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**AML1/ETO Downregulates the Transcription Factor  
PU.1 in Acute Myeloid Leukemia**

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# **Chapter 1**

## **Introduction**

## **Chapter 1. Introduction**

### **1.1. Clinical significance of AML1-ETO**

Acute myeloid leukemia (AML) is a heterogeneous disease, as reflected by a high variability in the clinical presentation, blast cell morphology, therapeutic response and long-term prognosis (1). This heterogeneity extends to the molecular genetics lesions underlying the pathogenesis of AML. Although non-random clonal chromosomal aberrations are present in the majority of cases, each abnormality affects only a limited subset of cases. Nonetheless, recent studies have demonstrated that several chromosomal rearrangements, and the molecular abnormalities they produce, identify distinct patient subgroups with predictable clinical features and therapeutic responses (2).

One of the most frequent cytogenetic abnormalities in AML is the translocation  $t(8;21)(q22;q22)$ , which is found in approximately 10-15% of all AML cases and 40% of the M2 subtype (1). The translocation  $t(8;21)$  results in fusion of the AML1 gene on chromosome 21 to the ETO gene on chromosome 8. The encoded fusion protein consists of the N-terminal 177 amino acids of AML1 fused in frame to nearly the complete ETO protein (3). Patients with this subtype of AML typically present with FAB AML-M2 morphology, in which leukemic blasts have prominent aurer rods, strong myeloperoxidase positivity, homogeneous salmon-coloured granules, cytoplasmic granules, cytoplasmic vacuolisation, and prominent bone marrow eosinophilia (1). In addition, the leukemic blasts frequently have a distinct immunophenotype, characterized by



positivity for B-cell-associated marker CD19, as well as CD13, CD34 and CD56 positivity (4). This combination of findings suggests the presence of t(8;21). In fact, >90 percent of t(8;21)-containing leukemias have FAB AML-M2 morphology, and as many as 30-40 percent of FAB-M2 cases have this chromosomal translocation (1, 5). Interestingly t(8;21)-containing leukemias frequently form extramedullary tumours or chloromas (6). Despite this tendency for bulk disease the presence of t(8;21) is associated with high remission rate and prolonged disease-free survival in patients treated with standard induction and consolidation chemotherapy (7, 8). Cloning and characterizing of AML1-associated t(8;21) translocation led to the identification of AML1, which encodes the DNA binding subunit of AML1/CBF $\beta$ , a transcription factor that regulates a number of haematopoiesis specific genes and is essential for normal development of haematopoietic system.

## **1.2. Structure and function of AML1 protein**

AML1 is a member of the *Runx*-like transcription factors (*Runx*-1, -2, and -3) (9) named after the *Runx* protein that regulates segmentation during *Drosophila* embryogenesis (10, 11). The highly conserved *Runx* homology domain (RHD) is the DNA binding motif in this family of proteins. AML1 appears to act as an “organizing” factor for many promoters and enhancers by interacting with various co-activators and other DNA binding transcription factors (10, 13, 14). The N-terminal amino acids of AML1 protein inhibit the intrinsic ability of RHD (15) to bind to DNA. The sequence to which viral

protein PEBP2/CBF binds is different from its homolog in humans, The sequence bound by PEBP2/CBF in the polyoma enhancer is PuACCPuCA (16, 17, 18) while the human AML protein was determined to bind a similar consensus TG(T/C)GGT (19). The RHD is a target of mutation in familial platelet disorder (FPD) associated with predisposition to leukemia (20) and mutated in sporadic cases of AML and MDS without chromosomal translocations (21, 22, 23).

### **1.3. AML1 target genes and transcriptional mechanisms**

The AML1 protein (24) has been shown to be able to regulate a number of genes relevant to haematopoietic differentiation (25, 26) including those of the T-cell receptor  $\beta$  chain, cytokines such as IL3 and GM-CSF (27, 28) and granulocyte proteins such as myeloperoxidase (29), neutrophil elastase (30), TNF $\alpha$  (31), and granzyme B. Studies of these promoters as well as those of viruses indicated that in general AML1 is ineffective as an activator in the absence of cooperating factors (32) bound to adjacent promoter sites (33, 34). For example on the TCR promoter (35, 36) AML1 synergistically activates transcription through the binding and recruitment of ETS-1 into a ternary DNA-protein complex (37). The formation of such a complex is facilitated by the presence of the DNA binding protein TCF/LEF. On the M-CSF receptor promoter AML1 synergistically activates in combination with C/EBP $\alpha$  and PU.1 (38, 39, 40). In the case of C/EBP $\alpha$  the two proteins bind to each other and cooperatively bind to DNA. In contrast PU.1 and AML1 also synergistically

activate transcription but bind to each other weakly and do not bind to DNA in a cooperative manner (40). In this case the two proteins might cooperate to recruit CBP/p300 (41) to the promoter. On the myeloperoxidase promoter AML1 is an effective transactivator only when co-expressed with c-myb (42). In the TCR $\beta$  promoter (43) AML1 and ETS-1 bind to DNA cooperatively and direct binding of Ets to AML1 augments DNA binding by AML1. In a reciprocal manner, binding of the AML1 to ETS activates DNA binding by that protein due to intermolecular interaction between inhibitory domains within the two proteins that usually act in an intramolecular manner to inhibit their transcriptional activity (37, 44, 45). Similarly AML1 and AML1-ETO protein bind to a newly described myeloid ETS factor (MEF) and this factor can cooperate with AML1 to activate the IL3 promoter (46). In addition an interaction between AML1 and SMAD protein was described implicating AML1 in TGF signalling pathway (47, 48). Many interactions between AML1 and other transcription factors are mediated by RHD and therefore are expected to be preserved in the AML1-ETO. AML1-ETO was also shown to interact with other transcription factors like ear-2 in a similar manner as AML1 and downregulate their functions (49). AML1 needs to interact with its non-DNA (50, 51) binding partner CBF $\beta$  for its normal function (52). These interactions might be disrupted by AML1-ETO (53, 54, 55) including the nuclear localization of these interacting genes (56, 57). Therefore protein-protein interaction of AML1-ETO define the molecular pathogenesis of t(8;21) leukemia.

#### **1.4. t(8;21) and disruption of AML1 in leukemia**

The (8;21) translocation is associated with about 40% of the cases of M2 AML with karyotypic abnormalities (1, 58) and represents the most frequent anomaly in leukemia (18-20%). The cloning of break point and discovery of AML1 gene helped to spur great focus on this transcription factor in normal haematopoiesis as documented above. As the result of this fusion the AML1 gene on chromosome 21 is fused to the ETO/MTG8 gene on chromosome 8. The breakpoint within the AML1 locus is between exons 5 and 6 and topoisomerase II cleavage sites are found near the breakpoint region (59, 60, 61, 62). In all cases the resulting fusion gene yields a fusion transcript that encodes the initial 177 amino acids of AML1 linked to ETO (eight\_twenty\_one oncogene) sequences (63, 64, 65). ETO is a subject to alternative splicing so that different forms of AML1-ETO have been isolated with varying amounts of ETO sequence (66). In addition a transcript encoding a truncated form of AML1 was cloned from these patients, representing an alternatively spliced form of the AML1-ETO gene (62, 67, 68, 69, 70, 71). An AML1-ETO fusion transcript can be detected by reverse-transcription /PCR (72, 73) and can be used for diagnosis of the syndrome (66, 68, 74, 75, 76, 77, 78, 79). A reciprocal ETO-AML transcript has not been found. A large amount of literature has been devoted to the use of PCR for diagnosis and monitoring of t(8;21)-associated AML (80, 81, 82, 83).

The presence of AML1-ETO transcript correlates with some distinct

phenotypic characteristics of the leukemic blasts including a single Auer rod, abnormal cytoplasmic granules high-level expression of CD34 and CD19 and low levels of CD33 (84, 85). This suggests that the AML1-ETO fusion inhibits differentiation at a particular stage of myeloid development. AML1-ETO can be detected in the peripheral blood and marrow of patients with long-term complete remissions after treatment with chemotherapy or stem cell transplantation (3, 86, 87, 88, 89). AML1-ETO positive, multipotent progenitor cells that yield normal blood cell colonies, including B cells can be demonstrated in these patients along with normal progenitors (82, 90, 91). This strongly suggests that the AML1-ETO fusion event occurs in an early stem cell or progenitor cell (92) and that additional events are required for the cell to become fully transformed. With more standardization and use of quantitative techniques such as real-time PCR, determination of level of AML1-ETO transcript in the peripheral blood and marrow of the patients may be a useful tool for prediction of remission. After long-term remission and after stem cell transplant patients in some studies were found to become PCR negative (93, 94). Some of the inter-study difference in the detection of the AML1-ETO transcript in the remission patients may be due to the fact that some groups used two rounds of nested PCR which may have increased sensitivity and that different primer sets may have been used.

### **1.5. Structure of AML1-ETO and ETO proteins**

The ETO gene yields proteins of 577 and 604 amino acids. The ETO protein sequence has very high homology to that of *Drosophila* nervy domains

(NHR1-4). The first region (NHR1) shares similarity with the TAF110 and related TAF proteins. The second, NHR2 has predicted coiled structure with a heptad repeat of hydrophobic amino acids. The third region has notable homology with Nery and the other ETO family members but no other similarities to offer clues to its function. The fourth region has also been termed the MYND domain (MTG8, Nery, Deformed). This region has two non-classical zinc fingers, the first of the form CxxC-CxxC, and the second CxxCHxxX (95). *Drosophila* Deformed is a transcription factor but the MYND domain of the protein is believed to be a protein-protein interaction motif and no sequence-specific DNA binding activity has been noted for ETO protein. The ETO protein has a high content of serine and threonine rich sequences are found in the N and C-terminus of the protein that could affect protein stability. Specific kinases that act upon ETO can be isolated from cell extracts but their identity and physiological relevance is not yet known (96, 97, 98).

### **1.6. Transcriptional function of AML1-ETO and ETO**

The t(8;21) fusion generated in AML maintains the RHD of AML1 protein (10) and N-terminal region whose importance is not clear and fuses it to virtually all of ETO. This yields a protein of 752 amino acids which can be detected as a ~95 kDa band in t(8;21) cells and in the nucleus by immunofluorescence (99, 100, 101). From the description above it is apparent that several important domains of AML1 are lost in the fusion protein including (i) The C-terminal activation domains which interact with specific co-activators;

as well as (ii) interaction sites for the sin3 and TLE co-repressors (102, 103, 104, 105, 106). Furthermore (iii) the nuclear localization signal outside of the RHD is lost, as is the nuclear matrix-targeting signal. It would be predicted that the chimeric protein would have very different properties from wild-type protein, a fact borne out in many experiments. These experiments have led to identification of the leukemogenic mechanism of the AML1-ETO protein and have given insight into the possible normal role of ETO and related proteins in cellular metabolism.

Initial work with the AML1-ETO fusion indicated that it could bind to cognate AML1 binding site. The AML1-ETO fusion could be detected as a DNA protein complex in EMSA experiments using t(8;21) leukemic cells and could heterodimerize with CBF $\beta$  (107) even more efficiently than wild-type AML1 (101), perhaps sequestering this critical component away from wild-type AML1. AML1-ETO was localized to nucleus, indicating that sequences within ETO could direct the protein to the nucleus in absence of AML1 nuclear localization signal (108). AML1-ETO failed to activate the TCR $\beta$  reporter and actually blocked the ability of wildtype AML1 to activate the reporter. This occurred even when 25-fold less AML1-ETO was co-transfected with AML1 vector. Subsequent experiments on the GM-CSF promoter showed that AML1-ETO not only blocked the ability of AML1 to activate the promoter but also decreased the expression of the promoter below baseline levels. Mutagenesis of AML1-ETO protein indicated that both RHD and sequences within the C-terminus of ETO

were required for ability of AML1-ETO to repress activation by AML1. These data strongly suggested that AML1-ETO was a dominant repressive form of AML1 that did not simply work by competing for AML1 binding site but actively repressed transcription of AML1 target genes. Furthermore AML1-ETO could also inhibit transactivation by the AML2/RUNX3 protein and likely blocks the activity of RUNX2 as well (109). Hence the AML1-ETO fusion might broadly inhibit the genetic effects of the entire family of RUNX proteins that can be co-expressed in haematopoietic cells.

The mechanism by which ETO actively represses AML1-mediated transcription became apparent with the findings that ETO protein can interact with specific domains of the highly related N-CoR and SMRT co-repressors as well as mSin3A (110, 111, 112). ETO itself was a potent transcriptional repressor when fused to the GAL4 DNA binding domain and this effect was suppressed by HDAC inhibitors (112, 113). Furthermore ETO and AML1-ETO could be co-immunoprecipitated with histone deacetylase activity and can complex with HDAC1 and HDAC2 (114). This indicated that AML1-ETO fusion is an active repressor that recruits a multi-protein complex including HDACs to AML1 target genes. This would replace the AML1 complex that contains co-activators including the p300/CBP histone acetyl transferases. This is very similar to the leukemogenic mechanism in acute promyelocytic leukemia where fusion of novel proteins to the RAR leads to increased affinity for co-repressors and active repression of RAR target genes (115). The MYND/Zinc



finger motifs of ETO were critical for interaction with N-CoR and repression of the MDR1 promoter (112, 116) although NH3 (117) appeared to assist in the binding of related SMRT repressor (118). On other promoters the MYND domain appeared dispensable for repression suggesting the presence of additional co-repressor sites in the ETO protein that could be utilized in certain contexts. Deletion of NHR3 and particularly the NHR2/heptad repeat inhibited the ability of AML1-ETO to repress transcription also indicating other important contacts for co-repressors. The ability of AML1-ETO to interact with co-repressors was shown to be critical for its biological function. The ability of AML1-ETO to repress a target promoter was blocked by HDAC inhibitors (111). U937 cells transduced with wild-type AML1-ETO were blocked in their ability to differentiate with vitamin D3 and TGF $\beta$ . Deletion of the C-terminal N-CoR/SMRT interaction domain yielded a protein no longer able to block differentiation (110).

Recent studies with the PML-RAR $\alpha$  (119, 120) and PLZF-RAR $\alpha$  (121, 122, 123, 124) fusion proteins suggested that an aberrant tendency for homodimerization and formation of high molecular weight complexes contributed to the ability of these proteins to repress RAR targets and induce leukemia (125, 126). ETO can be isolated by sucrose sedimentation as a high molecular weight complex of up to 600kDa (127, 128). This may represent homo-multimers or heteromers of ETO bound to the related MTGR1 or MTGR2 proteins (129). N-CoR, mSin3A and HDAC1 can also co-sediment with ETO.

AML1-ETO can also be purified under native conditions as a higher molecular weight complex (126). Deletion of the NHR/heptads repeat prevented the formation of this higher molecular weight complex and such proteins were defective in transcriptional repression and inhibition of myeloid cell differentiation. This was at first attributed to a lack of homodimerization of ETO moiety of AML1-ETO (118) protein but more recent studies indicate that the NHR2 site overlaps with a binding site for mSin3A on ETO (127). In addition replacement of the HHT/NHR2 region of ETO with GCN4 homodimerization motifs yielded an AML1-ETO protein defective in repression (111). Such a protein would be unable to heterodimerize with ETO family members. The full repressional complex of ETO or AML1-ETO may indeed be a high molecular weight multimer, but it appears that mSin3A is a critical component of the complex and ubiquitously expressed ETO family members may be part of the complex as well. Multimerization *per se* may not be as important for the repression activity of AML1-ETO as its ability to recruit an array of co-repressors to the promoter. Through interaction with mSin3A and N-CoR as well as by more direct interaction, ETO recruits a number of HDACs to its targets.

AML1-ETO as well as AML1 was also reported to activate the *bcl2* (130, 131, 132, 133) and M-CSF promoters (134). This effect required both the MYND and NHR2 domains. The other genes that were upregulated by overexpression of AML1-ETO were *TIS11b* (135) and G-CSF receptor (136). Given the strong association of the ETO protein with a co-repressor complex and

tendency of AML1 itself to bind to sin3A (137, 138) it seems unlikely that AML1-ETO can recruit co-activators to promoter sites. Activation of the M-CSF receptor promoter was only observed when wild-type AML1 and AML1-ETO are co-expressed (139). This suggests that AML1-ETO may be removing mSin3A from AML1 bound to the promoter, tipping the balance of wild-type AML1 action more towards activation. AML1-ETO can block activation by other transcription factors as well. While AML1 cooperates with the C/EBP $\alpha$  protein to activate the NP3/defensin promoter, AML1-ETO blocks the activation of the promoter by either AML1 or C/EBP $\alpha$ . AML1-ETO binds to C/EBP $\alpha$  and repression of C/EBP $\alpha$  action required the co-repressor binding moieties of ETO (140). AML1-ETO may exert a dominant effect on transcriptional activators arrayed on a promoter. For example TGF $\beta$  (141) activation of immunoglobulin  $\alpha$ -chain promoter mediated by SMAD binding sites can completely suppressed by AML1-ETO. Hence wide spread disruption of gene activation pathways might be expected in t(8;21)-associated AML (139). Similarly AML1-ETO binds to the MEF, an ETS domain protein through a C-terminal portion of RHD of AML1 and inhibits transcription through MEF DNA binding sites (46). The domain of interaction of AML1-ETO in MEF-2 was mapped to be the ETS DNA binding domain that is conserved in all ETS family members. PU.1 is important ETS transcription factor which might also be functionally altered in presence of AML1-ETO, therefore we wanted to study if AML1-ETO interacts with PU.1 and thus inactivates the function of PU.1.

## 1.7. PU.1 gene structure

PU.1 is a member of the transcription factor family ETS that is expressed selectively in B cells, myeloid cells and macrophages (142, 143, 144, 145). PU.1 regulates the expression of several genes (146, 147, 148), including those encoding immunoglobulins, receptors and enzymes. The expression of these genes is crucial for macrophage and B-cell differentiation and for the functional activity of neutrophils. The PU.1 cDNA sequence revealed an open reading frame of 816 bases, which codes for a protein of 272 or 266 amino acids, depending on which initiation codon is used. Within the PU.1 protein is the ETS DNA-binding domain of ~85 amino acids, which is located near the C-terminus of the protein. Other domains within PU.1 include a glutamine-rich domain and acidic residues towards the N-terminal half of the protein, which are necessary for *trans*-activation (149), and a central PEST (proline, glutamic acid, serine and threonine-rich region) domain that is important for protein–protein interactions (150, 151). PU.1 is the most distantly related member of the ETS family of DNA-binding factors.

The gene for the human homologue of PU.1 has 85% sequence identity with the murine counterpart (152). The human PU.1-coding gene is located on chromosome 11, region p11.22 (71) and the murine gene encoding PU.1 is located on chromosome 2, band E3 (153, 154). The crystal structure of the PU.1–DNA complex has been determined (155). The domain is similar to the  $\alpha+\beta$  (winged) helix–turn–helix motif, but it has a novel loop–helix–loop architecture.

Four of the amino acids that interact directly with the DNA are highly conserved in all ETS family members: two arginines (R232 and R235) from the recognition helix lie in the major groove, whereas one lysine (K245) from the wing binds upstream of the core GGAA sequence, and another lysine (K219) from the turn of the helix–turn–helix motif binds downstream to the opposite strand (150). When isolated by immunoprecipitation from cells, PU.1 appears as multiple species: on SDS-polyacrylamide gel electrophoresis the PU.1 protein migrates as five bands ranging in size from 38.5 to 46.5 kDa. Some of this heterogeneity might be due to the presence of two potential translational start sites, at positions 1 and 7. Moreover, conversion to the slower-migrating forms was found to be influenced by the degree of phosphorylation: the PU.1 protein is potentially phosphorylated *in vitro* on five serine residues that are within consensus sites for casein kinase II (CKII): Ser41 and 45 in the acidic *trans*-activation domain and Ser132, 133 and 148 in the PEST region. Mutation of these serines had no effect on the translocation of the PU.1 protein to the nucleus, nor on its binding to a target DNA sequence. The phosphorylation of Ser148 is increased by lipopolysaccharide (LPS) treatment of RAW264.7 cells (156), and it has been reported that the stress-activated protein Janus kinase JNK1 can also phosphorylate PU.1 (157).

### **1.8. Expression and distribution of PU.1**

Perhaps one of the most interesting aspects of PU.1 is that its tissue distribution is restricted specifically to cells of the haematopoietic lineage (158,

159, 160). These include B cells, macrophages, mast cells, neutrophils and early erythroblasts (152, 161, 162, 163, 164, 165, 166, 167). *In vitro* studies have shown that PU.1 is expressed in multipotent, interleukin 3 (IL-3)-dependent haematopoietic progenitor cell lines (168), with downregulation of expression during differentiation into non-expressing lineages. The extinction of PU.1 expression in some lineages might be due to the presence of negative regulatory mechanisms that operate during cell differentiation. This has been suggested following studies using hybrid cells in which the expression of PU.1 was lost (169). The regulation of expression of the PU.1-coding gene has been studied in both human and murine cells. The proximal 120 bp are sufficient to mediate a high level of activity specifically in B cells and macrophages (169, 170) and three important motifs have been identified within this region. Two of them, an Ets-binding site and a variant octamer motif, were found to be most important for cell-type-specific promoter activity. An additional Sp1 motif stimulates basal activity of this promoter element. In B cells, both the octamer motif and the Ets-binding site combine to mediate a high level of activity, whereas in macrophages it is predominantly the Ets-binding site that confers promoter activity (166, 171, 165). This might account for the differences in PU.1 expression between macrophages and B cells (145, 172). Co-transfection of PU.1 into PU.1-deficient cells specifically *trans*-activated a minimal promoter containing the PU.1-binding site (172). This indicates that PU.1 can activate its own promoter elements in a self-regulatory loop.

