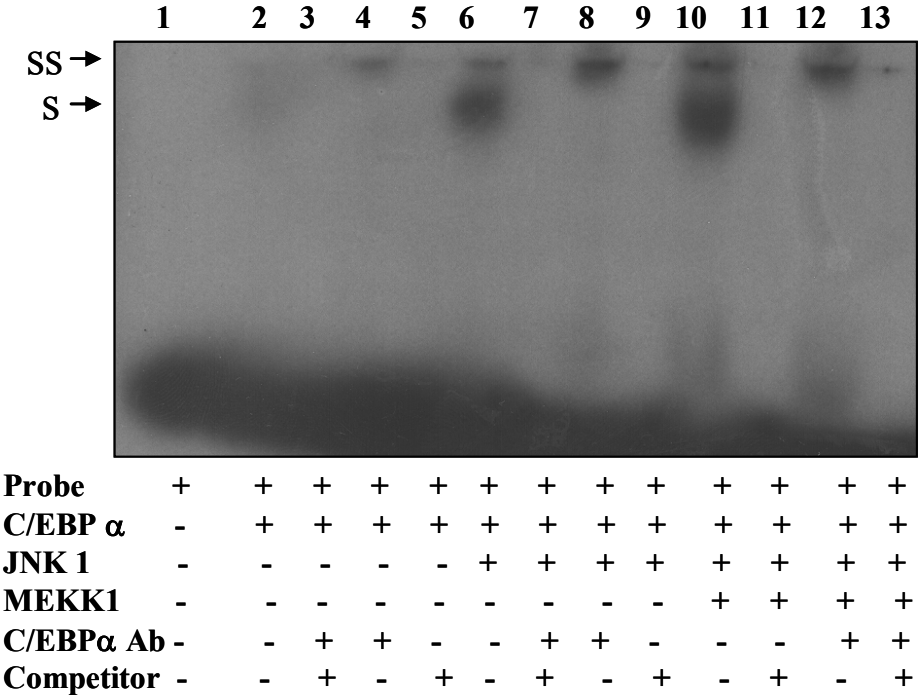

Fig 15. JNK1 enhances C/EBPα DNA binding activity in Electrophoretic Mobility Shift Assay (EMSA): EMSA analysis using 5μg of nuclear extract from 293T co-transfection of JNK1, C/EBPα (lane 6); JNK1, C/EBPα, MEKK1 (lane 10); and C/EBPα alone (lane 2); together with radiolabelled probe shows JNK1 enhances (S) C/EBPα DNA Binding Activity. Super shifted (SS) complexes are indicated with arrow (lane 4, 8 and 12), for competition analysis, a 50-fold molar excess of unlabelled oligonucleotide probe was used (lane 3,5,7,9, 11 and 13).

3.13 JNK1 mRNA expression is downregulated in AML subtypes: Affymetrics analysis of JNK1 mRNA expression from different AML subtypes

shows that JNK1 mRNA expression is downregulated in these patients in comparison with normal bone marrow mononuclear cells from healthy volunteers (figure 16). The affymetrix data for these AML patients has been previously reported (Schoch et al., 2002). Standard Affymetrics software (Micro array Suite, Version 5.0) and the HG-U133A set of normalization controls were used for data analysis. As recommended by the manufacturer, 100 human maintenance genes served as a tool to normalize and scale the data prior to performing data comparisons. Expression signal intensities are given as absolute numbers. AML patient samples included FAB M2 patients with a translocation t(8;21), normal karyotypes (NK), complex karyotype (CK), M3 with t(15;17), M4eo inversion 16 (inv 16), and MLL.

