Aus der Medizinischen Klinik und Poliklinik III
der Ludwig Maximilians Universität München

C/EBPα downregulates c-jun expression: Significance in myelopoiesis and AML

Dissertation
zum Erwerb des Doktorgrades der Humanbiologie
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Vorgelegt von
Janki Rangatia M.Sc
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Dedicated to

my parents

Contents

Preface:

Myeloid lineage commitment to form either the granulocytes or monocytes is one of the crucial regulatory mechanism which, if disrupted leads to various forms of leukaemia. C/EBPα is an important transcription factor that regulates the granulocytic lineage decision. Absence of C/EBPα leads to complete loss of mature granulocytes. c-jun has been well studied and its role is elucidated in various regulatory mechanisms viz. cell proliferation, apoptosis, cell cycle and differentiation. In hematopoietic system, c-jun's role has been shown to be important for monocyte/macrophage lineage decision. This report has taken the studies further in elucidating the mechanism by which C/EBPα governs the fate of hematopoietic progenitor cells. To drive granulocytic differentiation, C/EBPα represses c-jun expression and function. These findings are also implicated in leukaemia where a reciprocal expression of C/EBPα and c-jun is observed. The data presented here provides a model to understand the myeloid regulatory mechanism controlled by transcription factors and cofactors.

2. Introduction

2.1. C/EBPα

C/EBPα was the first member cloned in the C/EBP family transcription factors. C/EBPα, previously termed C/EBP, was identified originally as a heat-stable protein present in soluble extract of rat liver nuclei and having sequence-specific DNA binding activity (1,2,3). Purified C/EBPα selectively recognized CCAAT homologies and enhancer core sequences, implying that it might be a transcriptional regulatory protein (2). Expression pattern of C/EBPα mRNA are similar in the mouse and human with measurable levels in liver, adipose, intestine, lung, adrenal gland, peripheral blood mononuclear cells, and placenta (7,8). In liver and adipose, highest levels of C/EBPα mRNA are detected only in differentiated tissue (7,8). Autoregulation of C/EBPα mRNA occurs by different mechanisms in the mouse and in humans:

a). The murine C/EBPα promoter directly binds C/EBPα within 200 base pairs of the transcriptional start resulting in 3-fold activation (9).

b). Autoregulation of the human C/EBPα promoter occurs by C/EBPα-induced binding of USF, a ubiquitously expressed transcription factor, to its upstream site within the C/EBPα promoter (10).

2.1.1. Member of C/EBP family

The CCAAT/enhancer-binding proteins (C/EBPs) encompass a family of transcription factors with structural as well as functional homologies (11). Since the cloning of the family's original member, C/EBPα, five other C/EBPs have been identified that interact with each other and transcription factors in other

protein families to regulate mRNA transcription. The six C/EBP proteins are designated as C/EBPα, β, γ, δ, ε and CHOP 10 (4,5,6). Similarities between C/EBP family members suggest an evolutionary history of genetic duplications with subsequent pressure to diversify. The resulting family of proteins varies in tissue specificity and transactivation ability. The pleiotropic effects of C/EBPs are in part because of tissue- and stage-specific expression, leaky ribosomal reading, post-transcriptional modifications, and variable DNA binding specificities. These mechanisms result in variable amounts of the C/EBP isoforms, available to dimerize and bind to cognate sites in different tissues (11).

Each isoform, however, shows distinct but overlapping patterns of tissue-or stage- restricted expression. Modifications of a transcription factor, such as by phosphorylation, glycosylation, and reduction-oxidation, affects its binding activity and function (12). For example, phosphorylation of the DNA-binding domain of C/EBPα, containing Ser 299 in the basic region of the basic leucine zipper structure, which is the phosphorylation site by protein kinase C results in an attenuation of DNA binding (13,14).

CCAAT/enhancer-binding protein (C/EBP) family members are among the bZIP transcription factors, and they bind to specific DNA sequences as dimers. The basic leucine zipper (bZIP) regions of C/EBP isoforms show high similarity, and some cis-elements are recognized by each of the C/EBP isoform.

Recent work with mice genetically altered to abolish expression of C/EBPs underscores the role these factors play in normal tissue development and cellular function, cellular proliferation and functional differentiation.

2.1.2. Domains of C/EBPα

The prototypic C/EBP, like many transcription factors, is a modular protein, consisting of an activation domain, a DNA-binding basic region, and a leucine-rich dimerization domain.

2.1.2.1. The activation domain:

Domains responsible for transcriptional activation and/or repression are located in the N-terminal end of the protein. Two isoforms of C/EBPα are generated from its mRNA by a ribosomal scanning mechanism (15,16). The full-length protein is 42 kDa and contains three transactivation domains (TEI-III) (17-19). TEI and TEII mediated cooperative binding of C/EBPα to TBP (TATA-binding protein) and TFIIB, two components of the RNA polymerase II basal transcriptional apparatus (18).A fraction of ribosomes ignore the first two AUG codons and initiate translation at the third AUG, 351 nucleotides downstream of the first AUG (15,16). This shorter 30-kDa protein retains its dimerization and DNA-binding domains; however, it possesses an altered transactivation potential compared with the 42-kDa isoform (15,16). TE-III contains a negative regulatory domain, the function of which is alleviated when C/EBPα is bound to the albumin promoter (17).

2.1.2.2. The leucine zipper domain:

The dimerization domain, aptly termed the “leucine zipper”, is a heptad of leucine repeats that intercalate with repeats of the dimmer partner, forming a coiled coil of alpha-helices in parallel orientation (20-22). Leucine zipper (LZ) dimerization segment contribute to DNA-binding specificity by determining which subunits form stable dimers, and by appropriately positioning the basic region helices over the binding site (23). A high resolution x-ray structure shows that the leucine zipper of yeast GCN4 is a parallel, two-stranded coiled-coil (24). The primary sequence of the leucine zipper is a repeating heptad (a-b-c-d-e-f-g) with hydrophobic and apolar residues predominating at positions a and d, and polar and charged amino acids dominating the other positions of the repeat. Apolar a and d residues face the interior of the coiled-coil and form an extensive van der Waals surface (23). Leucines at position d and position d’ of the

apposing subunit pack in a side-by-side arrangement, forming layers that alternate with those formed by the alternate hydrophobic residues at positions a and a' of the heptad repeat. The neighbouring e and g residues shield the hydrophobic dimer interface from solvent, completing the knobs-in-holes packing arrangement of helices in a coiled-coil. Charged residues frequent the e and g positions, sometimes forming intrahelical or interhelical salt bridges that contribute to dimer stability. Residues along the interior of the coiled-coil (positions a, d, e, and g) provide most of the stability and specificity of leucine zipper dimerization. Despite the marked conservation of LZ sequences within the bZIP family, the dimerization preferences of these proteins are distinct and varied. Residues e and g of the LZ heptad repeat are particularly important in this regard. These residues may influence dimer stability by shielding residues a and d from solvent and/or by participating in electrostatic interactions with other surface residues (23).

Vision and co-workers (21) note that two alternative, interhelical salt bridges involving residues e and g are possible. They term electrostatic interactions between positions e and the succeeding g' of the apposing subunit 'i+2' salt bridges, and the interaction between g and the succeeding e', 'i+5' salt bridges. In the case of C/EBP and closely related proteins, consideration of potential attractive and repulsive i+5 electrostatic interactions is a useful predictor of dimerization potential. Moreover, the stability of the C/EBP homodimer can be modulated by changing the identities of e and g residues in a manner consistent with an 'i+5 rule' for dimerization specificity.

Formation of heterodimers within a transcription factor family has been well described, and there is now increasing evidence that transcription factor from different families, containing similar as well as unrelated DNA-binding domains, form composite regulators (25).

Cell type specificity of activation of the albumin promoter by C/EBPα is determined, to a large extent, by its leucine zipper, which appears to have a

negative regulatory function in the context of the albumin promoter that is especially pronounced in the nonhepatic HeLa cell line compared with HepG2a hepatoma cells. These results suggest cell-type specific cooperation with other transcription factors as a basis for differential activation of genes by C/EBPα in different cell types (26,27).

2.1.2.3. The DNA-binding domain:

C/EBP dimerization is a prerequisite to DNA binding. DNA binding specificity, however, is determined by the DNA contact surface, the “basic” region of approximately 20 amino acids, upstream of the leucine zipper, specifically by 3 amino acids lying along the protein-DNA interface (28). The bZIP domain comprises a carboxyl-terminal dimerizatioin segment and an amino-terminal DNA-binding segment (the basic region) consisting of two extended alpha-helices rich in basic residues (23). Residues along the ‘inner’ surface of the basic region helix contact the edges of base-pairs in the major groove, while neighbouring residues contact the phosphodiester backbone of the binding site (23). Alignment of the avian retroviral binding sequences with the published binding sites for C/EBPα in two CCAAT boxes and in the simian virus 40, polyoma, and murine sarcoma virus enhancer suggested TKNNGYAAK (K=T or G, and Y=C or T) as a consensus sequence for binding of C/EBPα (29) Transcription factors sharing a common DNA-binding motif may associate as homodimers or heterodimers with distinct DNA-binding and transcriptional activities. These alternative, dimeric combinations of proteins explain the regulatory potential of each family. In both GCN4/DNA complexes, the bZIP dimer is composed of two smoothly curving alpha-helices, associated in a coiled-coil (the leucine zipper) that spans the carboxyl-terminal thirty residues of each subunit. The overall appearance of the bZIP is that of an alpha-helical fork (23). The bZIP subunits diverge at the amino-terminus of the leucine

zipper and position a relatively straight basic region helix in the major groove over each half-site, where it contacts the edges of base-pairs and the phosphodiester backbone (23).

2.1.3. C/EBPα in hepatocytes

2.1.3.1. C/EBPα-deficient animal hepatic phenotypes:

Coordinate expression of specific C/EBP isoforms is essential for normal hepatic synthetic activity and response to injury (11); however, C/EBPα is the predominant nuclear signal regulating terminal hepatocyte differentiation and function (11). Elimination of C/EBPα in targeted mouse knockout models results in profound derangement of liver structure and function. C/EBPα -/- mice have disturbed hepatic architecture with acinar formation, resembling proliferative or pseudoglandular hepatocellular carcinoma (30,31). c-Myc and c-jun mRNAs are induced consistent with a proliferative liver (30). Metabolic derangements are pronounced with an impairment of hepatic glycogen storage, and the majority of mice die soon after birth because of hypogylcemia (30,31). Known targets of C/EBPα have decreased expression at birth, including albumin, glycogen synthase, phosphoenolpyruvate carboxykinase, and glucose-6-phosphatase (31).

2.1.3.2. Hepatocyte proliferation following partial hepatectomy is accompanied by profound changes in C/EBP expression patterns (32-34). C/EBPα:C/EBPβ heterodimers are replaced by increased amounts of C/EBPβ homodimers during the early G1 period after partial hepatectomy. C/EBPα mRNA decreases following partial hepatectomy (35,36). Necessary downregulation of C/EBPα expression during liver regeneration may be mediated by the increased binding of C/EBPβ homodimers to the C/EBPα promoter, normally transactivated by α:β heterodimers in the non-proliferative state (36).

2.1.3.3. Studies of the interplay of different C/EBP factors on the promoters of several AP (acute phase) genes in hepatocytes have shown that at steady state the majority of DNA-protein complexes contain various forms of C/EBPα homodimers and C/EBPα: C/EBPβ heterodimers. Upon AP induction, however, the amount of complexes containing C/EBPα is dramatically reduced, replaced by C/EBPβ and δ (37).

The duality of C/EBPα function in mediating cell cycle arrest and hepatic metabolism is clearly demonstrated in the C/EBPα knockout mouse.

2.1.4. C/EBPα in adipocytes

2.1.4.1. C/EBPα-deficient animal adipose phenotype:

Adipocytes grown in tissue culture and in animal models lacking C/EBPα fail to accumulate lipids (31). Uncoupling protein is responsible for uncoupled mitochondrial respiration and heat generation and is a marker for differentiation of brown adipose tissue. C/EBPα deficient mice have minimal levels of uncoupling protein expression at 2 h postpartum, which increases to 60% that of control mice by 32 h postpartum (31).

Gene transcription of fatty acid synthase, GLUT4, and 422/aP2 is unaltered in white adipose tissue of the C/EBPα-deficient mouse, which is inconsistent with transcriptional data from 3T3-L1 cell lines (31,38,39). Redundant transcriptional elements operative in the animal model may regulate the fatty acid synthesis pathway, compensating for the lack of C/EBPα.

2.1.4.2. Preadipocyte differentiation into functional adipocytes results from a highly regulated cascade of C/EBP isoform expression. C/EBPα expression increases during late phase of differentiation. Abrogation of C/EBPα expression, either by antisense interactions or hydrocortisone administration, prevents

terminal adipocyte differentiation (5,15).

2.1.4.3. Ectopic expression of C/EBPα in 3T3-L1 cells arrests mitotic growth. C/EBPα interacts with known regulators of cell cycle progression; it activates transcription and induces post-transcriptional stabilization of p21 (WAT-1/CIP-1/SDI-1) protein, an inhibitor of cyclin-dependent kinase (40-42). Additionally, c-Myc and C/EBPα share a reciprocal relationship, balancing proliferation versus growth arrest via C/EBPα-transactivated expression of gadd45 (growth arrest-associated gene), a target of p53 tumor suppressor protein at G1 (43,44).

2.1.4.4. Transient modulation of C/EBP levels in response to insulin and dexamethasone suggests a dynamic role in adipocyte metabolism (45). Insulin treatment decreased DNA-binding of C/EBPα while increasing nuclear C/EBPβ and C/EBPδ binding. Insulin also induced rapid dephosphorylation of C/EBPα and represses C/EBPα expression, modulating adipocyte gene transcription. Another target gene of C/EBPα, the obese gene, may be similarly regulated.

2.1.5. C/EBPα in hematopoiesis:

Pluripotent hematopoietic stem cells combine a capacity for self-renewal with the ability to differentiate along any one of at least eight possible lineages (46). Understanding the molecular processes underlying self-renewal and differentiation in the hematopoietic system is important for our appreciation both of similar developmental processes and of the genesis of leukemias, in which this balance becomes deregulated. Precursor cells divide and differentiate from the pluripotent hematopoietic stem cell and proceed to committed stem cells, which provide lineage-restricted progenies.

2.1.5.1. C/EBPα-deficient animal hematopoietic phenotypes:

Profound abnormalities of the hematopoietic system are seen in C/EBPα deficient mice (47). Mice deficient in C/EBPα display an early block in the maturation of granulocytes. Peripheral blood and bone marrow smears show only myeloblastic cells of the myeloid lineage. The G-CSF receptor expression is undetectable in these cells, suggesting a loss of G-CSF signal-directed maturation (47). In transient assays, C/EBPα contributes to tissue-specific expression of G-CSF and GM-CSF receptors (48-50) and neutrophil elastase (51,52). Evidence suggests that C/EBPα play an early, pivotal role in the granulocytic lineage.

2.1.5.2. Polymorphonuclear neutrophils (PMN):

PMNs are short-lived non-mitotic cells generated in large numbers in the bone marrow through a highly controlled process of myelopoiesis, where C/EBPα plays a central role (53). Polymorphonuclear neutrophils (PMNs) play a crucial role in host defence by phagocytosing and killing invading microorganisms. Mature PMNs are incapable of cell division, and their sustained generation by bone marrow at impressive numbers (10E11 cells per day in a normal adult) is the result of a highly controlled, yet incompletely understood, process of myelopoiesis. During maturation, PMNs acquire their granule products, which equip the activated PMNs to kill microorganisms.

2.1.5.2.1. Development of PMN granules:

CCAAT enhancer-binding proteins (C/EBP) play a role in PMN development, by inducing transcription of granule protein coding genres, either by direct binding to the promoters of the corresponding genes or by activating transcription of the granulocyte colony-stimulating factor receptor (G-CSFR) (53). Myelopoiesis in C/EBPα-knockout mice fails to proceed beyond the myeloblast stage; whereas transfection of C/EBPα in U937 promonocytic cells

induces PMN differentiation and synthesis of PMN granule proteins (47). C/EBPα works in concert with CCAAT displacement protein, a negative regulator that must be downregulated to allow expression of proteins confined to PMN-specific granules. Differences in timing of granule protein synthesis during maturation of PMN precursors results in formation of granule subsets with different protein content. One of these organelles is the secretory vesicle created by endocytosis late during PMN maturation. This organelle is particularly rich in receptors and provides the plasma membrane with receptors promoting attachment to activated endothelium (53).

2.1.5.2.2. Clearance of apoptotic PMNs:

Many of the daily produced neutrophils undergo apoptosis before leaving the bone marrow. Apoptotic neutrophils can be removed by macrophages (in which case, neutrophils are anonymously degraded) or by dendritic cells (leading to antigen presentation and autoimmunity). Anti-apoptotic signals generated by growth factors and cytokines can increase the number of neutrophils by prolonging the PMN life span (about 8 h after leaving the bone marrow), and accelerating neutrophil maturation (53).

2.1.5.2.3. One of the major decisions leading to PMN generation is made by stem cells committed to the myeloid lineage; genes coding for granule proteins are turned on to synthesize the granule constituents that will endow the PMN with its battery of microbicidal proteins. The high heterogeneity of PMN granules is due to the continued formation of granules from the myeloblast to the segmented PMNs, where granules are filled with proteins formed at a particular stage of PMN maturation. Mature PMNs are fully equipped with an NADPH oxidase and an arsenal of harmful agents stored in granules, ready to be used to destroy phagocytosed microorganisms. These cells constitute unique and perfect weapons to fight infection, and play a major role in the host organism’s

surveillance system against foreign invaders and in acute inflammation. In vitro and in vivo studies have revealed a great deal of cross-talk and complexity, as well as pleiotropic effects of signalling molecules and receptors in the differentiation stages of PMN differentiation and activation. Recent developments in the characterization of the molecular pathways involved in PMN differentiation and activation are providing a new basis for the treatment of infection and inflammation (53).

2.1.5.3. Lineage commitment decisions presumably involve modulations of regulatory circuits already active in precommitted cells and there are now various lines of evidence suggesting that this regulatory environment is unusually complex (46). For instance, the emergent haematopoietic lineages appear to be characterised not by unique transcription factors but by unique combinations of factors, each of which may occur singly in a number of lineages (54). Evidence that this combinatorial feature may extend back to multipotent progenitors has come both from preliminary studies of expression patter and from the multilineage defects resulting from null mutations of a number of transcription factors that are expressed only in a subset of mature cell types (55). Complexity does not necessarily indicate chaos and it is reasonable to assume that hierarchies and cross-talk between the transcription factors co-expressed in multi-potent progenitors co-ordinate commitment and differentiation and may also be involved in maintenance of the multipotent state (46). The analysis of regulatory sequences and their DNA-binding proteins has in some cases confirmed regulatory connections directly. Direct protein-protein interaction probably provides an important means of coordination during normal differentiation. Most notably, a combination of C/EBPα and PU.1 appears to be instrumental in directing expression of the granulocyte-specific genes G-CSF receptor (48), neutrophil elastase (52), and myeloperoxidase (56). In the latter study, priming for low level activity of the MPO gene in multipotent cells is

thought to be maintained by C/EBPα, whereas upregulation during granulocyte differentiation is associated with replacement by C/EBPβ and δ isoforms that translocate to the nucleus from a pool initially present in the cytoplasm (56). The succession of C/EBP isoforms binding to the MPO enhancer suggests a basis for the lineage-specific upregulation of a gene cohort from the multiplicity of genes expressed at low levels in the multipotent state (46).

2.1.6. C/EBPα in other systems:

C/EBPs role in the function of other organ systems is only beginning to be elucidated. A significant percentage of C/EBPα-deficient mice succumb to respiratory defects soon after birth (30). Histologic examination of C/EBPα-deficient lungs shows hyperproliferation of type 2 pneumocytes (30).

C/EBPα expression is temporally correlated with the appearance of surfactant A protein and is not present in A549 cells, a cell line that does not express surfactant proteins (57).

Normal ovarian physiology is dependent upon both C/EBPα and C/EBPβ. Rat ovarian follicles express C/EBPα in a cell-, time-, and hormonally specific manner (58). Attenuation of C/EBPα expression results in decreased responsiveness to exogenous gonadotropins and decreased ovulation rate (58).

In squamous cell carcinomas, the expression of C/EBPα was greatly diminished.

2.1.7. C/EBPα in Acute Myeloid Leukemia (AML):

Some types of acute leukemia, especially those associated with chromosomal translocations, involve blocks in differentiation at specific stages of hematopoiesis (59). The transcription factor C/EBPα is required for neutrophil differentiation, suggesting its function may be disrupted in acute myeloid leukemia (AML) (47).

2.1.7.1. The highest percent of mutations were found in patients with M2 AML, a myeloblastic leukaemia characterized by an early block in neutrophil differentiation (59). It has been reported that loss of C/EBPα expression or function in leukemic blasts, suggesting that a reduction in its activity contributes to a block in myeloid cell differentiation (60,61). In these leukemic cells, C/EBPα function was disrupted either through mutation of one allele of the CEBPA gene or transcriptional repression (60). CEBPA mutations were observed in leukemic cells lacking any chromosomal translocations (60). These mutations resulted in truncated forms of C/EBPα proteins that retained the DNA-binding domain. Unexpectedly, these proteins failed to bind DNA, but inhibited DNA binding by wild-type C/EBPα, suggesting that these mutants act as dominant inhibitors. However, further studies are needed to determine the reasons that these proteins fail to bind DNA while the murine counter-parts are capable of DNA-binding, and to determine how these proteins act at sub-stoichoimetric levels.

2.1.7.2. The t(8;21) creates a fusion protein between the amino-terminal DNA binding domain of the acute myeloid leukaemia-1(AML1) transcription factor and the eight-twenty-one (ETO) corepressor, suggesting that repression of AML1 target genes stimulates leukemogenesis (62,63). Moreover, the t(8;21) fusion causes an early block in granulocytic differentiation, and C/EBPα levels were reported to be unaffected in these leukemias (47). Therefore, these fusion proteins (also inv (16)) can act independently of C/EBPα. The different phenotypes associated with these translocations suggest that AML-1/ETO has a ‘modifier’ effect that causes the down-regulation of C/EBPα and blockade at a distinct stage of differentiation (59). Differentiation blocks are thought to contribute to tumorogenesis, along with mutations that affect cellular

proliferation, inactivate cell-cycle checkpoints, and block apoptosis. Thus, the loss of C/EBPα could represent one ‘hit’ in multi-step leukemogenesis (59). The observation that C/EBPα function can be deregulated by two separate mechanisms highlights the importance of this factor. Disrupting C/EBPα activity by either creating dominant negative forms or repressing its transcription may have the same effects, both leading to a block in myeloid progenitor-cell differentiation. The importance of C/EBPα in cell growth and differentiation suggests it may play a role of a tumour suppressor.

2.1.7.3. The mutations in the C/EBPα locus upon screening 408 patient samples can be divided into 4 classes concerning their effect on the predicted protein (64):

2.1.7.3.1. Termination of translation by introducing a nonsense codon; (The nonsense mutations would introduce termination codons before the bZIP domains. This would create polypeptides that are unable to localize to the nucleus, dimerize, and bind to the DNA)

2.1.7.3.2. Alteration of amino acid sequence by introducing a missense codon; (the mutation of Arg305Pro occurs in the fork region of bZIP domain)

2.1.7.3.3. Frame-shift by either deletion or duplication of nucleotides and an eventual termination; and

2.1.7.3.4. Inframe deletion or duplication that removes or inserts additional amino acid residues. (The inframe deletion and insertion mutations occur within the first conserved leucine finger)

Comparison of the predicted amino acid sequences of 11 bZIP family members reveals conservation between the basic and leucine zipper regions and

the exact spatial register or phasing between these regions, referred to as the fork. The phasing is important for a continued α-helical structure that progresses from the zipper into the basic region. The basic region starts exactly 7 amino acid residues amino-terminal to the first leucine zipper. When the phasing between these regions is altered by insertion or deletion of 2-, 4-, 5- or 6- amino acid residues, sequence-specific DNA-binding is eliminated although dimerization is reported to still occur via the leucine zippers.

These mutants did not attenuate transcriptional activation by wild-type C/EBPα. This may be due to a lack of heterodimerization between the wild type and mutant forms of C/EBPα. The data from this study are consistent with a model in which the mutations disrupt the dimerization interface formed by the leucine zipper and the proteins are unable to homodimerize or heterodimerize and bind to DNA (64).

2.1.8. C/EBPα regulates monocytic lineage commitment:

Ectopic expression of C/EBPα could prevent 12-O-tetradecanoylphorbol-13-acetate (TPA) induced monocytic differentiation of bipotential myeloid progenitor cells (67). In addition, TPA treatment of HL60 cells led to a decrease of C/EBPα mRNA levels by approximately 90% (67).

Recent reports indicate that C/EBPα exerts effects inhibiting growth and gene expression independent of DNA-binding (65,66):

The C/EBPα protein interacts with the p21 and CDK2 proteins, resulting in decreased CDK2 activity and inhibition of cell proliferation (65).

The C/EBPα protein disrupts E2F complexes in several cell lines, and this correlates with C/EBPα-mediated growth arrest. Interaction of C/EBPα with E2F appears to be the mechanism by which it downregulates c-myc expression during granulocytic differentiation (66).

These protein-protein interactions appear to involve several regions of the

C/EBPα protein, including the bZIP domain. Perhaps the duplication and deletion mutations in the bZIP domain may affect these interactions, thereby contributing to leukemogenesis (65).

2.2. c-jun:

The AP-1 transcription factor complex owes its initial definition to a fortuitous convergence of tumour virology and transcription research (68-70). AP-1 has been described as a 12-O-tetradecanoyl-phorphol-13-acetate (TPA) inducible transcription factor activity that addresses specific sequences in the enhancer of the metallothionien gene and in the 72-base pair repeat of the simian virus 40 enhancer region (68-70). Jun was found as a novel oncoprotein encoded by a cellular insert in the genome of avian sarcoma virus 17 (ASV17), an acutely oncogenic retrovirus isolated from a spontaneous tumour in chicken (71). The two findings became linked by the discovery of homology between Jun and the yeast transcriptional regulator GCN4 (72).