### **1.9. *in vivo* models for importance of PU.1 in myeloid differentiation**

CD34<sup>+</sup> cells, which are upregulated during early phases of granulocytic/monocytic differentiation, express PU.1. When a competitive oligonucleotide containing the binding site of PU.1 was added to CD34<sup>+</sup> cells, the formation of haematopoietic colonies was blocked (173). The granulocytic and monocytic differentiation of CD34<sup>hi</sup> cells is associated with distinct changes in the expression of the PU.1-regulated molecules CD64 (FcγPI) and M-CSFR (174). This demonstrates that the PU.1 function is crucial for the development of some haematopoietic cells. To understand the biological role of the PU.1 protein *in vivo*, knockout mice carrying a mutation in the PU.1 locus were generated by gene targeting. In one of the studies, PU.1<sup>-/-</sup> mouse fetuses died at a late gestational stage (142, 143, 144). They had a multilineage defect in the generation of progenitors for B and T cells, monocytes and granulocytes (175).

In a second study, PU.1<sup>-/-</sup> mice were born alive but died of septicaemia within 48h (176) as they lacked mature macrophages, neutrophils, dendritic cells, osteoclasts, B cells and T cells. However, if these PU.1<sup>-/-</sup> mice were maintained on antibiotics, they began to develop apparently normal T cells 3–5 days after birth. Mature B cells, however, remained undetectable and no mature macrophages were observed in the tissues, although a few F4/80<sup>+</sup> (specific for macrophages) cells were detected. A few cells with the characteristics of neutrophils also began to appear on day 3. These neutrophils had normal morphology and expressed neutrophil markers such as Gr-1 and chloroacetate

esterase. However, they failed to differentiate completely as shown by the absence of transcripts for neutrophil secondary granule components and the absence of cellular responses to chemokines; these cells did not generate superoxide ions and were ineffective at bacterial uptake and killing (144).

Interestingly, PU.1<sup>-/-</sup> mice showed no osteoclasts (177) and no dendritic cells. The reason for the different phenotypes of the two strains discussed above (175, 176) is not known and they give rise to contradictory conclusions on the role of PU.1 in haematopoiesis. The key question is how does PU.1 induce differentiation of myeloid and B cells? Two different mechanisms can be considered: either PU.1 induces the commitment or, once cells are committed to the myeloid/B-cell lineage, PU.1 regulates the expression of several specific genes required for terminal differentiation, i.e. Ig in B cells or M-CSFR in macrophages. In the first mouse model, PU.1 would play a role in the early stages of commitment.

By contrast, the second model suggests that, although the PU.1 protein is not essential for myeloid and lymphoid lineage commitment, it is required for the normal differentiation of mature B cells and macrophages and also for the normal activity of neutrophils. Although some subpopulations, such as CD5<sup>+</sup> B cells, seem to be closely related to macrophages (178), B cells and myeloid cells are considered to be distantly related, derived from separate lineages and to have unique, specialized functions. Many genes induced during myeloid differentiation are not induced in B-cell differentiation (177).



Although macrophages and neutrophils share a common progenitor, the culture of early progenitors from PU.1<sup>-/-</sup> mice in the presence of IL-3, IL-6 and stem cell factor become neutrophils but not macrophages (179, 190). Early genes such as GM-CSFR or G-CSFR are expressed (181). However, no expression of genes associated with terminal differentiation (CD11b, CD64 and M-CSFR) were found in differentiated PU.1<sup>-/-</sup> embryonic stem (ES) cells (182). For myeloid development, the glutamine-rich *trans*-activation domain and the PEST region of the PU.1 molecule are required but not the potent acidic *trans*-activation domain (183). During this process there is a suppression of a master regulator of non-myeloid genes such as GATA-1 (158, 184) and *c-myb* (185).

Macrophages transfected with an antisense PU.1 expression construct showed a decrease in proliferation compared with controls (186). By contrast, macrophages transfected with a sense PU.1 expression construct displayed enhanced M-CSF-dependent proliferation. Following transfection with either a sense or an antisense PU.1 construct, macrophages showed an increased or a reduced level, respectively, of surface expression of receptors for M-CSF. The overall conclusion is that PU.1 regulates macrophage proliferation through the expression of the M-CSFR-coding gene. This hypothesis is supported by a report on vascular smooth muscle cells (187). A combination of platelet-derived growth factor BB and epidermal growth factor induces the stable expression of M-CSF receptors in normal vascular medial smooth muscle cells. These factors induce the expression of PU.1 mRNA and protein in such cells, and the use of PU.1

antisense oligonucleotides inhibited such growth factor-induced M-CSFR expression.

Recently it has been shown that c-Jun an AP-1 transcription factor can bind to the ETS DNA binding domain of PU.1 and co-activate PU.1 in transactivating its target genes (188). Therefore it is important to study the effect of AML1-ETO in this positive regulatory effect by PU.1/c-Jun. The leukemic cell lines U937 and HL60 differentiate along the macrophage lineage in response to phorbol esters such as TPA (189). It has been shown previously that this differentiation correlates temporally with expression of the c-Jun proto-oncogene, as does macrophage differentiation induced by other (non phorbol esters) compounds such as vitamin D3, bryostatin and okadaic acid (189). Furthermore, studies of differentiation of normal blood monocytes have also demonstrated a marked increase c-Jun expression during macrophage or granulocyte-macrophage colony-stimulating factor treatment (189). Since c-Jun is elevated only during macrophage/monocyte differentiation and not during granulocyte differentiation in HL60 cells, its induced expression is lineage specific and not simply the result of terminal differentiation. These data suggest that c-Jun plays a pivotal role in monocytic differentiation.

Earlier studies have demonstrated that c-Jun, a member of the AP-1 transcription factor family, can interact with PU.1 at the  $\beta 3\beta 4$  region in the DNA binding domain and co-activate PU.1's transcriptional activity (188, 190). Usually, c-Jun forms heterodimers with c-Fos in AP-1 transcription factor complexes (188, 190,

191). However, c-Fos does not cooperate with c-Jun in its co-activator function. In contrast, c-Fos completely blocks the co-activation of PU.1 by c-Jun. Since c-Fos does not physically bind to PU.1, it might compete with PU.1 for binding partner c-Jun. The requirement of the leucine zipper domain of c-Jun for the activation of PU.1 suggests that a c-Jun homodimer or heterodimer with a non-c-Fos partner mediates activation of M-CSF receptor promoter. These results suggest that c-Jun mediates its effects through direct interaction with PU.1 and not by DNA binding to an AP-1 site. However, this co-activation of PU.1 by c-Jun was observed to be JNK-independent activity of c-Jun (188). Therefore c-Jun plays an important role in the normal myelopoiesis. We wanted to understand AML1-ETO's influence on PU.1/c-Jun synergy.

# **Chapter 2**

## **Materials and Methods**

## **Chapter 2. Materials and Methods:**

**2.1 Cell Lines and Cell Culture:** Human kidney 293T cells were maintained in Dulbecco's Modified Eagle's Medium (GIBCO) supplemented with 10% Fetal Bovine Serum (GIBCO). Human monoblastic U937, HL60 and Kasumi-1 cells having t(8;21) were cultured in RPMI 1640 medium (GIBCO) supplemented with 10% Fetal Bovine Serum.

Bone marrow cells were isolated from the femurs of Balb mice. The femurs were removed and stripped of the soft tissue, and then crushed to release cells within marrow cavity. The red blood cells were lysed with a 0.15-mol/L solution of ammonium chloride. The cells were incubated in 0.15-mol/L ammonium chloride at room temperature for 15 minutes and centrifuged at 1200rpm for 10 minutes. The pelleted cells were resuspended in PBS and subjected to low-density mononuclear cell separation by incubating with density gradient (Histopaque 1083; Sigma) for 10 minutes and centrifuged at 600rpm for 30 minutes. The low-density mononuclear cells in the upper layer were then washed twice in PBS solution and resuspended in IMDM media (Stem Cell Technologies) with 20% Fetal bovine serum, 25nm IL3, 10nm IL6, 10nm CSF, 10nm GM-CSF, 10nm M-CSF, 10nm G-CSF.

## **2.2 Co-immunoprecipitation assay:**

**2.2.1. Preparation of nuclear extracts:**  $1 \times 10^7$  Kasumi-1 cells were centrifuged at 1200rpm for 7 minutes and the RPMI medium was removed. The cell pellet was resuspended in 10ml PBS and spin down as mentioned above. This step was

repeated thrice to remove the excess medium. After the final washing the cells were suspended in buffer A (20mM Tris pH8.0, 10mM NaCl, 3mM MgCl<sub>2</sub>, 0.1% NP40, 10% Glycerol, 0.2mM EDTA, 1mM DTT, 0.4mM PMSF, 1µg/ml antipain, 1µg/ml antipapain, 1µg/ml leupeptin, 1µg/ml pepstatin, 1µg/ml chymotrypsin) and incubated on ice for 25 minutes. After 25 minutes the nucleus was spin down at 2200rpm for 5 minutes temperature being maintained at 4°C. After removing the supernatant (cytoplasmic extract), the cells were suspended in buffer C (20mM Tris pH8.0, 400mM NaCl, 0.2mM EDTA, 20% glycerol, 1mM DTT, 0.4mM PMSF, 1µg/ml antipain, 1µg/ml antipapain, 1µg/ml leupeptin, 1µg/ml pepstatin, 1µg/ml chymotrypsin) for 15 minutes and incubated on ice to break open the nucleus. The nuclear extracts were then spin down at 14000rpm for 30minutes at 4°C to clear the extracts from nuclear membrane. The supernatant was then used for co-immunoprecipitation assay.

**2.2.2 Co-immunoprecipitation:** 40µl of protein-A agarose beads mixed with 2µg of anti-AML1 or anti-ETO or anti-PU.1 or mouse IgG, rabbit IgG and 0.5µl of co-immunoprecipitation buffer (50mM Tris pH7.5, 150mM NaCl, 1mM EDTA, 5% glycerol, 0.5% NP40, 0.4mM PMSF, 1µg/ml antipain, 1µg/ml antipapain, 1µg/ml leupeptin, 1µg/ml pepstatin, 1µg/ml chymotrypsin). This mixture was rotated for 2 hours at 4°C. After rotation, the mixture was spin down at 2000 rpm for 2 minutes at 4°C and supernatant was removed. This pellet containing the antibodies bound to the protein-A agarose beads was washed in 1ml co-immunoprecipitation buffer. After the washing the beads-antibody

complex is resuspended in 450µl of co-immunoprecipitation buffer. To this mixture 100µg of above nuclear extract was added and rotated for 1 hour at 4°C. After rotating for 1 hour the antibody-beads-protein complex was washed in 1ml co-immunoprecipitation buffer for 4 times.

After washing the remaining pellet consisting of antibody-beads-protein (protein of the respective antibody bound to the beads) was resuspended in protein loading buffer and denatured at 95°C for 5 minutes before loading the sample on to an SD-page gel. After running an SDS-page of the samples the proteins are transferred on to a nitrocellulose membrane overnight at 25 volts at 4°C. The membrane was then blotted for interacting protein.

Antibodies used were, rabbit IgG (Santa Cruz Biotechnology Cat# 2027), goat IgG (Santa Cruz Biotechnology, Cat# sc-2028), anti-AML antibody (Calbiochem, Cat# PC284), anti-ETO antibody (Santa Cruz Biotechnology, Cat# sc-9737), anti-PU.1 antibody (Santa Cruz Biotechnology, Cat# sc-352), anti-Gal4 antibody (Santa Cruz Biotechnology, Cat# sc-577), anti-N-CoR (Santa Cruz Biotechnology, Cat# sc-1611) and protein-A agarose beads (Santa Cruz Biotechnology, Cat# sc-2001).

## **2.3 Western Blot:**

**2.3.1 Preparation of whole cell lysates:** To generate cell lysates, 293T cells were plated in 100mm plates and transfected using the LipofectAMINE Plus kit (GIBCO) following the manufacturer's protocol. 24h post transfection, cells were harvested by removing the medium by aspiration. The cells were washed

twice in PBS and finally resuspended in 2ml of PBS. The cells were centrifuged at 2000rpm and supernatant was removed followed by addition of RIPA lysis buffer (1% NP-40 or Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 molar NaCl, 0.01 molar sodium phosphate, pH 7.2). The cells with RIPA lysis buffer were rotated at 4°C for 30 minutes. After rotation for 30 minutes, the cell lysates were spin down at 14000rpm for 15 minutes at 4°C. The supernatant after spinning down was collected as whole cell lysate. The protein concentration of each sample was estimated by Bradford assay. These lysates were then mixed with protein loading dye and denatured at 95°C for 5 minutes. The denatured protein lysates were then loaded on to a SDS-page with equal amounts of protein being taken (100µg). After running on an SDS-page gel, the proteins were transferred on to a nitrocellulose membrane overnight at 4°C and 25 volts. Following the transfer on to a nitrocellulose membrane an immunoblot was done with anti-PU.1, anti-ETO antibodies. Mouse monoclonal anti-β-tubulin purchased from Roche (Cat No. 1 111 876,) was used for immuno-blot assay as internal control. The following secondary antibodies were used: Protein A, peroxidase linked (NA 9120, Amersham Pharmacia), rabbit anti-Goat Peroxidase-Conjugated Immunoglobulins (Code No. P0449, DAKO) and anti-mouse IgG HRP (sc-2005, Santa Cruz). After blotting with the respective primary antibodies, the same membrane was washed and probed with secondary antibodies. The proteins were then detected by chemiluminiscense kit supplied by Amersham Pharmacia.



**2.4. Reporter Constructs and Expression Plasmids:** The human monocyte-specific M-CSF receptor promoter with and without AML1 binding site ranging from bp -88 to +71 with respect to the major transcription start site was subcloned in the firefly luciferase vector pXP2. pTK with PU.1 sites is a dimer of both PU.1 binding sites from granulocyte colony-stimulating factor receptor promoter from bp +28 to +54 subcloned into pTK81luc, a pXP2-based luciferase construct with TATA box only as minimal promoter. PTK with mutated PU.1 sites is a dimer of both PU.1 binding sites in granulocyte colony-stimulating factor receptor promoter from bp +28 to +54 (primers: 5'-TCG AGT GGT TTC ACA AAC TTT TGT TGA CGA GAG-3' and 5'-TCG ACT CTC GTC AAC AAA AGT TTG TGA AAC CAC-3') subcloned into pTK81luc. As an internal control plasmid for co-transfection assays, the pRL-0 construct derived from a Renilla luciferase gene (Promega) was used (192). Other vectors used were pECE-PU.1-murine, pcDNA.1-PU.1, pGEX-2TK-PU.1 or  $\beta 3\beta 4$  were kindly provided by Michael Klemsz (Indiana University Medical Center, Indianapolis, IN) and Richard Maki (the Burnham Institute, La Jolla, CA and Neuroscience Biosciences, San Diego, CA), pS3H-c-Jun was a gift by Micheal Karin (University of California, San Diego) and pSP65-c-Jun was kindly provided by Elisabetta Mueller and Bruce Spiegelmann (Dana Faber Cancer Research Institute, Boston, MA). AML1B-pCMV5 and CBF $\beta$ -pCMV5 were gifted by D.E: Zhang (Harvard Medical School, Boston, MA). AML1-ETO $\Delta$ C, AML1-ETO-C488S, AML1-ETO-C508S, PINCO-GFP, and PINCO-AML1-ETO-GFP

were kindly provided by P.G. Pelicci (European Institute of Oncology, Milan, Italy). AML1-ETO mutants, AML1-ETO $\Delta$ ZnF, AML1-ETO $\Delta$ ND, AML1-ETO $\Delta$ HHR and AML1-ETO-1-540 were kindly provided by Dr. Scott W. Hiebert (Vanderbilt-Ingram Cancer Center, Nashville, Tennessee). AML1-ETO-pcDNA3 was constructed by enzymatic digestion of AML1-ETO-pCMV5 with XbaI and sub-cloning the resulting 2.258 kb fragment into the XbaI-site of pcDNA3 plasmid (Invitrogen). PGCsam-ires-NGFR and PGCsam-Flag-PU.1-ires-NGFR were provided by Atshushi Iwama (Japan).

## **2.5. Transfection assays:**

**2.5.1 Transfection of 293T cells:** A day before transfection, 293T cells were trypsinized and washed with DMEM medium twice to remove trypsin. After washing twice, the cells were resuspended in DMEM medium and counted, followed by dilution to  $2 \times 10^4$ /ml concentration. 0.5ml of these cells were plated on a 24 well plate or  $5 \times 10^4$ /10ml on 100mm plate to get 50-80% confluency on the day of transfection. The DNA (0.45 $\mu$ g for 24 well plate or 4 $\mu$ g for 100mm plate) containing 0.1 $\mu$ g of PU.1, 0.15 $\mu$ g of MCSF receptor tagged to luciferase reporter gene or pTK(4PU.1) or pTKmut(4PU.1) or pXP2 or Serum response element tagged to luciferase reporter gene, 0.05 $\mu$ g pRLnull internal control plasmid and expression plasmids of c-Jun (0.05 $\mu$ g) or AML1b (0.05 $\mu$ g) or 0.02 $\mu$ g AML1-ETO or mutants of AML1-ETO (0.02 $\mu$ g of all the mutants) or 0.05 $\mu$ g of CBF $\beta$ , 0.1 $\mu$ g of PU.1, 0.1 $\mu$ g of Ras were mixed with PLUS reagent (correspondingly the concentrations of each of the plasmids were increased for

100mm plates) and rest of the DNA was filled with pCMV5 empty vector (to make the final concentration to 0.45µg of DNA for 24 well plates or 4µg of DNA for 100mm plates). This DNA-PLUS mixture was then mixed with LipofectAMINE reagent and diluted with serum free DMEM medium to make the final volume to 50µl. This mixture was incubated at room temperature for 15 minutes. DNA-PLUS-LipofectAMINE reagent complexes were then added to each well containing fresh medium on cells and mixed gently before incubation at 37°C at 5% CO<sub>2</sub>, for 3 hours. After 3 hours of incubation the volume of the medium was increased with normal growth medium to make 10% serum concentration. 24 hours post transfection the cells were washed with PBS and lysed in 1x lysis buffer supplied by Promega. Transient transfection of 293T cells were carried out with LipofectAMINE Plus Transfection Kit (GIBCO) in 24- or 6-well plates (192).

**2.5.2. Transfection of myeloid cells:** A day before transfection U937, HL60 or Kasumi-1 cell lines were passaged to a cell number of  $1 \times 10^5$ /ml concentration to give the cells good growth conditions (as usually maintained in culture). On the day of transfection  $1 \times 10^7$  cells were taken and washed twice in PBS and 500µl of fresh RPMI medium was added without serum following the washing. 20µg of DNA was added to the cells. Transient transfection was carried out by electroporating in RPMI 1640 medium at 980µF and 280V. After electroporation the cells were resuspended with 10ml of regular growth medium medium and incubated at room temperature for 10 minutes and shifted to 37°C incubator.

Firefly luciferase activities from the constructs M-CSF receptor promoter luciferase, pXP2, p(PU.1)4TK and p(mutPU.1)4TK and *Renilla* luciferase activity from the internal control plasmid pRL0 were determined 24 hours post transfection using the Dual Luciferase Reporter Assay System (PROMEGA). Results are given as means + standard error of mean from at least 3 independent experiments. Kasumi-1 cells were electroporated as mentioned above with pGCsam-FLAG-PU.1-ires-NGFR or with pGCsam-ires-NGFR vectors and sorted for NGFR positivity by FACS sorting (with anti NGFR antiserum from Chemicon, Cat# MAB5264 and mouse IgG RPE from Dako, Cat# R0439). HL60 cells were electroporated in a similar way as Kasumi-1 cells with PINCO-GFP, PINCO-GFP-AML1-ETO, pGCsam-ires-NGFR and pGCsam-FLAG-PU.1-ires-NGFR vectors. A day after the transfection the cells were sorted for GFP in case of PINCO-GFP and PINCO-GFP-AML1-ETO and for NGFR as mentioned above in case of pGCsam-ires-NGFR and pGCsam-FLAG-PU.1-ires-NGFR.

## **2.6. Protein interaction assays:**

**2.6.1. *in vitro* translation of proteins:** PU.1, c-Jun and AML1-ETO were *in vitro* transcribed and translated in presence of [<sup>35</sup>S] methionine (Amersham pharmacia) using the T7/SP6/T3 coupled reticulocyte lysate system (Promega) in accordance with the manufacturer's instruction. Following reagents were mixed for *in vitro* translation of the required proteins: 25µl of rabbit reticulocyte lysate, 2µl of reaction buffer (supplied by Promega), 1µl of RNA polymerase (SP6

RNA polymerase in case of AML1-ETO and c-Jun, T3 RNA polymerase in case of PU.1), 1µl of amino acid mixture minus methionine in case of radioactive translation, 2µl of [<sup>35</sup>S] methionine (in case of radioactive labeling of the proteins translated), 1µl of RNasin (Ribonuclease inhibitor), 1µg of template DNA (PU.1 or c-Jun or AML1-ETO) and final volume of the mixture was made up to 50µl with nuclease-free water. This mixture was incubated at 30°C for 90 minutes. The *in vitro* translated proteins were then analyzed by SDS-page gel analysis. 2µl of each of the *in vitro* translated proteins were denatured by adding protein loading dye and loaded on to a 10% SDS-page gel. After running the gel, the gels were dried and kept with Biomax films for 5 hours and developed to check the proteins.