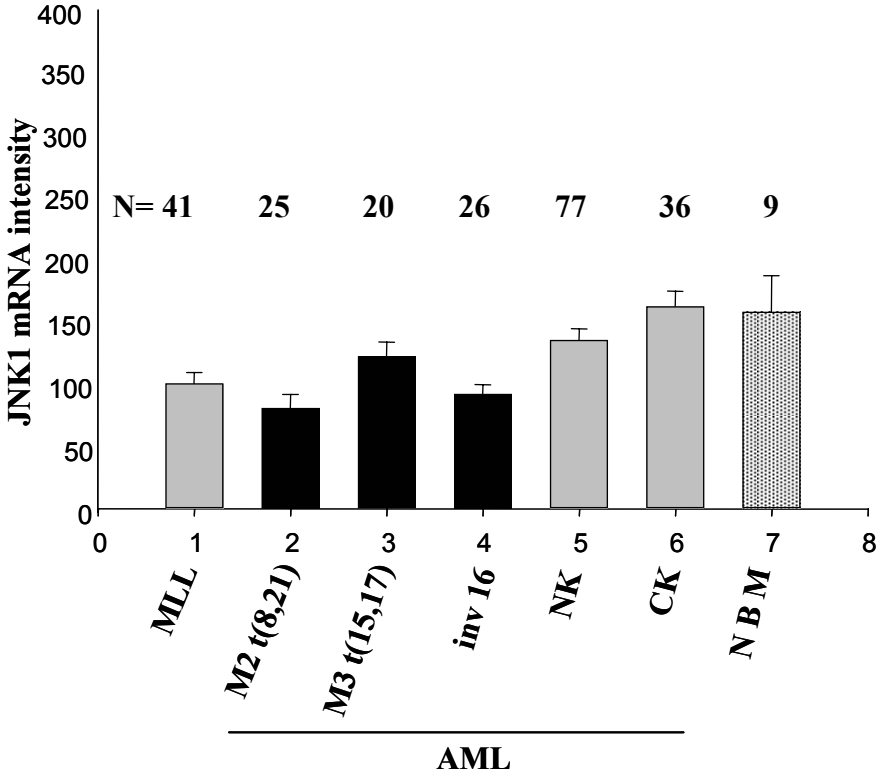

Fig 16. JNK1 mRNA expression is downregulated in AML subtypes: JNK1 mRNA expression is decreased in AML patient samples (*n* = 225) in comparison with normal bone marrow (nBM) mononuclear cells (*n* = 9). Total RNA was isolated and processed as described before (Schoch *et al.* 2002). Expression signal intensities are given as absolute numbers (Schoch *et al.* 2002). The error bars indicate the s.e.m., *n* indicates the number of patient samples analysed in each subgroup. AML patient samples included FAB M2 patients with translocation t(8;21), normal karyotype, complex karyotype, M3 with t(15;17), M4eo inversion 16, t(11q23), AML complex karyotype, and AML normal karyotype.

3.14 Phospho JNK1 expression and its kinase activity is reduced in AML subtypes: Because JNK1 mRNA expression is downregulated in AML subtypes we next analysed the JNK1 kinase activity from different AML patients. We assessed the phospho JNK1 protein expression which served as measure of JNK1 kinase activity. Patient samples were lysed in RIPA buffer and 50μg of protein from each sample was separated on 8% SDS PAGE. The immunoblot developed after blotting against an anti-phospho JNK1 antibody (Cell Signalling Technology, Germany) shows that phospho-JNK1 expression (figure17; upper panel) is slightly lower as compared to normal bone marrow except for CML patients where JNK is reported to be constitutively active. Further we analysed the phospho c-Jun expression (figure17; middle panel) by stripping and reblotting the same membrane, which is also consistent with a decreased JNK1 kinase activity in these patients. In AML-FAB subtypes (French-American-British nomenclature) M2 with t(8;21) and M3 with t(15;17) translocation, C/EBPα function is known to be inactive which is due to its inhibition by fusion proteins. However, in many cases the reason behind the inactivation of C/EBPα is not clear. Here we propose that the downregulation and inactivation of the JNK kinase activity may possibly lead to C/EBPα inactivation.

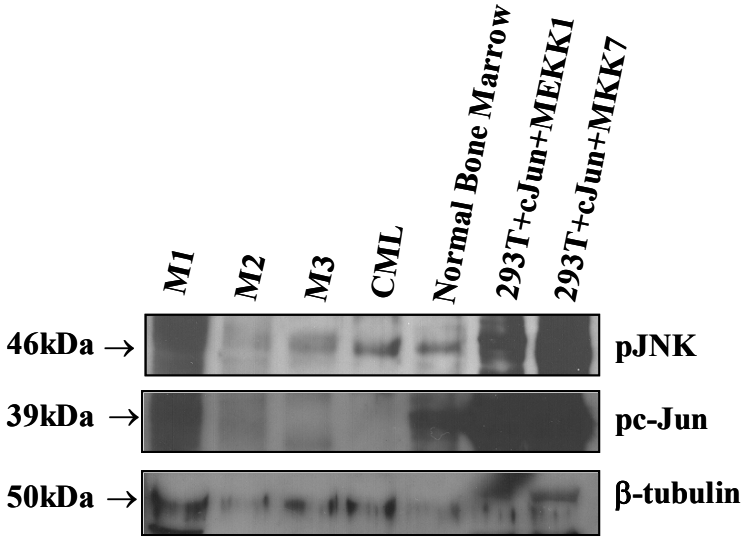

Fig 17. JNK kinase activity is decreased in the various AML subtypes: Phospho JNK1 expression is downregulated in the AML subtypes; Patients samples from different AML subtypes M2 t(8,21); M3 t(15,17); M1 and CML were lysed in RIPA lysis buffer. An equal amount of protein was separated on 10% SDS PAGE. Immunoblot against an anti phospho

JNK1 antibody shows lower phospho JNK1 expression in AML as compared to normal bone marrow. Subsequently the same membrane was stripped and blotted for phospho c-Jun which is also consistent with lower JNK1 kinase activity except for CML patients where JNK1 is reported to be constitutively active (middle panel), lower panel is loading control β–tubulin. 293T transfected with c-Jun, MKK7 and MEKK1 was used as positive control.

4. Discussion:

Protein-protein interactions are operative at every biological step and are important to the formation of complexes and signal transduction through protein pathways. Perturbation in such multi protein complexes often leads to improper functioning as seen in diseased conditions (Figeys, 2003). Recent studies have emphasized that the interaction of transcription factors with other nuclear proteins plays an important role in a combinatorial fashion in stem cell development, lineage commitment, differentiation in the haematopoietic system, and the pathogenesis of myeloid leukemia (Behre et al., 2002a; Nerlov, 2000). Inactivation of key lineage specific factors by mutations has previously been shown by us and others. In many cases inactivation of such factors in acute myeloid leukemia is not well defined. Direct antagonist protein-protein interaction of transcription factors and other regulatory molecules is a newly detected mechanism in the pathogenesis of leukemia (Nerlov, 2000; Rangatia et al., 2003; Reddy et al., 2002; Tenen, 2000). The inhibition of AML1, C/EBPα and PU.1 by direct protein-protein interactions has been implicated in AML. C/EBPα being a regulatory switch that is sufficient for the induction of granulocytic development from bipotential myeloid progenitors is often impaired in AML (Pabst et al., 2001a; Pabst et al., 2001b; Radomska et al., 1998) either by mutation or inhibition by other proteins (Rangatia et al., 2003). Negative protein-protein interactions are not only responsible for the inactivation of C/EBPα function in AML, but even C/EBPα itself seems to function via protein-protein interactions (Schaufele et al., 2001). Proteomics-based approaches, which enable the quantitative investigation of both cellular protein expression levels and protein-protein interactions involved in signalling

networks, promise to define the molecules controlling the processes involved in cancer (Simpson RJ, 2001). This prompted us to hypothesize that identifying C/EBPα interacting proteins might help in understanding the molecular mechanisms underlying leukemogenesis.