A second important step in the definition of AP-1 came with the discovery that the Fos-associated protein p39 is the product of the cellular jun gene. This finding identified cellular Fos as a partner of Jun and as another component of the AP-1 complex (73,74).

Cloning of the cellular Jun gene showed it to be without introns and with an atypical TATA box (75,76). The human jun gene is located on chromosome 1 at region p31-32 (77).

The mammalian AP-1 proteins are homodimers and heterodimers proteins that belong to the Jun (c-Jun, JunB and JunD), Fos (c-Fos, FosB, Fra-1 and Fra-2), Jun dimerization partners (JDP1 and JDP2) and the closely related activation transcription factors (ATF2, LRF1/ATF3 and B-ATF) subfamilies (78-82).

c-Jun protein can form stable dimers that bind AP-1 DNA recognition elements (5’TGAG/CTCA-3’), also known as TREs [phorbol 12-O-tetradecanoate-13-acetate (TPA) response elements] based on their ability to mediate transcriptional induction in response to the phorbol ester tumour promoter TPA (68). However, Fos proteins do not form stable dimers but can bind DNA by forming heterodimers with Jun proteins that are more stable than Jun: Jun dimmers (83,84). Heterodimerization with c-Fos further increases c-Jun’s

transcriptional capacity through formation of more stable dimers, while heterodimerization with JunB attenuates it (85,86).

2.2.1. Cellular transformation.

The transforming activity of c-Jun and c-Fos suggests that AP-1 complexes containing these components are involved in stimulation of cell proliferation (87). Deregulated expression of Jun and Fos proteins can induce transformation in-vivo (88). Interestingly, antisense oligonucleotides specific for c-fos or c-jun were found to inhibit the proliferation of mouse fibroblasts and erythroleukemia cells (89-91).

Differential expression of AP-1 proteins in response to extracellular stimuli was suggested as one of the major mechanisms that modulate AP-1 activity (85). Identification of c-Fos and c-Jun, the mammalian homologues of the retroviral oncoproteins v-Fos (92) and v-Jun (93), as components of AP-1 immediately linked AP-1 to cellular growth control and neoplasia.

Several lines of evidence suggest that AP-1 transmits signals from activated Ras to the nucleus in transformed cells. In non-immortalized rat embryo fibroblasts (REF) overexpression of mammalian or avian c-Jun was insufficient for induction of transformation (86). However, Ha-ras induced transformation of immortalized mouse fibroblasts requires c-Jun expression (94). Fos further enhanced the transforming capacity of c-Jun, whereas JunB attenuated it (86). These differences are consistent with higher DNA binding and transcriptional activity of c-Jun: c-Fos heterodimers in comparison to c-Jun: JunB heterodimers. Furthermore, by providing survival and growth promoting signals, Ha-Ras may suppress the effect of c-Jun-induced growth inhibitors or activators of apoptosis such as TGFβ (95) or Fas ligand (96).

2.2.2. Apoptosis and senescence.

Apoptosis or programmed cell death is commonly used to eliminate damaged or

non-essential cells during development, to maintain tissue homeostasis or to eliminate oncogene-expressing cells in adult organisms (97).

AP-1 transcription factors have also been implicated in the control of cell death and survival (87). Among growth regulators, AP-1 is somewhat unusual because, in addition to being responsive to growth factors, it is also upregulated by genotoxic stresses, such as UV or alkylating agents that cause growth arrest and/or cell death. Furthermore, the consequence of AP-1 activation seems to be cell type specific. While it may promote apoptosis in some cell types, it is required for the survival of others. Of course, given the many different forms of AP-1, its exact functions are likely to be dependent on its composition post translational modifications and presence of interacting factors (87).

AP-1 can trigger either pro- or anti-apoptotic signals depending on the dimer composition, the cell type or the death-inducing treatments (97).

2.2.2.1.c-Jun:pro-apoptotic gene:

c-jun expression is induced in response to treatments, which frequently triggers apoptosis, among them UV, ionising radiation, hydrogen peroxide and tumour necrosis factor α (TNFα) (98-100).

c-jun and c-fos expression is upregulated in lymphocytes that undergo apoptosis in response to IL6 depletion and c-jun or c-fos antisense oligonucleotides protect these cells from death response (101,102).

Amino-terminal phosphorylation of c-Jun is also required for ceramide-induced apoptosis of myeloid or lymphoid tumour cell lines and neuronal stress-induced apoptosis in vivo (103).

Inducible c-Jun protein transmits an apoptotic death signal to immortalized fibroblasts that can be antagonized by Bcl-2. This pro-apoptotic effect is specific for c-Jun (104).

Increased c-Jun activity mediates apoptosis in neurons, after removal of survival factors. Moreover, c-Jun accumulates in neurons undergoing apoptosis

by hypoxia in vivo. Studies using PC12 pheochromocytoma cells and cultured neuronal cells showed that inhibition of c-Jun activity with a dominant negative mutant that lacks the c-Jun N-terminal activation domain or by neutralizing antibodies, can protect the cells from apoptosis induced by withdrawal of nerve growth factor (NGF) or chronic depolarisation (105-108). Ectopic expression of c-Jun can induce apoptosis in sympathic neurons as well as in mouse fibroblasts, Syrian hamster embryo (SHE) cells and a human colorectal carcinoma (RKO) cell line (102,104,106).

An essential question is how does AP-1 mediate its proapoptotic effects in those cases where it is positively involved in the process. One possibility is that AP-1 directly activates the transcription of genes, whose products can trigger apoptosis. Indeed, several reports suggest that AP-1 induces expression of the Fas-ligand (FasL) gene (96,107,109). Another possibility is that AP-1 has a homeostatic function that reacts to changes in growth and environmental conditions to adjust the gene expression profile in a way that allows the cell to adapt to the new environmental conditions to adjust the gene expression profile in a way that allows the cell to adapt to the new environment. However, after excessive environmental stress, a conflict may arise between the AP-1 regulated program and other stress activated gene expression programs that may cause a catastrophe that culminates in cell death (87).

2.2.2.2.c-Jun: anti-apoptotic gene

c-Jun -/- embryos exhibit higher rates of apoptosis in liver than control animals (110), and c-Jun is not essential for programmed cell death and axon growth in the retina (111). Moreover, in Jurkat cells, AP-1 is not increased during Fas-mediated cell death although Fas cross-linking leads to Ras induction and JNK activation (112). Furthermore, AP-1 regulates the murine anti-apoptotic Bcl-3 gene, which acts as a survival factor for T cells in the absence of growth factors (113). The dominant negative c-Jun mutant could reduce

apoptosis in human monoblastic leukemia cells after exposure to various DNA damaging agents (103). The role of c-Jun in apoptosis is thus complex, mostly cell-type specific and depends on the nature of the death-inducing signal.

In contrast to apoptosis induced by survival factor withdrawal or genotoxic stress, AP-1 does not promote apoptosis during normal development. In vivo observations suggest that AP-1 proteins may in fact have a protective role during embryonic development, as c-Jun deficient embryo exhibit massive increase in liver cell apoptosis, which may be the cause of their lethality.

One current hypothesis to explain these apparent contradictory results links c-Jun containing AP-1 dimers to de novo protein synthesis. Hence, it has been shown that active pro-apoptotic machinery pre-exists in the cells. AP-1 activity would be required for apoptosis in cells in which the death effectors are present in limiting amounts and in which protein synthesis is required to initiate the apoptotic suicide program (80).

2.2.3. Cell cycle.

Several lines of evidence suggest that the Jun and Fos proteins are important targets for mitogenic signal transduction pathways. First, the AP-1 binding sequence was originally identified as a TPA responsive element (TRE) and was shown to respond to the tumour promoter, TPA. Treatment of cultured cells with mitogenic agents resulted in a strong increase in AP-1 binding to a TRE sequence. Upon serum stimulation, many quiescent cells displayed an increase in c-jun, junB and fos mRNAs. Based on their rapid, high and transient transcriptional upregulation in response to mitogenic agents, these agents were defined as ‘immediate early genes’ or ‘competence genes’ and encode products necessary for entry into G1 and S phases of the cell cycle.

Protooncogenes frequently participate in the regulation of cell proliferation through signal transduction pathways that convert extra-cellular stimuli into gene expression programs. Many of these genes are also activated during G0 to

G1 transition when quiescent cells are stimulated by mitogens to re-enter the cell cycle. Based on this study, the AP-1 proteins were classified into three subgroups according to their expression patterns (114):

1. c-Jun, JunD and Fra2 are expressed in cycling mouse fibroblasts and are only mildly induced upon serum addition to the growing cells. When cells become quiescent upon serum starvation, c-Jun level decreases and JunD level increases. Upon serum-induced re-entry into the cycle, c-Jun is strongly stimulated.

2. The basal levels of JunB, c-Fos, FosB and dFosB protein is low in cycling mouse fibroblasts but strongly and rapidly increases after serum stimulation.

3. Fra1 is the only member of the third group. Undetectable in cycling cells, Fra1 stimulation following serum addition is delayed to later time points and shown to be transcriptionally activated by c-Jun/c-Fos dimers.

Progression through the cell cycle is tightly controlled by the sequential activation of a genetic program including the synthesis of the cyclins and the activation of the cyclin-dependent kinases (cdk) (97). c-Jun and JunB or JunD display opposite effects on cell cycle progression. While c-Jun is a strong transcriptional activator of cyclin D1, JunD is a weak positive activator and JunB even an inhibitor of this promoter (82,115-117). Increasing the abundance of JunB or JunD relative to c-Jun decreases cyclin D1 transcription. The temporal changes in AP-1 composition and activity during the M-G1 transition may constitute an interesting way of controlling cell cycle progression. The cell cycle dependent variation in Jun protein levels constitutes a novel reciprocal link between the cell cycle machinery and a transcription factor.

Ras transformation increases the cyclin D1 protein level, a major regulator of the G1/S transition previously identified as an AP-1 target gene (118,119). Increased cyclin D1 expression in Ras-transformed cells shortens their G1 phase. Moreover, overexpression of cyclin D1 in immortalized fibroblasts accelerates G1 progression and decreases serum requirement for growth (120).

Thus, transformation by Ras leads to accumulation and activation of AP-1 transcription factor that in turn increases cyclin D1 protein level and accelerates G1 progression. AP-1-mediated induction of cyclin D1 explains how changes in AP-1 composition mimic in part the proliferative effects of activated Ras (115,121).

AP-1 activity is also required for serum stimulated cell cycle re-entry of serum-starved cells. Another study suggests that c-Jun might also have a role in allowing UV irradiated cells to re-enter the cell cycle from the G2/M interphase (122). Interestingly, c-Jun expression levels do not significantly change during the cell cycle, but the protein undergoes transient N-terminal phosphorylation as cells proceed from G2 to M that persists until the cells complete mitosis (121). It is not clear however, whether these changes result in cell cycle dependent regulation of AP-1 transcriptional activity.

Fibroblasts lacking a single Jun protein, with the exception of JunB, exhibit significantly altered growth properties. The most severe defects are exhibited in c-jun -/- fibroblasts, which can be passed only once or twice in culture before they exhibit a pseudo-senescent phenotype and their cell cycle transit time increases dramatically (82,123,124). Reintroduction of c-Jun into such cells results in increased proliferation, indicating that the proliferation defect is indeed due to the absence of c-Jun.

Many AP-1 binding sites have been characterized in promoters or enhancers of cellular genes encoding enzymes involved in tissue remodelling, like collagenase and stromelysin, in cell cycle controlling genes such as cyclin D1 or c-jun itself, in cytokine encoding genes like IL-2 (78).

2.2.4. Domains of c-Jun.

The dimerization, DNA binding, and transactivation domains of Jun are modular, exchangeable for functionally equivalent domains from other proteins. Such ‘cut and paste’ operations within the bZIP family usually preserve the

basic activity of the protein.

2.2.4.1. Dimerization: Importance of a partner

The Jun protein can form homodimers, though heterodimers with Fos are more stable and have a higher affinity for the DNA target sequence (83,125-127). Dimerization is a prerequisite for DNA binding of AP-1 members (83,127) Dimerization of Jun and Fos enhances their nuclear translocation (128). Dimerization requires the C-terminal region of Jun (129,130). This domain contains five heptad repeats of leucines forming an amphipathic helix that upon dimerization takes up a coiled coil confirmation with the leucines aligned along the contact surface. This dimerization domain is referred to as a leucine zipper (22). A given leucine zipper pairs with a limited set of specific leucine zipper partners; the specificity is dictated by the non-leucine residues of the zipper.

The consensus DNA binding sequence for AP-1 dimers is TGACTCA (69). Jun can also form dimers with bZIP proteins from outside the AP-1 complex. These dimerization partners include some members of the CREB/ATF proteins (the consensus DNA-binding sequence being TGACGTCA, is referred to as CRE). It bears close resemblance to the AP-1 target sequence. The binding of c-Jun: ATF2 heterodimers to divergent AP-1 sites within the c-jun promoter results in its transcriptional activation. Heterodimers of Jun and certain ATF proteins are able to recognize the CRE sequence, whereas heterodimers of Jun and Maf bind equally well to the AP-1 and CRE consensus (131-134). Thus, dimerization with diverse partners can function as a modulator of target gene specificity. Dimerization is a prerequisite for DNA binding.

2.2.4.2. DNA binding: Importance of an address

The basic region, serving as DNA contact surface, is located immediately N-terminal to the leucine zipper. This characteristic arrangement of DNA binding and dimerization domains is found in numerous transcriptional regulators and gave them the family name ‘bZIP proteins’ (135).

The consensus DNA binding sequence for AP-1 dimers is TGACTCA, it is referred to as TPA-response element (TRE) (69). The TRE consensus sequence of the AP-1 family is a minimal shared target of the various AP-1 dimers, but not all combinations bind to the TRE with the same affinity (136). Mutations in the Jun DNA binding domain that eliminate interaction with the TRE lead to a complete loss of oncogenicity; mutations that preserve TRE recognition may cause a reduction in oncogenicity (137).

The DNA binding domain of a transcription factor determines the spectrum of genes that are controlled by that protein. The dimeric structure and that different dimerization partners can significantly modulate the specificity of DNA targeting. DNA binding of Jun is also affected by interactions of the transactivation domain with regulatory proteins such as JAB-1 (136).

2.2.4.3. Transactivation.

The N-terminal half of Jun contains the major transactivation domain. The important regulatory phosphorylation sites of Jun are Ser63 and Ser73 near the N terminus (138). JNK connects c-Jun to incoming signals, responding to mitogens, stress signals, and genotoxic substances. JNK-mediated phosphorylation of c-Jun at serines 63 and 73 is required for two principal functions of the protein: transcriptional activation and co-transformation of mammalian cells in conjunction with Ras (139,140). N-terminal phosphorylation is also essential for transformation by Fos and for c-jun-dependent apoptosis.

The delta domain (amino acids 34-60 of c-Jun) is critical for the regulation of transcriptional activation by c-Jun.

2.2.5. c-Jun regulation and regulated genes

Transcription factors are the ultimate recipients of incoming signals and convert the signals into patterns of gene expression. Transformation is induced by corrupting the transcriptional regulation of specific target genes that are then

expressed at higher or lower levels in transformed cells.

In vitro, dimers formed by Jun and Fos bind with the highest affinity to an asymmetric heptanucleotide recognition sequence TGA(C/G)TCA (AP-1) and with slightly lower affinity, to a symmetric octanucleotide TGACGTCA (CRE) (73,126). The AP-1 site is a ubiquitous regulatory element that is found in a wide range of promoter and enhancer regions. Since the AP-1 site and variants thereof occur with a high frequency in the genome, it is unlikely that Fos-Jun family proteins regulate all genes that contain AP-1 recognition sequences. Conversely, many genes that are bona fide regulatory targets of Fos-Jun family proteins do not contain consensus AP-1 recognition sequences within their control regions (141).

In natural promoter and enhancer regions, the sequences of AP-1 regulatory elements often deviate from the optimal recognition sequence. This variation in recognition sequences may contribute to the differential functions of different Jun-Fos family dimers at various regulatory elements (141). The weaker binding affinities of Jun-Fos family members at these non-consensus recognition sites may also impose a requirement for interactions with other transcription factors.

Several mechanisms that may contribute to the cell type specificity of Jun-Fos family proteins can be envisioned. These include differential post-translational modifications, selective dimerization between different family members and interactions with other regulatory protein. The first two mechanisms modulate the activities of Jun-Fos proteins, but they have mostly indirect effects on selection of the genes that are regulated by Jun-Fos family proteins in a particular cell type. Interactions with other transcription factors can modify the regulatory specificities of Jun-Fos family proteins in a cell or tissue specific manner. Thus, Jun-Fos proteins have to be considered in the broad context of dynamically changing protein-protein interactions on and off DNA (141).

Jun-Fos interacting proteins can be subdivided into four groups:

1.structurally related basic region-leucine zipper proteins;

2.unrelated DNA binding proteins;

3.transcriptional coactivators that do not bind DNA directly; and

4.structural components of the nucleus.

In many cases, either the functional significance or the structural basis of the interaction remains to be investigated. Nevertheless, it is clear that interactions among many structurally divergent protein families can contribute to the functional specificity of Jun-Fos family proteins (141).

Among the genes that are differentially expressed in Jun-transformed cells, there is a group whose deregulation is essential for inducing and maintaining the neoplastic cellular phenotype, the oncogenic effector genes and a presumably larger group whose differential expression is of no consequence to the growth behaviour of the cell, the innocent bystanders (142).

Phosphorylation of AP-1 proteins further modulates their activity and provides another route for extracellular stimuli to regulate AP-1 activity. AP-1 was identified as a transcription factor that contributes to basal gene expression as well as TPA-inducible gene expression (143,144).

Many other stimuli, most notably serum (145,146), growth factors (146-148) and oncoproteins, such as v-Src or Ha-Ras (78) are also potent inducers of AP-1 activity.

The findings that growth factors and tumour promoters induce AP-1 activity, and c-Jun and c-Fos are encoded by protooncogenes immediately suggested that AP-1 activity is likely to be important in growth control as well as play a key role in transformation.

In addition, the response to proinflammatory cytokines (TNFα (98) and IL-1 (149,150)), as well as the finding of AP-1 target genes such as collagenase (68) and IL-2 (150) suggested that AP-1 is also likely to be involved in inflammation and innate immune response. Several mechanisms account for

stimulation of AP-1 activity by growth factors, proinflammatory cytokines, and UV radiation (143).

The most important mediator of the growth factor response is likely to be the ERK MAP kinase (MAPK) cascade, which through phosphorylation of ternary complex factors (TCFs) (151) causes induction of fos genes, whose products then heterodimerize with Jun proteins to form more stable AP-1 dimers. Through AP-1 site in the c-jun promoter, these newly formed Jun: Fos heterodimers can lead to increased c-jun transcription (152).

Erk activation may also contribute to c-jun induction through MEF2 proteins, another group of transcription factors that bind to the c-jun promoter.

Jun interacts with numerous other transcription factors, and this transcriptional cross talk modulates the activities of Jun and its partners (153). The following are some examples:

The glucocorticoid receptor interferes with Jun activity, but under certain conditions can also exert an enhancing effect (154-157). The inhibition of Jun induced by the glucocorticoid receptor affects oncogenic transformation.

A potentially highly significant interaction takes place between Jun and various SMADs, components of the TGFβ−signalling pathway. This interaction enhances transcription from AP-1 binding sites and interferes with transactivation from SMAD binding sites (158-161).

TGFβ can also enhance c-Jun expression (162).

Expression of transcriptional regulator called nuclear factor 1 (NF-1) strongly interferes with Jun-induced transformation in chicken embryo fibroblasts (CEF) (163).

A particularly interesting example of cross-talk is the interaction of Jun and Stat3 that involves both physical association of the proteins and binding to closely spaced sites on specific promoters (164).

Important interactions also take place between Jun and the transcription factor NFAT (nuclear factor of activated T-cells) family. NFAT proteins are

critical in the regulation of cytokine and other immune response genes (165-166). Dominant negative Jun inhibits NFAT transcriptional activation and interferes with the control of IL-2 expression (167).

Jun also binds to human papilloma virus type 16 protein E7. This association enhances AP-1 activity and may be part of the mechanism by which HPV transforms cells (168).

As part of the UV response, Jun interacts with p53, stimulating the activation of the mismatch repair enzyme MSH2 (169).

Jun also plays a part in the oncogenic transformation induced by the v-Rel oncoprotein. Rel belongs to the NF-kB family of transcriptional regulators (170). c-Jun is upregulated in Rel-transformed cells, and dominant negative Jun strongly interferes with transformation of avian lymphoid cells and of chicken embryo fibroblasts.

Negative modulation of Jun also occurs through binding to bZIP proteins, e.g. the Jun dimerization protein JDP2 (79).

The interaction of Jun/Fos dimers with promoter sequences is strongly influenced by the SWI/SNF chromatin-remodelling complex (171).

The response to proinflammatory cytokines and UV radiation, on the other hand, are mostly dependent on two other MAPK cascades, JNK and p38 (143). A major role in c-jun induction by UV is played by the JNKs as they phosphorylate and enhance the transcriptional activity of two major players in c-jun expression, c-Jun itself, and ATF-2 (143).

Cyclin D proteins are regulators of G1 to S phase transition that bind to the CDK4 cyclin dependent kinase and increase its kinase activity, thereby cause the phosphorlyation and inactivation of the retinoblastoma (Rb) tumour suppressor protein. The human cyclin D1 gene regulatory sequences contain two AP-1 binding sites to which several AP-1 proteins (including cJun, JunB, c-Fos and ATFs) were shown to bind. However, cyclin D1 overexpression in c-jun -/- MEFs could restore only 25-30% of the DNA synthesis induced by c-Jun. Thus,

suggesting that other c-Jun target genes are likely to be involved in its growth promoting activity (82,121,172,173).

Another important c-Jun target gene is p53 (122,124). p53 is a negative growth regulator (174,175). c-Jun is a negative regulator of both p53 expression and its ability to activate target gene transcription. The basal level of p53 expression is higher in c-jun -/- fibroblasts that in wild-type counterparts and stable expression of c-Jun in c-jun -/- cells reduce the level of p53 expression (124). Most importantly, the removal of p53 from c-jun -/- cells completely suppresses their proliferative defects (124). c-jun -/- cells express higher levels of p53 regulated gene products, especially p21, whereas cells that express c-Jun constitutively express very low levels of p53 target genes. By repressing p53-mediated p21 induction, high levels of c-Jun prevent UV induced growth arrest and channel most of p53 activity towards the induction of apoptosis (122).

Another AP-1 regulated gene coding for FasL was suggested to provide an explanation to the proapoptotic capacity of c-Jun. c-jun -/- fibroblasts, that are relatively resistant to apoptosis induced by UV radiation and alkylating agents, are also impaired in FasL expression (109). FasL expression is induced by DNA damaging agents including topoisomerase II inhibitors and UV irradiation, agents that activate JNK (96).

The biological function of AP-1 was initially restricted to the transmission of growth-promoting signals. Stimulation of different transcription programs and genes encoding Jun and Fos protein underwent similar, but not identical, regulation in response to various stimuli. The expression of jun and fos gene products is temporally modulated suggesting that the AP-1 composition vary in the cell as a function of time and stimulus but also according to the cellular differentiation state and its environment (97).

The c-jun gene is expressed in many different cell types at low levels and its expression is enhanced in response to many stimuli including TPA (in a protein-kinase C-dependent manner), growth factors (EGF, NGF, FGF), UV

irradiation, or cytokines (176,177).

The c-jun promoter region is highly conserved between mouse, rat and human The 200 nucleotides upstream of the murine and human transcription initiation site share 94% identity (178). The c-jun promoter contains potential binding sites for several transcription factors, including SP-1 (179), NF-cjun (Nuclear factor-cjun) (180), CTF (CCAAT Transcription factor) and AP-1 itself (152).

Induction of c-jun expression by serum, phorbolester, or TGFβ is mediated through a TRE-like site in the proximal region of the c-jun regulatory sequences. This TRE sequence differs from the consensus TRE by one base pair insertion. It is preferentially recognized by a c-Jun/ATF2 heterodimer rather than a conventional c-Jun/c-Fos AP-1 dimer (181).

In the distal part of the c-jun promoter, a second AP-1 site also mediates the c-jun responsiveness to TPA or insulin treatment and growth factor stimulation (182,183).

The distal AP-1 site also binds c-Jun/ATF2 heterodimers. As ATF-2 alone cannot confer TPA-inducibility of c-jun, the c-jun gene is thus upregulated by its own product. Despite its inducible expression, most cell types contain a certain basal level of c-Jun protein before stimulation and the TRE site in its promoter is constitutively occupied (152,179).

Following exposure to stimuli, the N-terminal Jun Kinases (JNK), members of the MAPK family, is activated leading to the rapid phosphorylation of pre-existing c-Jun and ATF-2 proteins (184). Phosphorylation of c-Jun on residues Ser63 and Ser73, located within its transactivation domain, potentiates its transactivation properties by recruiting the coactivator protein, CBP, a histone acetylase, thereby enhancing c-jun transcription (185). Thus, stimulation of AP-1 activity in response of JNK-mediated stimuli (such as UV irradiation, ras activation, NGF removal or TNFα treatment) proceeds through two distinct steps: endogenous basal c-Jun protein is first activated by post-translational

modifications and the phosphorylated form of c-Jun induces subsequently its own transcription by a positive auto-regulatory loop.

2.2.6. c-Jun phenotype

Besides the standard indicators of oncogenicity, such as multilayered growth on solid substrate, growth in low serum concentration, anchorage-independence, and tumorogenicity, Jun also induces more subtle changes.

Jun-expressing cells show increase motility and invasiveness (186,187). In mammary epithelial cells, Jun induces the loss of polarity (188). Although it is clear that the growth promoting functions of Jun can be stimulated by a variety of exogenous and endogenous factors, it is not known whether these factors merely cause a gain of Jun function or whether they provide complementary and independent activities that are qualitatively distinct from that of Jun.

Jun phenotype has been dissected and correlated with preferential binding of Jun to specific bZIP proteins (189). Mutated Jun that selectively dimerizes with members of the Fos family induces anchorage- but not growth factor-independence, while a mutated Jun that preferentially associates with ATF2 causes growth factor-independence but fails to support colony formation in nutrient agar. Combining the two mutants in the same cell leads to a fully transformed phenotype, indistinguishable from that induced by wild type Jun. These data probably reflect the different spectra of target genes and hence of genetic programs addressed by Jun-Fos and Jun-ATF2 dimers (189).