**2.6.2. Production of GST-PU.1 and GST-β3β4 proteins:** pGEX-PU.1 or pGEX-β3β4 vectors were transformed into DH5α bacteria. These bacteria were grown overnight in 10ml Luria Broth (LB medium) medium at 37°C. This 10ml culture was then added to 100ml of LB medium and grown for 2-3 hours at 37°C. After the bacteria has grown, 1M IPTG was added to make a final concentration of 0.5 mM for 3 hours of induction of fusion protein. After the induction of fusion protein for 3 hours, the bacteria were spun down at 4500rpm at 4°C and resuspended in 5ml of NETN buffer with protease inhibitors (20mM Tris pH8.0, 150mM NaCl, 1mM EDTA, 0.5% NP40, and 0.4mM PMSF, 1µg/ml antipain, 1µg/ml chymotrypsin, 1µg/ml Leupeptin). The cells are then sonicated 5-6 times, 15 seconds each time on ice to prevent overheat. The 5ml of the

bacterial lysate is split in four 1.7ml Eppendorf tubes and spin down at 14000rpm for 5 minutes at 4°C. The supernatant lysate is then incubated with GST beads.

0.5ml of prehydrated GST beads (beads volume) (Glutathione Crosslinked 4% beaded agarose, Sigma Catalog Nr: G4510) were washed thrice in NETN buffer and above bacterial supernatant was mixed with these beads and rocked at 4°C for 1 hour. The beads are then washed thrice again in NETN buffer (2000rpm for 5 minutes) and finally suspended in 1ml buffer. The GST, GST-PU.1 and GST-β3β4 proteins were run on a 12% SDS-Page gel and stained with comassie blue staining to check the amount of protein and purity of proteins.

**2.6.3. Protein-protein interaction assay:** Required amounts of *in vitro* translated proteins (c-Jun or AML1-ETO) were incubated with 25μl of GST-PU.1 or GST-β3β4 and incubated at 4°C for 1 hour. After 1 hour, the GST-PU.1 or GST-β3β4 and *in vitro* translated protein complexes were washed twice with 1ml of NETN buffer. After washing the complexes were denatured by adding protein loading dye and loaded on to 12% SDS-page gels. These gels after running were dried and incubated with Biomax films for 3 hours and developed to check the protein interactions.

## **2.7. Electrophoretic mobility shift assay:**

**2.7.1. Labeling of the DNA probes:** 20μg of single stranded probes were mixed with 10μl of 10X annealing buffer (20mM Tris pH7.5, 10mM MgCl<sub>2</sub>, 50mM NaCl, 1mM DTT). This mixture was then diluted to achieve 1X concentration of

annealing buffer with Dnase free water. DNA and annealing buffer mixture was then heated to 95°C for 5 minutes and allowed to cool down to room temperature slowly to allow the single stranded oligos to anneal together forming double stranded DNA. 10µl of above mixture containing double stranded DNA was mixed with 70µl of 1X annealing buffer to give a final concentration of 50ng/ml of double stranded oligonucleotides. The final labeling mixture has to following mixture: 1µl of 50ng/µl double stranded DNA, 4µl of GIBCO BRL 5X forward reaction buffer, 5µl of  $\gamma^{32}\text{P}$ -ATP (Life Technologies), 2µl of T4 polynucleotide kinase and 8µl of Dnase free water to make final volume of 20µl reaction mixture. This mixture was incubated at 37°C for 60 minutes. After incubation for 60 minutes, the reaction mixture was applied to Quickspin columns (Sephadex columns for radioabeled DNA purification, Boehringer Mannheim) and centrifuged for 5 minutes at 2800rpm. The collected volume after centrifugation contained the radiolabeled double stranded DNA.

**2.7.2. DNA binding assay:**  $\gamma^{32}\text{P}$ -ATP labeled double-stranded oligonucleotides of PU.1 DNA binding site (oligo 1:5'-TCG ACC CTA GCT AAA AGG GGA AGA AGA GGA TCA GC-3', oligo 2: 5'-GGA TCG TTT TCC CCT TTC TTC TCC TAG TCG AGCT-3') and AML-1 binding site (oligo 1: 5'-TCG CGT ATA GAT CAT CTT TGT GGT ACT ACG ACA AAC-3', oligo 2: 5'-AGC GCA TAT CTA GAA ACA CCA TGA TGC TGT TTC-3') were used for EMSA. 1µl of the radiolabeled probe was incubated with 2.5µl of *in vitro* translated proteins with or without respective antibodies (anti-PU.1 antibody for PU.1 or anti-ETO

antibody for AML1-ETO) and with or without 200X competitor (non-radiolabeled probe). This reaction mixtures were incubated at room temperature for 30 minutes and the loaded on to non-denaturing 5.2% placrylamide gel. This gel was dried and kept along with Biomax-MS films for 1 hour before developing to check the DNA binding of the *in vitro* translated proteins.

## **2.8. Retroviral transduction:**

**2.8.1. Production of retroviral particles:** A day before the transfection, ecotrophic Phoenix cells were plated in 100mm plates at a density of  $5 \times 10^6/10\text{ml}$  to obtain 80% confluency. 24 hours post plating the cells were transfected with as mentioned for 293T cells with PINCO, PINCO-AML1-ETO, PU.1 pGCsam-FLAG-PU.1-ires-NGFR or pGCsam-ires-NGFR retroviral vectors. After 12 hors of transfection the regular DMEM medium was removed and OPTI-MEM medium was added. 12 hours after addition of OPTI-MEM medium the cells were tripnized and washed in serum free DMEM medium. The cells were then resuspended in 5ml of regular DMEM medium and plated in 1:10 dilution on to 100mm plates. 12 hours after plating the cells,  $2\mu\text{g/ml}$  puromycin was added as a selection marker for 72 hours. After 72 hours the puromycin selection medium was removed and IMDM medium (growth medium for bone marrow cells) was added. After 2-3 days the virus particles were collected.

For estimation of the titer value of the virus collected, 293T cells were plated at  $1 \times 10^4/\text{ml}$  density in a 24 well plate. The virus collected was added on the cells 24 hours post plating. 24 hours post incubation with the virus particles,



the cells were trypsinized and checked for NGFR positivity (in case of pGCsam-FLAG-PU.1-ires-NGFR or pGCsam-ires-NGFR) or GFP positivity (in case of PINCO or PINCO-AML1-ETO). The percent of the cells positive were calculated and correspondingly the titer value was also calculated. The mice bone marrow cells were collected freshly from Balb/c mice and incubated with the virus particles for 3 hours, followed for spin culture for 1 hour at 2000rpm. After spin culture the cells were resuspended in fresh growth medium. This procedure of viral transduction was repeated for 3 following days and on 4<sup>th</sup> day the GFP or NGFR positive cells were selected by FACS analysis.  $1 \times 10^4$  GFP and NGFR positive cells were then plated on 60mm plate. After 3, 6, and 12 days of plating live cells were counted by Trypan blue staining.

**2.9. RNA extraction and Light Cycler Real-time-PCR analysis of patient samples:**  $1 \times 10^5$  patient cells were spin down and 800 $\mu$ l of lysis buffer (4M guanidinium thiocyanate, 25mM Sodium citrate, 0.5% N-Lauroyl sarcosine, 0.7% Mercaptoethanol) was added to lyse the cells. 500 $\mu$ l of acid phenol/chloroform was added to the lysed cells, followed by rotation for 15 minutes at room temperature. The lysate was then centrifuged at 13000rpm for 30 minutes at 4°C and the aqueous phase was collected. The RNA was precipitated by adding 5 $\mu$ l of glycogen/EDTA, 400 $\mu$ l of isopropanol for 30 minutes at -80°C and spin down at 13000rpm for 30 minutes at 4°C. The pelleted RNA was washed with 80% ethanol and resuspended in 20 $\mu$ l of RNase free water. The RNA of 10 patients, 5 AML M2 without any chromosomal

aberrations and 5 samples of AML M2 with t(8;21) as a single abnormality were collected. Equal amounts of RNA were reverse transcribed at 37°C for 90 minutes with 2µg of RNA. For amplification of PU.1, and G6PD, the Light Cycler-Primer set for Human SPI-1 (Search-LC Heidelberg) was used. Real-time PCR was performed using a Light Cycler Instrument (Roche) and final results are given as ratio of PU.1/G6PD calculated concentrations (193).

**2.10. FACS analysis:**  $1 \times 10^6$  cells (Kasumi-1, HL60, mice bone marrow cells) transfected or transduced with pGCsam-FLAG-PU.1-ires-NGFR or pGCsam-ires-NGFR were incubated with 10µl of recombinant PE-conjugated mouse monoclonal CD11b or FITC-conjugated mouse monoclonal CD14 antibodies (Pharmingen, Cat# 555388 and 555397 respectively) in 100µl PBS for 60 min on ice, washed in PBS and analyzed on a FACScan flow cytometer (Becton Dickinson) using Cellquest software.

293T cells (trypsinized and washed twice in PBS), Kasumi-1, HL60 and mice bone marrow cells were washed in PBS following by incubation with GFP or NGFR antibodies and similar FACS analysis was performed as above.

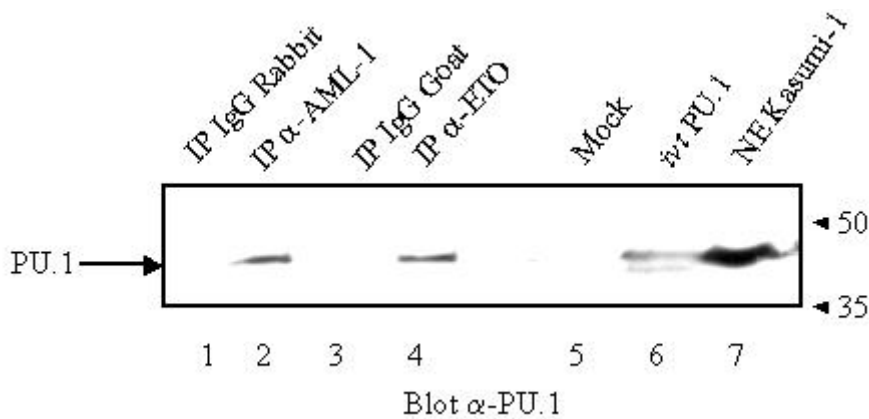
## **Chapter 3**

### **Results**

## Chapter 3. Results :

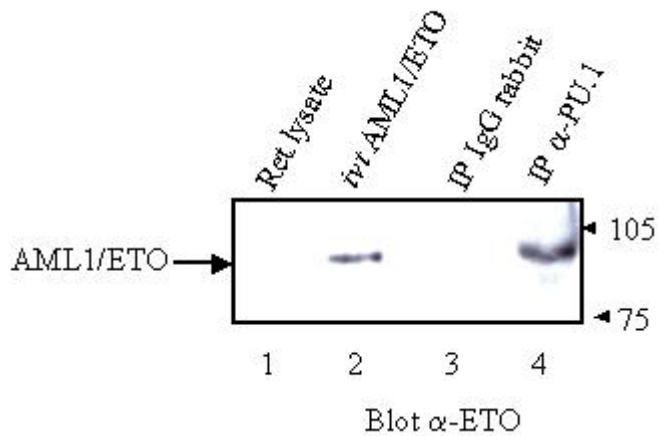
### 3.1 AML1-ETO interacts with PU.1 and blocks its transcriptional activity:

To determine whether PU.1 interacts with AML1-ETO, we performed co-immunoprecipitation assays in Kasumi-1 cells, a human cell line containing t(8;21). PU.1 co-precipitated with both the AML1 and ETO antibodies (Fig. 1a, lane 2 and 4), but not with the IgG control (Fig. 1a, lane 1 and 3), suggesting that PU.1 interacts with AML1-ETO *in vivo*.



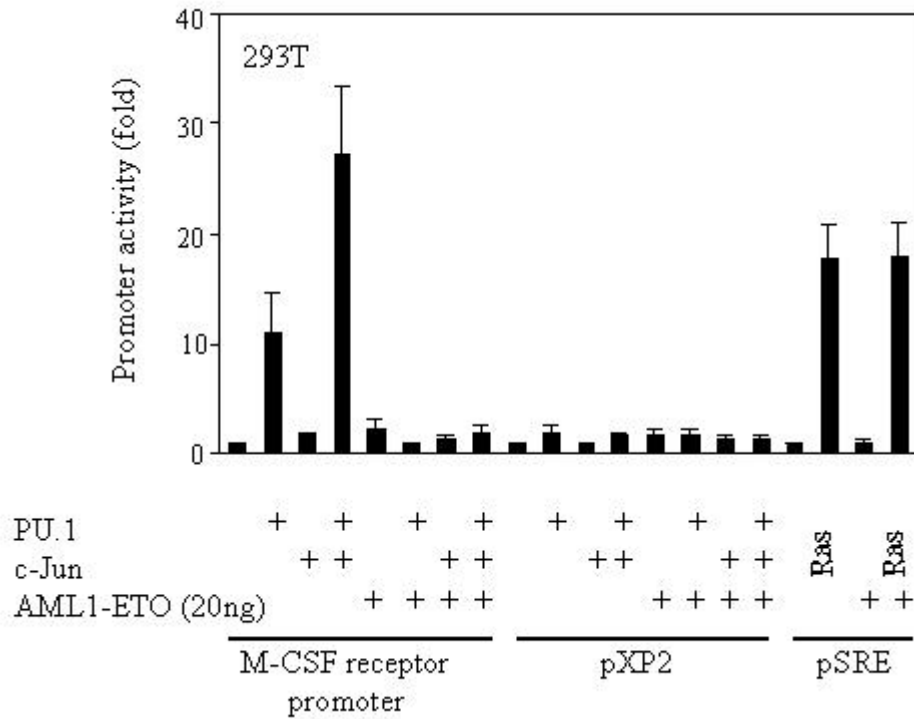
**Figure 1a: AML1-ETO binds to PU.1 *in vivo*:** Kasumi-1 nuclear lysates were co-immunoprecipitated with rabbit IgG (lane 1), anti-AML1 antibody (lane 2), goat IgG (lane 3), ETO antibody (lane 4), mock (lane 5). The immunoprecipitates (IP) were subjected to SDS-PAGE along with *in vitro* translated PU.1 (lane 6), and nuclear extract (NE) of Kasumi-1 cells (lane 7) and further subjected to immunoblotting with PU.1 antibody.

A similar experiment was performed with anti-PU.1 antibody (Fig. 1b), AML1-ETO co-precipitated with PU.1 antibody (Fig. 1b, lane 2) but not with rabbit IgG control (Fig 1b, lane 3).



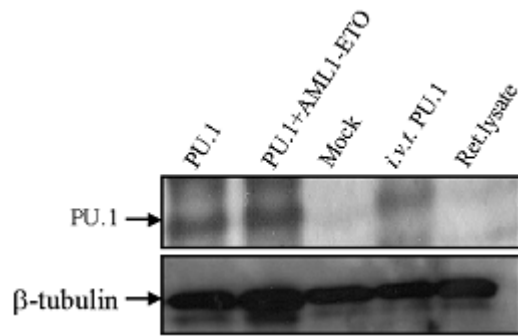
**Figure 1b: PU.1 binds to AML1-ETO *in vivo*:** Kasumi-1 nuclear lysates were immunoprecipitated with anti-PU.1 (lane 2), rabbit IgG (lane 3) and subjected to SDS-PAGE along with *in vitro* translated AML1-ETO (lane 1), blotted with anti-ETO antibody.

To understand the functional impact of this *in vivo* interaction, we performed transient transfection assays in 293T cells. A M-CSF receptor promoter luciferase reporter construct was transactivated 12-fold by PU.1 and 28-fold by PU.1/c-Jun (Fig. 1c), which was completely abrogated by AML1-ETO. AML1-ETO had no effect on Serum Response Element (pSRE)/Ras activity nor on the empty vector (pXP2).



**Figure 1c: AML1-ETO inhibits PU.1 s transactivation capacity:** 293T cells transiently transfected with M-CSF receptor promoter or the promoterless vector pXP2 or pSRE (Serum Response Element) and with expression plasmids of PU.1, c-Jun, AML1-ETO and Ras. Luciferase activities (fold) were determined 24h after transient transfection and normalized to the activities of the internal control plasmid pRL0.

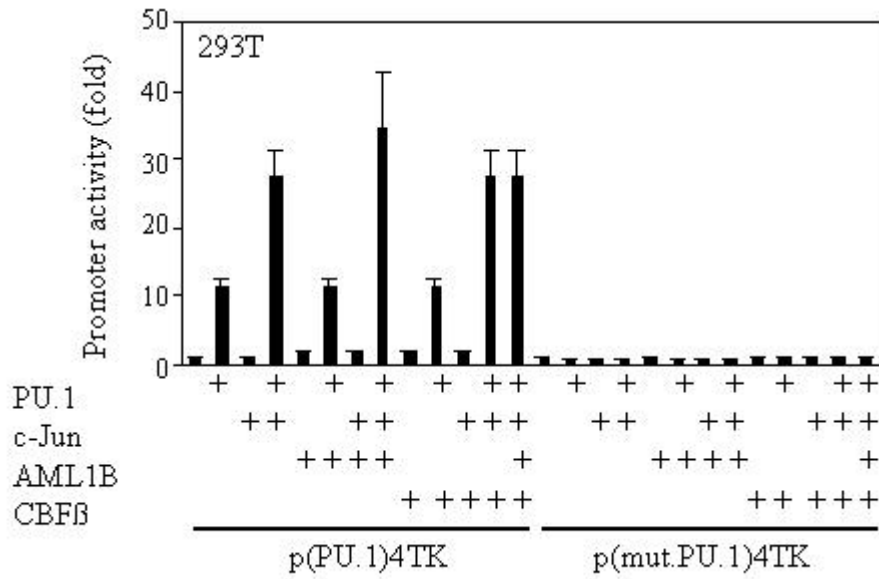
The expression levels of PU.1 did not change in presence of AML1-ETO (Fig. 1d) suggesting that the transcriptional activity of PU.1 is downregulated, but not the expression in the transient transfection assays.



**Figure 1d: AML1-ETO does not change the expression of co-transfected PU.1:** The lysates of above transfection (Fig. 1c) were subjected to SDS-PAGE followed by immunoblot assay with PU.1 antiserum. Lysate of the transfection with PU.1 and M-CSF receptor promoter (lane 1), lysate of transfection with PU.1, AML1-ETO and M-CSF receptor promoter (lane 2), mock transfection (lane 3), *in vitro* translated PU.1 (lane 4) and reticulocyte lysate (lane 5). As a loading control the same blot was stripped and blotted for  $\beta$ -tubulin.

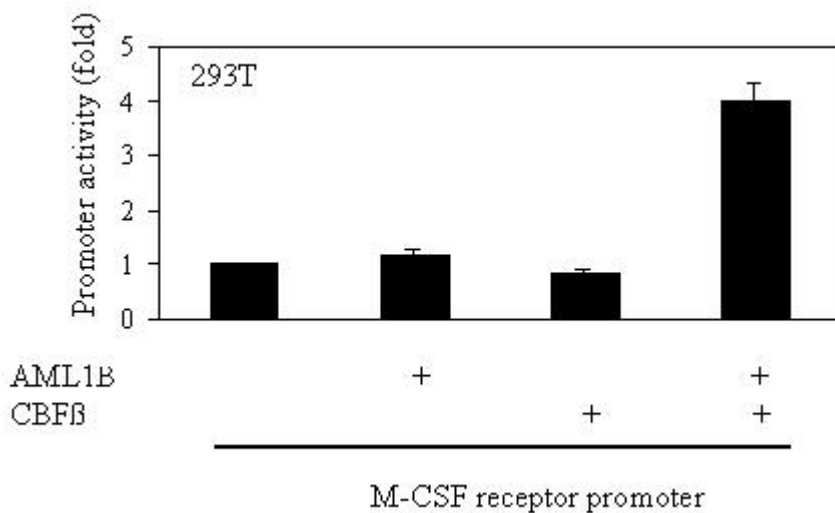
### **3.2 AML1 does not affect PU.1 or PU.1 c- un s transactivation capacity:**

AML1-ETO retains 177 N-terminus amino acids of AML1, suggesting that AML1 might also have similar affect on PU.1. Transient transfection assays in 293T cells were performed as mentioned above (Fig. 1c) with p(PU.1)4TK, p(mutPU.1)4TK (control vector), or M-CSF receptor promoter and expression plasmids of PU.1, c-Jun., AML1b, or CBF $\beta$ . Results (Fig. 1e) show that AML1 did not affect the PU.1 or PU.1/c-Jun transactivation capacity.



**Figure 1e: AML1B does not effect PU.1 s or PU.1 c- un s transactivation capacity:** 293T cells were transfected with p(PU.1)4TK, p(mutPU.1)4TK, M-CSF receptor promoter and expression plasmids of PU.1, c-Jun, AML1B, CBFβ. Luciferase activities (fold) were determined 24h after transient transfection and normalized to the activities of the internal control plasmid pRL0.