Identification of C/EBPα interacting proteins: High-throughput proteomics by mass spectrometric analysis of gel-separated proteins can identify multi-protein complexes and changes in the expression of multiple proteins simultaneously (Behre et al., 2002a). Due to its high throughput, MS-based proteomics has been extensively applied for probing molecular mechanisms in signal transduction, identifying cancer biomarkers and profiling various diseases (Wang et al., 2004). We performed affinity based C/EBPα multiprotein purification followed by subsequent MALDI TOF and / or TOF TOF mass spectrometric analysis to identify the gel separated proteins. We identified unknown as well as few known interacting proteins of C/EBPα such as retinoblastoma and SWI/SNF2 though with a low significance score. Here, we identified JNK1, a member of the MAP kinase family of proteins with a significant mowse score as an interacting protein of C/EBPα. Earlier studies have shown that JNK physically interacts with its associated substrates such as c-Jun, c-Myc, ATF2. Therefore, we validated the identified spot by western blotting and confirmed the physical interaction between JNK1 and C/EBPα *in-vitro* (figure 8) and *in-vivo* (figure 9) respectively. JNK1 and C/EBPα interaction was observed with various C/EBP basic region and leucine zipper mutants (figure 9B) in co-immunoprecipitation studies. This could be due to an intact phosphopeptide region in these mutants.

Biological implication of the JNK1 and C/EBPα interaction: Previous analyses have shown that Ras signalling enhances C/EBPα activity by phosphorylating at S248 (Behre et al., 2002b). Recently, C/EBPα has been reported to be regulated by post translational modifications such as phosphorylation, SUMOylation and ubiquitination (Ross et al., 1999; Ross et al.,

2004; Subramanian et al., 2003). In this report, we show that JNK1 phosphorylates C/EBPα in its DNA binding domain (DBD) while the JNK mutant (kinase domain dead) could not phosphorylate it (figure 10B). Furthermore, by mass spectrometric analysis we identified one phosphopetide [(K)SVDKNSNEYRVR (R) which starts from amino acid 277 and ends at 288)] corresponding to the BR-LZ region in DBD from tryptic peptides of phosphorylated C/EBPα (figure 11B). Still we can not rule out the possibility of more than one phosphopetide. There are two serine residues in the identified phosphopetide which could be phospho modified though they are not conventional S/TP MAP kinase phosphorylation sites; however, they could be phosphomodified by MAP kinases (S.Corbalan-Garcia, 1996). We constructed two point mutants S277-A (serine 277 mutated to alanine) and S282-A (serine 282 mutated to alanine) and performed *in-vitro* kinase assay where we observed some altered phosphorylation of these mutants (data not shown). Mutation of serine 282 to alanine had not much effect on C/EBPα phosphorylation while serine 277 to alanine surprisingly showed an increase in phosphorylation. Enhanced phosphorylation of the S277A mutant may be due to a conformational change and better accessibility of C/EBPα to JNK1. Thus our data indicate that C/EBPα is a substrate for JNK and probably more than one amino acid of C/EBPα is being targeted upon the JNK activation. However, extensive study is required to identify the amino acid residues being phospho modified.

In addition to its function as a transcription factor, C/EBPα has been reported to act as tumour suppressor gene which inhibits cell proliferation via a transcription-independent mechanism in which C/EBPα forms a complex with cdk2 and cdk4 preventing cyclin/cdk complex formation, E2F inhibition and cell cycle progression. The regulation of the C/EBPα protein level through an ubiquitin-proteasomal pathway would control both the transcription-dependent and transcription-independent activities of C/EBPα (Shim and Smart, 2003). C/EBP proteins and C/EBPα in particular have been reported to be regulated

both at mRNA (Perrotti et al., 2002; Timchenko et al., 2002) and protein levels (Smart, 2003). Recent findings suggest that the stabilization of the C/EBPα protein contributes to its transactivation and growth arrest activity (Wu et al., 2003a; Wu et al., 2003b); (Yoon and Smart, 2004). Wu et al. show that physical interaction between C/EBPα and the Epstein-Barr virus lytic-cycle transactivator protein ZTA prolongs C/EBPα and p21 protein levels through both transcriptional and posttranslational mechanisms and leads to growth arrest in kaposi´s sarcoma cells (Wu et al., 2003a).