Jun is a strong inhibitor of cellular differentiation. Myoblasts can be induced to fuse into multinucleated, postmitotic myotubes and to differentiate into muscle tissue that shows spontaneous contraction in cell culture. Expression of v-Jun in such myoblasts keeps the cells cycling, interferes with cell fusion, and prevents synthesis of muscle-specific proteins (190,191).

In avian chondrocytes, c-Jun is expressed during the early, immature

phases of development and is downregulated when the cell matures and expresses differentiation-specific markers, such as alkaline phosphatase. Overexpression of c-Jun in this cell system significantly retards differentiation and blocks the retinoic acid induced expression of alkaline phosphatase (192).

Jun is also active in lens differentiation. Overexpression of Jun in lens epithelial cells retards the formation of lentoid bodies and reduces levels of beta A3/A1 crystallin mRNA. A transdominant negative Jun enhances differentiation and alpha a crystalline expression (193).

In NIH3T3 cells, overexpression of Jun triggers programmed cell death (104). This occurs in the presence or absence of growth factors; it requires the bZIP and transactivation domains of Jun, suggesting apoptosis depends on transcriptional regulation. Overexpression of c-Jun induces apoptosis in endothelial cells (194). This process is preventable by a dominant negative Jun mutant.

Dominant negative Jun interferes with Jun-dependent transactivation and oncogenic transformation (195,196). Since Jun functions at the nuclear end of certain mitotic signals, dominant negative Jun interferes with upstream elements of such signals which include several oncoproteins : receptor tyrosine kinases, cytoplasmic tyrosine kinases, Ras, and Raf. Transformation by these oncoproteins is blocked by dominant negative Jun, and overexpression of the dominant negative mutant in cells already transformed by these oncoproteins forces reversion to the normal phenotype (197,198).

2.2.7. *in vivo* functions.

Inactivation of Jun and Fos family members generate various phenotypes spanning from embryonic lethality to post-natal survival associated with growth retardation or behavioural defects (97). Disruption of c-jun, junB or fra1 results in embryonic death whereas embryos lacking either junD, c-fos or fosB genes develop normally to birth.

c-jun deficient embryos die at 13.5 days from impaired hepatogenesis (116, 117). These embryos exhibit massive apoptosis in hepatoblasts and erythroblasts lineages and present malformation in the heart outflow tract (110).

The specificity and differences of the biological functions of each jun gene is also reflected by their differential expression patter during embryogenesis. c-Jun mRNA is detected throughout organogenesis in restricted cell populations within developing cartilage, gut and the central nervous system (97). AP-1 members are involved in various processes including hepatogenesis, spermatogenesis, and vascularization.

However, the lack of major defects in three AP-1 knockout mice suggests that a genetic redundancy may exist among some members of the AP-1 multi-gene family.

In conclusion, the AP-1 transcription factors are considered immediate-early response genes and are thought to be involved in a wide range of transcriptional regulatory processes linked to cellular proliferation and differentiation (199-206). A combination of in vitro and in vivo molecular genetic approaches has provided evidence to suggest that AP-1 transcription factors play multiple roles in functional development of haematopoietic precursor cells into mature blood cells along most, if not all, of the haematopoietic cell lineages. This includes the monocyte/macrophage, granulocyte, megakaryocyte, mastocyte and erythroid lineages (200,206,207). The c-jun protooncogene encodes for the transcriptional activator protein AP-1. c-jun is a member of the early-response gene family-genes that are rapidly and transiently activated in response to proliferative stimuli. Among the AP-1 members, c-jun is unique in its ability to positively regulate cell proliferation and to induce partial macrophage like morphology in U937 cells (208). TPA treatment of HL-60 and other human myeloid leukaemia cell lines like U937 is associated with the appearance of c-jun transcript (145,200,203,209). The level of c-jun expression is regulated by both transcriptional and posttranscriptional

mechanisms. Among important regulatory elements previously identified in the c-jun promoter, there are two AP-1 sites, a proximal AP-1 site (pAP-1) located between bp-71, bp-64 and a distal AP-1 site (dAP-1) located between bp-190 and bp-183 in the c-jun promoter (152,210,211). Importantly, c-jun is autoregulated by its product Jun/AP-1 (152).

2.3.C/EBP and c-jun members' interaction in hematopoiesis

The prevention of commitment: striking a balance

It is obviously important to balance stem cell commitment with sufficient self-renewal to maintain hematopoiesis throughout life. Moreover, for this, the formation of transcription factor complexes from different families has become a major area of interest. The resulting mixed complexes provide a means to integrate different signalling pathways. One possibility for association of transcription factors with related DNA-binding domains to interconnect pathways is by selective heterodimerization between two transcription factors that share a common dimerization motif. The resulting heterodimer is often imbued with properties that are distinct from those of either homodimer. For example, a c-jun/ATF-2 heterodimer juxtaposes members of the AP-1 and ATF families. The resulting heterodimer has a preferred specificity for the CRE/ATF element and confer TPA responsiveness to a CRE reporter (132).

Heterodimers between AP-1 and C/EBP members are present in the case of jun/fos and NF-IL6, also known as C/EBPβ (204). NF-IL6 is part of the interleukin (IL)-6 signal transduction pathway and can bind to both NF-IL6 and AP-1 sites. NF-IL6 associates with jun or fos through their respective bZIP domains (204). Consequently, DNA binding and activation from a NF-IL6 site is reduced. The bZIP region of NF-IL6 (C/EBPβ) isoforms mediates a direct association with the bZIP regions of Jun and Fos (to a lesser extent) in vitro (204). It was shown that NF-IL6-2 transactivation capacity is reduced in presence of c-jun. However, the N-terminal transactivation domain of NF-IL6-1 seems to be important in regulating the repressional effect by c-jun. However, the effect of this NF-IL-6-c-jun interaction on c-jun/AP-1 DNA binding capacity was not addressed (204).

ATF-2, another member of the AP-1 family also has cross-family dimerization capacity with C/EBP family protein (213). ATF-2-C/EBPα interaction diminishes transactivation capacity of C/EBPα through the C/EBP

consensus DNA-binding sites whereas this heterodimer complex could transactivate through chimeric DNA-binding site. However, the effect of this interaction on ATF-2 transactivation or DNA binding capacity was not addressed. Interaction between members of the ATF and C/EBP families has been described for C/ATF and C/EBPβ (212). The C/ATF-C/EBPβ heterodimer shows DNA-binding specificities distinct from those of the respective homodimers. It acts preferentially on a subclass of asymmetric CRE sequences as found in the promoter of the proenkephalin gene. C/ATF is closely related to AT-4, which has also been shown to heterodimerize with a member of the C/EBP family, IGEBP1 or C/EBPγ (21).

CHOP, a member of the C/EBP family (lacking the N terminal transactivation domain) acts as a dominant inhibitor of C/EBPα. CHOP interacts with c-jun via the leucine zipper domain of CHOP. CHOP-c-jun synergizes to activate transcription from an AP-1 site (214).

2.4. Model for C/EBPα and c-Jun regulation in myeloid differentiation-

Commitment and differentiation:

Indeed, though the lineage choice is downstream, bipotent (granulocyte/macrophage) progenitors can indeed be influenced by myeloid growth factors and signalling events, as well as by single transcription factors. In general, the available data are consistent with haematopoietic lineage commitment being an essential intrinsic process, in which lineage choice is dependent on attainment of a threshold level of important transcriptional regulator(s). These would subsequently feed back to consolidate activation of a specific cohort of genes and inactivation of others. The degree to which lineage-specific growth factors can impinge on these decisions may well increase with progressive development of the multipotent cell.

Here we propose that the proliferation arrest (215) and granulocytic lineage commitment function of C/EBPα (67,215) involves inactivating c-jun function via attenuation of its DNA binding activity. Inactivation of c-jun might be important for the multifunction of C/EBPα i.e. to drive granulocytic differentiation, block monocytic lineage commitment and for proliferation arrest.

3. METHODS

3.1. Transfection.

3.1.1.Transfection of adherent cells using lipofectamine plus reagent.

1. The day before transfection, trypsinize and count the cells, plating them so that they are 50-80% confluent the day of transfection.
2. Pre-complex the DNA with the PLUS reagent: dilute DNA inot serum-free dilution medium. Mix PLUS reagent before use, add to DNA, mix again, and incubate at room temperature for 15 min.
3. Dilute LIPOFECTAMINE Reagent into serum-free medium in a second tube; mix.
4. Combine pre-complexed DNA (from step 2) and diluted LIPOFECTAMINE (from step 3); mix and incubate for 15 min at room temperature.
5. While the complexes are forming, replace medium on the cells with serum-free transfection medium.
6. Add the DNA-PLUS-LIPOFECTAMINE Reagent complexes to each well containing fresh medium on cells (from step 5). Mix complexes into the medium gently; incubate at 37ºC at 5% CO_2 for 3 h.
7. After 3 h incubation, increase volume of medium to normal volume; add serum to bring the final concentration to that of normal growth medium.
8. Assay cell extracts for reporter gene activity 24-48 h after the start of transfection, depending on the cell type and promoter activity. The same procedure can be used to transfect DNA for stable expression: Instead of harvesting the culture, passage into fresh culture medium 1 day after the start of transfection and at 2 days add the appropriate antibiotic to select for expression of the transfected antibiotic-resistance gene.

3.1.2.Transfection of suspension cells

3.1.2.1. Effectene protocol

1. Split the cells the day before transfection.
2. On the day of transfection, harvest cells by centrifugation, remove the medium, and wash the cells once with PBS in a 10 ml Falcon tube.
3. Seed 1.5-3.5 x 10^6 cells (depending on the cell type) per well in 6 well plate in 2 ml growth medium containing serum and antibiotics.
4. Dilute 1 µg DNA dissolved in TE buffer pH 7 with the DNA-condensation buffer, Buffer EC, to a total volume of 150 µl. Add 10 µl of Enhancer and mix well.
5. Incubate at room temperature for 5 min.
6. Add 25 µl of Effectene Reagent to the DNA-Enhancer solution. Mix well by pipetting up and down 5 times.
7. Incubate the samples for 10 min at room temperature to all transfection-complex formation.
8. Add 500 µl growth medium (containing serum and antibiotics) to the tube containing the transfection complexes. Mix by pipetting up and down twice then immediately and the transfection complexes dropwise onto the cells in the 6 well plates. Gently swirl the plates to ensure uniform distribution of the complexes.
9. Incubate the cells with the transfection complexes under their normal growth conditions for an appropriate time (usually 24-48 h) for maximal expression of the transfected genes.
10. Assay for expression of the transfected genes using the Dual luciferase assay system.

3.1.2.2. Electroporation:

1. Use 1 x 10^7 cells per cuvette.
2. Spin cells at 1000 rpm for 5 min.

3. Discard supernatant medium and resuspend cells in fresh growth medium (containing serum and antibiotics).
4. Spin again at 1000 rpm for 5 min.
5. Resuspend cell pellet in 400 µl of growth media (containing serum and antibiotics).
6. Add DNA to final concentration of 10-20 µg per cuvette.
7. Electroporate. (Using prestandardized conditions for each cell line).
8. Keep at cells at room temperature for 10 min.
9. Transfer cells to 100 mm Petridishes with 10 ml growth medium and incubate at 37ºC.
10. Depending on the cell line and expression of the transfected gene, spin down the cells at 1000 rpm for 5 min.
11. Wash with 10 ml PBS and give one more round of spin.
12. Proceed with assay to detect the transfected gene.

3.1.3 Dual luciferase assay

3.1.3.1. Solutions: (Promega Kit)

1. Lysis buffer: Prepare 1X lysis buffer from the 5X concentrate using distilled water. The buffer can be stored at 4ºC for one month.
2. Luciferase Assay Reagent II (LAR II): Resuspend Luciferase Assay Substrate in 10 ml Luciferase Assay buffer. Make 1 ml aliquots. The aliquots can be stored at –20ºC for one month or –70ºC for one year.
3. Stop & Glo Reagent: Transfer 200 µl of Stop & Glo Substrate Solvent to the dried Stop & Glo powder. This 50x solution is stored at –20ºC. While using, always keep on ice. Prepare 1x solution using Stop & Glo buffer. 1x should be prepared fresh.

3.1.3.2.Protocol:

1. Wash the cell with PBS.
2. Lyse the cells in 20-500 µl of lysis buffer, depending on the amount of cells.
3. Take 20 µl of the lysate.
4. Add 100 µl of LAR II and measure the Firefly luciferase.
5. Add 100 µl of Stop & Glo, vortex, and measure the Renilla luciferase.

Firefly luciferase activities were normalized to the *Renilla* luciferase values of pRL-null (232,233). The fold promoter activity was calculated as the ratio between the promoter and promoter plus C/EBPα, assigning a value of 1 for the promoter alone. Results are given as mean +S.E.M. of atleast three independent experiments.

3.2. Northern blot analysis.

3.2.1.RNA preparation:

1. Thaw RNA on ice.
2. Take required µg (10-30 µg). (If needed lyophilise for ~20 min.)
3. Add 15-20 µl of RNA loading buffer. Mix well and pulse spin.
4. Incubate at 70ºC for 10 mins.
5. Transfer to ice for 5 min and then spin down.

3.2.2.RNA gel electrophoresis:

1. Carefully load RNA samples prepared as described above into the respective wells of the RNA gel.
2. Load 0.2 Kb RNA ladder at one end of the gel.
3. Run gel overnight at 35-40 V in 1X MOPS.
4. Once the run is complete, take a photo of the gel under UV light with different exposure time. Place a scale along the side of the gel to use for calculating the size of the desired RNA.

3.2.3.RNA transfer:

1. Cut the gel area to be transferred to a membrane: from the loading wells and including the dye front of the gel. Also, mark an orientation of the gel to identify the loading pattern of the samples.
2. Cut the Hybond N+ (from Amersham Biosciences) membrane of same dimensions (and same orientation mark) as the gel.
3. Cut Whatman filter paper of similar dimensions. Soak in DEPC water until ready to use.
4. Wash the gel in DEPC water.
5. Set up the transfer in the following sequence:

   Tray with 10X SSC

   Glass plate covered with filter paper soaking in the tray solution.

RNA gel

Hybond N+ membrane

Whatman filter paper: 3X

Stack of blotting paper.

Glass plate

Weight (enough to keep the blotting paper pressed to the Whatman filter paper.

6. Let the transfer proceed overnight (or at least 16 hrs.)
7. After transfer, wash membrane in 5X SSC for 1 min. Air-dry at room temperature for 5 mins.
8. UV crosslink the RNA onto the nylon membrane.
9. Store dry till ready for hybridisation.

3.2.4. Hybridisation:

3.2.4.1. Prehybridization

1. Prewarm Church-Gilbert buffer to 60ºC.
2. Prehybridize the membrane in Church-Gilbert buffer at 60ºC for 2-4 h.

3.2.4.2.Probe labelling:

1. Dilute the DNA to be labelled to a concentration of 2.5-25 ng in 45 µl of sterile water or 10 mM TrisHCl pH 8.0, 1mM EDTA.
2. Denature the DNA sample by heating to 95ºC for 5 min.
3. Centrifuge briefly to bring the contents to the bottom of the tube.
4. Add the denatured DNA to the labelling mix and reconstitute the mix by gently flicking the tube until the blue colour is evenly distributed.
5. Centrifuge briefly.
6. Add 5 µl of Redivue [γ-^{32}P] dCTP and mix by gently pipetting up and down 4-6 times.
7. Centrifuge briefly.
8. Incubate at 37ºC for 60 min.

3.2.4.3. Probe purification:

1. Take Nick column (Amersham) and remove the solution from inside (by gravity).
2. Add 3 ml of TE and wash the same way.
3. Place 50 µl of the labelled probe in the centre of the column.
4. Add 400 µl, 400 µl, and 100 µl of TE and collect these fractions.
5. Measure the counts in each fraction.
6. Take appropriate amount from the second fraction that is equivalent to $30X10^6$ DPM.
7. Incubate at 95ºC for 10 min.
8. Cool on ice for 5 min.
9. Pulse spin and it is ready to be used for hybridisation.

3.2.4.4. Hybridisation:

1. Add the purified probe from above to prewarmed fresh hybridisation buffer.
2. Discard the prehybridisation buffer from the membrane.
3. Transfer the labelled buffer to the membrane.
4. Keep for hybridisation at 55-65ºC overnight.
5. Proceed for washing the next day.

3.2.5. Washing:

1. Prewarm the washing buffer to 55-65ºC.
2. Remove the radioactive hybridisation buffer from the membrane tubes.
3. Add wash buffer to membrane and give first wash for 15 min.
4. Measure the counts and depending on that set the number of washes and wash time.
5. After final wash, air dry the membrane, wrap in saran-wrap and expose to ^{32}P sensitive film at –80ºC.
6. Develop the autoradiograph and decide if more or less exposure time is required for good signal.

3.2.6. Reagents:

3.2.6.1.RNA loading buffer:

50% Formamide

15% Formaldehyde

10% 10X MOPS

10% Bromophenol Blue.

5 µl of Ethidium bromide per 1ml of the buffer

3.2.6.2.RNA gel composition:

4 gm Agarose

300 ml DEPC water

Boil until agarose melts.

Cool to 50-60ºC in water bath.

Add 40 ml 10X MOPS

67 ml Formaldehyde (37% stock)

* Mix under fume hood.

* Pour into casting tray.

* Leave it to solidify for ~1 h.

3.2.6.3.Church-Gilbert buffer:

1M NaH_2PO_4 100 ml

1M Na_2HPO_4 400 ml

Bring to pH 7.2 using these 2 solutions only.

20% SDS 350 ml

100 mM EDTA 10 ml

DEPC H_2O to 1 litre.

3.2.6.4.Wash buffer:

1X SSC

0.1% SDS

3.3.Immunoblotting:

3.3.1.Sample preparation

1. Aliquot equal concentration of the samples to be loaded. Keep on ice.
2. Add equal amount of 2X loading dye. If the sample volume is in excess, use 6X-loading dye.
3. Mix well and boil to 95ºC for 5 min.
4. Cool on ice, centrifuge briefly and now the sample is ready for loading.

3.3.2.Western blotting procedure

1. Prepare gel apparatus.
2. Pour in running gel, and then add isopropanol. Allow gel to polymerise and then remove the organic top phase with filter paper.
3. Pour in stacking gel and insert comb. Avoid bubbles formation.
4. Insert apparatus into tank and fill with 1X Tank Buffer.
5. Wash wells using a pipette and the 1X Tank Buffer.
6. Load samples, and run gel at 110 Volts for 1 h.

3.3.3.Western immunoblot procedure

1. Pre-soak the nitrocellulose membrane in methanol until it is completely wet (generally 30 sec is enough).
2. Transfer membrane to transfer buffer until ready to use. Keep the buffer cold before use.
3. Separate glass plates and place one piece of filter paper on gel.
4. Order of layering for protein transfer:

    Black panel of transfer apparatus

    Sponge

    Filter paper

    Gel

    Nitrocellulose membrane

    Filter paper

Sponge

White panel of transfer apparatus

5. Place transfer apparatus in tank so that black panel is against the black of apparatus.
6. Place ice pack and stir rod in the tank.
7. Fill tank with Transfer buffer.
8. Run at 100 Volts for 90 min. Replace ice pack to maintain cold tank.

3.3.4.Chemiluminescence analysis

1. Blot the membrane for 1 h at room temperature in 5% non-fat skim milk powder in TBST (termed Blotto).
2. Wash 3 x 5 min with 2.5% Blotto.
3. Incubate with primary antibody (1:5000) in 2.5% Blotto overnight at 4ºC with constant agitation.
4. Wash 3 x 5 min with 2.5% Blotto.
5. Incubate with secondary antibody HRP (1:10,000) in 2.5% Blotto for 1-2 h at room temperature.
6. Wash 3 x 5 min 2.5% Blotto and 2 x 5 min in TBST.
7. Detect using 1:1 ratio luminol/oxidant for 1 min, blot dry on filter paper and expose for 30 sec to get an idea as to optimise the result.

3.3.5.Reprobing with another antibody.

1. Wet nitrocellulose membrane thoroughly in methanol.
2. Rinse in d.H_2O.
3. Strip in 10-50 ml of Strip Buffer at 60ºC for 30-40 min with constant agitation.
4. Wash 2 x 10 min in TBST at room temperature.
5. Block and probe at mentioned above.

3.3.6.Reagents

1. 2X Loading dye:

   2.5 ml 0.5M Tris pH 6.8

   4.0 ml 10% SDS

   2.0 ml glycerol

   1.0 ml 2-mercaptoethanol

   Pinch of bromophenol blue

   Final volume to 10 ml with distilled water

2. Protein gel recipies:

10% Running gel (10 ml)		Stacking gel (5 ml)	
d.H_20	2.8 ml	d.H_20	3.0 ml
Acrylamide mix	3.0 ml	Acrylamide mix	0.65 ml
1.5 M Tris pH 8.8	3.8 ml	0.5 M Tris pH 6.8	1.25 ml
10% SDS	0.1 ml	10% SDS	0.05 ml
10% APS	0.2 ml	10% APS	0.05 ml
TEMED	5.0 µl	TEMED	5.0 µl

   * Add TEMED only when ready to pour, and shake after adding TEMED.

   * Use freshly prepared APS.

   * This recipe will make two gel plates.

3. 4X Tank Buffer

   48 g Tris base

   230.4 g Glycine

   16 g SDS

   * Bring volume up to 4 litres with distilled water and set pH to 8.5

   * For 1X, use 500 ml 4X Tank Buffer and bring volume up to 2 lit.

4. Transfer Buffer

   3 g Tris

   14.1 g glycine

   1 g SDS

   200 ml methanol

* Dissolve Tris, glycine and SDS in water before adding methanol.
* Make up volume to 1 litre with d.H_2O.

5. 10X TBS

    121 g Tris

    176 g NaCl

* Dissolve salts in 1.6 litre d.H_2O.
* Adjust to pH 8.0 with conc. HCl and make up to 2 litre.

6. TBST

    200 ml of 10X TBST pH 8.0

    0.5 ml TWEEN-20

* Make up to 2 litre with d.H_2O.

7. Strip Buffer

    0.7 ml 2-mertaptoethanol (14 M)

    2 gm SDS

    1 M Tris pH 6.8

    * Make volume to 100 ml with d.H_2O

3.4. GST PULL DOWN ASSAY

3.4.1 GST-Fusion protein:

1. Grow bacterial culture in 10 ml LB with appropriate antibiotics overnight.
2. Incoulate the above 10 ml culture in 100 ml LB and grow at 37ºC for 2-3 h.
3. Add IPTG to final concentration of 0.5 mM.
4. Grow bacterial culture for another 3-5 h.
5. Centrifuge the bacteria.
6. Resupend the bacterial pellet in 10 ml of NETN lysis buffer.
7. Sonicate at maximum setting (40-50 Duty Cycle; 20 Volts), 3-5 times, 0.7 min each time on ice to prevent overheating.
8. Centrifuge at 14,000 rpm for 5 min.
9. Transfer the supernatant (lysate) to new tube.
10. Add 0.5-1 ml of prehydrated Glutathione cross-linked agarose beads (beads volume). Pre-wet beads in PBS, wash thrice in same buffer and store at 4ºC with an equal volume of the buffer on top of the beads.
11. Rotate the lysate plus beads at 4ºC for 3-5 h.
12. Wash the beads 4-5 times in NETN wash buffer and centrifuge at 2,000 rpm for 5 min at 4ºC. These beads can be now stored in the same buffer at 4ºC.

3.4.2. *in vitro* protein-protein interaction analysis:

1. *in vitro* translate the interacting protein of interest with ^{35}S-labeled Methionine.
2. Incubate the in vitro translated protein with GST-fusion protein and rock the samples at 4ºC for 2-3 h.
3. Wash beads three times with the same buffer.

4. After final wash, resuspend beads in the SDS sample loading buffer, boil for 5 min, centrifuge briefly.
5. Load the supernatant to SDS-PAGE.
6. Autoradigraph the gel.

3.4.3. Reagents:

1. NETN lysis buffer
   20 mM Tris
   200 mM NaCl
   1 mM EDTA
   0.5 % NP40
   1 mM DTT
   1% Triton X-100
   +Protease inhibitors
2. NETN wash buffer
   20 mM Tris
   200 mM Nacl
   1 mM EDTA
   +Protease inhibitors

3.5. Coimmunoprecipitation assay:

3.5.1. Preparation of Nuclear extracts

1.1.1. Protocol:

1. Centrifuge 1x 10E7 cells at 1000 rpm for 5 min.
2. Wash cell pellet twice in 5 ml ice cold PBS.
3. Resupsend cell-pellet in 1 ml ice cold PBS and transfect to eppendorf tube. Centrifuge briefly to bring the cells to the bottom of the tube. Remove PBS completely.
4. Resuspend cell pellet in 100 µl of Buffer A. Incubate on ice for 10-15 min with occasional tapping.
5. Pellet the nuclei by centrifugation at 2000 rpm for 5 min at 4ºC. The supernatant cytoplasmic fraction can be snap frozen in liquid nitrogen and stored at –80ºC.
6. Resuspend the nuclei in 50 µl of ice cold Buffer C. Mix thoroughly.
7. Break open the nuclei by incubation in liquid nitrogen followed by incubation at 37ºC. Repeat thrice.
8. Centrifuge the nuclear extract at 13,000 rpm for 15 min at 4ºC.
9. Aliquot the nuclear extract supernatant, snap freeze in liquid nitrogen and store at –80ºC.

3.5.2. Coimmunoprecipitation

1. Incubate the nuclear extract with 40 µl of protein A-Agarose beads and 2 ug specific antibody in 500 µl of coIP buffer.
2. Rock at 4ºC for 3 h.
3. Spin down the protein-A-agarose bound protein complex at 4ºC and 2000 rpm for 5 min.
4. Wash the beads complex with coIP buffer for 3-5 times.
5. After final wash, denature proteins by sample loading buffer and run a SDS-PAGE.