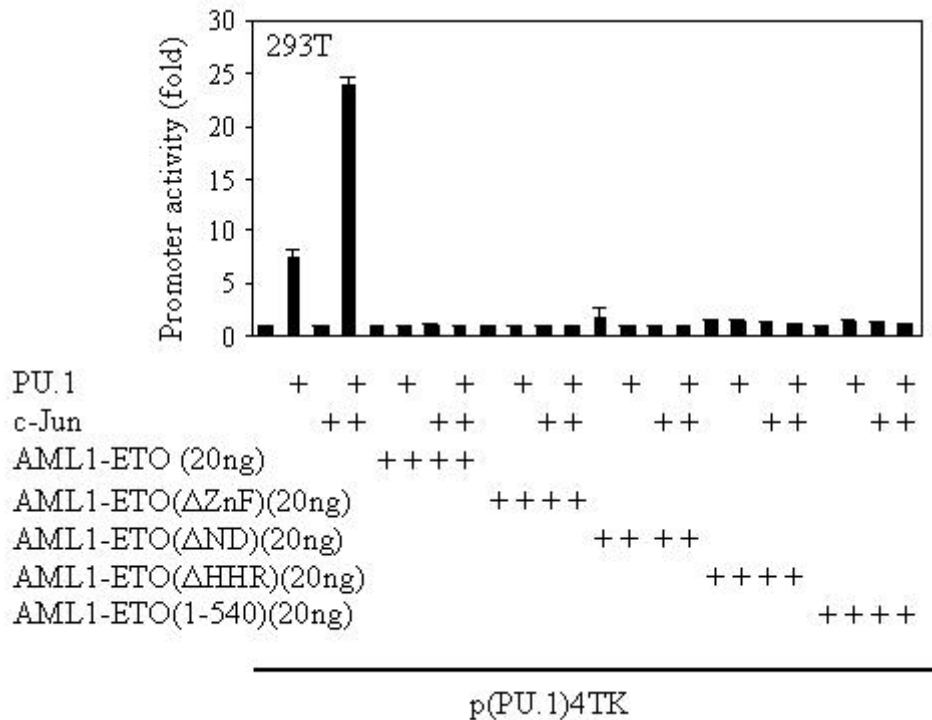
AML1 could transactivate the M-CSF receptor promoter 4-fold in presence of CBFβ (Fig. 1f).





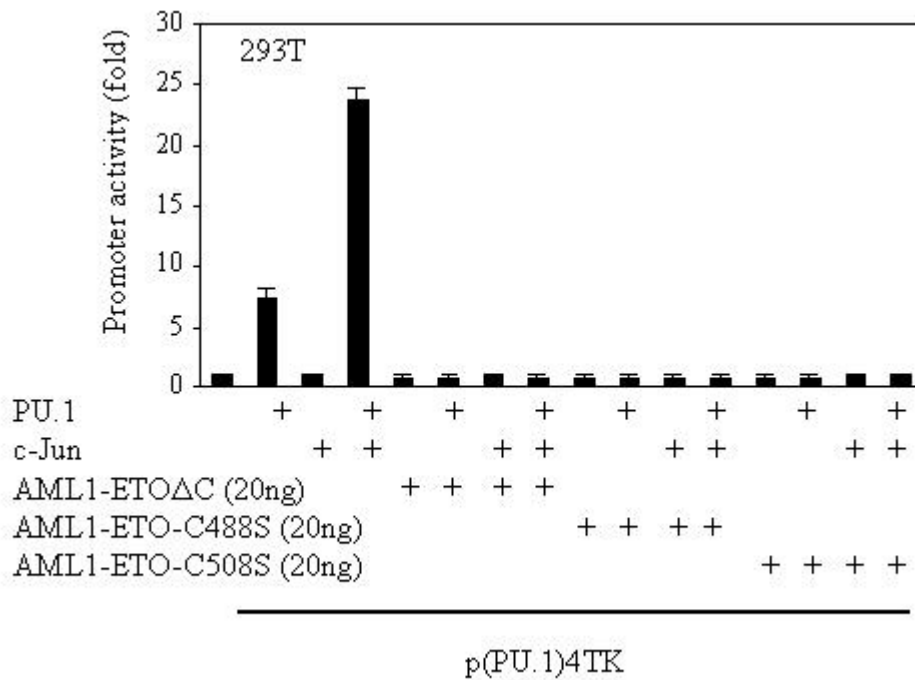
**Figure 1f: AML1b and CBF $\beta$  transactivate the M-CSF receptor promoter:** In the same experiment of Fig. 1e, the M-CSF receptor promoter luciferase reporter construct and expression plasmids of AML1b and CBF $\beta$  were transfected. AML1b and CBF $\beta$  transactivated the M-CSFR promoter four fold.

**3.3. Co-repressor binding domains in AML1-ETO are not required for downregulation of PU.1's transactivation capacity:** To investigate if co-repressor recruiting domains of AML1-ETO were necessary to downregulate PU.1's transactivation capacity, we used various mutants of AML1-ETO which were shown not to recruit co-repressors. Transient transfection assays with AML1-ETO $\Delta$ ZnF (zinc finger domain), AML1-ETO $\Delta$ ND (nervy domain), AML1-ETO $\Delta$ HHR and AML1-ETO-1-540 (Fig. 2a) (110) show that these regions are not important for downregulating PU.1's transactivation capacity



**Figure 2a: Co-repressor binding domains in AML1-ETO are not required for downregulation of PU.1's transactivation capacity:** AML1-ETO mutants lacking ZnF (Zinc finger domain), ND (nervy domain), HHR (hexa heptad repeat) and AML1-ETO 1-540, which does not have any co-repressor binding sites, were transfected as mentioned in Fig. 1c. All the mutants downregulated PU.1's transactivation capacity.

We have performed transient transfection assays with other mutants AML1-ETOΔC, AML1-ETO-C488S and AML1-ETO-C508S, which were shown not to interact with mSin3A and HDAC1 (110). These mutants were also found to downregulate PU.1's activity (Fig. 2b). The above data (Fig. 2a, Fig. 2b) suggests that the co-repressor recruiting domains in AML1-ETO are not necessary for downregulating PU.1's transcriptional activity.

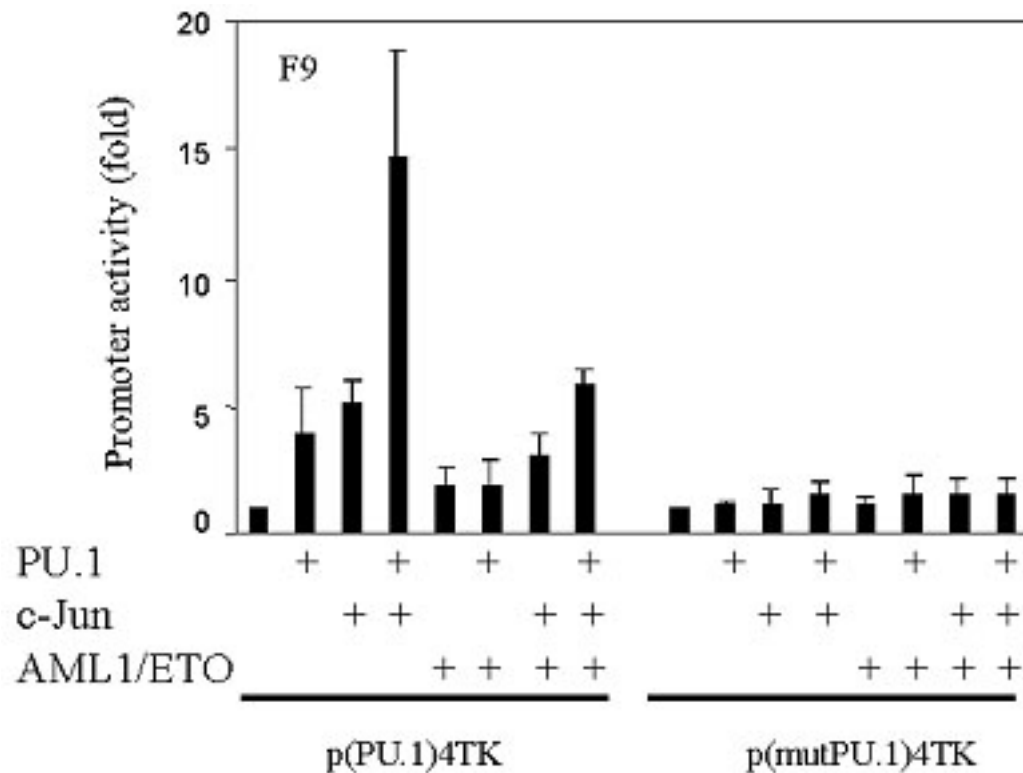


**Figure 2b: N-CoR DAC1 binding sites are not important for downregulating PU.1 s transactivation capacity:** AML1-ETO mutants which are shown not to interact with N-CoR and HDAC1 were transfected in a similar way as in Fig. 2e. These mutants still downregulated PU.1's transactivation capacity.

### 3.4. AML1-ETO competes away c- Jun by interacting at the $\beta 3\beta 4$ region of

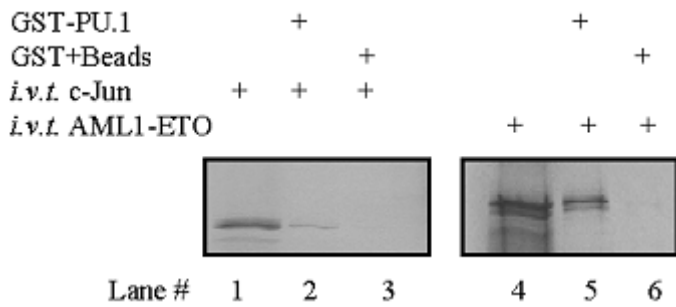
**PU.1:** Since our experiments suggest that the co-repressor recruiting domains of AML1-ETO are not necessary for downregulation of PU.1's transactivation, we asked whether PU.1's co-activator c-Jun (188) is disrupted for co-activating PU.1. We performed transient transfection assays in F9 cells, which lack endogenous c-Jun expression thus serving as a tool for investigating if co-activation of PU.1 by c-Jun is affected. We used a minimal promoter with multimerised binding sites of PU.1, p(PU.1)4TK, and similar results (Fig. 3a) suggest that AML1-ETO downregulates the co-activation of PU.1 by c-Jun. C-Jun upregulated p(PU.1)4TK promoter in F9 cells (Figure 3a), which might be

due to presence of noncanonical sites in the promoter construct or unknown factors in these cells collaborating with c-Jun. However, this does not influence the final conclusion.



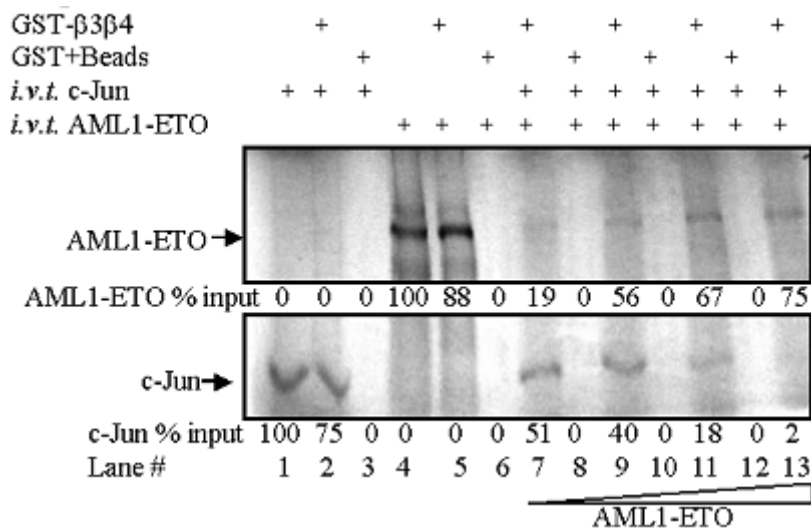
**Figure 3a: AML1-ETO downregulates the co-activation of PU.1 by c-Jun:** F9 cells were transfected in a similar way as in Fig. 1c. PU.1/c-Jun transactivate the minimal promoter p(PU.1)4TK but in presence of AML1-ETO this co-activation was downregulated.

The above results also suggest that AML1-ETO might bind to the same region of PU.1 as c-Jun binds to, thus preventing co-activation of PU.1 by c-Jun. Upon performing the *in vitro* protein-protein interaction assay, we found that AML1-ETO interacted with PU.1 (Fig. 3b, lane 5).



**Figure 3b: AML1-ETO interacts with PU.1 *in vitro*:** GST-PU.1 (full length) was incubated with *in vitro* translated c-Jun (lane 2) and *in vitro* translated AML1-ETO (lane 5).

As suggested by the transient transfection assays AML1-ETO might interact with the same domain as c-Jun does in PU.1 and displace c-Jun from interacting to PU.1, thus downregulating the co-activation of PU.1 by c-Jun. To verify this, the  $\beta 3\beta 4$  domain of PU.1 fused to GST and *in vitro* protein-protein interaction assays were performed. AML1-ETO interacted with GST- $\beta 3\beta 4$  (Fig. 3c, lane 5).



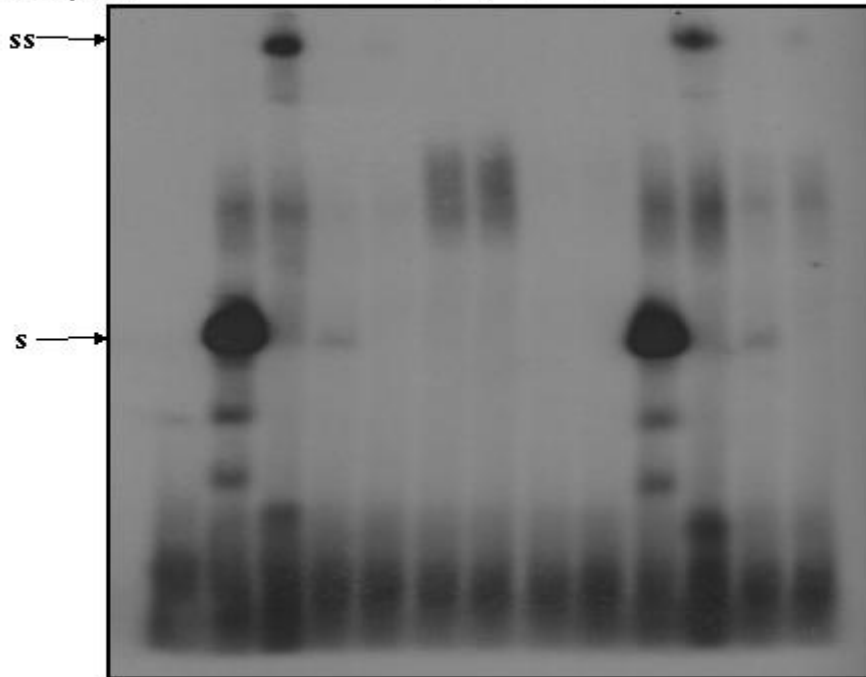
**Figure 3c: AML1-ETO competes away c-Jun from binding to  $\beta 3\beta 4$  domain of PU.1:** GST- $\beta 3\beta 4$  was incubated with *in vitro* translated c-Jun (10 $\mu$ l) and AML1-ETO (5 $\mu$ l) (lane 2 and 5 respectively). Increasing amounts of AML1-ETO (7.5 $\mu$ l-12.5 $\mu$ l) were incubated with

GST- $\beta 3\beta 4$  (lane 7-13) keeping the amount of c-Jun constant (10 $\mu$ l). The band intensities were quantified by densitometry.

Increasing amounts of *in vitro* translated AML1-ETO competed away almost all of c-Jun bound to GST- $\beta 3\beta 4$  (Fig. 3c, lane 7-13). We found that AML1-ETO binds to PU.1 (Fig. 3b) at the same region as c-Jun binds and competes away c-Jun from binding to GST- $\beta 3\beta 4$  (Fig. 3c). We have further quantified the amount of c-Jun and AML1-ETO proteins bound to GST- $\beta 3\beta 4$  (given as % input of proteins), which showed that amount of c-Jun was almost reduced to 2%.

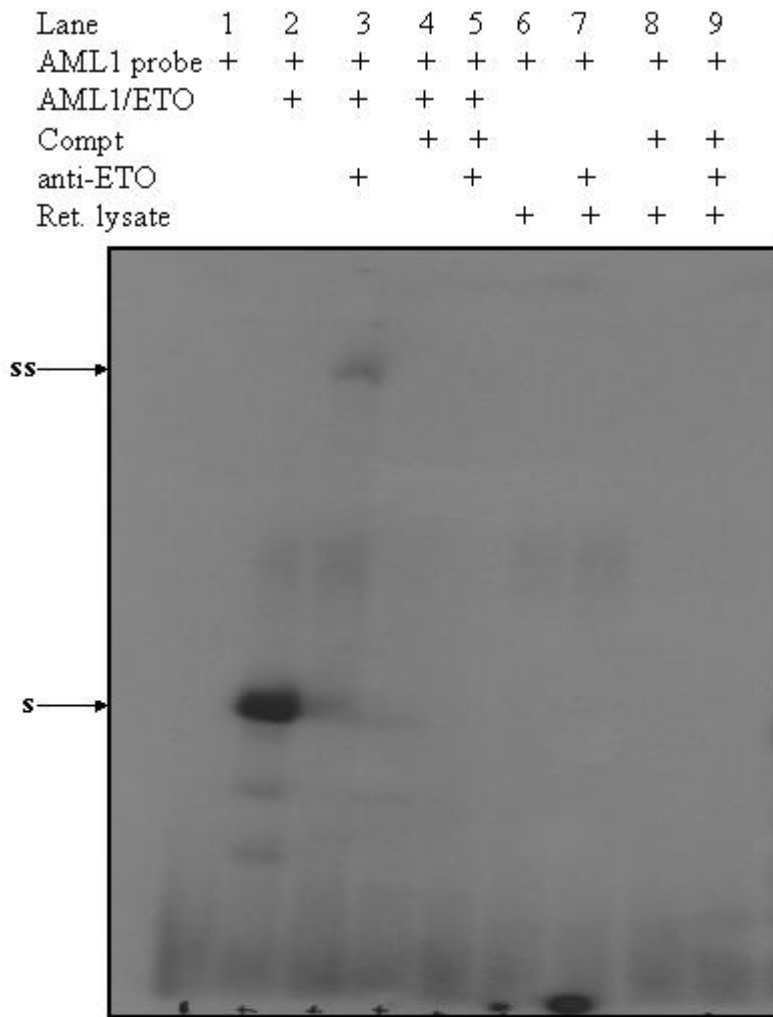
**3.5. AML1-ETO does not change the DNA binding of PU.1:** We next addressed the question if AML1-ETO could change the DNA binding capacity of PU.1, which is essential for PU.1's transcriptional activity. We performed an electrophoretic mobility shift assay (EMSA) with *in vitro* translated proteins and respective oligonucleotides having DNA binding sequences for PU.1 and AML1-ETO. The *in vitro* translated proteins bind specifically to their respective oligonucleotides (Fig. 4a and Fig. 4b), and even in presence of AML1-ETO, no change in the DNA binding of PU.1 was observed (Fig. 4a).

Lane	1	2	3	4	5	6	7	8	9	10	11	12	13
PU.1 probe	+	+	+	+	+	+	+	+	+	+	+	+	+
PU.1		+	+	+	+					+	+	+	+
anti-PU.1			+		+		+		+		+		+
Compt				+	+			+	+			+	+
AML1/ETO										+	+	+	+
Ret. Lysate						+	+	+	+				



**s** Shift  
**ss** Super shift

Figure 4a: **AML1-ETO does not change the DNA binding of PU.1:** PU.1 consensus binding sequence from CD11b promoter was chosen and labelled with  $\gamma$ -dATP (lane 1), incubated with *in vitro* translated PU.1 (lane 2), *in vitro* translated PU.1 and anti-PU.1 antibody (lane 3). As a competitor, unlabelled probe was used in 100 molar excess with (lane 5) and without anti-PU.1 antibody (lane 4). To check if this binding and supershift is specific for PU.1, similar experiments were performed with rabbit reticulocyte lysate (lanes 6, 7, 8, 9). In presence of AML1-ETO, PU.1 could still bind to its DNA binding sequence (lane 10) and supershifts with anti-PU.1 antibody (lane 11).



s Shift  
 ss Super shift

Figure 4b: **AML1-ETO binds to AML1 binding site:** In a similar experimental condition, AML1-ETO binds to an AML1 binding sequence (lane 2), and supershifts with an anti-ETO antibody (lane 3); as a competitor 100 molar excess of unlabelled probe was used (lane 4), with anti-ETO antibody (lane 5). The same experiment was also performed with rabbit reticulocyte lysate (lanes 6, 7, 8, and 9).

### 3.6. AML1-ETO downregulates PU.1 s transcriptional activity in U937

**cells:** We next investigated if AML1-ETO could also downregulate PU.1's transcriptional activity in myeloblastic U937 cells. U937 cells were transfected



with the wild type M-CSF receptor promoter, M-CSF receptor promoter without AML1 binding site, minimal promoter having PU.1 binding sites (p(PU.1)4TK), minimal promoter with mutated PU.1 binding sites (p(mutPU.1)4TK) and empty vector with or without AML1-ETO. We observed that all the promoters were downregulated by AML1-ETO without any effect on the empty vector (Fig. 5a).