The stress responsive kinase JNK has been shown to regulate the ubiquitination dependent degradation of a different subset of substrates by acquiring a specific phosphorylation pattern that affects conformation, stability, subcellular localization and transcriptional activation (Fuchs et al., 1998d; Musti et al., 1997; Ronai, 2004). In non-stressed cells i.e., inactive JNK functions as a targeting molecule for ubiquitination, an activity which depends not on the fact that it is a kinase, but its ability to associate tightly with its substrate. This association attracts the enzymes of the ubiquitination machinery to the substrate, thereby marking it for the proteasome-dependent degradation. However, in stressed cells i.e. when JNK1 is active, ubiquitination is inhibited (Fuchs et al., 1998d; Fuchs et al., 1997). The interaction of inactive JNK with c-Jun targets its ubiquitination which is inhibited upon phosphorylation of c-Jun by JNK. However, it is noteworthy that physical association of JNK with its substrates is the only prerequisite for targeting ubiquitination; this is evidenced by the fact that Elk1, which is not capable of associating with JNK, can not be targeted for ubiquitination and moreover its phosphorylation by JNK does not prevent its ubiquitination (Gupta et al., 1995). Our data is consistent with the above findings. Here we report that the physical association between JNK1 and C/EBPα, targets ubiquitination of C/EBPα (figure 12A); which is inhibited upon activation of JNK by MKK7 in an *in-vivo* HA-Ubiquitination assay (figure 12A). We also show in a pulse chase labelling assay that activation of JNK

enhances half life of C/EBPα protein which shows that the activation of JNK is indeed required for the inhibition of JNK targeted C/EBPα ubiquitination.

The inhibition of ubiquitination of its associated substrates by JNK prolongs their half life and hence expression (Fuchs et al., 1997). Our result is in accordance with these findings. We also show that the anisomycin induced activation of JNK1 in various leukemic cell lines significantly enhances C/EBPα protein expression (figure 13). Further, we show that JNK1 affects the C/EBPα protein level rather than its mRNA via an immunoblot against C/EBPα from cells lysates of U937 after treatment with cycloheximide alone and together with anisomycin (figure 13E). An increase in C/EBPα protein expression was consistent with the increase in its transactivation and DNA binding activity (figure 14 and 15) which suggests that the regulation of C/EBPα protein stability has an impact on its activity. More recently, Yoon K *et al.* have shown that C/EBPα is a DNA damage-inducible p53-regulated mediator of the G1 checkpoint in keratinocytes. Their study shows that UVB irradiation of keratinocytes enhances the C/EBPα protein expression by 70 fold which is accompanied by an upregulation of p53 and p21 expression leading to a G1 phase of cell cycle growth arrest. However, UVB (Ultra Violet Radiation B) is a potent inducer of the JNK kinase activity which strengthens our data that the physical association of active JNK with C/EBPα inhibits its ubiquitination and stabilizes it (Yoon K et al., 2004).

An affymetrics analysis of JNK mRNA from different AML subtypes shows that JNK1 mRNA expression in various AML subtypes M2 with t(8;21), M3 t(15;17), and inversion 16 is lower in comparison with normal bone marrow mononuclear cells from healthy volunteers, Further we show that not only JNK1 mRNA expression but also phospho JNK kinase expression (which- served as a

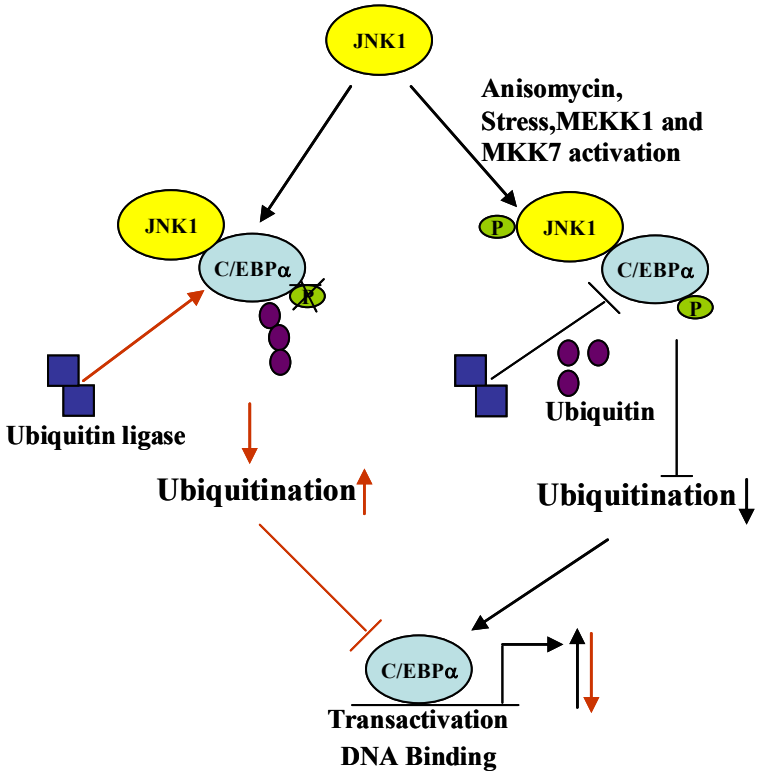

Fig 18. Model: Functional relevance of JNK1 physical interaction with C/EBPα: On the basis of our data we propose a hypothetical model where interaction of active JNK1 with C/EBPα prolongs half life of C/EBPα by inhibiting its ubiquitination. JNK induced increase in C/EBPα protein results in transcriptional activation. In a symmetrical manner interaction of inactive JNK with C/EBPα leads to degradation of C/EBPα and hence results in transcriptional repression.

measure of kinase activity) is decreased in such patients (figure 17). Moreover, the JNK signal transduction pathway has been implicated in many pathological conditions (Manning and Davis, 2003), including cancer. This implicates that a JNK activity is required for the C/EBPα activation in myeloid cells and that a loss of JNK regulated C/EBPα expression may contribute to leukemogenesis.