6. Immunoblot using antibody directed against the interacting partner.

3.5.3. Reagents

1. Buffer A

Components	Stock	Final (100 ml)
20 mM Tris pH 8.0	1 M	2 ml
10 mM NaCl	5 M	0.2 ml
3mM MgCl2	1 M	0.3 ml
0.1% NP40	10%	1 ml
10% glycerol	100%	10 ml
0.2 mM EDTA	0.5 M	0.04 ml
0.4 mM PMSF		10 µl
1µg/ml antipain		10 µl
1µg/ml leupeptin		10 µl
Sterile water to make final volume to		100 ml

2. Buffer C

Components	Stock	Final (100 ml)
20 mM Tris pH 8.0	1 M	2 ml
400 mM NaCl	5 M	8 ml
20% glycerol	100%	20 ml
1 mM DTT	1 M	0.1 ml
0.4 mM PMSF		10 µl
1µg/ml antipain		10 µl
1µg/ml leupeptin		10 µl
Sterile water to make final volume to		100 ml

3. coIP buffer

50 mM Tris.HCL pH 7.5

150 mM NaCl

1 mM EDTA

5% Glycerol

0.25% NP-40

+ Proteinase inhibitors

3.6. Electro-Mobility-Shift-Assay (EMSA):

3.6.1 Probe stock

1. Add 20 µg of Oligo A and Oligo B each to a final volume of 100 µl in 1X Annealing buffer. The final concentration of the oligo is now 0.4 µg/µl.
2. Boil the oligo mix to 95ºC for 5 min in water bath.
3. Cool to room temperature slowly.
4. Dissolve 10 µl of the oligo mix in 70 µl of 1X annealing buffer. This stock will now have 50 ng/ µl ds-oligo concentration.
5. Store the oligo stocks at –20ºC.

3.6.2 Probe labelling

1 µl	50 ng/ul ds oligo stock
4 µl	5X Forward reaction buffer (Gibco)
5 µl	γ-^{32}P ATP (50 µCi)
8 µl	d.H_2O
2 µl	T4 Polynucleotide kinase

* Incubate at 37ºC for 60 min.
* Use Gibco Quickspin column to purify the labelled oligo. Briefly, centrifuge the column at 1,000 rpm for 2 min. Add the labelled probe and centrifuge again at 2,700 rpm for 5 min.
* Store the labelled probe at –20ºC until futher use.

3.6.3. Gelshift reaction

1. Cast a 4.8% native polyacrylamide gel.
2. Meanwhile prepare the reaction mix. One can use in vitro translated protein or nuclear extract as the source of protein binding to the oligo probe. A short example of a typical reaction mix is as follows:
    a) Probe alone
    b) Probe + protein

c) Probe + protein + 200X cold oligo competitor
d) Probe + protein + specific antibody
e) Probe + protein + non specific antibody
f) Probe + protein + 200X cold oligo comp. + specific antibody

3. Make the binding reaction master mix which consists of 2 µl of poly.dI.dC and 4 µl of 1X BBS buffer and d.H_2O to 20 µl for every reaction.
4. Sequence of adding the reaction components should always be: BBS buffer->poly.dI.dC->d.H2O->protein->cold comp.->probe->antibody.
5. Incubate the probe with protein for 30 min on ice, and then incubate with the anibody for further 30 min on ice.
6. Load the samples and run the gel at 155 Volts until the dye 2/3rd of the gel (usually take 5-7 h).
7. Vacum dry the gel.
8. Develop an autoradiograph with first 2 h exposure at –80ºC. Depending on the results, decide on better exposure time.

3.6.4. Reagents

1. 10X Annealing buffer

   10 mM Tris pH 7.5
   10 mM MgCl2
   50 mM NaCl
   1 mM DTT

2. 4.8% Polyacrylamide gel

6 ml	1:19 bis:acrylamide (40%)
5 ml	10X TBE
0.5 ml	10% APS
38.5 ml	d.h2O

0.035 ml TEMED

* Mix and cast a 1 mm thick gel and insert an appropriate comb. Allow to polymerise.

3.7. Retroviral transduction

Retroviral transduction was performed as described by Grignani et al. (234)

3.7.1. Viral particles production

1. Grow the Phoenix retroviral producing cell line in DMEM plus 10% FBS. Every 6 months do a 14 day selection with 1 μg/ml Hygromycin and 1 μg/ml Diphteria Toxin.
2. Transfection - DAY O: Split cells into 10 cm dishes at 2 x 10^6 cells in the DMEM growth medium.
3. Transfection – DAY 1: Transfect each 10 cm dish with 5 μg of DNA using Lipofectamine-Plus reagent and protocol (Promega).

   x μl DNA (5 μg) + medium = 125 μl

   20 μl PLUS reagent + medium = 125 μl

   30 μl LIPO reagent + medium = 250 μl

   Medium to cells = 2 ml

   Total transfection volume = 2.5 ml
4. After 3 h add 2.5 ml of 20% FBS medium.
5. 12 h post transfection, replace the transfection medium with regular DMEM growth medium, and incubate for further 12 h.
6. Trypsinize cells, wash in PBS and resuspend in 5 ml growth medium.
7. Plate 1:10 from 5 ml.
8. After 24 h, start selection with the appropriate antibiotics.
9. Replace the selection medium with regular growth medium for viral particle harvest.
10. After 2-3 days, collect the medium, centrifuge at 1000 rpm for 5 min.
11. Aliquot and store at –80ºC.

3.7.2.Viral titre estimation

1. Plate NIH3T3 cells in 6 well plate at 5 x 10E4 cells per well per 2 ml

medium.

2. 24 h post-plating, remove medium and replace with increasing volume of the viral medium (aliquot ranging from 125 µl to 1 ml of the viral medium).
3. 48 h post-infection count GFP positive cells under UV light microscope and compute the titre value for that batch of viral medium.

3.7.3.Retroviral transduction

1.DAY 1: 1 x 10^7 of cells to be transduced are centrifuged at 1000 rpm for 5 min.

2.Resuspend the cells in 2.5 ml of the viral medium for 1-2 h; spin down after adding 8ug/ml of Polybrene (Sigma) for 3 h.

3.Resuspend in regular growth medium and incubate under normal growth conditions for the cells.

4.Repeat step b & c for DAY 2 and DAY 3.

5.DAY 4: sort for GFP positive cells or start selection using appropriate antibiotic.

6.Proceed for further analysis of the transduced cells.

3.8. FACS ANAYLSIS:

1. 10^6 were washed twice in washing buffer (PBS, 0.1% [wt/vol] NaN_3, 1% FBS)
2. Resuspend cells in 100 µl of washing buffer with 2 µl of the respective antibody.
3. Incubate at room temperature for 30 min.
4. Remove non-bound antibody by washing in 10 volumes of the wash buffer.
5. Resupend cells in 1 ml of the buffer and proceed for FACS analysis.
6. Minimum of 10^4 cells were analysed by flow cytometery. (CD15 PE (clone H198), its isotype control IgM κPE (clone G155-228), CD 11b PE (clone ICRF44) and its isotype control IgG1 κPE (clone MOPC-21) were purchased from BD Biosciences.)

3.9. REAL TIME PCR:

Real-time quantitative PCR was performed using the light cycler technology (Roche Diagnostic) as described previously (235).

3.9.1. RNA isolation

1. Centrifuge the appropriate number of cells for 5 min at 300 x g. Discard supernatant, completely removing all the media.
2. Disrupt cells by addition of Buffer RLT. Ensure β-ME is added to Buffer RLT before use. Add 350 µl of Buffer RLT for cell number up to 5×10^6.
3. Pipette lysate directly onto a QIAshredder column (Quiagen) sitting in the 2-ml collection tube, and centrifuge for 2 min at maximum speed to homogenize.
4. Add 1 volume of 70% ethanol to the homogenized lysate, and mix well by pipetting.
5. Apply up to 700 µl of sample, including any precipitate which may have formed, to an RNeasy mini spin column sitting in a 2-ml collection tube, and centrifuge for 15 sec at ~8,000 x g.
6. Pipette 700 ul Buffer RW1 onto the RNeasy column, and centrifuge for 15 sec at ~8,000 x g.
7. Transfer RNeasy column into a new 2-ml collection tube. Pipette 500 µl Buffer RPE onto the RNeasy column, and centrifuge for 15 sec at ~8,000 x g to wash.
8. Pipette 500 µl Buffer RPE onto the RNeasy column. Centrifuge for 2 min at maximum speed to dry the RNeasy membrane.
9. Transfer RNeasy column into a new 1.5 ml collection tube, and pipette 30-50 ul of RNase-free water directly onto the RNeasy membrane. Centrifuge for 1 min at ~8,000 x g to elute.

3.9.2. Real time PCR

1. 2 µl of cDNA template was used for the real time PCR.
2. For amplification of the housekeeping gene Glucose-6-phosphate dehydrogenase (G6PD), the primers used were: forward 5'CCG GAT CGA CCA CTA CCT GGG CAA C 3' and reverse 5' GTT CCC CAC GTA CTG GCC CAG GAC CA 3'.
3. The primers for c-jun amplification were: forward 5'GCA TGA GGA AAC GCA TCG CTG CCT CCA AGT '3 and reverse 5'GCG ACC AAG TCC TTC CCA CTC GTG CAC ACT '3.
4. G6PD plasmid was serially diluted from 1ng to 100 fg and 2 µl of the plasmid dilution was used for standard curve plotting.
5. The Real Time PCR was performed using 2 µl of master mix (Light Cycler FastStart DNA Master SYBR Green I, Roche Diagnostics).
6. PCR protocol for each sample was:
   (i) Denaturation: 10 min at 95ºC.
   (ii) Amplification: 95ºC 10 sec denaturation
   64ºC 10 sec annealing
   72ºC 25 sec extension
   Fluorescence of SYBR Green I was measured after each amplification cycle at 530 nm by F1 channel.
7. Melting curve analysis was performed by cycles of denaturation at 95ºC for 0.1 sec followed by annealing the samples at 65ºC for 10 sec to confirm the PCR product. Fluorescence at 530 nm by F1 channel was monitored by measurement at every 0.2ºC temperature increment.
8. The concentration of G6PD from samples was calculated using the G6PD standard curve. The concentration of c-jun was also calculated with reference to the G6PD standard curve.
9. The estimated concentration of c-jun was normalized to the G6PD concentration from the same sample to get the quantitative value for c-jun mRNA from each sample.

3.10. *in vitro* translation

(USING TNT COUPLED RETICULOCYTE LYSATE SYSTEM):

1. Remove the reagents from storage at –70ºC. Immediately place the TNT RNA Polymerase on ice. Rapidly thaw the TNT Reticulocyte Lysate and place on ice. Thaw other components at room temperature and store on ice.
2. DNA is prepared using the Quiagen DNA amplification protocol.
3. Assemble the reaction components, appropriate for the label being used, in an eppendorf tube. After all the components are added, gently mix the lysate by pipetting and stirring the reaction with the pipette tip. If necessary, centrifuge briefly to return the sample to the bottom of the tube.

Component	Std. Reaction	
	[^{35}S]methionine	cold reaction
TNT Rabbit Reticulocyte lysate	25 µl	25 µl
TNT Reaction buffer	2 µl	2 µl
TNT RNA Polymerase (SP6, T3 or T7)	1 µl	1 µl
Amino acid mixture, minus leucine, 1 mM	-	1 µl
Amino acid mixture, minus methionine	1 µl	1 µl
[^{35}S]methionine	4 µl	-
RNasin Ribonuclease Inhibitor (40u/ µl)	1 µl	1 µl
DNA template (0.5 µg/µl)	2 µl	2 µl
	----	----
Nuclease-free water to a final volume of	50 µl	50 µl

4. Incubate the translation reaction at 30ºC for 90 min.

5. Analyse the results of translation either by running SDS-PAGE and developing autoradiograph for [^{35}S] labelled samples, or by immuno-blotting with specific antibody directed against protein of interest.

3.11.DNA amplification

3.11.1.DNA MINIPREP (QIAGEN KIT PROTOCOL)

1. Resuspend pelleted bacterial cells in 250 µl of Buffer P1 and transfer to eppendorf tube. Ensure that Rnase A has been added to Buffer P1.
2. Add 250 µl of Buffer P2 and gently invert the tube 4-6 times to mix. Do not let the lysis reaction to proceed for more than 5 min.
3. Add 350 µl of Buffer P3 and invert the tube immediately by gently 4-6 times.
4. Centrifuge for 10 min.
5. Apply the supernatants from the step 4 to the QIAprep column.
6. Centrifuge 30-60 sec. Discard the flow-through.
7. Wash QIAprep spin column by adding 750 µl of Buffer PE and centrifuge 30-60 sec.
8. Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer.
9. Place QIAprep column in a clean 1.5 ml eppendorf tube. To elute DNA, add 50 µl of Buffer EB or water to the center of the QIAprep column, let stand for 5 min, and centrifuge for 1 min.

3.11.2.MAXI PREP (QUIAGEN KIT)

1. Pick a single colony from a freshly streaked selective plate and inoculate a starter culture of 2-5 ml LB medium containing the appropriate selective antibiotic. Incubate for ~8 h at 37ºC with vigorous shaking (~250 rpm).
2. Dilute the starter culture 1/500 into selective LB medium. Grow at 37ºC for 12-16 h with vigorous shaking (~250 rpm).
3. Harvest the bacterial cells by centrifugation at 6000 x g for 15 min at 4ºC.
4. Resuspend the bacterial pellet in 10 ml of Buffer P1.
5. Add 10 ml of Buffer P2, mix gently but thoroughly by inverting 4-6 times, and incubate at room temperature for 5 min.

6. Add 10 ml of chilled Buffer P3, mix immediately but gently by inverting 4-6 times, and incubate on ice for 20 min.
7. Centrifuge at ~20,000 x g for 30 min at 4ºC. Remove supernatant containing the plasmid DNA promptly.
8. Equilibrate a QIAGEN-tip 500 by applying 10 ml Buffer QBT, and allow the column to empty by gravity flow.
9. Apply the supernatant from step 7 to the QIAGEN-tip and allow it to enter the resin by gravity flow.
10. Wash the QIAGEN-tip with 2 x 30 ml Buffer QC.
11. Elute DNA with 15 ml of Buffer QF.
12. Precipitate DNA by adding 10.5 ml (0.7 volumes) room temperature isopropanol to the eluted DNA. Mix and centrifuge at 15,000 x g for 30 min at 4ºC. Carefully decant the supernatant.
13. Wash DNA pellet with 2 ml of 70% ethanol and centrifuge at 15,000 x g for 10 min. Carefully decant the supernatant without disturbing the pellet.
14. Air-dry the pellet for 5-10 min, and redissolve the DNA in a suitable volume of buffer TE or water.

3.12.Whole cell lysate (RIPA)

3.12.1.Solutions

RIPA Lysis Buffer:

Final	Stock	For 100 ml
1% (w/w) NP40	10%	10 ml
0.5%/w/v) Na. Deoxycholate	5%	10 ml
0.1%(w/v) SDS	10%	1 ml
0.15 M NaCl	5 M	3 ml
5mM EDTA	0.5 M	1 ml
50 mM Tris pH 8.0	1 M	5 ml
Sterile water		70 ml

+ Phosphatase inhibitors

10 mM Na. Pyrophosphate

50 mM Na. Floride

0.2 mM Na. Vanadate

+ Proteinase Inhibitors

1 mM PMSF

10 µg/ml Pepstatin A

10 µg/ml Leupeptin A

10 µg/ml Aprotinin

10 µg/ml Antipain

10 µg/ml Chymostatin

3.12.2.Protocol

1. Spin cells at 1000 rpm for 5 min at 4ºC.
2. Wash in 10 ml ice cold PBS twice.
3. Resupend in 1 ml ice cold PBS and transfer to 1.5 ml eppendorf tube. Centrifuge briefly.

4. Decant PBS completely and resupend the cell-pellet in 20-100 µl of RIPA buffer depending on the cell number.
5. Keep on ice for 30 min with occational vortexing.
6. Centrifuge at 13,000 rpm for 30 min at 4ºC.
7. Aliquot the supernatant, snap freeze in liquid nitrogen and store at –80ºC.

3.13. Cell culture

3.13.1.Human myeloid U937 cells stably transfected with a zinc inducible C/EBPα construct (U937 α#2) or vector alone (U937EV) have been described previously (67).

3.13.1.1.Cells were cultured in RPMI 1640 medium supplemented with 10% foetal calf serum, 1% L-glutamine (Gibco), 1% PenStrep (Gibco) and 850μg/ml of G418 (Gibco).

3.13.1.2.C/EBPα expression from the metallothionine promoter was induced upon adding 100μM $ZnSO_4$ (Sigma).

3.13.2.Human promyelocytic HL-60 cells (DSMZ no. ACC 3) were grown in RPMI 1640 media containing 10% FBS, 1% PenStrep (Gibco) and 1% L-glutamine (Gibco).

3.13.3.CV-1 (CCL-70), NIH 3T3 (ACC 59), 293 clone constitutively expressing the proteins encoded by E1A (293E1A), CHO (CCL-61), 293T and phoenix-A cells were maintained in DMEM (Gibco) supplemented with 10% FBS, 1% Penstrep and 1% L-Glutamine.

3.13.4.HeLa cells were grown in RPMI 1640 supplemented with 10% FBS, 1% Penstrep and 1% L-Glutamine.

3.14.Plasmids

3.14.1.A human c-jun promoter bp-1780/+731 construct was generated by amplifying the c-jun promoter fragment along with XhoI half sites at each end from human genomic DNA and ligated into pGL3 basic vector (Promega) XhoI site.

3.14.2.A series of 5' deletions were generated as mentioned in reference 47. The bp-79/+170 human c-jun promoter construct, bp-79/+170 AP-1/CRE mutant and pGL2 basic vector were a gift from Dr. Vedekis (216).

3.14.3.The pcDNA3 C/EBPα construct was generated by releasing a BamHI/ EcoRI fragment of rat C/EBPα cDNA from the pUC18 vector and ligating this fragment into pcDNA3 (Invitrogen).

3.14.4.The reporter construct p(C/EBP)2TK contains two consensus C/EBPα binding sites linked in tandem and cloned into pTK81 luciferase.

3.14.5.AP-1 x7 luciferase reporter construct containing seven repeats of AP-1 DNA binding sites was purchased from Stratagene.

3.14.6.Gal-4 (1-147), Gal-4 Tel and Gal-4 DNA binding domain in eukaryotic expression vector SGS424 were kindly provided by Dr. Bohlander, Göttingen.

3.14.7.C/EBPαΔBR (lacking the basic region) and C/EBPαΔLZ (lacking the leucine zipper domain) were a kind gift from Dr. Friedman (22,40,217).

3.14.8.c-junΔRK (DNA binding domain deletion) and c-junΔLZ (leucine zipper domain deletion) have been described in reference 218.

3.15.May-Grünwald-Giemsa staining:

1. Spin down cells to be stained onto a glass slide.
2. Dip the slides in May-Grünwald solution for 7 mins.
3. Wash the slides in staining buffer.
4. Dip the slides in diluted Giemsa solution for 30-40 mins.
5. Wash the slides in staining buffer.
6. Air dry the slides and observe under microscope
7. Reagents:

   a. Undiluted May-Grünwald solution (with fixative).

   b. Diluted Giemsa solution:

   37.5 ml Giemsa in 500 ml of staining buffer pH 6.8

3.16. Transformation Mutagenesis Protocol:

Clontech Transformer Site-Directed Mutagenesis kit was used for constructing the required mutatants. Here is a brief description of the protocol:

3.16.1 Denaturation of plasmid DNA and annealing of primers to the DNA template.

1. Pre-warm a water bath to boiling (100ºC).
2. Set up the primer/plasmid annealing reaction in a 0.5 ml eppendorf tube as follows:

10X Annealing buffer	2.0 µl
Plasmid DNA (0.05 µg/µl)	2.0 µl
Selection primer (0.05µg/µl)	2.0 µl
Mutagenic primer (0.05 µg/µl)	2.0 µl

   Adjust with water to a total volume of 20 µl. Mix well and centrifuge briefly.
3. Incubate at 100ºC for 3 min.
4. Chill immediately in an ice water bath for 5 min. Centrifuge briefly.

3.16.2 Synthesis of the mutant DNA strand.

1. To the primer/plasmid annealing reaction add:

10X Synthesis buffer	3.0 µl
T4 DNA polymerase (2-4 units/µl)	1.0 µl
T4 DNA ligase (4-6 units/µl)	1.0 µl

Sterile water 5.0 µl

2. Mix well and centrifuge briefly. Incubate at 37ºC for 1-2 hr.
3. Stop the reaction by heating at 70ºC for 5 min. Cool the tube to room temperature.

3.16.3 Primary selection by restriction digestion.

1. Add 1µl of the selection unique enzyme and incubate the tube at 37ºC for 1-2 hr.
2. After the primary restriction digestion, heat the mixture to 70ºC for 5 min.

3.16.4 First transformation

1. Preheat a heating block or water bath to 42ºC.
2. Add 5-10 µl of the plasmid/primer DNA solution from above step to 100 µl of competent BMH 71-18 mutS cells and incubate on ice for 20 min.
3. Transfer to 42ºC for 1 min.
4. Immediately add 1 ml of LB medium (without antibiotic) to each tube.
5. Incubate at 37ºC for 60 min with shaking at 220 rpm.
6. Add 4 ml of LB medium containing the appropriate selection antibiotic. Incubate the culture at 37ºC overnight with shaking at 220 rpm.
7. Isolate the plasmid DNA using Quiagen mini prep kit.

3.16.5 Selection of the mutant plasmid

1. To 100 ng of the isolated plasmid, add 10-20 units of the selection unique enzyme with the appropriate buffer supplied along with the kit.
2. Adjust the final volume to 20 µl with sterile water.
3. Mix well. Incubate at 37ºC for 2 hr.
4. Add additional 10 units of the appropriate restriction enzyme, and continue incubation at 37ºC for another 1 hr.

3.16.6 Final transformation

1. Use 5.0 µl of the digested plasmid DNA (approximately 25 ng) for transformation as described above.
2. After transformation, immediately add 1 ml of LB medium (without antibiotics).
3. Incubate at 37ºC for 60 min with shaking at 220 rpm.
4. Perform a serial dilution as follows in order to obtain several different concentrations of cells.
    a. Place 111 µl of the transformed cells into a fresh tube (labelled 1X)
    b. Remove 11 µl from tube 1X and add to a fresh tube containing 99 µl of LB broth (labelled 0.1X); mix contents of tube 0.1X.
    c. Remove 10 µl from tube 0.1x and add to a fresh tube containing 90 µl of LB broth (labelled 0.01X); mix contents of the tube 0.01X.

d. If colour conversion is expected as a result of transformation, add:

   40 µl of 20 mg/ml X-gal solution

   10 µl of 20 nM IPTG solution

e. Mix well and spread each suspension evenly on LB plates containing the appropriate antibiotic for selection of transformants.

3.16.7 Characterization of mutant plasmids

1. In case of transformations using the control pUC19M plasmid, the efficiency of mutagenesis is estimated by the number of blue (mutated) colonies divided by the total number of blue and white (unmutated) colonies. An efficiency rate of 70-90% is expected if the mutagenesis is performed successfully.
2. For mutagensis experiments that do not involve a visible phenotype, such as colony colour, resistance to another antibiotics, or hybridisation to a particular DNA probe, it is necessary to isolate the plasmid DNA to characterize the mutation.
3. Depending on the type of mutation generated (such as large deletion), the putative mutant plasmids may be screened by digestion with appropriate restriction enzymes.
4. The mutations should be further verified by directly sequencing the mutagenized region(s).

1.17. Immunofluorescence:

1. HeLa cells were plated on glass coverslips and transfected with the respective DNA plasmids using lipofectamine transfection protocol.
2. 24 hr post transfection, cells were washed in PBS and fixed with 3% paraformaldehyde for 10 min followed by incubation in 2% glycine for 15 min.
3. Cells were permeablized with 0.2% Triton X100 for 10 min. Cells were blocked in 0.2% BSA for 15 min followed by incubation in the primary antibody (1:100 dilution) for 1 hr.
4. 1:100 dilutions for FITC and 1:500 dilution for Cy3 was used for secondary antibody.
5. After incubation of the cells with secondary antibody for 1 hr., cells were washed, dried and mounted on coverslip.
6. Fluorescence for the respective secondary antibody was observed using Leica Confocal microscope.

4.RESULTS:

4.1. Reciprocal C/EBPα and c-jun expression.

4.1.1. C/EBPα knockout mice model

The macrophage colony-stimulating factor (M-CSF), required for growth and differentiation of mononuclear phagocytes (macrophages) regulates c-jun expression, which suggests that expression of this gene could contribute to nuclear signaling mechanisms that regulate a specific program of monocyte differentiation (219). Reciprocal expression pattern of C/EBPα and c-jun has been mentioned before in other cell types (30), but was not investigated in detail.