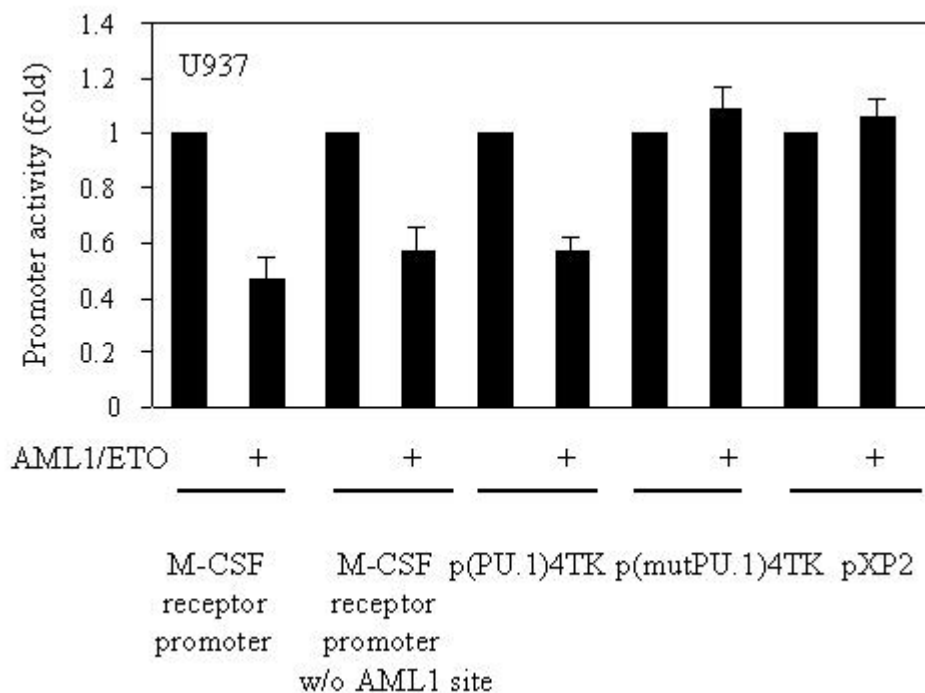


Figure 5a: **AML1-ETO downregulates PU.1 s transactivation in U937 cells:** U937 cells were electroporated with wild type M-CSF receptor promoter, M-CSF receptor promoter without (w/o) AML1 binding site, p(PU.1)4TK, p(mutPU.1)4TK or pXP2 with and without AML1-ETO.

**3.7. Low expression of PU.1 target genes in patients with t(8;21):** Due to the fact that PU.1 is a self-regulatory gene (8), we hypothesized that, if AML1-ETO can downregulate the transcriptional activity of PU.1, the expression of PU.1 itself might be decreased. We measured PU.1 expression levels by real-time PCR in AML M2 patients with and without t(8;21). The expression of PU.1 was

found to be 2.7 fold less in patients with t(8;21) than in patients without translocation t(8;21) (Fig. 5b).

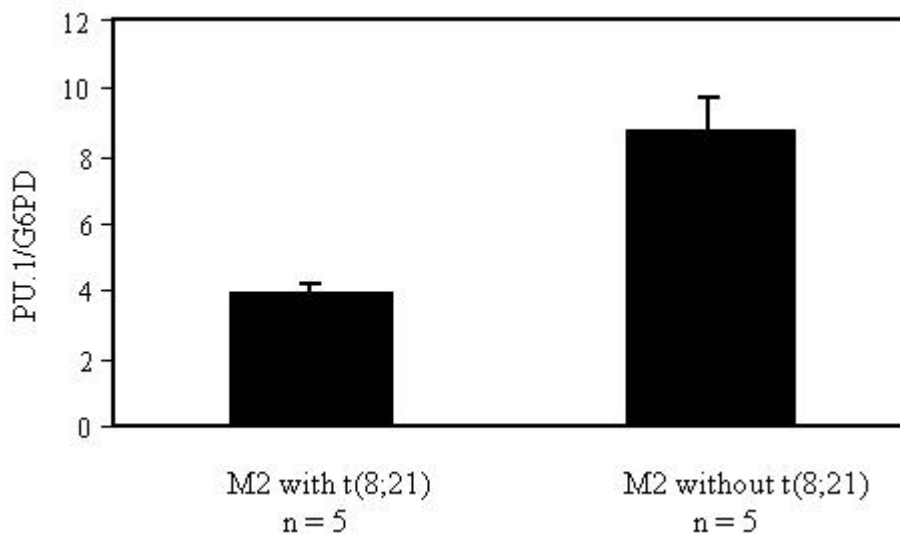
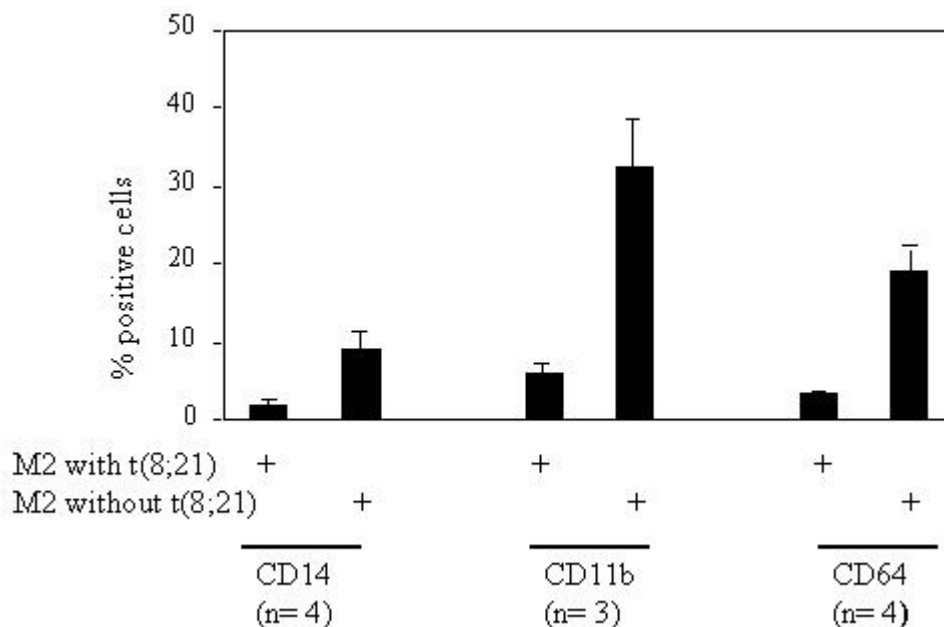


Figure 5b: **PU.1 mRNA is selectively downregulated in AML1-ETO positive leukemic blasts:** Real-Time PCR for PU.1 expression in AML patients (n = number of patient samples) was performed with G6PD as an internal control. Exact amounts of PU.1 mRNA was calculated and plotted. The Y-axis of the plot denotes the PU.1/G6PD ratio and X-axis is number of patients with or without AML1-ETO.

To verify the above results in patients with other target genes of PU.1, the cell surface markers (CD14, CD11b and CD64) that are regulated by PU.1 (2, 43, 40) were chosen for FACS analysis. In patients with t(8;21), CD14, CD11b and CD64 were 4.6, 5.4, and 5.8 fold less expressed in comparison to patients with normal karyotype (Fig. 5c). Regulation of CD11b promoter by PU.1 has been shown and further analysis (by TRANSFAC analysis to identify potential transcription factor binding sites in the promoter) of the promoter showed that CD11b promoter has C/EBP $\alpha$  and AML1 binding sites. Similar analysis of CD14 and CD64 promoters showed that these promoters have PU.1 binding

sites but not C/EBP $\alpha$ , AML1 or MEF binding sites. Therefore CD14 and CD64 downregulation might be through downregulation of PU.1's activity by AML1-ETO.



**Figure 5c: Low expression of PU.1 regulated genes in AML M2 patients with t(8;21) compared to AML M2 patients without translocation:** The patients (n = number of patients) with t(8;21) have less positive cells for cell surface markers regulated by PU.1 as compared to patients without t(8;21). CD14 and CD64 promoters have no putative C/EBP $\alpha$ , AML1 or MEF binding sites but have PU.1 binding sites.

**3.8. AML1-ETO causes proliferation in mouse bone marrow cells:** We transduced mouse bone marrow cells with PU.1 (pGSAM-NGFR-Flag-PU.1), AML1-ETO (PINCO-AML1-ETO-GFP), PU.1 and AML1-ETO. The cells transduced with AML1-ETO increased in cell number rapidly over 12 days, as did the cells overexpressed with AML1-ETO and PU.1 (Fig. 6a).

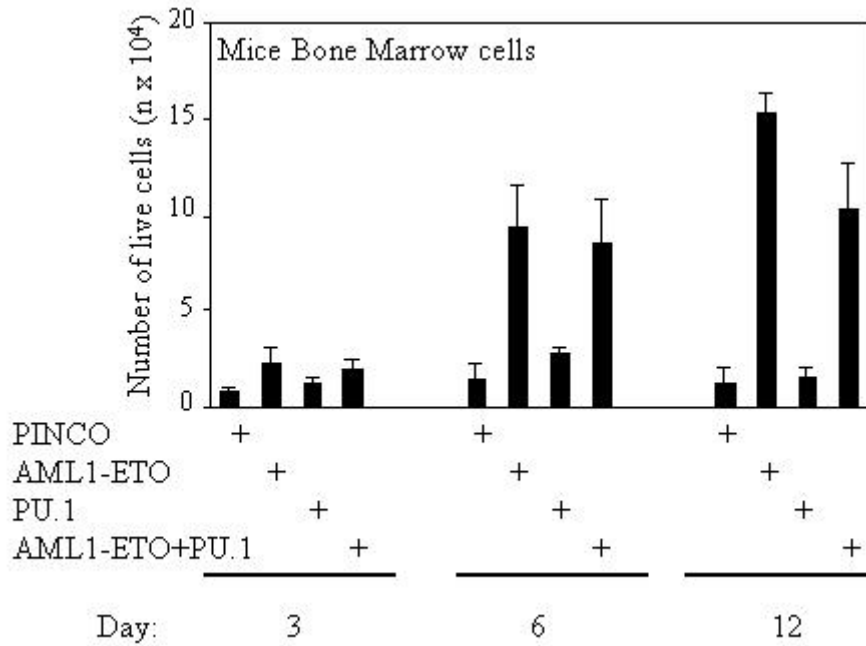


Figure 6a: **AML1-ETO causes proliferation in mouse bone marrow cells:** Live transduced mouse bone marrow cells with PU.1, AML1-ETO or PU.1 and AML1-ETO were counted on 3, 6, and 12 days after Trypan blue staining.

Expressions of the transduced genes are shown in Fig. 6b.

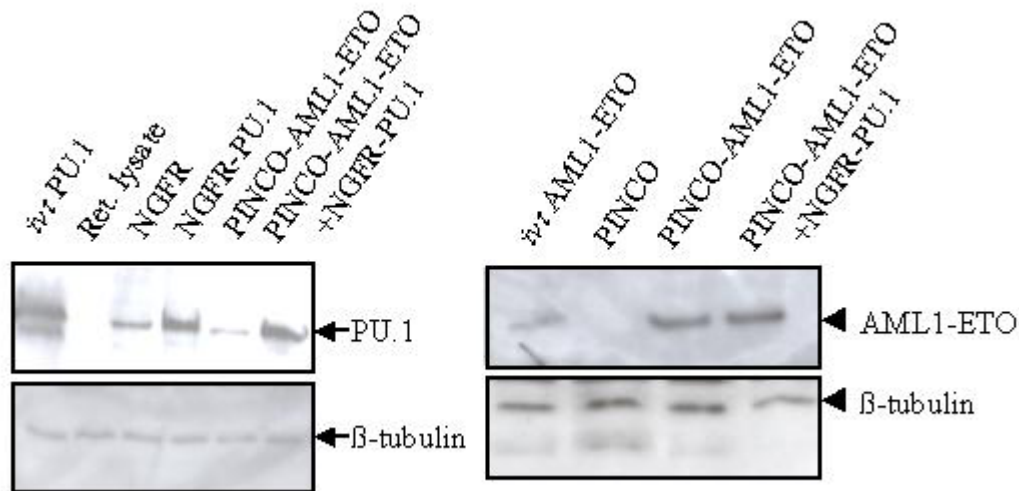


Figure 6b: **Expression of PU.1 and AML1-ETO in mice bone marrow cells:** The cells from the above transduction were lysed and immunoblot analysis was performed for expression of PU.1, AML1-ETO and  $\beta$ -tubulin in parallel with equal amounts of protein loaded.

These results suggest that AML1-ETO blocks the anti-proliferative capacity of

PU.1 resulting in the aberrant proliferation of hematopoietic progenitor cells.

### **3.9. AML1-ETO blocks the differentiation caused by PU.1 in HL60 cells:**

We next addressed if PU.1 can induce differentiation in other bipotential myeloid cell lines, which might be blocked by AML1-ETO. We overexpressed PU.1 and AML1-ETO in HL60 cells by electroporation and sorted for NGFR or GFP, respectively. After three days, FACS analysis for CD11b receptor, a marker for myeloid differentiation, showed that PU.1 transfected HL60 cells (side scatter [SS] of 2<sup>nd</sup> panel) were 64.9% positive in comparison to empty vector (SS of 1<sup>st</sup> panel) 1.1%. When AML1-ETO was co-transfected (SS of 3<sup>rd</sup> panel), CD11b positive cells were less (0.8%) in comparison to PU.1 alone (SS of 2<sup>nd</sup> panel) 64.9%. Similar FACS analysis for CD14, marker for monocyte differentiation showed that PU.1 transfected (SS of 2<sup>nd</sup> panel) HL60 cells were 14.2% positive in comparison to empty vector (SS of 1<sup>st</sup> panel) 0.7%. When AML1-ETO was co-transfected, CD14 positive cells were (SS of 3<sup>rd</sup> panel) 2.7%.

## HL60

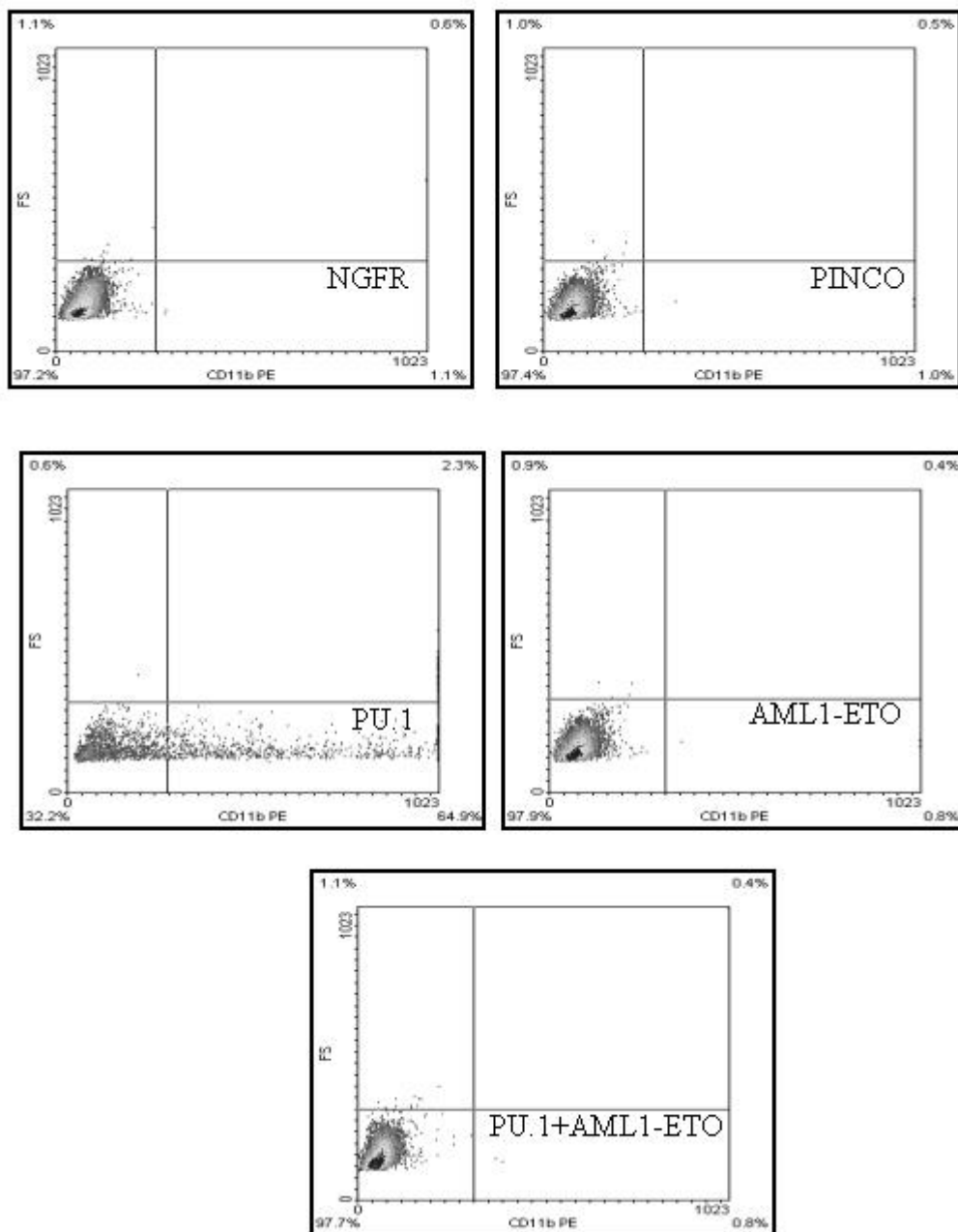


Figure 7a: **CD11b FACS analysis of PU.1 AML1-ETO transfected HL60 cells:** CD11b positive cells in PU.1 transfected (side scatter (SS) of 2<sup>nd</sup> panel) HL60 cells were 64.9% in comparison to empty vector (SS of 1<sup>st</sup> panel) 1.1%. When AML1-ETO was co-transfected (SS of 3<sup>rd</sup> panel), CD11b positive cells were less (0.8%) in comparison to PU.1 alone (SS of 2<sup>nd</sup> panel) 64.9%.

## HL60

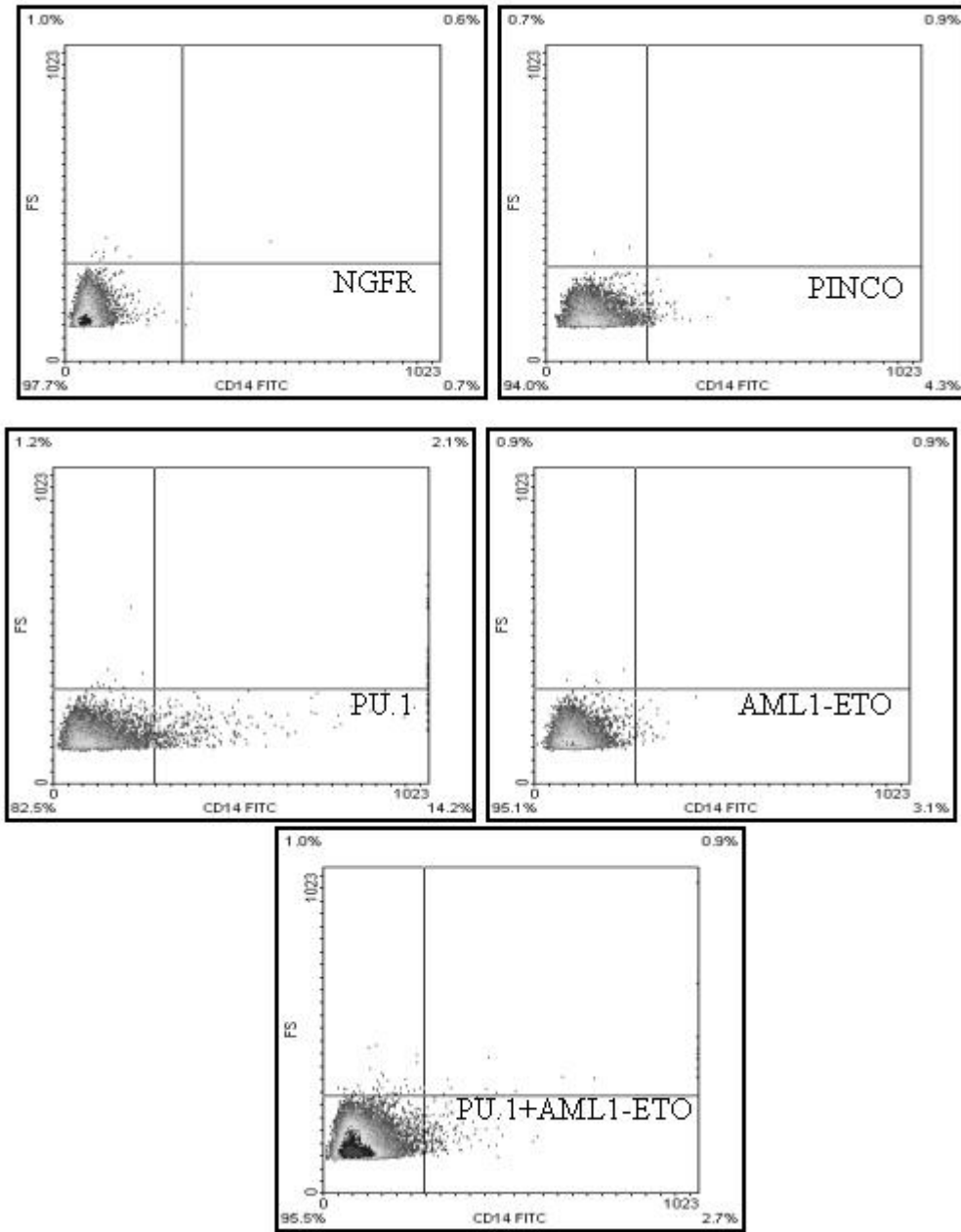


Figure 7b: **CD14 FACS analysis of PU.1 and AML1-ETO transfected HL60 cells:** CD14 positive cells in PU.1 transfected (SS of 2<sup>nd</sup> panel) HL60 cells were 14.2% in comparison to empty vector (SS of 1<sup>st</sup> panel) 0.7%. When AML1-ETO was also transfected, CD14 positive cells were (SS of 3<sup>rd</sup> panel) 2.7%.

The expression of transfected genes is shown in Fig. 7c.