In conclusion, we propose a hypothetical model (figure 18) of the physical interaction of active JNK with C/EBPα. JNK targeting of C/EBPα ubiquitination requires the tight interaction with C/EBPα; whereas phosphorylation of C/EBPα by JNK inhibits its ubiquitination, presumably due to an altered conformation of C/EBPα, which is likely to inhibit ubiquitination machinery. This results in an increased C/EBPα stability and availability which

is reflected by an enhanced DNA binding and transcriptional activation of C/EBPα.

5. Summary:

Functional inactivation of the transcription factor CAAT Enhancer Binding Protein Alpha (C/EBPα) either by mutation or direct protein-protein interaction leads to acute myeloid leukemia (AML), whereas the activation of C/EBPα restores normal myeloid cell differentiation. We and others have shown that protein-protein interactions of C/EBPα play a pivotal role in myeloid differentiation and AML. In the present study we applied proteomics based mass spectrometry to identify C/EBPα interacting proteins on a proteome-wide scale. For this, the GST and GST-DNA binding domain of C/EBP (GST-DBD) was incubated with nuclear extracts of U937 cells. Interacting proteins separated by two-dimensional gel electrophoresis were identified by MALDI-TOF mass spectrometry. Using this approach we could identify PAK6, MADP-1, ZNF45 and the c-Jun N-terminal kinase 1 (JNK1) as C/EBPα interacting proteins. Since JNK1 activates c-Jun, the contra-player for C/EBPα, we hypothesized that the JNK1 and C/EBPα interaction might have some biological relevance. We could show that JNK1 binds to the DNA binding domain of C/EBP in GST-pull-down and to the C/EBPα in co-immunoprecipitation assays *in-vitro* and *in-vivo* respectively. JNK1 phosphorylates and increases the half life of the C/EBPα protein via inhibiting its ubiquitination and thereby enhances its transactivation and DNA binding activity. Furthermore, JNK mRNA expression as well as its kinase activity is lower in the AML patients in comparison to normal bone marrow mononuclear cells which implicates a possible mechanism of C/EBPα inactivation in certain acute myeloid leukemia subtypes. Thus our data suggest that a JNK activity is required for C/EBPα activation in myeloid cells and a loss of JNK regulated C/EBPα expression may contribute to leukemogenesis.

6. ZUSAMMENFASSUNG

Die funktionelle Inaktivierung des Transkriptionsfaktors CAAT Enhancer Binding Protein alpha (C/EBPα) entweder durch Mutation oder durch direkte Protein-Protein-Interaktion führt zu akuter myeloischer Leukämie (AML), wohingegen die Aktivierung von C/EBPα eine normale myeloische Differenzierung gewährleistet. Wir und andere konnten zeigen, dass Protein-Protein-Interaktionen von C/EBPα eine entscheidende Rolle in der myeloischen Differenzierung und AML spielen. In der vorliegenden Studie haben wir die auf Proteomik basierende Massenspektrometrie genutzt, um die mit C/EBPα interagierenden Proteine in einem Proteom-weiten Maßstab zu identifizieren. Zu diesem Zweck wurde ein Fusionsprodukt aus GST und der DNA-bindenden Domäne von C/EBPα mit Kernextrakten von U937 inkubiert. Mit C/EBPα interagierende Proteine wurden mittels zweidimensionaler Gelelektrophorese separiert und durch MALDI-TOF-Massenspektrometrie identifiziert. Mit diesem Ansatz konnten wir PAK6, MADP-1, ZNF45 und die c-jun N-terminale Kinase 1 (JNK1) als interagierende Proteine von C/EBPα identifizieren. JNK1 aktiviert c-jun, einen Gegenspieler von C/EBPα. Daher stellten wir die Hypothese auf, dass die Interaktion von JNK1 und C/EBPα eine biologische Relevanz haben könnte. Wir konnten mittels GST-Pull-Down-Experimenten *in-vitro* zeigen, dass JNK1 mit der DNA-bindenden Domäne von C/EBP interagiert. Die Interaktion von JNK1 und C/EBPα konnte *in-vivo* durch Koimmunopräzipitation bestätigt werden. JNK1 phosphoryliert C/EBPα und erhöht dessen Halbwertszeit durch die Hemmung der Ubiquitinylierung. Dadurch wird die Stärke der Bindung von C/EBPα an die DNA und seine Fähigkeit zur Transaktivierung erhöht. Interessanterweise ist die Expression der JNK1-mRNA und die Kinaseaktivität von JNK1 bei AML-Patienten verglichen mit den entsprechenden Werten in mononukleären Zellen aus dem Knochenmark gesunder Kontrollpersonen erniedrigt. Dies weist auf einen möglichen Mechanismus zur funktionellen Inaktivierung von C/EBPα in bestimmten Subtypen der AML hin.

Zusammengefasst weisen unsere Daten darauf hin, dass die Aktivität von JNK1 für die Aktivierung von C/EBPα in myeloischen Zellen nötig ist, und dass ein Verlust der durch JNK1 regulierten Aktivität von C/EBPα einen möglichen Beitrag zur Leukämie-Entstehung leistet.

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8. Acknowledgements:

With a deep sense of gratitude, I wish to express my sincere thanks to my supervisor Prof. Dr. med Wolfgang Hiddemann for providing me the opportunity and lab space in his group for this research work.

I am deeply indebted to my mentor PD Dr. Gerhard Behre for his supervision, skilful guidance and financial support along the completion of this work. His company and encouragement during the course of this research work will be life long appreciated.