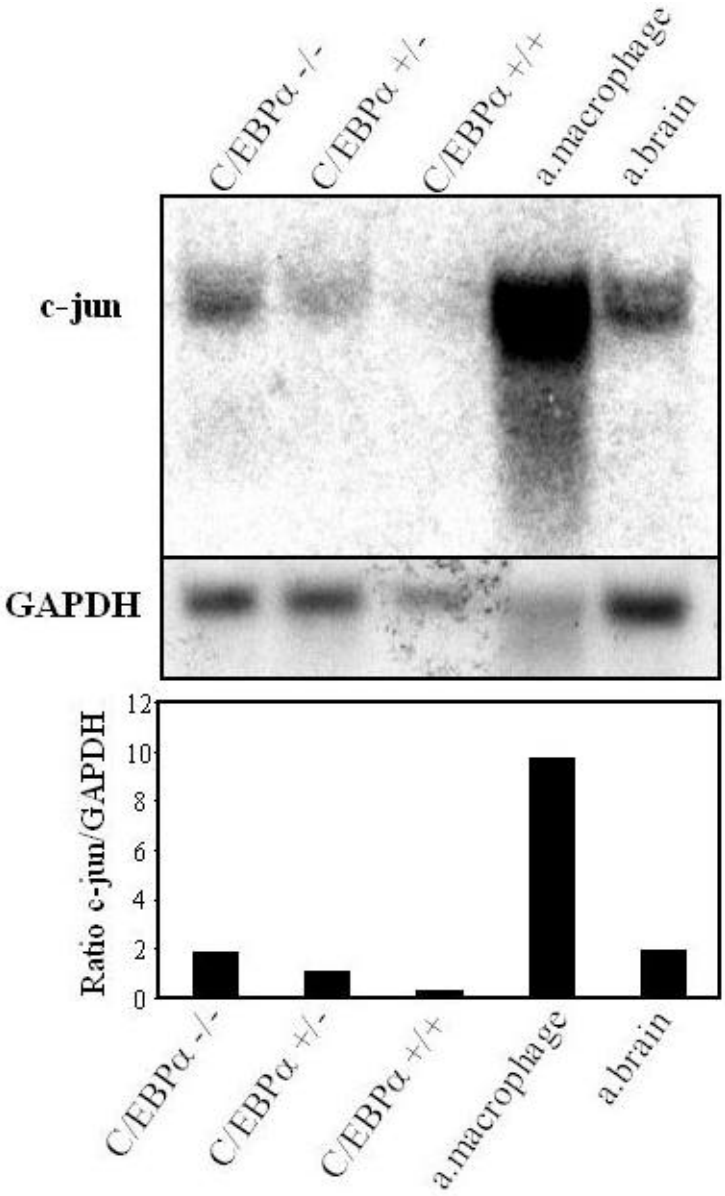

Figure 1A: Reciprocal expression of C/EBPα and c-jun. Northern blot analysis showing the expression of c-jun in day 19 fetal livers from C/EBPα +/+, +/- and -/- fetus, adult mouse brain and peritoneal macrophage. Northern blot analysis was performed as described in methods. Briefly, total RNA (10μg) for each sample was electrophoresed, transferred, and hybridized with α-^{32}P-labeled c-jun 1.1 Kb BamHI-EcoRI cDNA fragment and G6PD control fragment. Densitometric quantification ratio of c-jun/G6PD is shown below.

In C/EBPα heterozygous and homozygous knockout mice, we investigated the level of c-jun expression in fetal liver compared to expression in wild type adult macrophages (Fig. 1A).

Consistent with previous publications, high level of c-jun mRNA was detected in adult macrophages. In addition, various groups (220,221) had observed that induction of macrophage differentiation by LPS, TNFα, IFNγ or IL-1 was associated with decrease in C/EBPα expression. In comparison to wild type C/EBPα mice, high c-jun expression was observed in heterozygous mice whereas the maximum expression was observed in the homozygous, suggesting that the expression is controlled by both alleles of C/EBPα.

4.1.2. Myeloid cell line model

In the U937 cell line model with inducible C/EBPα expression, the decrease in c-jun mRNA level is reciprocal to the increase in C/EBPα expression as seen in Fig. 1B and 1C. c-jun protein level decreases between 0 to 4 hrs of C/EBPα induction (Fig. 1C).

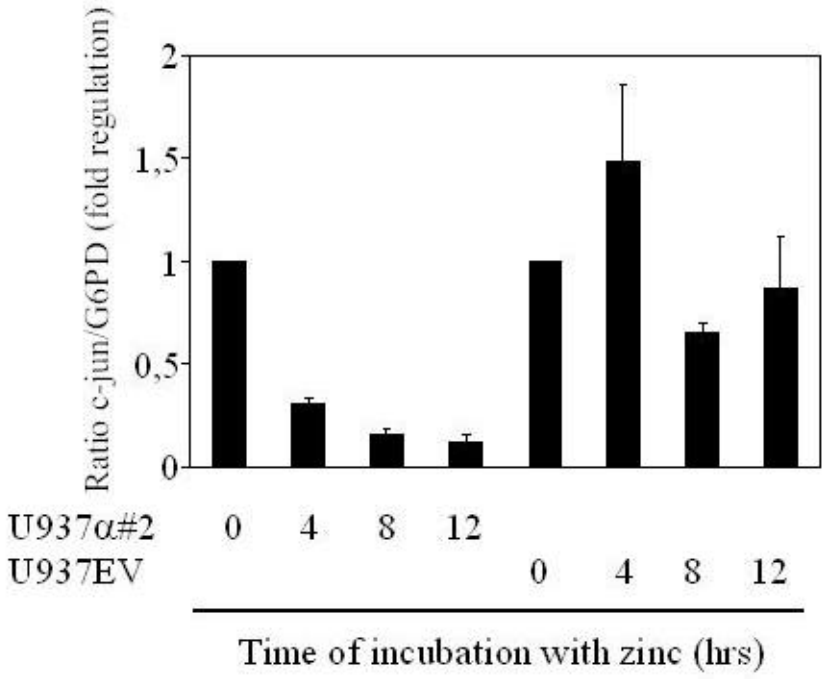

Figure 1B: Reciprocal expression of C/EBPα and c-jun. U937 α#2 and U937 EV cell lines were induced with 100μM of zinc sulphate and total RNA was collected at 0, 4, 8, 12 and 16 hrs. cDNA from 1μg total RNA was used for real time PCR using c-jun and G6PD specific primers.

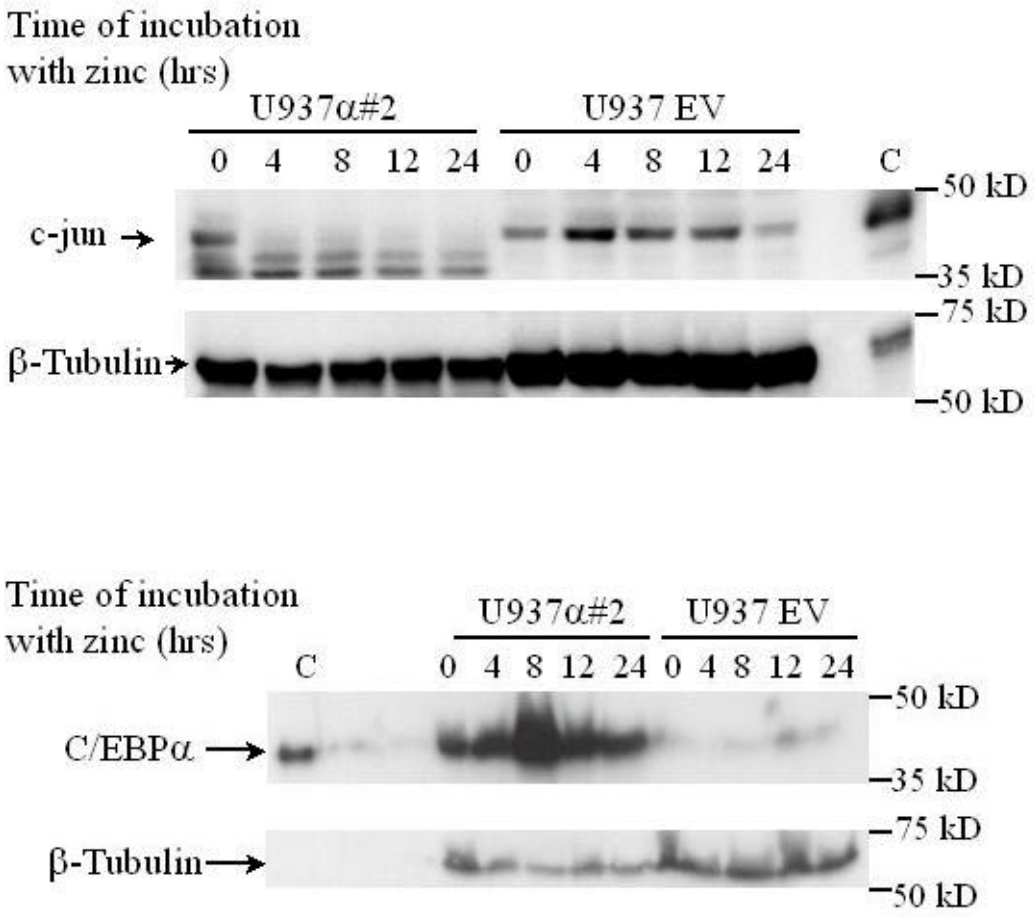

Figure 1C: Reciprocal expression of C/EBPα and c-jun. Western blot analysis showing the expression of c-jun and C/EBPα in protein extracts from U937 α#2 and U937 EV cell lines at 0, 4, 8, 12 and 24 hrs time points. Immunodetection was performed using c-jun and C/EBPα specific antibody. *in vitro* translated C/EBPα was used as a positive control. β-tubulin expression from the same blot is shown as a loading control.

4.2. C/EBPα downregulates c-jun promoter activity.

4.2.1. Adherent cell lines

To investigate the ability of C/EBPα as a negative regulator of c-jun expression, we first asked the question whether it was through transcriptional regulation of the c-jun promoter. Human c-jun promoter (216) bp-1780 to bp+731 in pGL3 basic luciferase vector was cotransfected with C/EBPα expression vector in various non-myeloid cell lines (Fig. 2A).

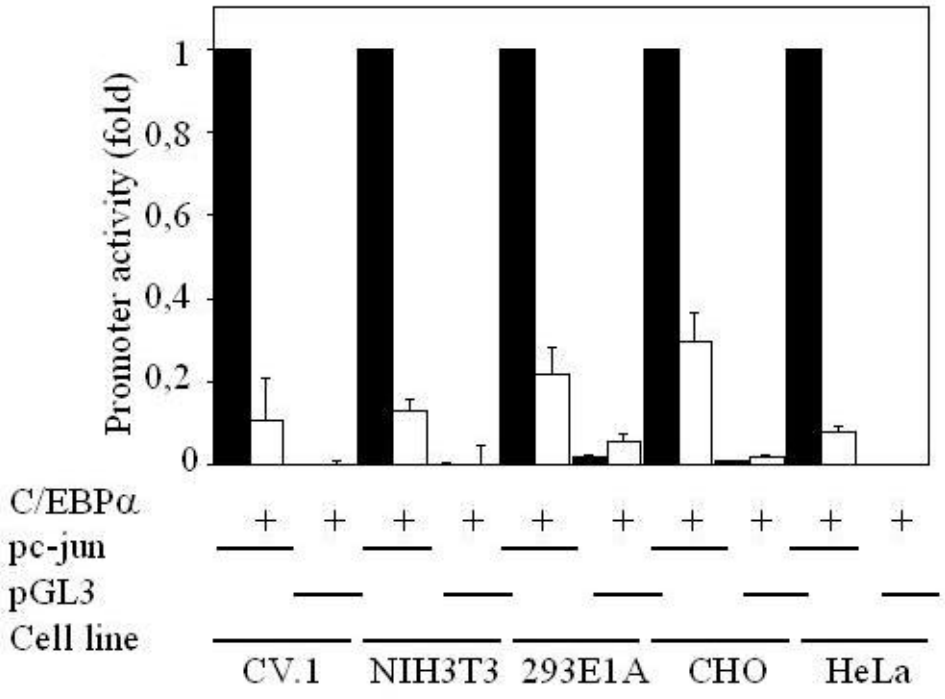

Figure 2A: C/EBPα downregulates the c-jun promoter activity. Transient cotransfection of a c-jun promoter reporter construct (bp-1780 to bp+731) and pGL3 with or without C/EBPα in various cell lines such as CV.1, NIH3T3, 293E1A, CHO and HeLa. Solid bars indicate the promoter alone values; whereas cotransfection with C/EBPα is shown as open bars. pRL-0 Renilla luciferase construct was cotransfected to normalize for transfection efficiency.

The pGL3 basic luciferase reporter vector into which the c-jun promoter was cloned served as vector alone control. Since we addressed the repressional activity of C/EBPα, we also used the p(C/EBP)2TK promoter containing two repeats of C/EBP consensus DNA binding site as a positive control for C/EBPα transcriptional activity under the same experimental conditions in order to rule out toxic effect of C/EBPα in transient transfection experiments (Fig. 2B).

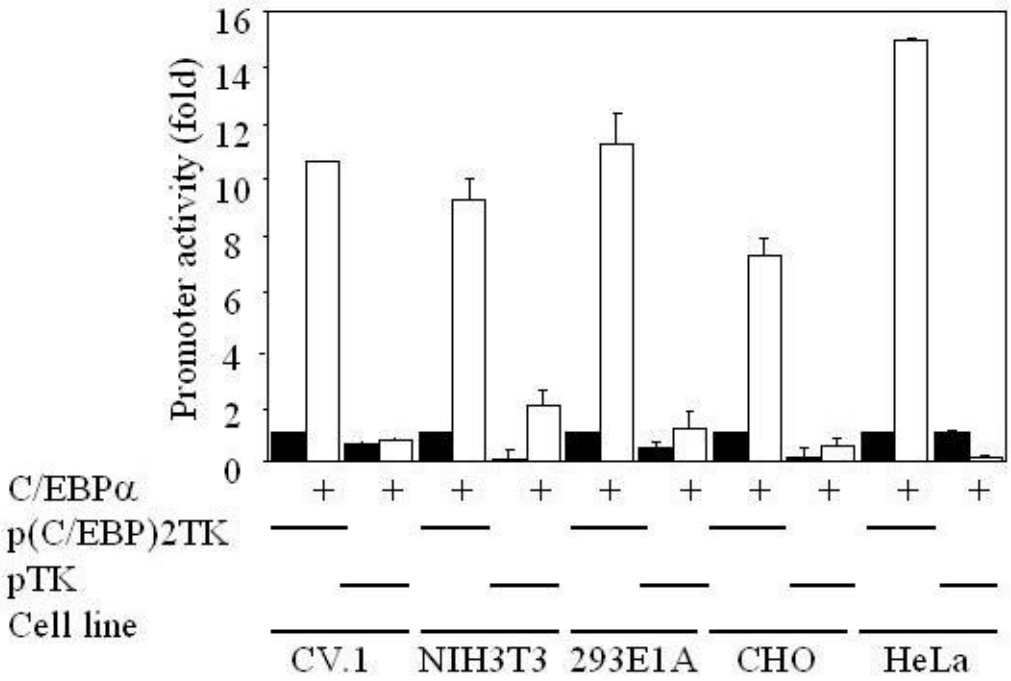

Figure 2B: C/EBPα downregulates the c-jun promoter activity. Effect of transient cotransfection of C/EBPα on the positive control p(C/EBP)2TK-luciferase reporter construct indicating the transactivation capacity of C/EBPα in these cell lines. pTK-luciferase reporter construct serves as negative control. Promoter alone values are indicated as solid bars, whereas the fold promoter activities in presence of C/EBPα are depicted in open bars.

These transient transfection experiments were carried out in various fibroblast cell lines as shown in Fig. 2, to demonstrate that it was a general

phenomenon and not cell line dependent. At least 8 fold c-jun promoter activity downregulation in presence of C/EBPα was observed, whereas the p(C/EBP)2TK promoter was transactivated about 10 fold upon transient expression of C/EBPα.

4.2.2. U937 myeloid cell line

Dose-dependent c-jun promoter downregulation was also observed in U937 myeloid cells in presence of C/EBPα (Fig. 2C)

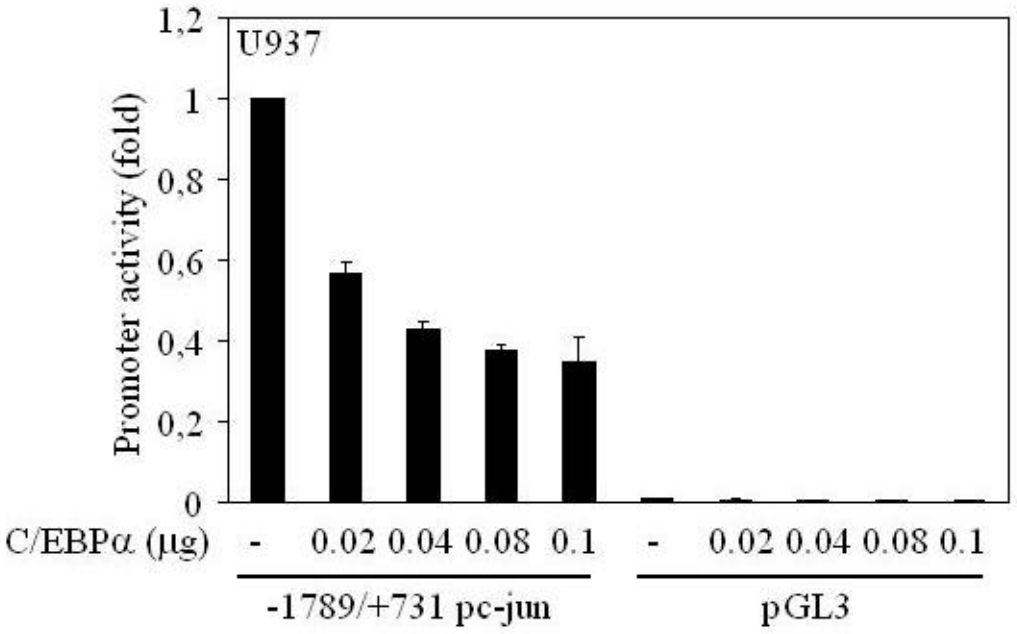

Figure 2C: C/EBPα downregulates the c-jun promoter activity. 1 x 10^6 U937 cells per well in 6 well plates were transfected with 0.55 μg c-jun promoter construct (bp-1780 to bp+731) or pGL3, with or without increasing concentrations of C/EBPα expression plasmid and 0.05μg pRL-0. The cells were transfected using the effectene protocol (Qiagen). The results are the mean of three independent experiments and error bars represent the standard error of mean values for each set.

4.3.1. C/EBPα does not recruit a TSA-sensitive co-repressor complex.

To address the possibility of C/EBPα mediated c-jun promoter downregulation by recruiting co-repressors, transient transfection experiments of the c-jun promoter were carried out with C/EBPα in the presence of Trichostatin A (TSA), a potent inhibitor of histone deacetylase-corepressor complex formation on an open transcription promoter machinery. Downregulation of c-jun promoter activity by C/EBPα is retained even in presence of 100nM TSA (Fig. 3A).

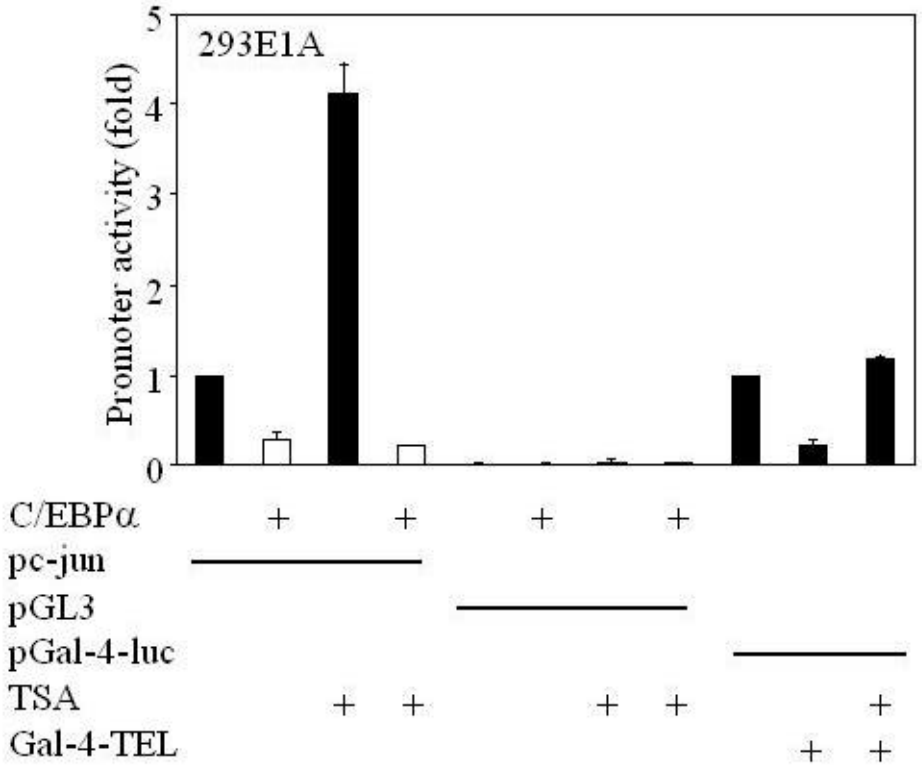

Figure 3A: C/EBPα does not recruit a TSA-sensitive co-repressor complex. Transient cotransfection experiments in 293E1A cell line with the c-jun promoter construct and C/EBPα in presence or absence of Trichostatin A (TSA) (100nM). pGal-4-luc with Gal-4-TEL and TSA was used as a positive control for functionally active TSA.

TSA increases the c-jun promoter activity by itself. This could be because TSA is known to increase histone H3 acetylation on c-jun associated

nucleosomes (222). A positive control for TSA showing the loss in recruitment of co-repressor complex by transcription factor TEL in presence of TSA was also included.

4.3.2. C/EBPα blocks TPA induced c-jun promoter activity.

TPA, a potent inducer of monocytic differentiation in myeloid bipotential

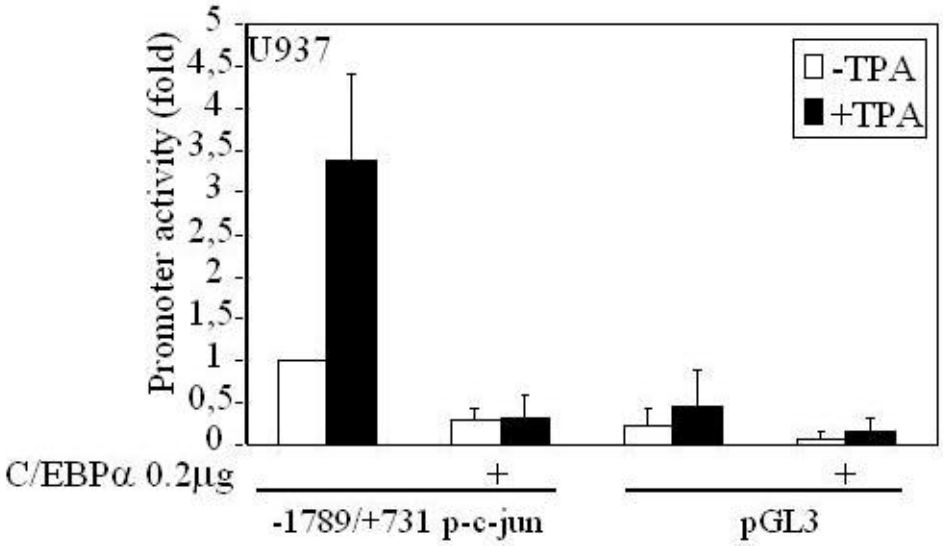

Figure 3B: C/EBPα blocks TPA induced c-jun promoter activity. 1 x 10^6 U937 cells in 6 well plates were transfected with 0.55μg of c-jun promoter construct (bp-1780 to bp+731) or pGL3, with or without 0.4μg of C/EBPα expression plasmid or empty vector, and 0.05μg pRL-0. The cells were transfected using the effectene protocol (Qiagen). 12 h post transfection, TPA (100nM) was added to the respective wells and further incubated at 37ºC for 24-30 hrs. pRL-0 Renilla luciferase construct was cotransfected to normalize for transfection efficiency. The results are the mean of three independent experiments and error bars represent the standard error of mean values for each set.

cell lines has been known to increase c-jun expression (145). Radomska et al. (67) had earlier demonstrated that C/EBPα can block TPA induced monocytic differentiation in U937 myeloid cells. We, therefore, asked the question if

C/EBPα could inhibit TPA induced monocytic differentiation capacity by blocking c-jun expression and activity. As shown in Fig. 3B, human c-jun promoter activity was downregulated by C/EBPα and, interestingly, TPA-induced increase in the c-jun promoter activity was blocked by C/EBPα.

4.4. Mapping of the region in the c-jun promoter that is important for C/EBPα mediated promoter downregulation.

4.4.1. Since C/EBPα was not recruiting a TSA-sensitive co-repressor complex to the c-jun promoter, we further asked whether C/EBPα could exert this repressional activity via some specific transcription factor binding sites in the c-jun promoter. Schematic presentation of various 5' c-jun promoter deletion constructs described by Wei et al. (217) are shown in Fig. 4A.

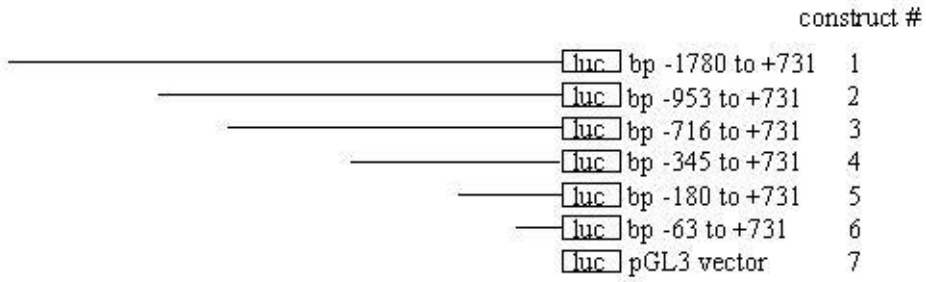

Figure 4A: c-jun promoter mapping to identify the region important for C/EBPα mediated downregulation. Schematic presentation of various c-jun promoter 5' deletion constructs used for the transient transfection experiment.