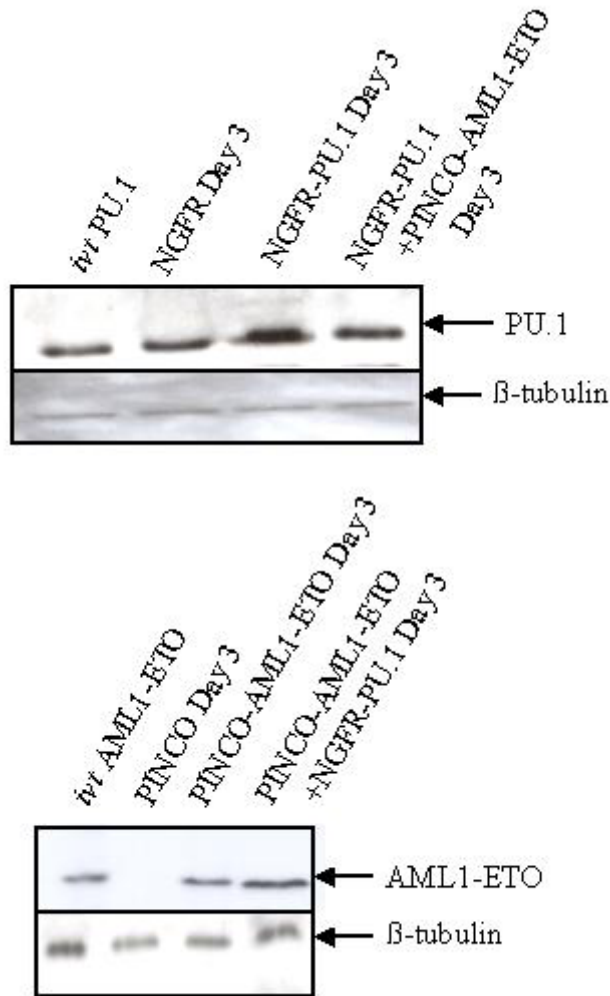


Figure 7c: **Expression of PU.1 and AML1-ETO in transiently transfected L60 cells:** Immunoblot analysis was performed from lysates of above transfected cells for PU.1, AML1-ETO and  $\beta$ -tubulin in parallel with equal amounts of protein loaded.

### 3.10. Overexpression of PU.1 initiates differentiation in t(8;21) positive

**asumi-1 cells:** Due to the protein-protein interaction of PU.1 and AML1-ETO, the transcriptional activity of PU.1 is downregulated. This might be an important cause for block in differentiation. Therefore, we investigated whether overexpression of PU.1 can differentiate cells having t(8;21). We transiently transfected Kasumi-1 cells carrying t(8;21) with NGFR-PU.1 or with the empty



vector (NGFR). Five days after transfection the morphological changes were observed by Wright-Giemsa staining of cells. PU.1-transfected cells differentiated to the monocytic lineage, whereas the mock-transfected cells did not (Fig. 8a).

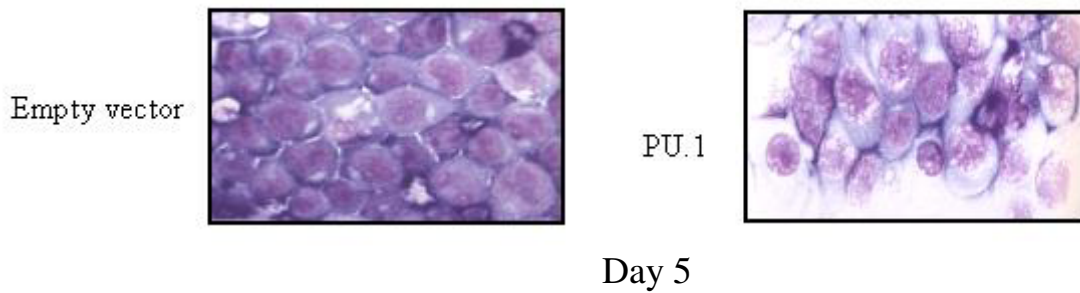


Figure 8a: **PU.1 induces differentiation in t(8;21) positive Kasumi-1 cells:** t(8;21) positive Kasumi-1 cells were transiently transfected with PU.1 (pGCsam-FLAG-PU.1-ires-NGFR) or the empty vector (pGCsam-ires-NGFR) and morphological changes were observed on day 5. FACS sorting of transfected cells for NGFR showed the expression of PU.1, which was further shown by immunoblot analysis of sorted cells for PU.1 expression (Fig 8b).

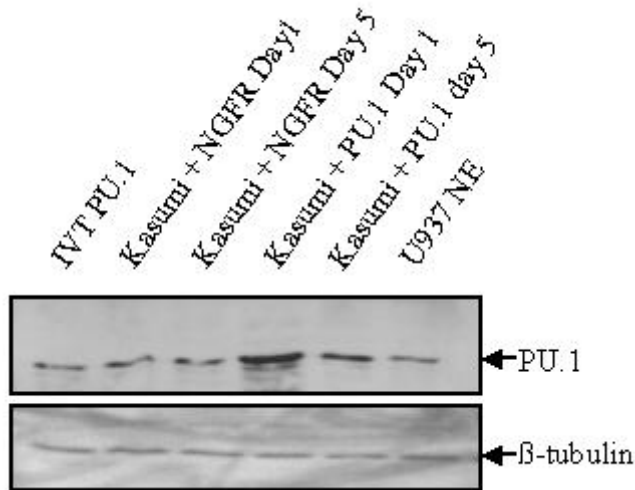
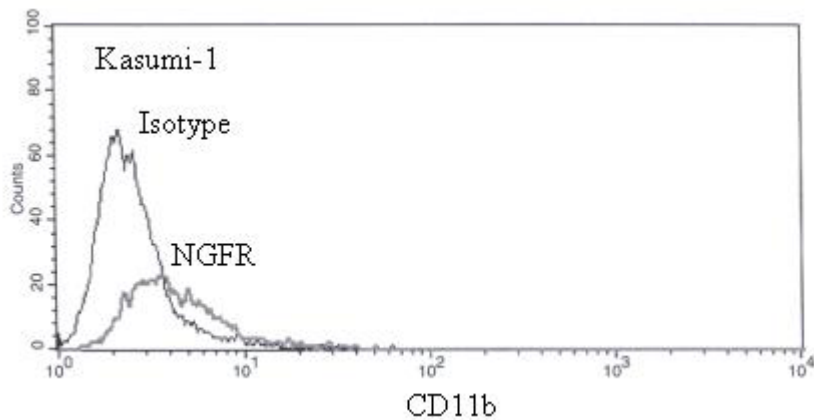


Figure 8b: **Western blot showing PU.1 expression:** Kasumi-1 cells lysates transfected with NGFR-PU.1 after day 1 and day 5 were subjected to immunoblot for PU.1 and  $\beta$ -tubulin in parallel with equal amounts of protein loaded. PU.1 expression was observed to be more on day 1 than on day 5.

The PU.1-transfected Kasumi-1 cells showed an increase of the cell surface markers CD11b (Fig. 8c) a marker for differentiation.



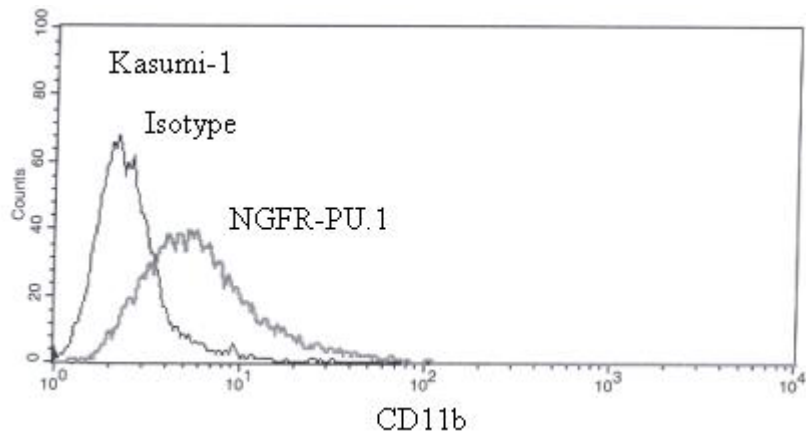
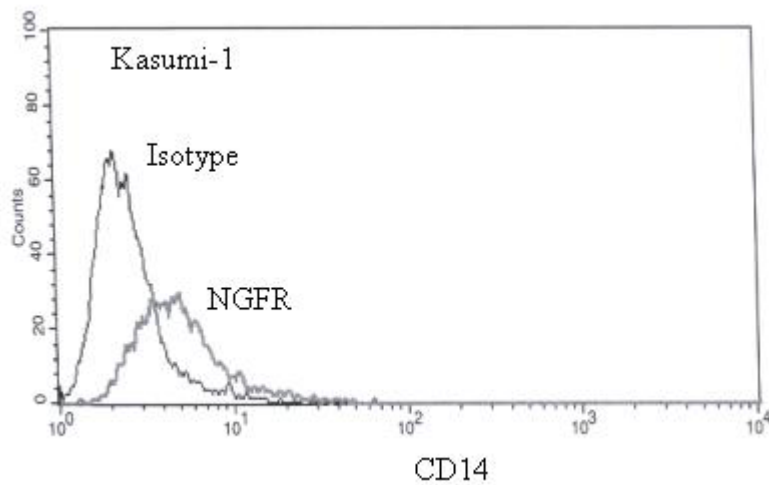


Figure 8c: **PU.1 induces CD11b expression in Kasumi-1 cells:** FACS analysis for the cell surface expression of CD11b in Kasumi-1 cells transfected with empty vector (top panel) and PU.1 (bottom panel).

CD14 (Fig. 8d) a marker for monocytic lineage was also seen to increase in expression in PU.1 transfected Kasumi-1 cells.



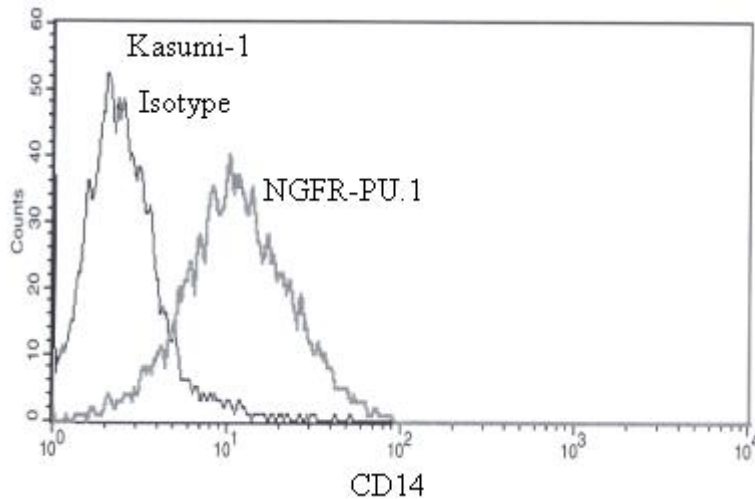


Figure 8d: **PU.1 induces CD14 expression in Kasumi-1 cells:** In the same experiment FACS analysis for the cell surface expression of CD14, in Kasumi-1 cells transfected with empty vector (top panel) or PU.1 (bottom panel).

PU.1 transfected cells did not show an increase in cell number, but on the contrary a decrease in cell number was observed (Fig. 8e).

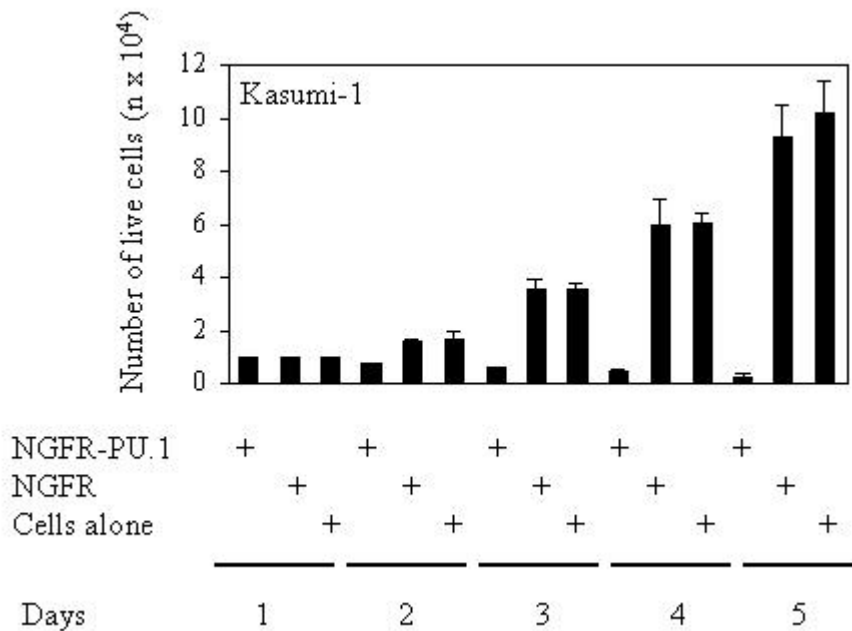


Figure 8e: **Kasumi-1 cell number decreases in PU.1 transfected cells:** The above transfected cells (Fig. 9a) were counted by trypan blue staining on day 1, day 2, day 3, day 4, and day 5 after transfection.

This also suggests that PU.1 overexpression might lead to cell death. However, this mechanism needs still to be elucidated.

## **Chapter 4**

### **Discussion**

## Chapter 4. Discussion

### 4.1. *in vivo* interaction of PU.1 and AML1-ETO and downregulation of PU.1's transactivation capacity by AML1-ETO

Although AML1-ETO has a potent dominant repressor activity over wild type AML1B, the biological effects of AML1-ETO may also relate to its ability to bind and inhibit the function of other cellular regulatory proteins. Recently, AML1-ETO was shown to repress MEF-2, an ETS transcription factor, to transactivate the IL-3 promoter (46). In the present report, we demonstrate *in vivo* (Fig. 1a, 1b) interaction between PU.1 and AML1-ETO. PU.1 is a key transcription factor for normal myeloid development as demonstrated by a complete block of myeloid development in PU.1<sup>-/-</sup> mice (142, 143). Fetal or newborn PU.1<sup>-/-</sup> mice have no detectable monocytes/macrophages or neutrophils (142, 143). On checking the functional impact of *in vivo* interaction of PU.1 and AML1-ETO by transient transfection assays, we found that PU.1's transactivation capacity was downregulated by AML1-ETO (Fig. 1c). AML1-ETO could block activation by other transcription factors like C/EBP $\alpha$  (195). While AML1 cooperates with the C/EBP $\alpha$  protein to activate the NP3/defensin promoter, AML1-ETO blocks activation of the promoter by either AML1 or C/EBP $\alpha$ . Similarly c-Jun co-activates PU.1 (Fig. 3a) but AML1-ETO downregulated this PU.1/c-Jun transactivation capacity (Fig. 3a). These results suggest that protein-protein interactions of AML1-ETO define its functional impact.

AML1-ETO retains the 177 N-terminus amino acids of AML1, suggesting that AML1 might also have an influence on transactivation of PU.1 or PU.1/c-Jun. However our data suggests that AML1B does not change the transactivation capacity of PU.1 or PU.1/c-Jun (Fig. 1e). AML1B was shown to activate many promoters like CD11a (26), M-CSF receptor promoter (196, 197) in synergy with other transcription factors like PU.1 and C/EBP $\alpha$ . PU.1 and AML1B synergistically activate transcription but bind to each other weakly and do not bind to DNA in a cooperative manner on promoter having PU.1 and AML1 binding sites in near proximity (194). In this case the two proteins might cooperate to recruit co-activators like CBP/p300 to the promoters. In contrast, AML1B does not affect PU.1's transactivation capacity on a promoter, having only PU.1 binding sites (Fig. 1e). These data taken together suggests that AML1B and PU.1 synergy was possible in the promoters having the respective binding sites in near proximity, like M-CSF receptor promoter.

The ETO portion of AML1-ETO has intrinsic capacity to bind to other co-repressors, which might not be sensitive to TSA. However the domains that are shown to be important for interaction with all the classes of co-repressors were HHR and MYND domains for N-Cor and mSin3A (111). NHR2 and NHR4 (nervy homology domains) domains were shown to be important for oligomerisation and recruiting more co-repressors. Zinc finger domains for other interactions with HDACs. The point mutations of C488S and C508S in ETO depleted the AML1-ETO interaction with N-Cor and mSin3A (110). Therefore



to further elucidate the function of co-repressor binding domains in AML1-ETO in downregulation of PU.1's transactivation capacity, transient transfection assays with various co-repressor recruiting domain mutants of AML1-ETO were performed. All the mutants still downregulated PU.1's transcriptional activity (Fig. 2a, 2b). These results suggest that the downregulation of PU.1's transcriptional activity by AML1-ETO does not need co-repressor binding regions but might compete with c-Jun in binding to PU.1.

**4.2. AML1-ETO inactivates PU.1 s function by competing away PU.1 s co-activator c- Jun from binding to PU.1:** c-Jun a member of AP-1 transcription factor complex was shown to interact with PU.1 at the  $\beta3\beta4$  domain and co-activate its transactivation capacity. Our data (Fig. 3a) suggest that AML1-ETO could block this co-activation capacity. The competitive protein-protein interaction experiments with *in vitro* translated proteins indicate that AML1-ETO disrupted PU.1/c-Jun interaction in a competitive manner (Fig. 3c), thus blocking c-Jun from co-activating PU.1. AML1-ETO interacted with PU.1 (Fig. 3b) at the  $\beta3\beta4$  domain, which is a part of the DNA binding domain of PU.1. Therefore we investigated if AML1-ETO interaction with PU.1 changed the DNA binding of PU.1. However, AML1-ETO interaction with DNA binding domain of PU.1 did not abolish the DNA binding capacity of PU.1 (Fig. 4a). This is as expected due to the structural properties of PU.1's DNA binding domain. The  $\beta3\beta4$  region in the DNA binding domain of PU.1 was shown not to interact with DNA but is exposed to the solvent atmosphere (155), thus the

interaction of AML1-ETO with the  $\beta 3\beta 4$  of PU.1's DNA binding domain does not change the DNA binding capacity of PU.1. The interaction of c-Jun at the same domain, the  $\beta 3\beta 4$  region might enhance the recruitment of excess co-activators to PU.1 thus increase PU.1's capacity to transactivate its target promoter sequences. However, in presence of AML1-ETO this interaction of PU.1/c-Jun was competed away (Fig. 3c) thus downregulating the transactivation capacity of PU.1.

#### **4.3. AML1-ETO downregulates PU.1 function in myeloid cells and clinical**

**significance:** All the experiments mentioned above were performed in fibroblast cell lines (293T or NIH3T3), which do not express PU.1 or AML1B. To understand the functional consequences of the PU.1/AML1-ETO interaction in the context of myeloid development we performed further experimentation in myeloid cells that express endogenous PU.1 and AML1B. AML1-ETO downregulated the PU.1 target genes in transient transfection assays (Fig. 5a). HL60 cells differentiated to monocytic lineage upon ectopic expression of PU.1, which was blocked by AML1-ETO (Fig. 7a, 7b). In patients with t(8;21), expression of the cell surface markers CD11b, CD14 and CD64 were less in comparison to patients without t(8;21) (Fig. 5c). CD14 and CD64 promoters have putative PU.1 binding sites but not AML1, C/EBP $\alpha$  or MEF binding sites suggesting that downregulation of PU.1's function by AML1-ETO could possibly be an important step in progression towards leukemia. CD11b, another marker for differentiation was also less expressed in patients with t(8;21) in

comparison to patients without t(8;21) (Fig. 5c). CD11b is regulated by PU.1 (198) and its promoter contains putative binding sites of AML1. In this case AML1-ETO's interaction and downregulation of important myeloid transcription factors like PU.1 and AML1 could explain the lower CD11b expression. The phenotype of PU.1<sup>-/-</sup> mice suggests that PU.1 is critical for myeloid differentiation and development (142, 143). Interaction of PU.1 with AML1-ETO and subsequent suppression of PU.1 target genes (Fig. 5c) might contribute to the phenotypic changes seen in t(8;21). Furthermore, PU.1 is a self-regulatory protein (199) and in patients with t(8;21) the expression levels of PU.1 were observed to be less in comparison to patients with out t(8;21) (Fig. 5b).

Because AML1<sup>-/-</sup> mice lack PU.1 expression (200), AML1-ETO could downregulate PU.1 expression through repressing AML1 function. However, in a diseased condition or in presence of AML1-ETO, like in Kasumi-1 cells, the expression of AML1 and PU.1 genes was still observed. Therefore, the presence of AML1-ETO does not completely repress the expression levels of these genes, but may block their functions by protein-protein interactions. AML1 does not have a downregulatory effect on PU.1 (Fig. 1e). To analyse the functional impact of AML1-ETO on PU.1 in presence of wild type AML1B protein we performed experiments in cells expressing endogenous AML1B protein. Overexpression of PU.1 in mouse bone marrow cells leads to a block in proliferation, but in presence of AML1-ETO this function of PU.1 was

abrogated (Fig. 6a). Recently, it was shown that AML1-ETO expression in human progenitor cells leads to expansion of human haematopoietic stem cells (201). Therefore, the block of differentiation and increase in abnormal proliferation of haematopoietic stem cells could be due to downregulation of PU.1's activity in t(8;21) leukemia. PU.1 could drive the HL60 myeloid cells to differentiate towards monocytic lineage but AML1-ETO could block this function of PU.1 (Fig. 7a, 7b). The anti-proliferative and differentiation capacities of PU.1 were downregulated by AML1-ETO, thus suggesting that downregulation of PU.1's functions in a leukemic condition by AML1-ETO might be a major mechanism in AML.