I am highly grateful to the Deutsche José Carreras Leukämie-Stiftung e.V. for providing me with financial support for the research and courses that I attended during the period of the whole research work.

I will never forget the company that I had from my fellow research scholars of the KKG leukemia, GSF hematologikum and some friends of my hall. In particular, I am thankful to Sheo, Abdul, Venkat, Rajani, Janki, Max, Alex Deepak, Mulu, John, Mumtaz, Aniruddha, Vijay, Monica, Natalia, Tobias, Kosti and Roman for their healthy discussion and suggestions during the lab meetings. This episode of acknowledgement would not be complete without the mentioning of my forever friends Mahendra, Ankita, Sudhanshu, Sanjay Gupta, Pallavi, Dharmraj and members of the Cricket Club of Bayern (CCB), Munich for their timely help to me and my family whenever needed.

My thanks are further due to members of the KKG Leukemia and the GSF hematologikum especially Mrs. Neumann and Mrs. Magalkammer for their cooperation, help and management.

I also want to thank my parents, who taught me the value of hard work by their own example. The encouragement, inspiration and motivation given by my father Late Mr. Sita Ram Tiwari to carry out my research work will be life long remembered.

Last but not least, I am grateful to my mother, brothers and sisters Mridula, Kamana, and Rachana for the inspiration and moral support they provided throughout my research work. Their patience was tested to the utmost by a long period of separation when they needed me most after my father's demise. Without their loving support and understanding I would never have completed my present work. Particularly, I owe to my mother for her silent prayer for my work at the time when she needed my company most.

Finally, I would like to thank all whose direct and indirect support helped me completing my thesis in time.

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Arun Kumar Trivedi
S/O Late Mr. Sita Ram Tiwari
Q No. 9II197 Obra Colony
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Academic Qualification:

1984-1991 Primary and middle schooling

1991- 1993 High School (Xth), Central Board of Secondary Education, Passed with **84.6%** marks with Science, Mathematics, Social Studies, English and Hindi as main subjects in last year of High School from Jawahar Navodaya Vidyalaya Patehara Kalan Mirzapur Uttar Pradesh, 231309 India.

1994 - 1995 Intermediate (XIIth), Central Board of Secondary Education, passed with **75.8% marks** with Biology, Mathematics, Chemistry, Physics and English as main subjects in last year of intermediate (XIIth) from Jawahar Navodaya Vidyalaya Patehara Kalan Mirzapur Uttar Pradesh 231309, India .

1995 - 1998 Bachelor of Science (B.Sc.), Government Post Graduate Degree College Obra, Purvanchal University passed with **78.0%** marks with Botany, Zoology and Chemistry, Obra Sonbhadra 231219, India

1999 - 2001 Master of Science (M.Sc.), School of Biotechnology, passed with **73.8%** with the following subjects: Genetics and Molecular Biology, Immunology, Biophysics and Biochemistry, Microbiology, Cell Biology and Virology, Animal cell culture, Plant tissue culture, Biostatics and Computer Application, Genetic Engineering, Enzymology and Enzyme Technology, Bioprocess Engineering and Technology, Environmental Biotechnology from Banaras Hindu University BHU Varanasi, India 221005.

Oct 2001-April 2005 PhD student worked on the topic "*Proteomic indentification of C/EBPα multiprotein complex reveals JNK1, an activator of C/EBPα is downregulated in patients with acute myeloid leukemia"* at Medizinischen Klinik und Poliklinik III, der Ludwig-Maximilians-Universitäts München and the GSF hematologikum KKG leukemia, Munich Germany.

Fellowships:

*Awarded with stipendium, **Deutsche José Carreras Leukaemie Stiftung (DJCLS)** for three years from 2004 onwards provided with extension every year.

*Awarded with **Junior Research Fellowship Dec 2000 (JRF)** conducted **by Council of Scientific and Industrial Research (C.S.I.R.) Govt. of India**, Also qualified for first Shyama Prasad Mukharjee fellowship interview being among top 20% qualifiers.

*Awarded with **Junior Research Fellowship by Indian council of medical research**, April 2001, Ministry of Health, Govt. of India.

*Qualified **Graduate Aptitude Test in Engineering with 99.05 percentile** having All India Ranking of 0017th conducted by Indian Institute of Technology Kanpur Feb2001.

Research Experience:

*Completed one year project from July 2000 to March 2001 during Master of Science course entitled as *´´Application of Reporter genes in the study of Plant Microbial Interaction´´* Under the supervision of Dr. A.K.Tripathi (Reader) School of Biotechnology, Faculty of science Banaras Hindu University, Varanasi 221005, India.

Completed Six weeks summer training on the topic´´ Screening and isolation of alkalostable and thermostable carbamoylases from the Indian biodiversity´´*Under the supervision of Hemarj Nandanwar (Scientist) at Institute of Microbial Technology (IMTECH) Chandigarh, India.

*Presently working as PhD student at Medizinischen Klinik und Poliklinik III, der Ludwig-Maximilians-Universitäts München and the GSF hematologikum KKG leukemia, Munich Germany on the topic entitled as "*Proteomic identification of C/EBPα multiprotein complex reveals that JNK1, an activator of C/EBPα is downregulated in patients with acute myeloid leukemia*". Manuscript submitted.