4.4.2. These constructs were used for promoter mapping experiments in 293E1A cells (Fig. 4B). As observed also by Wei et al., each 5' deletion construct had different transcriptional activity as compared to the longest (bp-1780/+731) promoter construct (Fig.4B). As seen in Fig. 4B, the promoter activity of each c-jun promoter deletion construct was downregulated by C/EBPα except for the

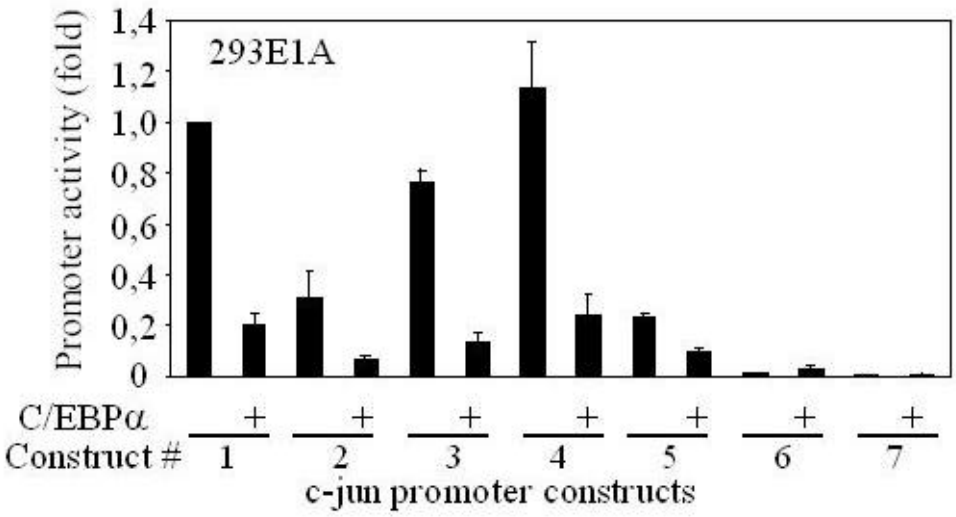

Figure 4B: c-jun promoter mapping to identify the region important for C/EBPα mediated downregulation. 1 x 10^4 293E1A cells per well in 24 well plate were transfected with 0.25μg of 5' c-jun promoter deletion constructs: bp-1780/+731; bp-953/+731; bp-716/+731; bp-345/+731; bp-180/+731; bp-63/+731 and pGL3 with or without 0.2μg of C/EBPα or empty vector and 0.05μg of pRL-0.

bp-63/+731 construct. This construct lacks most of the regulatory regions identified so far. Since the bp-180/+731 c-jun promoter construct was still downregulated by C/EBPα, we concluded that the site important for C/EBPα mediated transcriptional downregulation should be between bp-180 to bp-63.

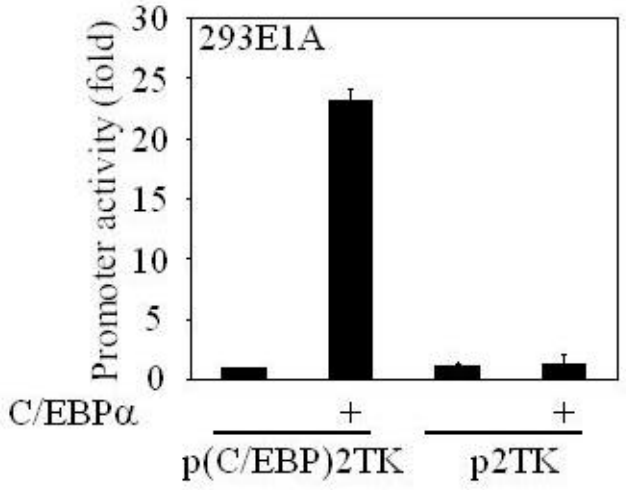

Figure 4C: c-jun promoter mapping to identify the region important for C/EBPα mediated downregulation. Effect of transient co-transfection of C/EBPα on the positive control p(C/EBP)2TK-luciferase reporter construct indicating the transactivation capacity of C/EBPα in this cell line.

In the same experiment, p(C/EBP)2TK was transactivated by C/EBPα (Fig. 4C).

4.4.3. Partial mapping of the c-jun promoter in U937 yielded similar results (Fig. 4D). The full length (bp-1780) and bp-180 c-jun promoter constructs were downregulated by C/EBPα whereas the bp-63 construct was not.

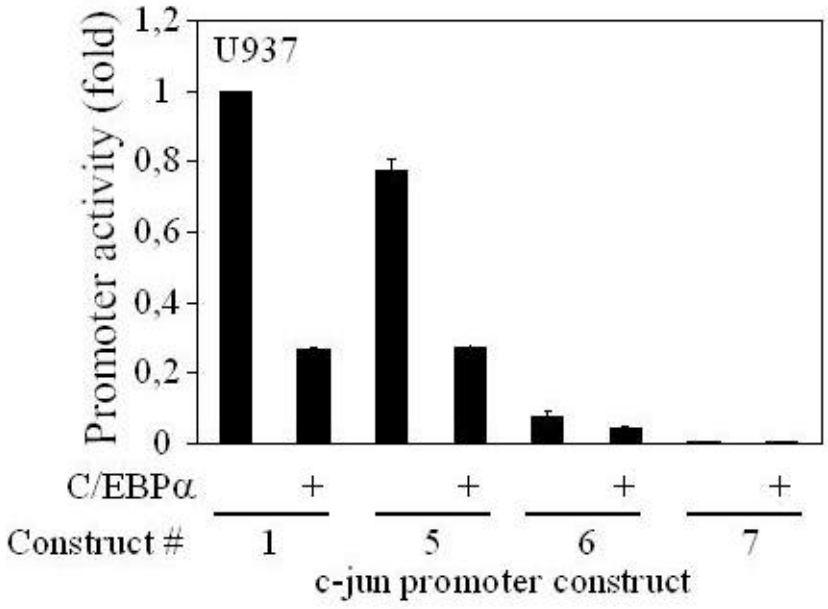

Figure 4D: c-jun promoter mapping to identify the region important for C/EBPα mediated downregulation. 1 x 10^6 U937 cells per well in 6 well plates were transfected with 0.55μg of 5' c-jun promoter deletion constructs: bp-1780/+731; bp-180/+731; bp-63/+731 and pGL3 with or without C/EBPα expression plasmid or empty vector, and 0.05μg pRL-0. The cells were transfected using the effectene protocol. pRL-0 Renilla luciferase construct was cotransfected to normalize for transfection efficiency.

4.4.4. Schematic presentation of the c-jun promoter spanning the bp-180 to bp-63 region (Fig. 4E) shows the binding sites for various transcription factors. This region includes pAP-1 (proximal AP-1), CTF, and Sp-1 sites.

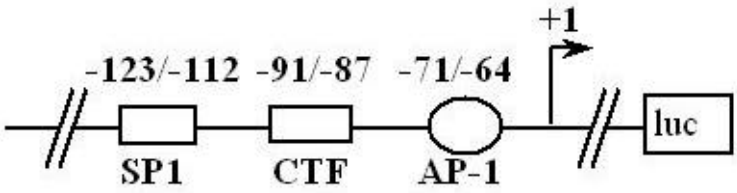

Figure 4E: c-jun promoter mapping to identify the region important for C/EBPα mediated downregulation. Schematic presentation of the c-jun promoter region between bp-180 and bp-63. This region contains a proximal AP-1 site, CTF site and SP-1 site.

4.5. C/EBPα does not bind to the CTF site in the c-jun promoter.

The CTF (C/EBP Transcription Factor) binding site in the c-jun promoter has been identified. We first addressed if C/EBPα can bind to this site for downregulating c-jun promoter activity. Using either U937 cells nuclear extract (Fig. 5A) or in vitro translated C/EBPα (Fig. 5B), no binding to the CTF site was observed. Moreover, mutating the CTF binding site also had no effect on the transcriptional downregulation of the c-jun promoter (Fig. 5C).

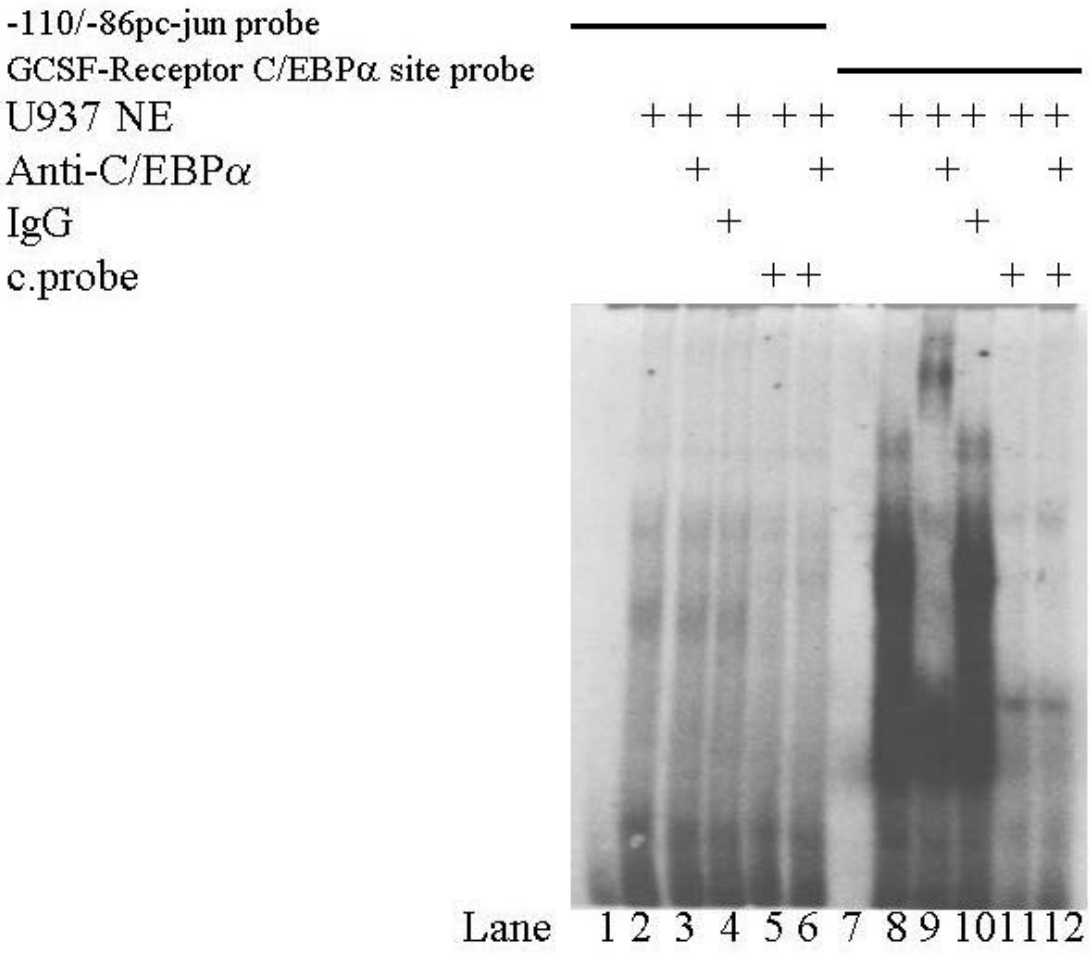

Figure 5A: C/EBPα does not bind to the CTF site in the c-jun promoter. EMSA using U937 nuclear extract can shift and supershift the control oligo (G-CSFR promoter C/EBPα binding site), but no such shift or supershift specific for C/EBPα was observed with the c-jun promoter CTF site.

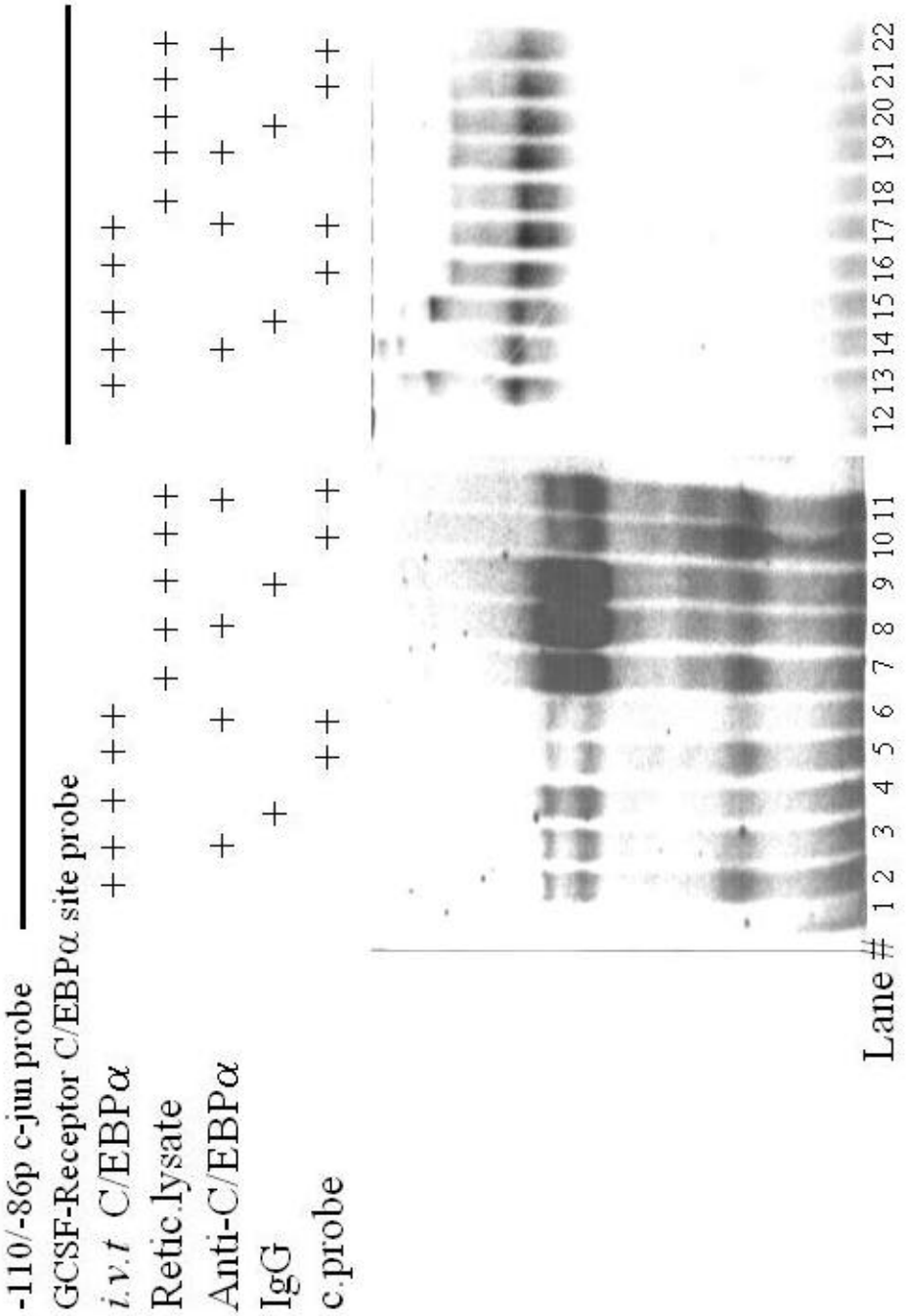

Figure 5B: C/EBPα does not bind to the CTF site in the c-jun promoter. EMSA using in vitro translated C/EBPα protein can shift and supershift the control oligo (G-CSFR promoter C/EBPa binding site), but no such shift or supershift specific for C/EBPα was observed with the c-jun promoter CTF site.

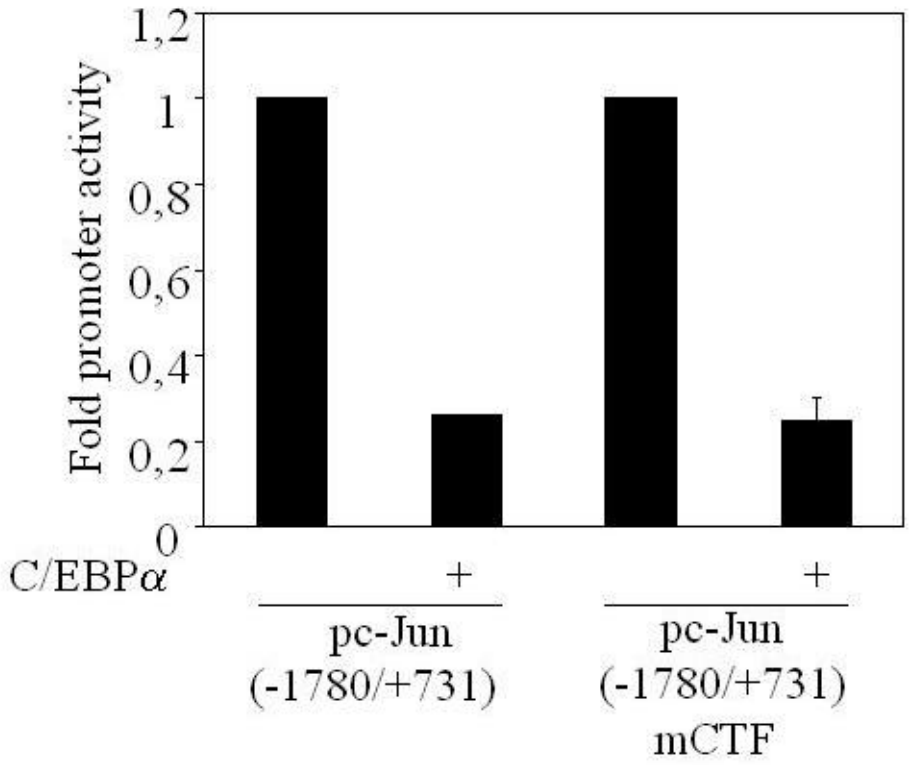

Figure 5C: C/EBPα does not bind to the CTF site in the c-jun promoter. Transient transfection with mutated CTF binding site in the c-jun promoter along with C/EBPα. The c-jun promoter activity downregulation for the wild type full length (bp-1780 to bp+731) and mut.CTF full length c-jun promoter were compared in presence and absence of C/EBPα.

4.6. C/EBPα blocks the autoregulatory capacity of c-jun by preventing c-jun from binding to the proximal AP-1 site in the c-jun promoter.

4.6.1. In transient transfections in U937 myeloid cells, c-jun transactivates its own promoter – an autoregulatory mechanism that was identified by Angel et al. (223). Our data (Fig. 6A) suggests that transactivation of the c-jun promoter by c-jun is blocked in presence of C/EBPα.

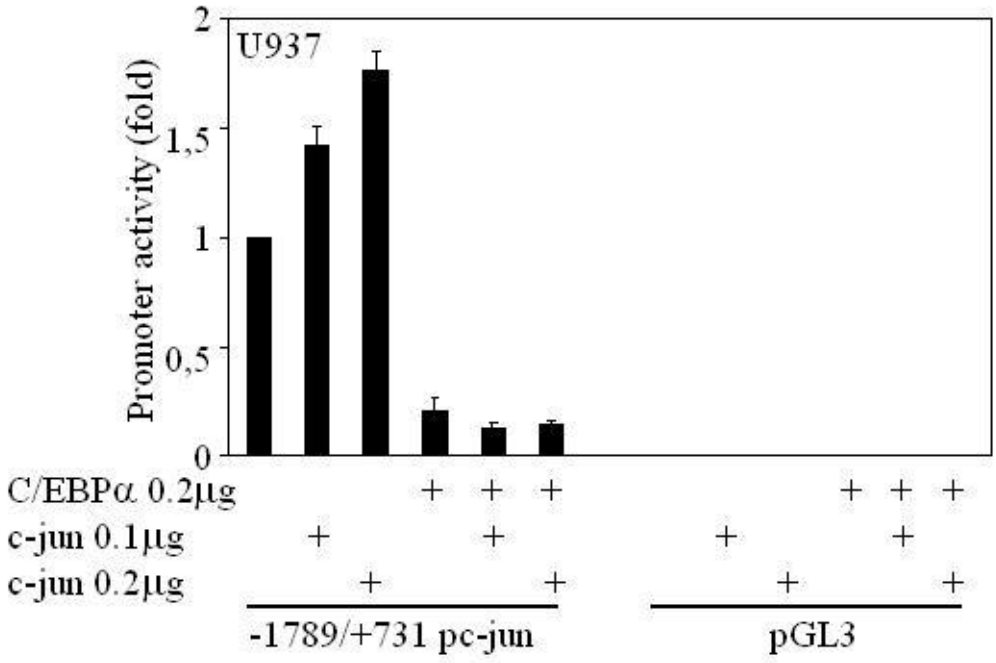

Figure 6A: C/EBPα blocks the autoregulatory capacity of c-jun by preventing c-jun from binding to the proximal AP-1 site in the c-jun promoter. Transient transfections in U937 myeloid cells were performed using a bp-1780/+731 c-jun promoter construct, pGL3 vector and 0.2μg C/EBPα expression plasmid and increasing concentrations of c-jun expression plasmid (0.1μg and 0.2μg).

4.6.2. Based on results from the previous promoter mapping experiments and TPA experiments, we decided to address the importance of the proximal AP-1 site in C/EBPα mediated c-jun promoter downregulation. We asked the question

whether C/EBPα could block the transactivation capacity of c-jun through the proximal AP-1 site in the c-jun promoter. Using the bp-79/+170 promoter (161) containing only the proximal AP-1 site of the c-jun promoter (Fig. 6B), we observed that the autoregulatory capacity of c-jun through the proximal AP-1 binding site was lost in presence of C/EBPα. Increasing the concentration of c-jun could not overcome the block by C/EBPα.

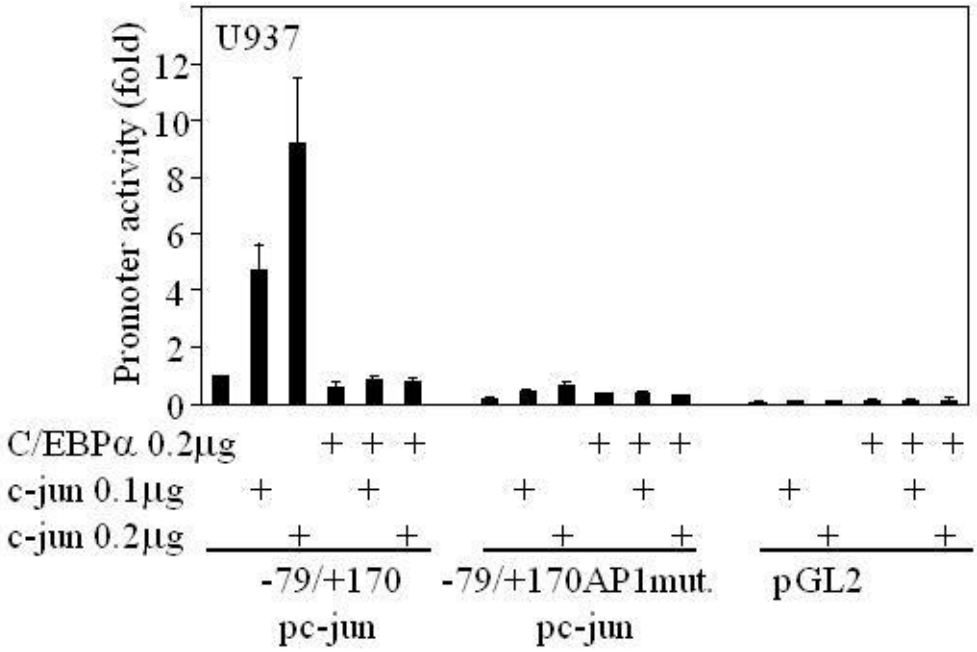

Figure 6B: C/EBPα blocks the autoregulatory capacity of c-jun by preventing c-jun from binding to the proximal AP-1 site in the c-jun promoter. bp-79/+190 and bp-79/+190 mutated AP-1 site c-jun promoter constructs and pGL2 were transiently transfected with 0.2μg C/EBPα expression plasmid and increasing concentrations of c-jun expression plasmid.

4.6.3. Using an artificial AP-1 construct containing 7 repeats of the consensus AP-1 site (Fig. 6C), similar results were obtained, indicating that C/EBPα blocks autoregulation of c-jun by preventing latter from binding to the AP-1 site in the c-jun promoter.

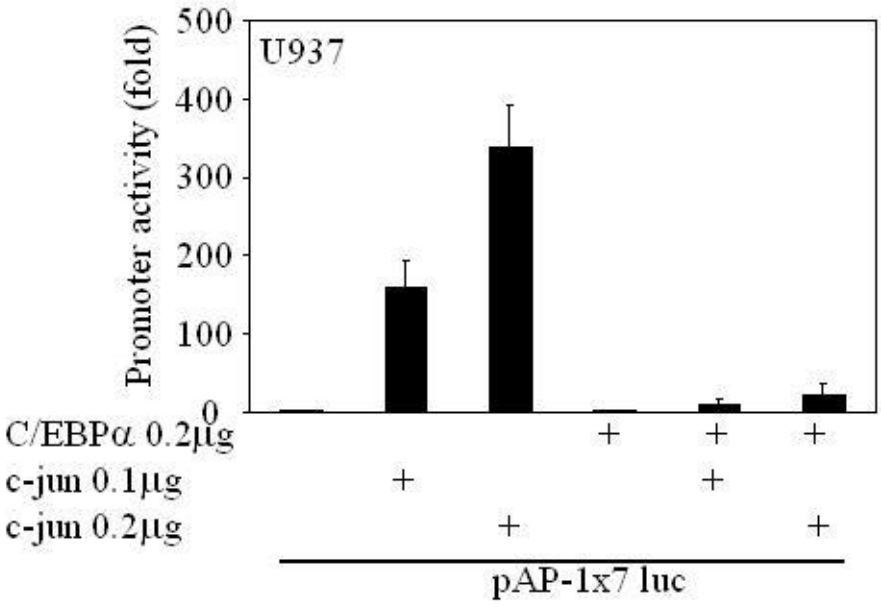

Figure 6C: C/EBPα blocks the autoregulatory capacity of c-jun by preventing c-jun from binding to the proximal AP-1 site in the c-jun promoter. AP-1 luciferase construct containing seven repeats of an AP-1 binding site was used for transient transfection with c-jun and C/EBPα in U937 myeloid cells.

4.6.4. The control experiment for the transactivation capacity of C/EBPα (Fig. 6D) suggested that c-jun could also partially suppress the transactivation capacity of C/EBPα. This effect could be similar to C/EBPα-c-jun interaction effect on former's transcriptional activity (204).