Since AML1-ETO downregulated the most important functions of PU.1, restoring the expression of PU.1 in AML1-ETO positive cells might give a potential clinical application. Overexpression of PU.1 in t(8;21) positive Kasumi-1 cells differentiates them towards the monocytic lineage (Fig.8a). Morphologically cells did not appear to be terminally differentiated even though the cell surface markers CD11b (Fig. 8c) and CD14 (Fig. 8b) were increased in expression. It has been earlier shown that short-term activation of PU.1 in multipotent haematopoietic cells leads to immature eosinophils (158). However, stable overexpression of PU.1 could lead to myeloid lineage in haematopoietic progenitor cells (158). Therefore, higher and stable expression of PU.1 in Kasumi-1 cells might be needed to terminally differentiate towards the monocytic lineage. The cell number of PU.1 transfected Kasumi-1 cells

decreased over a course of time (Fig. 8e) showing that PU.1 functions as anti-proliferative factor upon overexpression in Kasumi-1 cells. However, this mechanism needs to be further elucidated. Our data suggest that the ectopic expression of PU.1 in Kasumi-1 cells overcomes the functional block by AML1-ETO. PU.1 and C/EBP $\alpha$  are important factors for myeloid differentiation and AML1-ETO downregulating these two factors could be an important step towards leukemia. The abnormal regulation of major transcription factors required for normal differentiation of cells might also add to one of the molecular mechanism for AML (109, 144). Apart from regular chemotherapeutic approaches, one more approach would be to find an applicable methodology (159, 180, 241, 250) of increasing the expression levels of important transcription factors like PU.1 or C/EBP $\alpha$ . We have earlier described C/EBP $\alpha$  as potential therapeutic factor (195), here we also demonstrate the importance of PU.1 for the same purpose. This might also suggest the possibility of using these two factors independently or in combination for therapy of AML1-ETO positive leukemias.

## **Chapter 5**

### **Summary**

## Chapter 5.

### 5.1 Summary (English):

AML1-ETO is a fusion protein encoded by the translocation t(8;21) and found in 15% of acute myeloid leukemia patients. Here, we report a negative functional impact of AML1-ETO on the transcriptional activity of PU.1, an important transcription factor for normal myeloid differentiation. We have demonstrated that AML1-ETO interacts with PU.1 by immunoprecipitation assay in Kasumi-1 cells having t(8;21). On mapping the region of interaction in PU.1, we found that AML1-ETO binds to the  $\beta 3\beta 4$  region in the DNA binding domain of PU.1 and displaces the co-activator c-Jun from PU.1, thus downregulating PU.1's transcriptional activity. In doing so AML1-ETO does not change the DNA binding capacity of PU.1. The expression levels of PU.1 target genes in acute myeloid leukemia (AML)-M2 patients with t(8;21) were lower than in patients without t(8;21). Conditional expression of AML1-ETO causes proliferation in mouse bone marrow cells and inhibits PU.1 induced differentiation in HL60 cells. Overexpression of PU.1 differentiates AML1-ETO carrying Kasumi-1 cells to the monocytic lineage. Thus, PU.1's function is downregulated in presence of AML1-ETO in acute myeloid leukemia, whereas overexpression of PU.1 can restore normal differentiation.

## 5.2. Zusammenfassung (Deutsch):

AML1-ETO ist ein Fusionsprodukt, das durch Umlagerung von genetischem Material im Rahmen der Translokation t(8;21) zustande kommt und bei 15% aller Patienten mit akuter myeloischer Leukämie nachgewiesen werden kann.

In dieser Arbeit berichten wir über den negativen Einfluss dieses Proteins auf die Aktivität des Transkriptionsfaktors PU.1, der in der physiologischen Differenzierung der myeloischen Zellreihe eine wichtige Rolle spielt. In Lysaten von Kasumi-Zellen, die die t(8;21) positiv sind, konnten wir mit Hilfe der Immunoprecipitation eine physikalische Bindung zwischen PU.1 und AML1-ETO nachweisen. Des Weiteren konnte als Bindungsstelle in PU.1 die 3'/4-Region, die innerhalb der DNA-bindenden Domäne liegt, identifiziert werden. Die Bindung von AML1-ETO an dieser Stelle führte zur Verdrängung des Coaktivators c-Jun und damit zu einer verminderten Wirkung von PU.1 als aktivierender Transkriptionsfaktor. AML1-ETO rekrutierte weder TSA-empfindlichen Corepressoren, noch änderte es die DNA-Bindungskapazität von PU.1. Bei der Untersuchung von Patientenmaterial konnte ein vermindertes Expressions-Niveau von verschiedenen PU.1-Zielgenen in den AML M2-Proben mit t(8;21) im Vergleich zu M2 Proben ohne chromosomale Aberrationen beobachtet werden. Konditionale Expression von AML1-ETO verursachte in Knochenmarks-Zellen der Maus Proliferation; in HL60 Zellen hemmte es die PU.1-induzierte Differenzierung. Überexpression von PU.1 führte dagegen zu einer Differenzierung von AML1-ETO exprimierenden Kasumi-Zellen zur



monozytischen Zellreihe. Zusammenfassend ist also die Funktion von PU.1 in Anwesenheit von AML1-ETO gehemmt, wogegen die überexpression von PU.1 die normale Differenzierung wieder herstellen kann.

**Chapter 6**  
**Reference List**

## Chapter 6. Reference List

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## **Chapter 7**

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**Chapter 8**  
**Curriculum vitae**

## Curriculum vitae

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1992 **High school Diploma** (Biology, Physics and Chemistry), Nagarjuna Junior College, Hyderabad, India, Note 1.

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1997 **Master of Science** (Biotechnology), University of Hyderabad, Hyderabad, India, Note 1.

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Aug. 1995-Aug. 1997: **M.Sc Thesis**, University of Hyderabad, Department of Animal Sciences, India: Sequence and structural analysis of thermophilic and mesophilic proteins.

Aug. 1996-Aug. 1997: **Summer fellowship**, Centre for Cellular and Molecular Biology, India: Understanding the protein folding and refolding, isolating the molten globule state and studying the folding kinetics by Circular Dichroism and fluorescence spectrophotometry.

May 1997-Feb.1999: **Junior Research Fellow**, National Centre for Biological Sciences, Tata Institute of Fundamental Research, Bangalore, India. The project was characterising a transgenic drosophila model for Human Papilloma Virus (16), expressing the Viral oncoproteins E6/E7.

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### **Publications:**

1. The myeloid master regulator transcription factor PU.1 is inactivated by AML1-ETO in t(8;21) myeloid leukemia. **Raani . angala**, Marion S.Heiss-Neumann, Janki S.Rangatia, Sheo M.Singh, Annika Elsser, Claudia Schoch, Daniel G.Tenen, Wolfgang Hiddemann and Gerhard Behre. Blood. 2003 Jan 1;101(1):270-7.
2. Downregulation of c-Jun expression by transcription factor C/EBPalpha is critical for granulocytic commitment. Janki Rangatia, **Raani . angala**, Nicolai Treiber, Pu Zhang, D.G. Tenen, Wolfgang Hiddemann, Gerhard Behre. Mol Cell Biol. 2002 Dec;22(24):8681-94.
3. The granulocytic inducer C/EBP $\alpha$  inactivates the myeloid master regulator PU.1: possible role in lineage commitment decisions.



Venkateshwar A. Reddy, Atsushi Iwama, Guergana Iotzova, Mathias Schulz, Annika Elsasser, **Ra ani . angala**, Daniel G. Tenen, Wolfgang Hiddemann, Gerhard Behre. *Blood*. 2002 Jul 15;100(2):483-90.

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## **Chapter 9**

**Published manuscript**

**The myeloid master regulator PU.1 is inactivated by AML1-ETO in t(8;21)  
myeloid leukemia**

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**Abstract:**

The transcription factor PU.1 plays a pivotal role in normal myeloid differentiation. PU.1<sup>-/-</sup> mice exhibit a complete block in myeloid differentiation. Heterozygous PU.1 mutations were reported in some patients with acute myeloid leukemia (AML), but not in AML with translocation t(8;21), which gives rise to the fusion gene AML1-ETO. Here we report a negative functional impact of AML1-ETO on the transcriptional activity of PU.1. AML1-ETO physically binds to PU.1 in t(8;21)-positive Kasumi-1 cells. AML1-ETO binds to the  $\beta 3\beta 4$ -region in the DNA binding domain of PU.1 and displaces the co-activator c-Jun from PU.1, thus downregulating PU.1's transcriptional activity. This physical interaction of AML1-ETO and PU.1 did not abolish the DNA binding capacity of PU.1. AML1-ETO downregulates the transactivation capacity of PU.1 in myeloid U937 cells, and the expression levels of PU.1 target genes in AML FAB subtype M2 patients with t(8;21) were lower than in patients without t(8;21). Conditional expression of AML1-ETO causes proliferation in mouse bone marrow cells and inhibits anti-proliferative function of PU.1. Overexpression of PU.1, however, differentiates AML1-ETO expressing Kasumi-1 cells to the monocytic lineage. Thus, PU.1's function is downregulated by AML1-ETO in t(8;21) myeloid leukemia, whereas overexpression of PU.1 restores normal differentiation.

*ey ords* PU.1, AML1-ETO, c-Jun, myeloid, leukemia.

## Introduction:

The Ets family of transcription factors play a key role in the growth, survival, differentiation and activation of hematopoietic cells. This family of proteins is characterized by presence of an 85 amino acid, <sup>1</sup> winged helix-turn-helix DNA-binding domain. PU.1 is one of the most important Ets transcription factors. <sup>2</sup> Its expression is limited to hematopoietic cells, including primitive CD34+ cells, macrophages, B-lymphocytes, neutrophils, mast cells, and early erythroblasts. <sup>2 3</sup> *in vitro* studies suggest that PU.1 regulates the activity of a number of myeloid and lymphoid-specific promoters and enhancers. <sup>4 5 6 7 8 9 10</sup> PU.1 is a key transcription factor for normal myeloid development as demonstrated by a complete block of myeloid development in PU.1-/- mice. <sup>11 12</sup> Fetal or newborn PU.1-/- mice have no detectable monocytes/macrophages or neutrophils. <sup>11 12</sup> We have recently shown that PU.1 is mutated in AML patients. <sup>13</sup> These studies all point to the crucial role of PU.1 in both normal myeloid differentiation and leukemogenesis.

AML1 is a member of the *Runt*-like transcription factors (*Runx*-1, -2 and -3) named after the *Runt* protein that regulates segmentation during *Drosophila* embryogenesis. <sup>14 15 16</sup> AML1 appears to act as an “organizing” factor for many promoters and enhancers by interacting with various co-activators and DNA binding transcription factors. <sup>17 18 19 20 21 22</sup> The AML1 gene is one of the most frequently translocated or mutated genes in human cancer. <sup>23 24 25</sup> The t(8;21)(q22;q22) translocation fuses residues 1-177 of AML1 (including the DNA binding domain) to nearly all of ETO (also known as CBF2T1). <sup>26</sup> ETO is the human homolog of *Drosophila* NERVY protein. <sup>27 28 29</sup> The t(8;21) belongs to the most common chromosomal abnormalities in AML, accounting for 10% of all AML cases and 40% of the AML French-American-British (FAB) M2 phenotype. <sup>30 31 32 33</sup> AML1 activates transcription from enhancer core motifs (TGT/cGGY), which are present in a number of genes relevant to myeloid development, including the M-CSF receptor, GM-CSF, myeloperoxidase and

neutrophil elastase.<sup>34 35 36 37 38 39</sup> Like AML1, AML1-ETO can act as a transcriptional activator,<sup>40 41 42 43</sup> but is also a transcriptional repressor in other contexts.<sup>44</sup> Only one allele of AML1 is altered in leukemia cells expressing t(8;21), and AML1-ETO can efficiently repress AML1-dependent transcriptional activation. Therefore, AML1-ETO has been postulated to act as dominant inhibitor of AML1 function.<sup>34 37 44</sup>

Recently, we have shown that AML1-ETO blocks C/EBP $\alpha$ -dependent activation of its own promoter thus blocking normal granulocytic differentiation of myeloid cells.<sup>36</sup> Furthermore, AML1-ETO was shown to repress AML1 and MEF-2-dependent gene activation.<sup>45</sup> In our earlier studies we demonstrated that c-Jun, a member of AP-1 transcription factor family, can interact with PU.1 at the  $\beta$ 3- $\beta$ 4 domain in PU.1 and co-activate PU.1's transcriptional activity.<sup>46</sup> Here we show that AML1-ETO blocks the transcriptional activity of PU.1 by displacing its co-activator c-Jun.

## Materials and Methods:

**Cell lines and cell culture:** Human kidney 293T, mouse embryonal carcinoma F9 and ecotrophic Phoenix cells were maintained in Dulbecco's Modified Eagle's Medium (GIBCO) supplemented with 10% Fetal Bovine Serum (GIBCO), 1% Glutamine (GIBCO) and 1% Penstrept (GIBCO). Human monoblastic U937 cells and t(8;21) positive Kasumi-1 cells were cultured in RPMI 1640 medium (GIBCO) supplemented with 10% Fetal Bovine Serum (GIBCO).

Bone marrow cells were isolated from the femurs of Balb/C mouse. The femurs were removed and stripped of the soft tissue, and crushed to release cells within marrow cavity. The red blood cells were lysed with a 0.15-mol/L solution of ammonium chloride. The pelleted cells were subjected to low-density mononuclear cell separation by incubating with density gradient (Histopaque 1083; Sigma) for 10 minutes and centrifuged at 600 rpm for 30 minutes, washed twice in PBS, followed by culturing in IMDM medium (Stem Cell Technologies) supplemented with 10% FBS (Stem cell technologies), 50 ng/mL stem cell factor (R and D systems), 50 ng/mL IL-6 (R and D systems), and 50 ng/mL Flt-3L (R and D systems).

**Co-immunoprecipitation assay:**  $2 \times 10^7$  Kasumi-1 cells were lysed and 200 $\mu$ g or protein was used to perform immunoprecipitation as mentioned by Mao *et al.*<sup>45</sup> The following antibodies were used: rabbit IgG (Santa Cruz Biotechnologies, Cat# sc2027), goat IgG (Santa Cruz Biotechnologies, Cat# 2028), anti-AML1 antibody (Calbiochem, Cat# PC284), anti-PU.1 (Santa Cruz Biotechnologies, Cat# sc-352), and Protein-A agarose beads (Santa Cruz Biotechnologies, Cat# sc-2001).

**Western blot:** 293T cells (after plating in 100mm plates) were transfected using the LipofectAMINE Plus kit (GIBCO) as per the manufacturer's protocol. 24h post transfection, cells were harvested and lysed in RIPA lysis buffer, and immunoblot for PU.1 was performed with 100 $\mu$ g protein as described earlier.<sup>46</sup>

<sup>47 48</sup> To generate protein lysates,  $1 \times 10^6$  F9, Kasumi-1 or 293T cells were lysed and nuclear extracts were prepared and immunoblot was performed with 100 $\mu$ g protein for c-Jun (Santa Cruz Biotechnologies, Cat# sc45). Mouse bone marrow cells transduced with PU.1, AML1-ETO or respective empty vectors were similarly lysed (RIPA lysis) and 100 $\mu$ g of protein was used for immunoblot analysis for PU.1 and AML1-ETO (anti-ETO antibody, Santa Cruz Biotechnologies, Cat# sc9737). Mouse monoclonal anti- $\beta$ -tubulin purchased from Roche (Cat# 1111876) was used for immunoblot assay as internal control. Protein-A peroxidase-conjugated for anti-rabbit (Amersham Pharmacia, cat# NA 9120), or anti-goat peroxidase-conjugated Immunoglobulins (DAKO, code no. p0449) were used as secondary antibodies.

**Reporter constructs and expression plasmids:** The human monocyte-specific M-CSF receptor promoter with or without AML1 binding site, p(PU.1)4TK, and p(mutPU.1)4TK (PU.1 binding sites and mutated PU.1 binding sites subcloned into pTK61luciferase) were described earlier.<sup>46</sup> As an internal control plasmid for transient transfection assay, we used the pRL-null construct driving a Renilla luciferase gene (Promega).<sup>49</sup> Other vectors used were pECE-PU.1-murine, pcDNA.1-PU.1, pGEX-2TK-PU.1 or  $\beta 3\beta 4$ , pS3H-c-Jun and pSP6-c-Jun, as described previously.<sup>46 50</sup> AML1B-pCMV5 and CBF $\beta$ -pCMV5 were described earlier.<sup>42</sup> AML1-ETO-pcDNA3 was constructed by enzymatic digestion of AML1-ETO-pCMV5<sup>42</sup> with XbaI and sub-cloning the resulting 2258bp fragment into the XbaI site of pcDNA3 plasmid (Invitrogen).

**Transfection assays:** Transient transfections in 293T or F9 cells were carried out with LipofectAMINE transfection kit (GIBCO) in 24 well plates as described earlier.<sup>46 47 49</sup> U937 cells were transiently transfected by electroporation in RPMI medium at 980 $\mu$ F and 280V. *ire ly* luciferase activities from the constructs M-CSF receptor promoter luciferase, pXP2, p(PU.1)4TK, p(mutPU.1)4TK and *Renilla* luciferase activity from internal control plasmid pRL0 were determined 24h post transfection using Dual



Luciferase Reporter Assay System (Promega). Results are given as means + standard error of mean from at least 3 independent experiments.

**Protein interaction assays:** c-Jun and AML1-ETO were *in vitro* transcribed and translated in presence of [<sup>35</sup>S] methionine (Amersham Pharmacia) using the T7/SP6 coupled reticulocyte system (Promega) in accordance with the manufacturer's instruction. Glutathione-S-transferase precipitation assays were performed as described earlier.<sup>46 48</sup>

**Electrophoretic mobility shift assay:**  $\gamma^{32}$ P-ATP (Amersham Pharmacia) labelled double-stranded oligonucleotides of PU.1 DNA binding site<sup>51</sup> and AML1 binding site<sup>52</sup> for EMSA were prepared. The assay was performed with *in vitro* translated proteins as mentioned earlier.<sup>47 11</sup> For supershift experiments 3 $\mu$ l of either anti-PU.1 or anti-ETO-antibodies were added to the reaction mixture.

**Retroviral transduction assay:** 5x10<sup>6</sup> ecotrophic Phoenix cells were plated in 10cm plates and transfected with 5 $\mu$ g of PINCO-GFP, PINCO-AML1-ETO-GFP, pGsam-PU.1-ires-NGFR or pGsam-ires-NGFR vectors using LipofectAMINE transfection kit (GIBCO). 24hrs post-transfection the transfection medium was replaced with IMDM medium (supplemented with 10% FBS, 50 ng/mL stem cell factor, 50 ng/mL IL-6, and 50 ng/mL Flt-3L) for collection of the virus particles. After the viral particle production freshly isolated mouse bone marrow cells were incubated with viral medium on Fibronectin coated plates and centrifuged for 30minutes at 1000 x g (this step was repeated every 12hours).<sup>53</sup> At 60hr after first transduction NGFR and/or EGFP positive cells were isolated by FACS analysis (Becton Dickinson). To detect the expression of tNGFR on the cell surface, cells were stained with mouse anti-human NGFR (Chemicon, cat# MAB5246)) followed by phycoerythrin (PE)-conjugated rabbit anti-mouse immunoglobulins (mouse IgG RPE from Dako, cat# R0439). 1x10<sup>4</sup> transduced cells sorted for NGFR positivity were plated in 1.2ml of mouse colony forming medium (Stem Cell

Technologies). After 3, 6, and 12 days of plating live cells were counted by Trypan blue staining.

**Patient material and FACS analysis:** Bone marrow cells from AML-M2 patients with or without t(8;21) were obtained after the consent of the patients. Mononuclear cells were isolated from the bone marrow by density gradient centrifugation with Histopaque (Sigma). FACS analysis was performed with CD11b (Pharmlngen, cat# 555388), CD14 (Pharmlngen, cat# 555397) and CD64 (Pharmlngen, cat# 555527).

**Transfection of asumi-1 cells and FACS analysis:** Kasumi-1 cells were electroporated as mentioned above with pGsam-PU.1-ires-NGFR or pGsam-ires-NGFR vectors and sorted 24h post transfection for NGFR positivity by FACS (with anti-NGFR antibody from Chemicon, cat# MAB5246 and mouse IgG RPE from Dako, cat# R0439). Five days after sorting for NGFR expression, morphological changes were observed by Wright-Giemsa staining of cells.  $1 \times 10^6$  NGFR positive Kasumi-1 cells were incubated with 10 $\mu$ l of recombinant PE-conjugated mouse monoclonal CD11b (Pharmlngen, cat# 555388) or FITC-conjugated mouse monoclonal CD14 (Pharmlngen, cat# 555397) in 100 $\mu$ l PBS for 60 min on ice, washed in PBS followed by analysis on a FACScan flow cytometer (Becton Dickinson) using Cellquest software. The cells were also analysed for the isotype controls, PE conjugated mouse IgG<sub>1</sub>, $\kappa$  (Pharmlngen, cat# 554680) for CD11b-PE and FITC conjugated mouse IgG<sub>1</sub>, $\kappa$  (Pharmlngen, cat# 555748) for CD14-FITC. 24hrs post transfection,  $5 \times 10^4$  NGFR positive cells were plated in a 6-well plate and passaged with fresh medium every 24hrs. Cell count for live cells was performed by trypan blue staining every 24hrs.