Techniques dealt with during Masters and PhD thesis:

a. Microbiological Technique: Routine microbiological experiments such as isolation and staining of various microorganisms using different medium and various biochemical parameters, Isolation of cyanobacterial pigments, Purification of microbial lipids by TLC, Nitrogenase activity by GLC, Carbon assimilation in different wave length of light.

b. Immunological Technique: Immunodiffusion, Preparations of spleanic cells, Spleen cell counts, *In-vivo* generation of antibodies through Freund's complete adjuvant, Western blotting, and Affinity chromatography.

c. Enzymology: Purification of enzyme (Urease), Enzyme kinetic studies (effects of Temp., pH, Inhibitor, different substrate concentration), Immobilization of enzyme in gel beads, Comparison of kinetics of immobilized and soluble enzyme.

d. Bacterial Genetics: Transformation, Transduction, Conjugation and their uses in genetic mapping, Induced mutation by UV and NTG.

e. Biochemical Engineering: Immobilization of whole cells, Determination of K_La mixing time, Growth kinetics, Estimation of residual sugar, Ethanol production.

f. Animal cell culture: Isolation and culture of macrophages from PEC, Ficol-Hypaque test for isolation of mononuclear cells, MTT assay, chromosome staining, Isolation of splenocytes, Cryopreservation,

g. Plant tissue culture: Spheroplast isolation, seed sterilization, and culturing cotyledon and hypo cotyledon.

h. Molecular Biological Technique: Plasmid and DNA isolation, Genomic DNA isolation from bacteria, Gene cloning, ARDRA, Bacterial transformation, Restriction digestion, Polymerase chain reaction, Purification of DNA fragments from Agarose gel, Ligation, Electrophoresis, Electroporation, Southern hybridization, GST Pulldown, *In-vitro* synthesis of proteins, Electrophoretic mobility gelshift assay (EMSA), Luciferase Assay, *In vivo* labelling of cells, Pulse chase assay, *In vitro* kinase assay and *in-vivo* ubiquitination assay.

i. Environmental Biotechnology: Total and faecal coli form count, Turbidity measurement, Calculation of COD, BOD, DO, Estimation of Chromium-III, Total suspended particles.

j. Biochemistry: Estimation of protein by folin's, biurate and BSA, Estimation of Glucose by GOD-POD method, Co- immunoprecipitation and paper Chromatography,

k. Proteomics: 1D and 2D electrophoresis, Peptide Mass Finger Printing **(PMF)** using Mass spectrometer **REFLEX III** (Brucker Daltonics) and LC- TOF/TOF from AB bio systems **(AB4700)** for MS/MSP measurements. Post Source Decay Measurements **(PSD)** using Mass Spectrometry with MALDI –TOF.

Talk, poster presentation and seminars attended:

- Presented poster in **German Society of Hematology and Oncology (DGHO)** at Basel, Switzerland 2003; abstract published in Onkologie 2003;26 (sonderheft 5):1-235 ;
"Interacting proteins of AML1/ETO: proteomic approach"
Singh SM, **Trivedi AK**, Hiddeman W, Behre G.

- Presented poster about my work at **DGHO** meeting held at Basel, Switzerland 2003 and abstract published in Onkologie 2004; (suppl 3):1-230; *Proteomic identification of the C/EBPα Multiprotein Complex Reveals that Downregulation of c-Jun N-Terminal Kinase 1 (JNK1) Activity Leads to loss of C/EBPα function in Patients with Acute myeloid Leukemia.*
Trivedi AK, Bararia D, Peerzada AA, Hiddemann W, Behre G.

- Presented poster about my work at the meeting **Americam Society of Hematology (ASH) Dec 2003 held at San Diego**, California USA. Abstract in Blood 2003, vol 102,number 11; 346a; *Proteomic identification of the C/EBPα Multiprotein Complex Reveals that Downregulation of c-Jun N-Terminal Kinase 1 (JNK1) Activity Leads to loss of C/EBPα function in Patients with Acute myeloid Leukemia*.
Trivedi AK, Bararia D, Peerzada AA, Hiddemann W, Tenen DG, Behre G

- Presented poster about my work at **DGHO meeting held at Innsbruck Austria**, 2004 and abstract published in Onkologie 2005; (supple): *Proteomic identification of the C/EBPα Multiprotein Complex Reveals that Downregulation of c-Jun N-Terminal Kinase 1 (JNK1) Activity Leads to loss of C/EBPα function in Patients with Acute myeloid Leukemia*.
Trivedi AK, Bararia D, Peerzada AA, Hiddemann W, Tenen DG, Behre G

- Presented poster about my work at the meeting **Americam Society of Hematology (ASH) Dec 2004 held at San Diego**, California USA, Abstract in Blood 2004, vol 104, number 11; 561a; *Proteomic identification of the C/EBPα Multiprotein Complex Reveals that*

Downregulation of c-Jun N-Terminal Kinase 1 (JNK1) Activity Leads to loss of C/EBPα function in Patients with Acute myeloid Leukemia.
Trivedi AK, Bararia D, Peerzada AA, Hiddemann W, Tenen DG, Behre G

- Attended "Proteomic Forum 2003" conducted by Angelika Görg from Technical University and Mathias Mann at Munich.

- Participated in European Society of Hyperthermia and Oncology, ESHO meeting held at Munich in 2003

- Attended practical proteomics course form 17-22nd April 2005 held at University of York, Heslington UK.