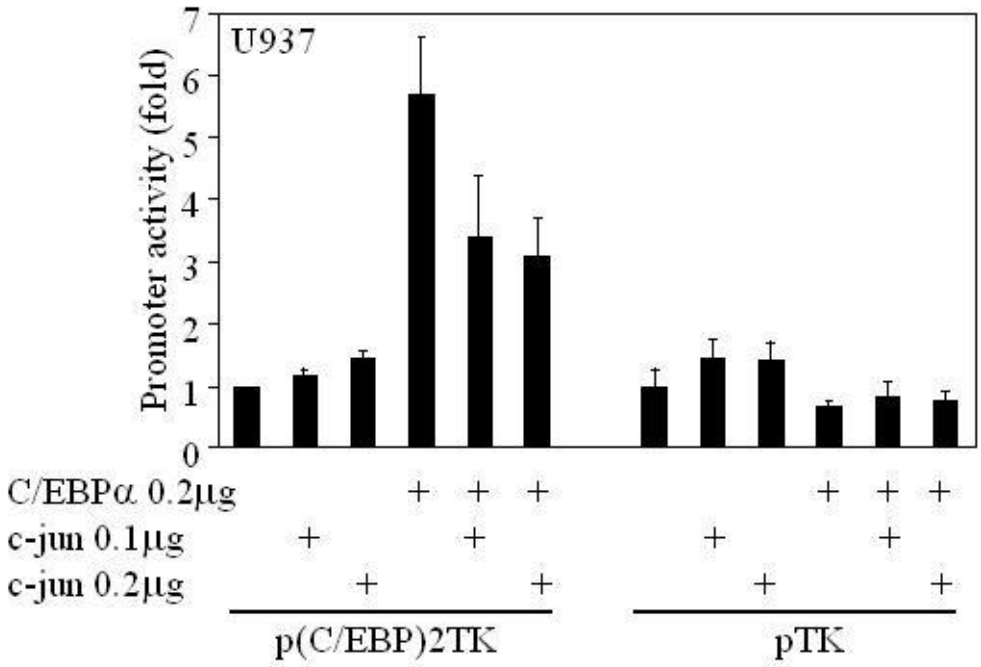

Figure 6D: C/EBPα blocks the autoregulatory capacity of c-jun by preventing c-jun from binding to the proximal AP-1 site in the c-jun promoter. As positive control for the transactivation capacity of C/EBPα, p(C/EBP)2TK construct and empty vector pTK were used under similar experimental conditions as mentioned above. pRL-0 Renilla luciferase construct was cotransfected to normalize for transfection efficiency. The results are the mean of three independent experiments and error bars represent the standard error of mean values for each set.

4.6.5. To understand how C/EBPα blocks the autoregulatory capacity of c-jun, we determined the DNA binding capacity of c-jun to the proximal AP-1 site in the presence of C/EBPα. In a band shift mobility assay using the bp-79 c-jun promoter oligo probe, c-jun binding to the proximal AP-1 site was blocked by in vitro translated C/EBPα, but not by reticulocyte lysate alone (Fig. 6E). Under similar experimental conditions, C/EBPα could bind to CEBP consensus DNA binding site in the G-CSFR promoter and the C/EBPα band shift was supershifted in presence of C/EBPα specific antibody (Fig 5A, Fig. 5B).

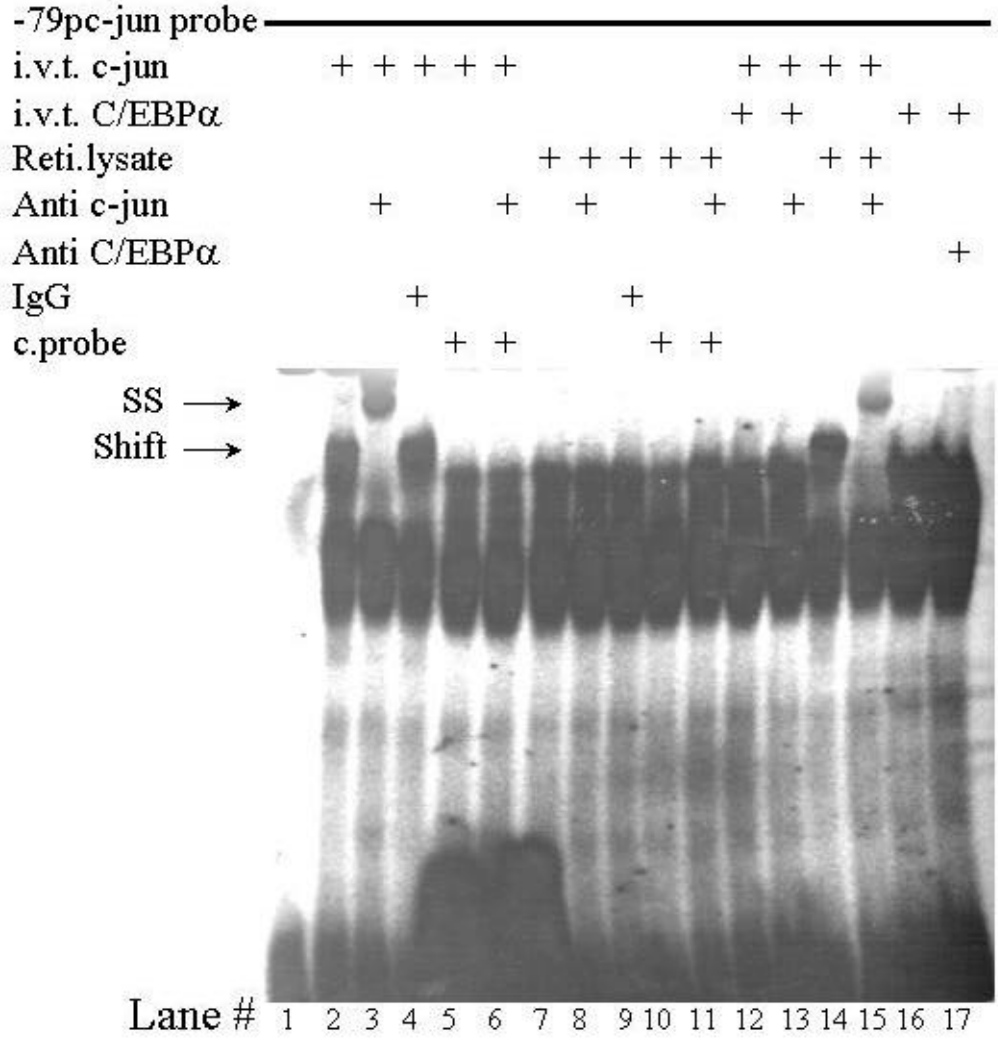

Figure 6E: C/EBPα blocks the autoregulatory capacity of c-jun by preventing c-jun from binding to the proximal AP-1 site in the c-jun promoter. EMSA using γ-^{32}P ATP labeled bp-82/-53 c-jun promoter oligo spanning the proximal AP-1 site was performed using *in vitro* translated c-jun (lanes 2-6,12-15) and C/EBPα (lanes 12,13,16,17) proteins, rabbit reticulocyte lysate (lanes 7-11,14,15), c-jun specific antibody (lanes 3,6,8,11,13,15), normal rabbit IgG (lanes 4,9), C/EBPα specific antibody (lane 17) and self unlabeled competitor probe (lanes 5,6,10,11). Arrows show (1) the c-jun shifted band and (2) super shifted higher band with c-jun specific antibody.

4.7. C/EBPα and c-jun interact through their leucine zipper domains.

4.7.1. *in vitro* interaction

We then asked the question if c-jun could interact with C/EBPα. An in vitro GST pull down assay (Fig. 7A, 7C) and an in vivo co-IP (Fig. 7D, 7E) indicate that C/EBPα and c-jun interact *in vitro* and *in vivo*.

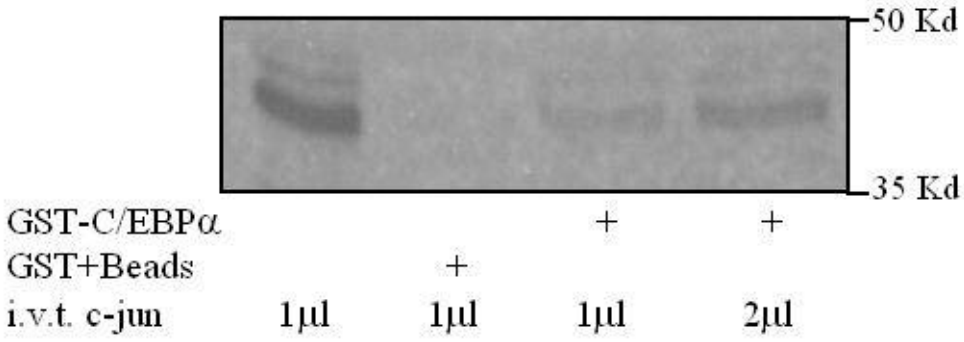

Figure 7A: C/EBPα and c-jun interact in vitro. GST-C/EBPα and GST+beads were incubated with *in vitro* translated c-jun as described in methods.

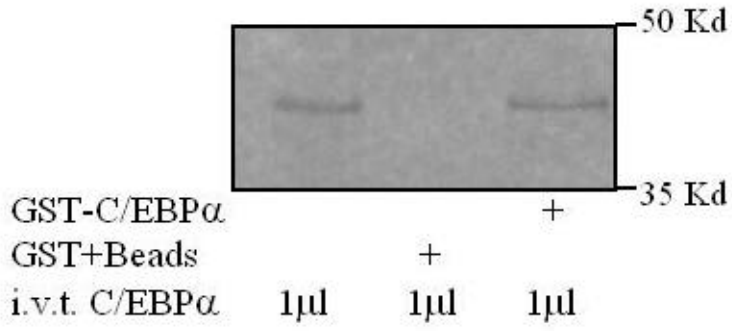

Figure 7B: C/EBPα and c-jun interact *in vitro*. As positive control for GST-C/EBPα, it was incubated with *in vitro* translated C/EBPα.

Fig. 7B shows a control for GST-C/EBPα, which can form homodimers with *in vitro* translated C/EBPα.

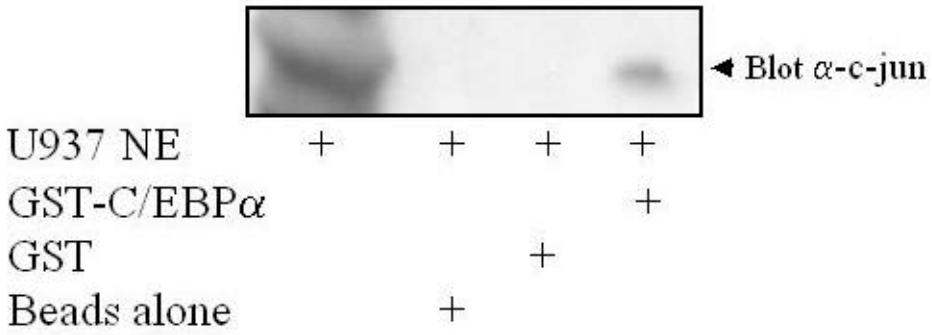

Figure 7C: C/EBPα and c-jun interact *in vitro*. GST-C/EBPα was incubated with 85μg of U937 nuclear extract. Immunodetection was carried out using c-jun antibody. GST and glutathione-agarose beads alone incubated with U937 nuclear extract to determine the specificity of this interaction.

4.7.2. *in vivo* interaction

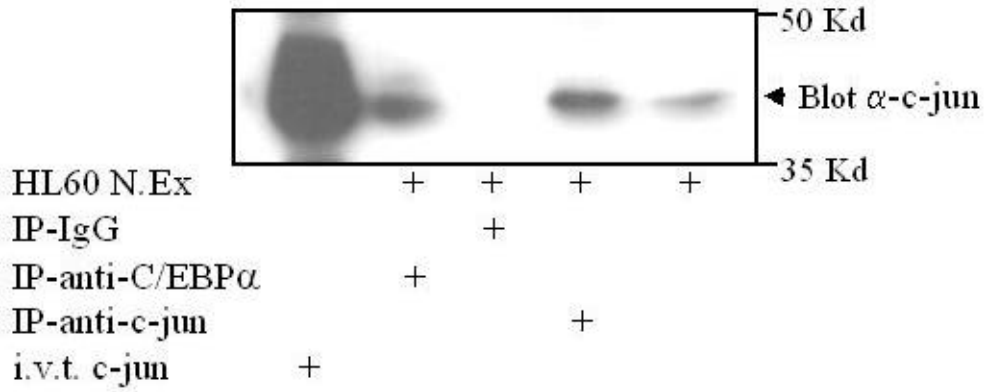

Figure 7D: C/EBPα and c-jun interact in HL60 myeloid cells. Coimmunoprecipitation assays from 85μg of U937 or 60μg of HL-60 nuclear extract were performed using C/EBPα, c-jun specific antibody or normal rabbit IgG.

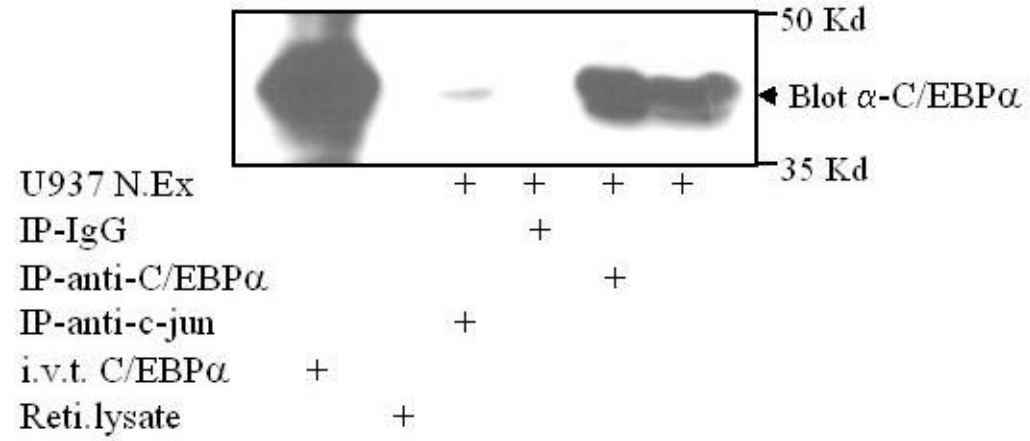

Figure 7E: C/EBPα and c-jun interact in U937 myeloid cells. Coimmunoprecipitation assays from 85μg of U937 or 60μg of HL-60 nuclear extract were performed using C/EBPα, c-jun specific antibody or normal rabbit IgG. Immunodetection was carried out using C/EBPα or c-jun specific antibody. *in vitro* translated C/EBPα and c-jun were included as positive controls.

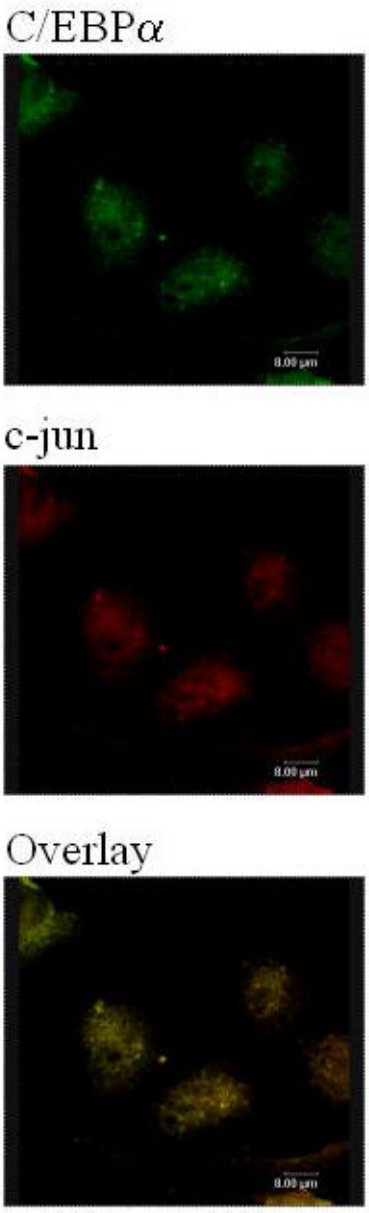

Figure 7F: C/EBPα and c-jun co-localize in HeLa cell nucleus. Respective plasmid DNA was cotransfected in HeLa cells. FITC staining shows C/EBPα expression within the cell nucleus. Cy3 staining denotes c-jun expression pattern in the same cells. Overlay of the same confocal image shows the co-localization of C/EBPα and c-jun in HeLa cell nucleus.

4.8. C/EBPα and c-jun interact with their leuzine zipper domains.

4.8.1. *in vitro* c-jun interacting domain mapping

We further wanted to investigate which domain of C/EBPα and c-jun were important for this interaction. GST pull down assay using GST-C/EBPα and in vitro translated c-junΔRK and c-junΔLZ suggested that leucine zipper domain of c-jun was required to interact with C/EBPα (Fig. 8A).

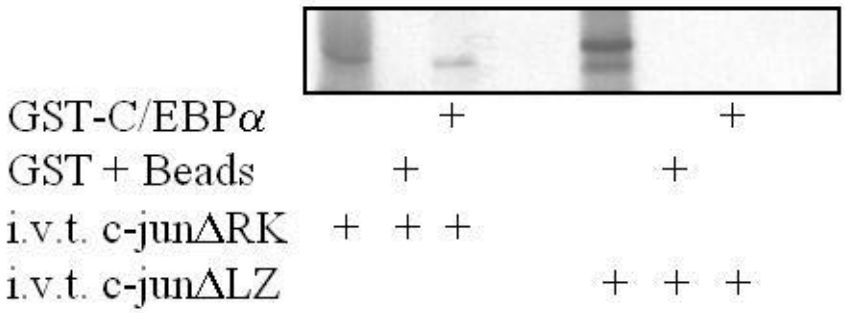

Figure 8A: C/EBPα and c-jun interact with their leuzine zipper domain. GST-C/EBPα was incubated with ^{35}S *in vitro* translated c-junΔRK and c-junΔLZ. GST plus beads alone incubated with these *in vitro* translated proteins served as negative control.

4.8.2. *in vivo* c-jun interacting domain mapping

In addition, 293T cells were transfected with various combinations of c-junΔRK, c-junΔLZ, C/EBPαΔBR, and C/EBPαΔLZ. Nuclear extracts from these cells were used for coimmunoprecipitation assays with C/EBPα specific antibody and control IgG. We observed that the leucine zipper domain of c-jun was required for its interaction with C/EBPα (Fig. 8B).

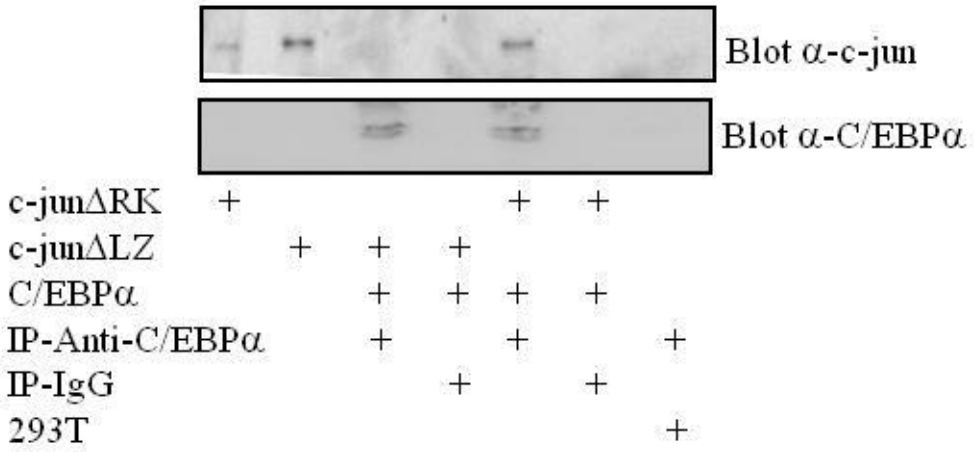

Figure 8B: C/EBPα and c-jun interact with their leuzine zipper domain. 293T cells were transfected with c-junΔRK, c-junΔLZ, C/EBPα or mock, and 24 hr post-transfection nuclear extract from these sets were used for coimmunoprecipitation assays using either C/EBPα specific antibody or normal rabbit IgG. The samples were probed with c-jun and C/EBPα specific antibodies.

4.8.3. *in vivo* C/EBPα interacting domain mapping

In addition, the leucine zipper of C/EBPα was required for its interaction with c-jun (Fig. 8C). C/EBPα expression in the same experiment was determined by immunodetection using C/EBPα specific antibody (Fig. 8B).

Figure 8C: C/EBPα and c-jun interact with their leuzine zipper domain. 293T cells were transfected with c-junΔRK, c-junΔLZ, C/EBPαΔBR, C/EBPαΔLZ and mock. 24 hrs post-transfection, nuclear extracts from these sets were used for coimmunoprecipitation assay using either C/EBPα specific antibody or normal rabbit IgG. The samples were probed with c-jun specific antibodies. (C/EBPα blot – data not shown).

4.9.Overexpression of c-jun blocks C/EBPα induced granulocytic differentiation.

4.9.1. C/EBPα and c-jun expression in transduced cells.

Since earlier studies have shown that C/EBPα could block monocytic lineage commitment, we addressed the effect of c-jun on C/EBPα induced granulocytic differentiation. Retroviral transduction of C/EBPα and c-jun expression vectors along with their empty vectors was performed in HL-60 cells.

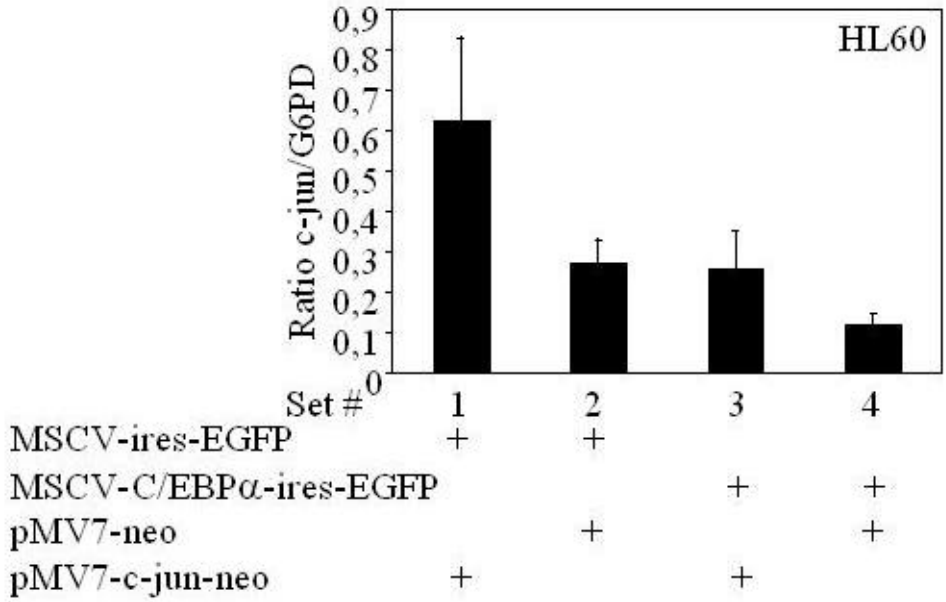

Figure 9A: Overexpression of c-jun blocks C/EBPα induced granulocytic differentiation. Real time PCR for c-jun and G6PD was performed for HL-60 cells which were transduced with pMV7-c-jun-neo (lane 1 and 3), pMV7-neo (lane 2 and 4), pMSCV-C/EBPα-ires-EGFP (lane 3 and 4) and pMSCV-ires-EGFP (lane 1 and 2) to estimate c-jun expression in each set.

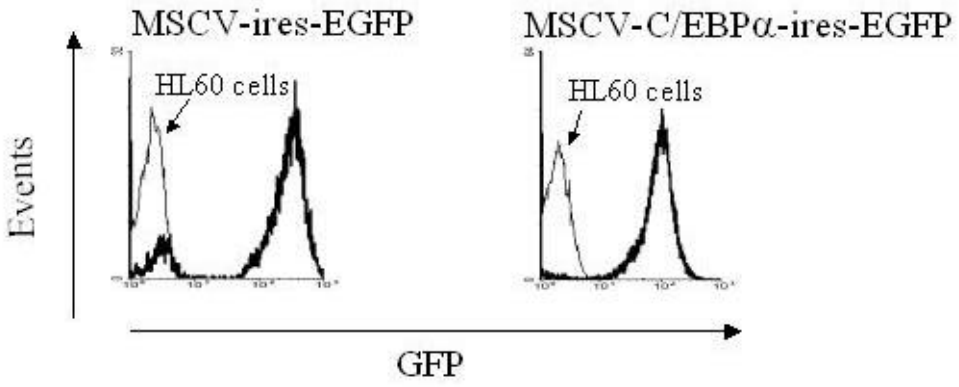

Figure 9B: Overexpression of c-jun blocks C/EBPα induced granulocytic differentiation. GFP expression in HL-60 cells transduced with MSCV-ires-EGFP and MSCV-C/EBPα-ires-EGFP as analyzed by the fluorescence in FL1 channel.

Real time PCR for c-jun and G6PD was performed from pool of transfected HL-60 cells with the respective DNAs for each set as shown in Fig. 9A. HL-60 cells transduced with pMV7-c-jun-neo showed 2 fold higher c-jun mRNA levels as compared to the pMV7-neo vector alone. These two vectors were transduced along with pMSCV-ires-GFP vector. However, when pMV7-c-jun-neo and pMV7-neo vectors were transduced along with pMSCV-C/EBPα-ires-EGFP, there was a marked decrease in c-jun mRNA. The basal level of c-jun mRNA from set 2 decreases to almost its half in set 4 (in presence of C/EBPα. A similar decrease from set 1 to set 3 was also observed.

However, the difference between set 3 and 4 is about 2 fold – same as the difference observed from set 1 and 2. The low expression in set 3 and 4 may be due to presence of C/EBPα (since C/EBPα decreases endogenous c-jun mRNA level). GFP expression of the pMSCV-ires-EGFP vector and pMSCV-C/EBPα-ires-EGFP vector was measured using FACS-analysis program (Fig. 9B).

4.9.2. CD15 marker expression in transduced cells.

Expression of CD15, a marker for granulocytic differentiation from HL-60 and U937 transduced cells was analyzed. As seen in Fig. 9C, CD15 expression increases in presence of C/EBPα transduction (left panels) and pMV7-neo vector alone had no effect on C/EBPα induced granulocytic differentiation (middle panels). The panels on the right shows the CD15 expression in HL60 and U937 cells transduced with MSCV-C/EBPα-ires-EGFP and pMV7-c-jun-neo. As expected, a negative shift of the CD15 peak was observed. This indicates that the increase in CD15 expression by C/EBPα was blocked in presence of c-jun (Fig. 9C).

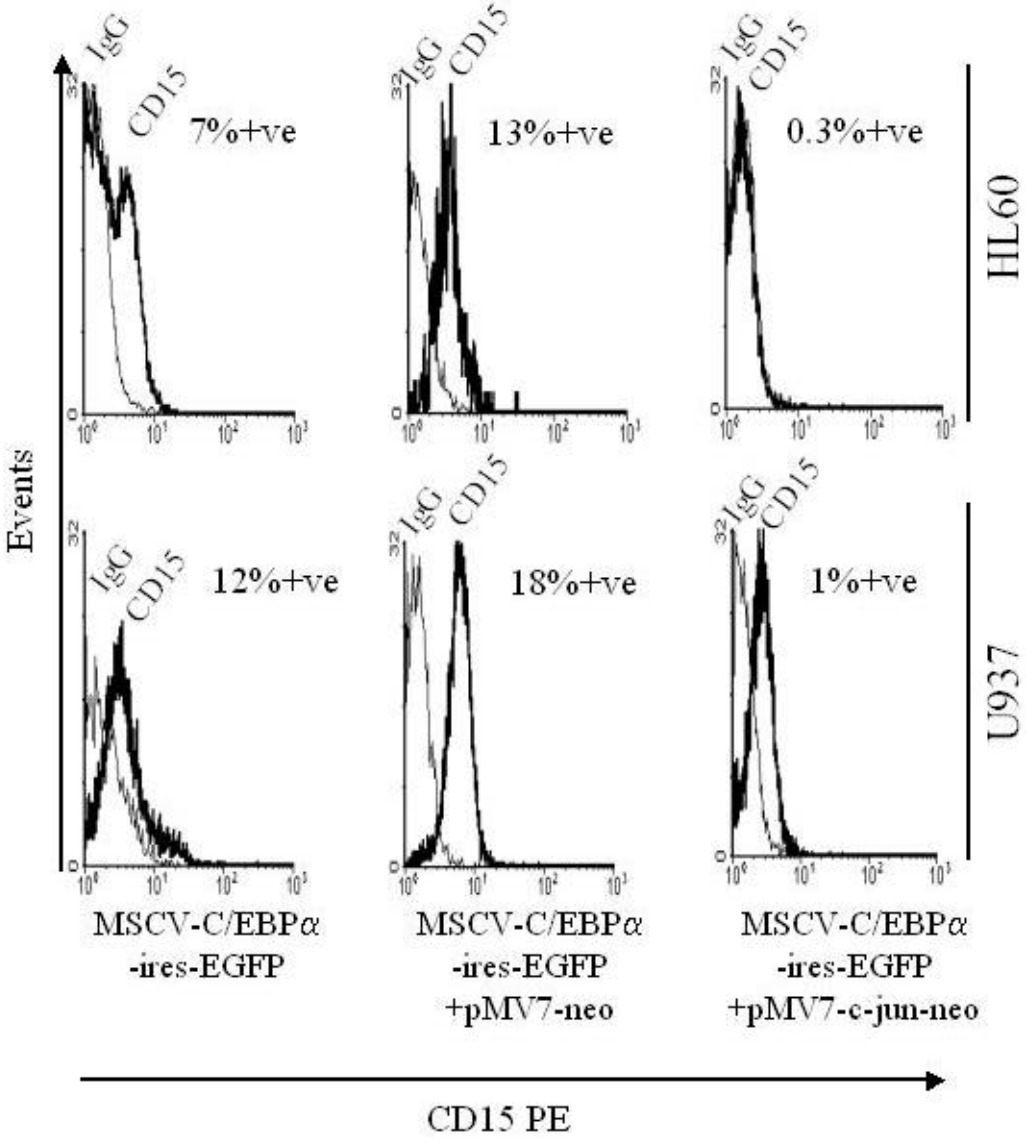

Figure 9C: Overexpression of c-jun blocks C/EBPα induced granulocytic differentiation. FACS analysis for CD15-PE from the HL-60 and U937 transduced cells along with its isotype control.

4.9.3. CD11b marker expression in transduced cells.

Similar results with CD 11b marker were also observed in HL-60 cells (Fig. 9D).

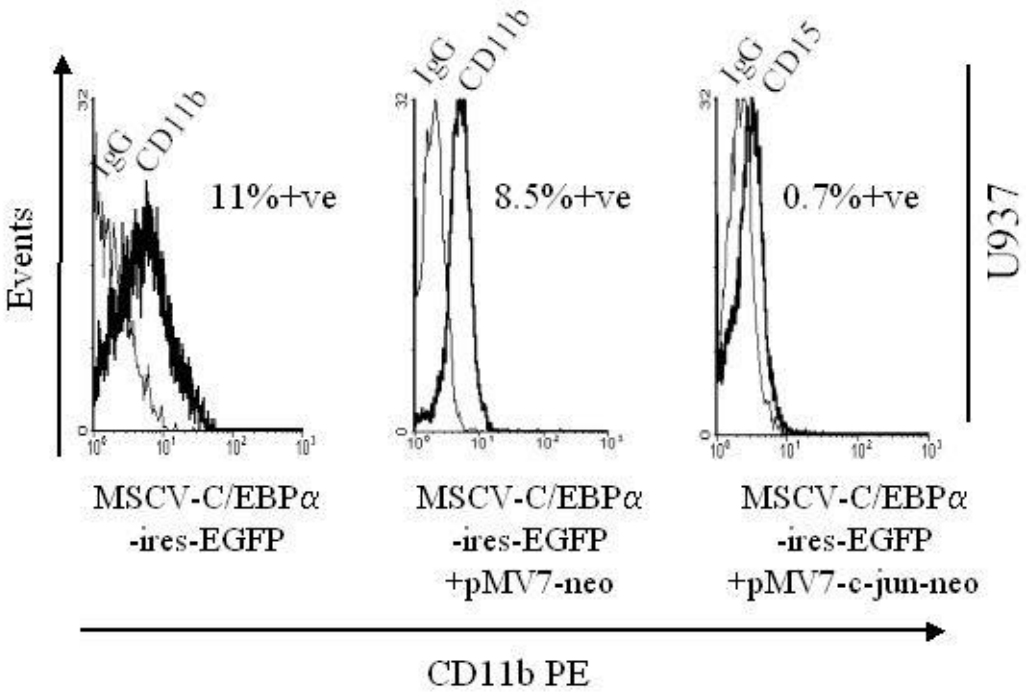

Figure 9D: Overexpression of c-jun blocks C/EBPα induced granulocytic differentiation. FACS analysis for CD11b-PE from U937 transduced cells along with its isotype control.

4.9.4. Morphology of the transduced myeloid cells.

In a similar experiment, cytospins from the transduced cells were made and the cells were stained using the May-Grunwald-Giemsa staining protocol.

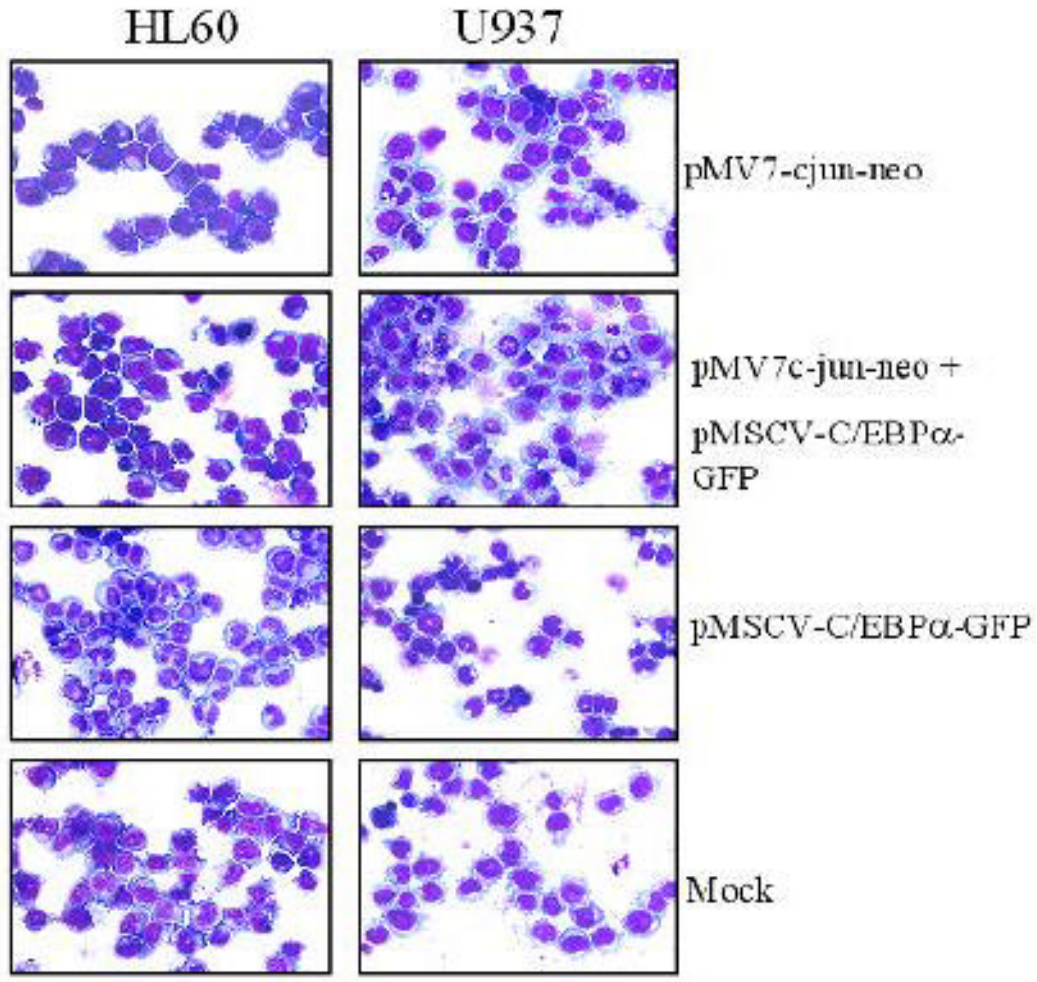

Figure 9E: Overexpression of c-jun blocks C/EBPα induced granulocytic differentiation. Morphological analysis of the transduced cells (HL60 and U937).

5. Discussion:

5.1.C/EBPα downregulates c-jun expression.

In this study, we have investigated the role of C/EBPα as a negative regulator of c-jun expression and transcriptional activity and its significance in the myeloid lineage commitment. Inducers of monocytic differentiation such as TPA, bryostatin 1, 1,25-dihydroxyvitamin D_3, okadaic acid were shown to increases c-jun activity by posttranslational events and increased synthesis (152,200,207,209,224). Some reports also state that expression of c-fos, another AP-1 member also increases on induction of monocytic differentiation. However, the increase in c-fos mRNA was found to be transient and not myeloid lineage specific (200,225). In addition, c-fos expression was not sufficient for the process of macrophage differentiation (205,226). Mice lacking c-fos have normal hematopoietic stem cells but exhibit altered B-cell differentiation due to an impaired bone marrow environment (226,227). These findings suggest the importance of c-jun in the myeloid differentiation program. Overexpression of c-jun in bipotential myeloid cells leads to macrophage like morphology (208). Using a C/EBPα inducible U937 cell line, we show that increase in C/EBPα expression results in a significant decrease in the levels of endogenous c-jun mRNA (Fig. 1B). c-jun protein level also decreases in the first 4 hrs of C/EBPα expression (Fig. 1C). At the same time, no change in c-fos expression was observed upon induction of C/EBPα expression (data not shown). The reciprocal pattern of expression for C/EBPα and c-jun was observed in C/EBPα knockout mice model and hepatocytes (Fig. 1A) (30,228). No detectable c-fos level was observed in the fetal liver samples, whereas adult macrophage and adult brain RNA samples showed a minimum c-fos expression (data not shown). The C/EBPα protein negatively regulates the human c-jun promoter in transient transfection assays in fibroblast as well as in myeloid cell lines (Fig. 2), thus stating that it was a general phenomenon and not cell line specific.

5.2.Mechanism of c-jun expression downregulation by C/EBPα through the proximal AP-1 site of the c-jun promoter.

Results from Fig. 3 suggest that C/EBPα does not recruit TSA-sensitive corepressor complexes to the c-jun promoter (Fig. 3A). Moreover, C/EBPα blocks the TPA-induced transactivation of c-jun promoter (Fig. 3B). Promoter mapping experiments (Fig. 4) suggested that region between bp-180 to bp-63 in the c-jun promoter was responsible for C/EBPα mediated c-jun promoter downregulation. Important transcription factor binding sites in this region were AP-1, CTF and SP-1 site. The CTF site has been shown to bind C/EBP transcription factors. However, it is not clear which of the C/EBP member could not bind to this site. C/EBPα was unable to bind to this CTF site (Fig. 5). This ruled out the possibility of direct regulation of the c-jun promoter by C/EBPα.

The human c-jun promoter has 2 AP-1 sites to which c-jun homo/hetero-dimer can bind. The proximal AP-1 site (pAP-1) in the promoter lies within the bp-180 to bp-63 regions. Earlier studies with the human c-jun promoter addressed the importance of the proximal AP-1 site in c-jun promoter to be sufficient for a maximal response to various signals (phorbol-12-myristate-13-acetate (TPA), serum, UV, E1A, and IL-1) (202,211). Using the human c-jun promoter, we show that C/EBPα blocks the autoactivation capacity of c-jun through the proximal AP-1 site (Fig. 6). On mutating this proximal AP-1 site, C/EBPα was unable to downregulate the c-jun promoter activity anymore (Fig. 6B). As observed in the gelshift experiment (Fig. 6E), c-jun was unable to bind to the proximal AP-1 site in the presence of C/EBPα. These results let to the idea that C/EBPα and c-jun might interact.

This is the first report showing C/EBPα and c-jun interaction in myeloid cells (Fig. 7). Furthermore, the leucine zipper domains of both proteins are required for this interaction (Fig. 8). DNA binding experiments (Fig. 6E) suggest that C/EBPα binding to c-jun inhibits latter from binding to its

consensus AP-1 site in the c-jun promoter. This was also confirmed by transient transfection experiments using the bp-79 c-jun promoter having only the proximal AP-1 site (Fig. 6B). It still needs to be addressed if the C/EBPα-c-jun interaction can block latter from binding to the AP-1 site in other c-jun regulated promoters as well. We think that the presence of other interacting partners of both these proteins (e.g. PU.1, AML-1, p300, C/EBPα etc.) might play an important role for such interactions in a promoter specific context.

5.3.Biological implication of C/EBPα-c-jun interaction in normal myelopoiesis and leukemia.

Previous reports have addressed the indispensability of C/EBPα in driving granulocytic differentiation, as well as its role in AML (47,60,61,67,229,230,236). Results shown in Fig. 9 indicate the importance of c-jun expression downregulation by C/EBPα in myeloid lineage commitment. If c-jun expression was high at the time of lineage commitment, it could block C/EBPα from committing these cells to the granulocytic lineage. c-jun is an important regulator of TPA mediated or independent macrophage lineage commitment (203,208). TPA induced monocytic differentiation was blocked by C/EBPα (67,236). These reports gave a hint that block in c-jun expression by C/EBPα was also necessary for preventing macrophage differentiation so as to commit the cells to the granulocytic lineage. Here we report the functional significance of the c-jun block by C/EBPα (Fig. 9). The normal granulocytic differentiation capacity of C/EBPα, as observed by increase in the CD15 and CD11b markers, was abolished upon overexpressing c-jun (Fig. 9). Thus, C/EBPα needs to inhibit c-jun expression and function to regulate granulocyte lineage specific genes. In addition, the morphological data (Fig. 9E) clearly shows that in presence of c-jun, C/EBPα is unable to make granulocytes. Concerning the importance of c-jun in myeloid-especially monocyte/macrophage lineage, it has been earlier shown that c-jun can induce partial macrophage like differentiation. Here, c-jun transduced cells do not show a clear monocyte like morphology. This could either be due to insufficient level of ectopic c-jun expression in the experiment performed, or, due to lack of additional transcription factor(s) that could help in full monocyte lineage commitment. Thus, C/EBPα needs to inhibit c-jun expression and function to regulate granulocyte lineage specific genes.

C/EBPα-c-jun interaction (Fig. 7,8) suggests that former might pull away c-jun from its other interacting partners, thereby, inhibiting c-jun's function. E.g., c-jun interaction with C/EBPα in monocytic differentiation by TNFα (231). Ubeda et al. (214) have reported that CHOP, a dominant negative regulator of C/EBP family members, can interact with c-jun through its leucine zipper domain. By such interaction, CHOP synergizes with c-jun to activate transcription through AP-1/TRE site. In contrast to these findings, we observe that the C/EBPα-c-jun interaction prevents c-jun from binding to the AP-1 site in c-jun promoter. The C/EBPα-c-jun complex formation probably pulls away c-jun from c-jun regulated genes.

Moreover, c-jun can also function as a co-activator of the PU.1 transcription factor important for the monocytic lineage (199). In presence of higher C/EBPα expression and TPA, U937 myeloid cells were unable to undergo macrophage differentiation (67,236). The explanation for this observation could be that C/EBPα inhibits c-jun from performing its co-activator role for PU.1 or from independent regulation of monocyte specific genes. This could be either by preventing c-jun from binding to the AP-1 site in the promoter, or by disrupting c-jun interaction with other transcription factors important for monocytic lineage commitment.

Pabst et al. have recently reported the important role of C/EBPα in acute myeloid leukemia (60,61). Dominant negative mutations in C/EBPα were observed in AML FAB-M2 patient samples in the absence of the AML1-ETO fusion protein. Higher c-jun mRNA levels in patient samples with C/EBPα mutations as compared to samples without C/EBPα mutations has been observed (data not shown). These results emphasize the importance of C/EBPα in controlling c-jun expression/transcriptional activity in AML. When c-jun is expressed in a deregulated manner, it has the potential to act as a protooncogene, and thus lead to hyperproliferation of the leukemic blasts.

The function of each transcription factor is differentiation stage dependent. Concentration of the protein plays an important role in deciding the cell fate, i.e. lineage commitment versus proliferation state. Few transcription factors, e.g. PU.1, program the cell for a specific lineage depending on its expression level. Similarly, c-jun could function either as a co-activator for PU.1 leading to differentiation or as a protooncogene causing proliferation. As observed in AML blasts, c-jun might act as a hyperproliferating agent, or in case of TPA induced macrophage differentiation c-jun might act as a transcription factor driving partial macrophage differentiation. The C/EBPα-c-jun interaction might disrupt the function of c-jun depending on the expression level of both these proteins.

5.4. Model for C/EBPα and c-jun regulation

In conclusion, we propose a model for the importance of C/EBPα in blocking c-jun expression and c-jun transactivation capacity (Fig. 10). Bipotential myeloid cells differentiate towards the granulocytic lineage upon overexpression of C/EBPα whereas the same cells have the potential for monocytic lineage commitment in presence of inducers such as TPA. TPA is known to transactivate c-jun and increase its expression. One of the important roles of c-jun is to act as a co-activator of transcription factor PU.1. c-jun on its

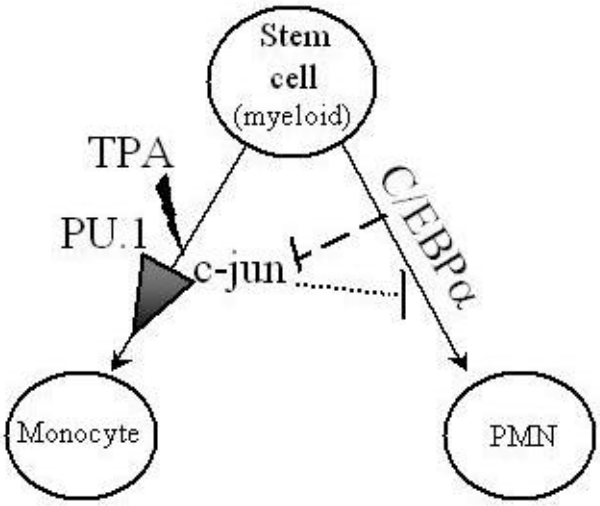

Figure 10A: Model for C/EBPα inactivating c-jun in granulocytic differentiation. Diagrammatic representation of myeloid bipotential stem cells that can differentiate to monocytes/macrophages on induction with TPA or become polymorphonuclear neutrophils (PMN) on overexpression of C/EBPα. TPA induction for macrophage differentiation requires increase in c-jun expression and c-jun transcriptional activity. c-jun acts as a co-activator of PU.1 leading to monocytic differentiation commitment. C/EBPα blocks the expression and transcriptional activity of c-jun, thus preventing TPA induced monocytic lineage commitment. At the same time, c-jun also blocks C/EBPα driven granulocytic lineage commitment.

own could also drive partial macrophage like differentiation. However, when C/EBPα and c-jun interact through their leucine zipper domains, the former prevents c-jun from functioning as a macrophage differentiation regulator. At the same time, such interaction could also arrest C/EBPα driven granulocytic lineage commitment (Fig. 10A).

Data so far suggests that this interaction blocks c-jun from binding to the AP-1 site of its own promoter, thereby inhibiting its expression and transcriptional activity (Fig. 10B). The significance of C/EBPα blocking c-jun DNA binding capacity on other c-jun regulated genes still needs to be addressed. Because of such sequestering of c-jun, C/EBPα might not only commit bipotential myeloid cells to granulocytic lineage, but also prevent these

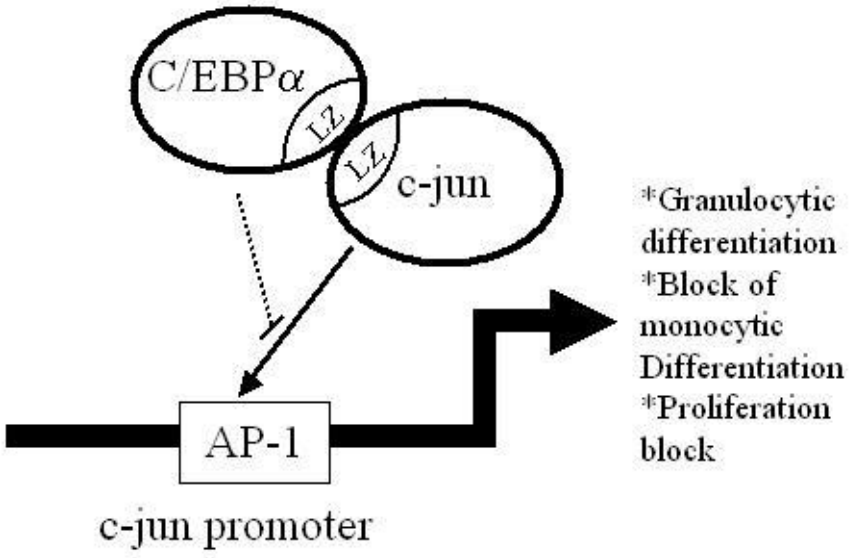

Figure 10B: Model for C/EBPα inactivating c-jun in granulocytic differentiation. Schematic representation showing interaction between C/EBPα and c-jun via their leucine zipper domains. This interaction prevents c-jun from binding to the proximal AP-1 site in its own promoter. c-jun interaction with C/EBPα and block in binding to its own promoter leads to downregulation of c-jun expression. This C/EBPα-c-jun interaction may lead to a block in monocytic lineage differentiation and proliferation.

cells from becoming monocytes/macrophages. We also suggest that the C/EBPα-c-jun interaction might abrogate the protooncogenic role of c-jun in causing proliferation.

6.1.SUMMARY.

The transcription factor C/EBPα is crucial for the differentiation of granulocytes. Conditional expression of C/EBPα triggers neutrophilic differentiation and C/EBPα can block TPA induced monocytic differentiation of bipotential myeloid cells. In C/EBPα knockout mice, no mature granulocytes are present. A dramatic increase of c-jun mRNA in C/EBPα knockout mice foetal liver was observed. c-jun, a component of the AP-1 transcription factor complex and a co-activator of the transcription factor PU.1, is important for monocytic differentiation. Here we report that C/EBPα downregulates c-jun expression to drive granulocytic differentiation. Ectopic increase of C/EBPα expression decreases c-jun mRNA level, and the human c-jun promoter activity is downregulated 8 fold in presence of C/EBPα. C/EBPα and c-jun interact through their leucine zipper domains and this interaction prevents c-jun from binding to DNA. This results in downregulation of c-jun's capacity to autoregulate its own promoter through the proximal AP-1 site. Overexpression of c-jun prevents C/EBPα induced granulocytic differentiation. c-jun expression was higher in AML M2 patients with dominant negative C/EBPα mutations in comparison to AML M2 patients without C/EBPα mutations. Thus, we propose a model in which C/EBPα needs to downregulate c-jun expression and transactivation capacity for promoting granulocytic differentiation.

6.2.ZUSAMMENFASSUNG

Der Transkriptionsfaktor C/EBPα ist essentiell für die Differenzierung von Granulozyten. Die konditionelle Expression von C/EBPα induziert die neutrophile Differenzierung. Überdies kann C/EBPα die TPA-induzierte Differenzierung von myeloiden Vorläuferzellen zu Monozyten blockieren. In C/EBPα knockout Mäusen gibt es keine reifen Granulozyten. In der fötalen Leber von C/EBPα knockout Mäusen konnte eine stark erhöhte Expression der c-jun mRNA beobachtet werden. c-jun ist eine Komponente des AP-1 Transkriptionsfaktorkomplexes, ein Koaktivator des Transkriptionsfaktors PU.1 und ist wichtig für die Differenzierung von Monozyten. In dieser Arbeit zeigen wir, dass C/EBPα die c-jun Expression herunterreguliert und somit die Differenzierung von Granulozyten induziert. Die Überexpression von C/EBPα reduzierte die c-jun mRNA Menge und die Aktivität des humanen c-jun Promotors war in der Gegenwart von C/EBPα 8-fach herunterreguliert. C/EBPα und c-jun interagieren über ihre Leucin-Zipper Domänen und diese Interaktion verhindert die DNA-Bindung von c-jun. Dies resultiert in einer verminderten Kapazität von c-jun, den eigenen Promotor über die proximale AP-1 Stelle zu regulieren. Die Überexpression von c-jun blockiert die durch C/EBPα induzierte granulozytäre Differenzierung. Die c-jun Expression war in AML-M2 Patienten mit dominant-negativen Mutationen in C/EBPα im Vergleich zu AML-M2 Patienten ohne Mutationen in C/EBPα erhöht. Aufgrund dieser Daten schlagen wir ein Modell vor, in dem C/EBPα die Expression und Transaktivierungskapazität von c-jun herunterregulieren muß, um die granulozytäre Differenzierung zu induzieren.

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Curriculum vitae

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