- ***"****Proteomic identification of C/EBPα multiprotein complex reveals that JNK1, an activator of C/EBPα is downregulated in patients with acute myeloid leukemia"*. Manuscript submitted.
Trivedi AK, Bararia D, Peerzada AA, Hiddemann W, Tenen DG, Behre G

- *Proteomic Identification Of Histone Acetyltransferase TIP60 As A Coactivator Of Myeloid Transcription Factor C/EBPalpha.* Manuscript submitted to experimental hematology
Deepak Bararia*; **Arun K. Trivedi***; Abdul PeerZada; Max Christopeit; Hermann Behre; Gerhard Behre
*Authors contributed equally

- *Proteomics of Acute Myeloid Leukemia: Cytogenetic Risk Groups Differ Specifically in their Proteome, Interactome and Posttranslational Protein Modifications*. Mannuscript submitted to Oncogene.
Mumtaz Y Balkhi, **Arun K. Trivedi**, Mulu Geletu, Maximilian Christopeit, Hermann M Behre, Gerhard Behre

- *Proteomic identification of Max as a novel interacting partner of C/EBPalpha*. Manuscript under preparation.
Abdul A Peer Zada, Deepak Bararia, Mulu Geletu, John A Pulikkan, **Arun K Trivedi**, Hermann Behre, Gerhard Behre

Extra Curricular Activities:

1. Received merit certificate for outstanding performance being among top 0.1% students by getting highest marks (95/100) in the subject Social Studies in class X in all India.

2. Received II and III prize in debate and essay competitions conducted by Regional Prachar Nideshalaya, Govt. of India.

3. Played Soccer and Kho-Kho at national Level of Jawahar Navodaya Vidyalaya.

4. Received certificate for playing Dumble and Lezim at police lines Mirzapur, UP. India.

5. Won award for general knowledge contest conducted by UNESCO.

6. Played cricket and Soccer at inter hostel level in University at BHU Varanasi.

Personal information:

Name	Arun Kumar Trivedi
Father's Name	Late Mr. Sita Ram Tiwari
Mother´s Name	Mrs. Rajeshwari Devi
Citizen	Indian
Sex	Male
Marital status	Single
Date of Birth	15.06.1978
Languages known	English, Hindi
Place of Birth	Obra, Sonbhadra India

Place: München
Date: 26.09.2005 Arun Kumar Trivedi

10. Appendix:

Buffers and their Composition:

Blocking Buffer: 5.0% milk in TBST (1X TBST: Tris 12.1g/L, NaCl 17.6g/L, 0.02% Tween-20)

Colloidal Coomassie Blue Staining:
Fixing: 50% Methanol and 10% Acetic acid
Staining: 0.1% Colloidal Coomassie Blue G-250, 2% H_3PO_4, 10% Ammonium Sulphate and 20% Methanol
Destaining: 25% Methanol

Coimmunoprecipitation Buffer: 1% TBS, 0.5% NP40 and Protease inhibitors

DHB Matrix Solution: 9:1 ratio of DHB: HMB
20 mg/ml 2,5-Dihydroxy-Benzoicacid (DHB); (Merck, Germany)
20 mg/ml 2-Hydroxy-5-Methoxy-Benzoicacid (HMB); (Merck, Germany)

Kinase Buffer: 150mM NaCl, 1mMEDTA, 50mM Tris-HCl, pH 7.5,
10mM $MgCl_2$, and 10 mM DTT,

Immobilin Strip Equilibration Buffer:
i) Reducing Buffer/Strip (1M Tris pH 6.8, Urea, 80% Glycerol 20% SDS and 0.2g DTE)
ii) Alkylating Buffer (1M Tris pH6.8, Urea, 80% Glycerol 20% SDS and 0.25g Iodoacetamide)

Laemmli Buffer (SDS loading buffer 2X, 1L): 125mM Tris pH 6.8, 4% SDS, 20% Glycerol, 10% 2-Mercapto β-Ethanol and a pinch of Bromophenol blue

Membrane Stripping Buffer: 100mM β-ME, 2%SDS and 62.5mM Tris pH 6.8

Nuclear Extract Buffer A: 20mM HEPES, 10mM NaCl, 3mM $MgCl_2$, 1% NP-40, 10%Glycerol, 0.2mM EDTA, 1mM DTT and Protease inhibitors

Nuclear extract Buffer C: 20mM HEPES, 400mM NaCl, 20% Glycerol, 0.2mM EDTA, 1mM DTT, and Protease inhibitors

RIPA Lysis Buffer: 1% NP40, 0.5% Sodium Deoxycholate, 0.1% SDS, 0.15M NaCl, 5mM EDTA and 50mM Tris pH8.0

Running Buffer (1x, 1L): 12g Tris base, 47.5g Glycine and 16g SDS in dH_2O

Silver staining:
Fixing: 50% Ethanol, 12%Acetic Acid
Sensitising: 200mg/L Sodium Thiosulphate ($Na_2S_2O_3$ $5H_2O$) and 30% Ethanol
Silver Reaction: 2g/L Silver Nitrate ($AgNO_3$)
Developing Buffer: 5mg/L Sodium Thiosulphate ($Na_2S_2O_3$ $5H_2O$), 60g/L Sodium Carbonate (Na_2CO_3) and 500µl Formaldehyde 37% (HCHO)
Stopping: 14.60g/L Sodium-EDTA

Transfer Buffer (1x, 1L): 3g Tris base, 14.1g Glycine, 200ml Methanol and dH_2O

Urea Lysis Buffer: 9.8M Urea, 1% DTE, 4% CHAPS, 2.5mM EDTA and 2.5mM EGTA

Washing Buffer: 2.5% milk in TBST