## **Results:**

**AML1-ETO interacts with PU.1 *in vivo* and inhibits its transcriptional activity:** To determine whether PU.1 interacts with AML1-ETO, co-immunoprecipitation assays were performed in Kasumi-1 cells, a human cell line containing t(8;21). PU.1 co-precipitated with both AML1 and ETO antibodies but not with IgG control, suggesting that PU.1 interacts with AML1-ETO *in vivo* (Fig. 1a). A similar experiment was performed using a PU.1 specific antibody: AML1-ETO co-precipitated with PU.1, but not with rabbit IgG control (Fig. 1a).

To investigate the functional impact of this *in vivo* interaction, we performed transient transfection assays in 293T cells. A M-CSF receptor promoter luciferase reporter construct which was transactivated 12-fold by PU.1 and 28-fold by PU.1/c-Jun, is completely downregulated by AML1-ETO (Fig. 1b). AML1-ETO had no effects on Serum Response Element (pSRE)/Ras activity nor on the empty vector (pXP2) as negative controls. The expression levels of co-transfected PU.1 did not change in presence of AML1-ETO indicating that the transactivating capacity, but not the expression of co-transfected PU.1 was downregulated (Fig. 1b).

**AML1 does not affect PU.1 or PU.1 c- Jun s transactivation capacity:** The M-CSF receptor promoter has adjacent AML1 and PU.1 binding sites.<sup>45</sup> AML1-ETO retains the 177 N-terminus amino acids of AML1, suggesting that AML1 might also have an influence on transactivation of PU.1 or PU.1/c-Jun. Therefore we addressed if AML1 had any functional impact on PU.1's or PU.1/c-Jun's transactivation capacity using a promoter containing only PU.1 binding sites (p(PU.1)4TK). Transient transfection assays in 293T cells were performed with p(PU.1)4TK and expression plasmids of PU.1, c-Jun, AML1 and CBF $\beta$ . Results (Fig. 1c) show that AML1 did not affect the PU.1 or PU.1/c-Jun transactivation capacity. In the same experiment AML1 could transactivate the M-CSF receptor promoter 4-fold in presence of CBF $\beta$  (data not shown).

AML1, PU.1, c-Jun and CBF $\beta$  had no effects on control vectors (p(mutPU.1)4TK and pXP2) in the experiments above (data not shown).

**AML1-ETO inhibits the co-activation of PU.1 by c-Jun:** We have earlier shown that c-Jun can co-activate PU.1's transactivation in a JNK-independent manner.<sup>46</sup> PU.1 induced strong transactivation of p(PU.1)4TK in 293T cells (Fig. 1c). This is possibly due to high expression of its co-activator c-Jun in these cells. Immunoblot assay for c-Jun indicated that 293T cells have high amounts of c-Jun (Fig. 2a, lane 1) comparable to Kasumi-1 cells (Fig. 2a, lane 3). However, F9 cells had no detectable c-Jun protein (Fig. 2a, lane 2). Therefore further experiments were carried out in F9 cells, which served as a model cell line for understanding how AML1-ETO might interfere with c-Jun's capacity in co-activating PU.1. PU.1/c-Jun could transactivate p(PU.1)4TK (Fig. 2b) and also the M-CSF receptor promoter (Fig. 2c) in F9 cells as described earlier.<sup>46</sup> In presence of AML1-ETO, PU.1/c-Jun's capacity of transactivating the target promoters (Fig. 2b, 2c) was downregulated.

**AML1-ETO displaces c-Jun by binding to the  $\beta 3\beta 4$  region in PU.1:** The results in F9 cells (Fig. 2b, 2c) suggest that AML1-ETO interferes with co-activation of PU.1 by c-Jun. To investigate this, *in vitro* protein-protein interaction assays were performed. c-Jun and AML1-ETO bind to the full length PU.1 fused to Glutathione-S-transferase (GST) (Fig. 3a). c-Jun was shown to interact at  $\beta 3\beta 4$  region of the DNA binding domain of PU.1.<sup>48</sup> Therefore, we performed protein-protein interaction assays using GST- $\beta 3\beta 4$  and found that AML1-ETO also binds to GST- $\beta 3\beta 4$  (Fig. 3b). In competitive protein-protein interaction assays upon increasing the AML1-ETO protein, c-Jun protein bound to GST- $\beta 3\beta 4$  was reduced (Fig. 3b). These results indicate that AML1-ETO competes c-Jun away from binding to the  $\beta 3\beta 4$  domain of PU.1. Thus c-Jun's co-activation function of PU.1 is downregulated and this in turn downregulates PU.1's transcriptional activity.

**AML1-ETO does not change the DNA binding of PU.1:** The protein-protein interactions described above demonstrate that AML1-ETO directly interacts with PU.1. The physical interaction of AML1-ETO/PU.1 might downregulate the DNA binding capacity of PU.1. To address this possibility, we performed an electrophoretic mobility shift assay (EMSA) using *in vitro* translated PU.1 and AML1-ETO and oligonucleotide probes having respective DNA binding sequences.<sup>51 52</sup> *in vitro* translated PU.1 binds specifically to the PU.1 binding oligonucleotide (Fig. 4). Even in presence of AML1-ETO, no change of DNA binding of PU.1 was observed (Fig. 4), indicating that AML1-ETO blocks the transactivation capacity, but not PU.1's DNA binding. In the same experiment *in vitro* translated AML1-ETO was found to bind to the AML1 probe (data not shown).

**AML1-ETO downregulates PU.1 transcriptional activity in myeloid cells:** All the above transfections were performed in non-myeloid 293T or F9 cells. We asked whether the same effects were also observed in myeloid cells. Therefore we performed transient transfection assays in myelomonocytic U937 cells. U937 cells were transfected with wild type M-CSF receptor promoter, M-CSF receptor promoter without AML1 binding site, minimal promoter having PU.1 binding sites (p(PU.1)4TK), minimal promoter with mutated PU.1 binding sites (p(mutPU.1)4TK) as control and empty vector with or without AML1-ETO expression plasmid. We observed that all the promoters were downregulated by AML1-ETO without any effect on the empty vectors (Fig. 5a). These data confirm that also in myeloid cells AML1-ETO downregulates the transcriptional activity of PU.1.

**Low expression of PU.1 target genes in patients with t(8;21):** To further understand if the downregulation of PU.1/c-Jun's transactivation capacity by AML1-ETO leads to downregulation of PU.1's target genes, we performed FACS analysis of PU.1 target cell surface markers.<sup>7 51</sup> In AML-M2 patients with t(8;21), CD14, CD11b and CD64 were 4.6, 5.4, and 5.8 fold less

expressed in comparison to patients with normal M2 karyotype (Fig. 5b). Regulation of CD11b promoter by PU.1 has been shown<sup>51</sup> and further analysis (by TRANSFAC analysis to identify potential transcription factor binding sites in a promoter) of the promoter revealed potential AML1 binding sites were present (data not shown). Downregulation of CD11b might be also due to downregulation of AML1 in addition to PU.1's transactivation capacity by AML1-ETO. Similar analysis of CD14 and CD64 promoters showed (data not shown) that these gene promoters have PU.1 binding sites but no C/EBP $\alpha$ , AML1 or MEF binding sites. Therefore, CD14 and CD64 downregulation could be due to specific downregulation of PU.1's transactivation capacity by AML1-ETO in these patients.

**AML1-ETO causes proliferation of mouse bone marrow cells by inhibiting PU.1:** To investigate the functional consequences of AML1-ETO downregulating PU.1's transactivation capacity, we transduced mouse bone marrow cells with PU.1 (pGsam-NGRF-PU.1), and AML1-ETO (PINCO-AML1-ETO-GFP). The cells transduced with AML1-ETO rapidly increased in number over 12 days, as did the cells overexpressed with AML1-ETO and PU.1 (Fig. 6a). The cells transduced with PU.1 showed no increase in cell number (Fig. 6a). Furthermore, transduction of AML1-ETO blocks PU.1 induced monocytic differentiation in mouse bone marrow cells (data not shown). The expression of transduced genes are shown in Fig. 6b and 6c. Densitometric quantification of the PU.1 protein expression in the same experiment revealed downregulation of endogenous PU.1 expression upon overexpression of AML1-ETO (Fig. 6b). This could be due to AML1-ETO preventing the autoregulation of PU.1.<sup>54</sup> The expression of AML1-ETO was also quantified (data not shown).

**Overexpression of PU.1 initiates differentiation in t(8;21) positive asumi-1 cells:** Our data so far shows that AML1-ETO interacts with PU.1 at the  $\beta 3\beta 4$  region in the DNA binding domain of PU.1 and displaces c-Jun from binding and co-activating PU.1 (Fig. 2 and 3). Moreover, overexpression of

AML1-ETO downregulated the PU.1 expression in mouse bone marrow cells (Fig. 6b). It is important to note that Kasumi-1 cells shown high levels of c-Jun protein expression (Fig. 2a). Hence, we asked whether overexpression of PU.1 could overcome the functional block of PU.1 by AML1-ETO. Transient overexpression of PU.1 (pGsam-NGFR-PU.1) in t(8;21) bearing Kasumi-1 cells was performed. FACS sorting (for NGFR) of the transfected cells showed the PU.1 expression, which was further shown by immunoblot analysis of sorted cells for PU.1 expression (Fig. 7b). 4-fold over expression was observed after transfection (Fig. 7b).

Five days post-transfection of PU.1, morphological changes (Fig. 7a) were observed by Wright-Giemsa staining of cells. PU.1-transfected cells differentiated to the monocyte like cells, whereas the empty vector (pGsam-NGFR) transfected cells showed no morphological change. The PU.1-transfected Kasumi-1 cells also showed increase in cell surface markers CD11b (Fig. 7c) (marker for myeloid differentiation) and CD14 (Fig. 7d) (marker for the monocytic lineage). 24hrs post transfection, the NGFR sorted cells were further plated and counted for live cells every 24hrs. In PU.1 transfected cells a decrease in cell number was observed (Fig. 7e).

## Discussion:

The importance of PU.1 in myeloid differentiation is well established. Recently we have reported that PU.1 is mutated in AML patients<sup>13</sup> similar to C/EBP $\alpha$ <sup>55</sup> suggesting that PU.1 also plays a major role in leukemogenesis. However, PU.1 was not found to be mutated in AML patients with t(8;21) which suggest that distinct pathways of inactivation of PU.1 might be occurring in t(8;21) leukemia. We show here that PU.1 plays a major role in leukemogenesis in t(8;21) leukemia as it interacts with fusion protein AML1-ETO (Fig. 1a). We have previously reported a similar phenomenon for C/EBP $\alpha$ ,<sup>36</sup> an important transcription factor in granulocytic differentiation. Physical interaction of PU.1 and AML1-ETO results in inactivation of PU.1's transactivation activity by displacing PU.1's co-activator c-Jun (Fig. 1b, 2b, 2c and 5a). AML1B was shown to interact with PU.1 and synergize on M-CSF receptor promoter.<sup>56,10</sup> In contrast, AML1B does not influence PU.1's transactivation capacity on a promoter driven by PU.1 binding sites only (Fig. 1c). These data taken together suggest that AML1B and PU.1 synergy is possible in the promoters having the respective binding sites in near proximity for physical interaction, like the M-CSF receptor promoter.

We observed that AML1-ETO downregulates the transcriptional activity of PU.1 in myeloid cells (Fig. 5a) and physically interacts at the  $\beta 3\beta 4$  region in the DNA binding domain of PU.1 (Fig. 3b). Our earlier data show that c-Jun, an AP-1 transcription factor complex member, bind to the  $\beta 3\beta 4$  region and co-activates PU.1 in a JNK-independent manner.<sup>46</sup> The competitive protein-protein interaction experiments with *in vitro* translated proteins indicate that AML1-ETO disrupted PU.1/c-Jun interaction in a competitive manner (Fig. 3b), thus blocking c-Jun from co-activating PU.1. We have described a similar mechanism for GATA-1<sup>50</sup> and C/EBP $\alpha$ .<sup>48</sup> The role of c-Jun in myeloid differentiation was shown to be rather important, as it could enhance the extent of differentiation in U937 cells.<sup>57</sup> In other studies AP-1 and C/EBP $\beta$  were



shown to cooperate in regulation of common target genes, including the human TSG-6,<sup>58</sup> collagenase-1 gene,<sup>59</sup> and TNF $\alpha$ .<sup>60</sup> This data suggest that c-Jun's capacity to co-activate PU.1 is also a very important mechanism, which is downregulated by AML1-ETO.

We observed that physical interaction between AML1-ETO and PU.1 did not abolish the DNA binding capacity of PU.1 (Fig. 4a), although AML1-ETO interacted with the PU.1 DNA binding domain. Interestingly, in PU.1's crystal structure,<sup>1</sup> the  $\beta 3\beta 4$  domain does not interact with DNA, but is exposed to the solvent. This structural ability allows PU.1 to retain its DNA binding though being functionally repressed. We show here that the normal interaction between co-activators and transcription factors are altered in presence of AML1-ETO, which could be one of the important mechanisms for disrupted myelopoiesis in t(8;21) positive leukemia (Fig. 7f).

AML1B and AML1-ETO have been shown to transactivate the M-CSF receptor,<sup>42</sup> suggesting that interaction between AML1B and AML1-ETO could be important for leukemogenesis. To investigate the importance of AML1-ETO/PU.1 interaction in leukemogenesis, transactivation, proliferation and differentiation assays were performed in cells expressing wild type AML1B protein. In presence of AML1-ETO the M-CSF receptor promoter was downregulated and similarly the AML1 site mutated M-CSF receptor promoter and minimal promoter containing only PU.1 binding sites in U937 cells (Fig. 5a). This could be explained by a dual function of AML1-ETO in regulation of the M-CSF receptor expression. During normal myeloid differentiation, M-CSF receptor expression is required for G1-S phase transition, which could be downregulated by AML1-ETO through the functional interaction with PU.1, and then AML1-ETO cooperates with AML1B to upregulate the M-CSF receptor expression for transformation and proliferation of abnormal progenitor cells.

In patients with t(8;21), expression of the cell surface markers CD11b, CD14 and CD64 was less in comparison to patients without t(8;21) (Fig. 5b).

CD14 and CD64 promoters have putative PU.1 binding sites but not AML1, C/EBP $\alpha$  or MEF binding sites suggesting that downregulation of PU.1's function by AML1-ETO could possibly be an important step in progression towards leukemia. CD11b, another marker for differentiation was also less expressed in patients with t(8;21) in comparison to patients without t(8;21) (Fig. 5b). CD11b is regulated by PU.1 and its promoter contains putative binding sites of AML1. In this case AML1-ETO's interaction and downregulation of important myeloid transcription factors like PU.1 and AML1 could explain the lower CD11b expression. The phenotype of PU.1<sup>-/-</sup> mice suggest that PU.1 is critical for myeloid differentiation and development.<sup>4 11 12</sup> Interaction of PU.1 with AML1-ETO and subsequent suppression of PU.1 target genes (Fig. 5b) might contribute to the phenotypic changes seen in t(8;21). Furthermore, PU.1 is a self-regulatory protein<sup>54</sup> and AML1-ETO overexpression in mouse bone marrow cells downregulated the endogenous PU.1 expression (Fig. 6b).

Because AML1<sup>-/-</sup> mice lack PU.1 expression,<sup>61</sup> AML1-ETO could downregulate PU.1 expression through repressing AML1 function. However, in a diseased condition or in presence of AML1-ETO, like in Kasumi-1 cells, the expression of AML1 and PU.1 genes was still observed. Furthermore, AML1 does not have a downregulatory effect on PU.1 (Fig. 1c). Therefore, the presence of AML1-ETO does not completely repress the expression levels of these genes, but may block their functions by protein-protein interactions. To analyse the functional impact of AML1-ETO on PU.1 in presence of wild type AML1B protein we performed experiments in cells expressing endogenous AML1B protein. Overexpression of PU.1 in mouse bone marrow cells leads to a block in proliferation, but in presence of AML1-ETO this function of PU.1 was abrogated (Fig. 6a). Recently, it was shown that AML1-ETO expression in human progenitor cells leads to expansion of human hematopoietic stem cells.<sup>62</sup> Therefore, the block of differentiation and increase in abnormal proliferation of

hematopoietic stem cells could be due to downregulation of PU.1's activity in t(8;21) leukemia.

Overexpression of PU.1 in t(8;21) positive Kasumi-1 cells differentiates them towards the monocytic lineage (Fig.7). Morphologically cells did not appear to be terminally differentiated even though the cell surface markers CD11b and CD14 were increased in expression. It has been earlier shown that short-term activation of PU.1 in multipotent hematopoietic cells leads to immature eosinophils.<sup>63</sup> However, stable overexpression of PU.1 could lead to myeloid lineage in hematopoietic progenitor cells.<sup>63</sup> Therefore, higher and stable expression of PU.1 in Kasumi-1 cells might be needed to terminally differentiate towards the monocytic lineage. The cell number of PU.1 transfected Kasumi-1 cells decreased over a course of time (Fig. 7e) showing that PU.1 functions as anti-proliferative factor upon overexpression in Kasumi-1 cells. However, this mechanism needs to be further elucidated. Our data suggest that the ectopic expression of PU.1 in Kasumi-1 cells overcomes the functional block by AML1-ETO. PU.1 and C/EBP $\alpha$  are important factors for myeloid differentiation and AML1-ETO downregulating these two factors could be an important step towards leukemia. This also suggest the possibility of using these two factors independently or in combination for therapy of t(8;21) myeloid leukemias.

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## Figure legends:

### Figure 1: AML1-ETO binds to PU.1 and downregulates PU.1 s transactivation capacity

- a. **AML1-ETO binds to PU.1 *in vivo*:** 200µg of Kasumi-1 cell nuclear extracts were immunoprecipitated with rabbit IgG (lane 1), anti-AML1 antibody (lane 2), goat IgG (lane 3), or anti-ETO antibody (lane4). The immunoprecipitates were subjected to SDS-PAGE along with *in vitro* translated PU.1 (lane 6) and nuclear extracts (NE) (lane 7) and further subjected to immunoblotting with PU.1 antibody. In the lower panel Kasumi-1 nuclear extracts were immunoprecipitated with anti-PU.1 (lane 2) or IgG (lane 3) and subjected to SDS-PAGE along with nuclear extracts of Kasumi-1 cells (lane 4) and blotted with anti-ETO antibody.
- b. **AML1-ETO inhibits PU.1 s transactivation capacity:** 293T cells were transiently transfected with human monocyte-specific M-CSF receptor promoter or promoterless vector pXP2 or pSRE (Serum Response Element) and with expression plasmids of PU.1 (100ng), c-Jun (50ng), AML1-ETO (20ng) and activated Ras (50ng). Promoter activities (fold) were determined 24h post transfection and normalized to the activities of the internal control plasmid pRL0. In the lower panel, it is shown that AML1-ETO does not change the expression of co-transfected PU.1. 293T cells were transfected as above and whole cell lysates were subjected to SDS-PAGE followed by immunoblot assay with PU.1 specific antibody.
- c. **AML1 does not affect PU.1 s transactivation capacity:** 293T cells transfected with p(PU.1)4TK-luc and expression plasmids of PU.1 (100ng), c-Jun (50ng), AML1 (50ng) or CBFβ (50ng). PU.1, c-Jun, AML1 and CBFβ had no effects on negative control p(mut.PU.1)4TK (data not shown).

**Figure 2: AML1-ETO inhibits co-activation of PU.1 by c- Jun**

- a. **F9 cells do not express c- Jun:** 100µg of nuclear extracts of 293T, F9 and Kasumi-1 cells along with *in vitro* translated c-Jun were subjected to SDS-PAGE and immunoblotted for c-Jun.
- b. **AML1-ETO inhibits PU.1 c- Jun transactivation capacity:** F9 cells were transfected with p(PU.1)4TK, a minimal TK promoter driven by PU.1 DNA binding sites only or control vector p(mut.PU.1)4TK along with expression plasmids of PU.1 (100ng), c-Jun (50ng) and AML1-ETO (20ng).
- c. **AML1-ETO downregulates the PU.1 regulated M-CSF receptor promoter activity by inhibiting PU.1 c- Jun function:** F9 cells were transfected with M-CSF receptor promoter and PU.1 (100ng), c-Jun (50ng) and AML1-ETO (20ng). PU.1, c-Jun and AML1-ETO had no effects on control vector pXP2 (data not shown).

**Figure 3: AML1-ETO displaces the co-activator c- Jun from PU.1 by binding to the  $\beta 3\beta 4$  region of PU.1**

- a. **AML1-ETO physically binds to PU.1 *in vitro*:** GST pulldown assay was performed using [<sup>35</sup>S]Met-labelled *in vitro* translated c-Jun (lane 1) or AML1-ETO (lane 4) incubated with equal amounts of bacterially expressed GST-PU.1 (lanes 2 and 5) or GST+beads (lane 3 and 6). GST-PU.1 or GST were recovered using glutathione-agarose beads and separated by SDS-PAGE prior to autoradiography.
- b. **AML1-ETO displaces c- Jun from binding to the  $\beta 3\beta 4$  domain of PU.1:** Saturating amounts of *in vitro* translated c-Jun (20 $\mu$ l) were incubated with GST- $\beta 3\beta 4$  and increasing amounts (from lane 7-lane 13) of *in vitro* translated AML1-ETO (7.5-12.5 $\mu$ l) were incubated. Densitometric quantification was also performed (given as % input of the proteins).



**Figure 4: AML1-ETO does not change the DNA binding of PU.1**

The PU.1 binding sequence from the CD11b promoter was chosen and labelled with  $\gamma$ -dATP (lane 1), incubated with *in vitro* translated PU.1 (lane 2) or *in vitro* translated PU.1 and anti-PU.1 antibody (lane 3). As a competitor, unlabelled probe was used in 100 molar excess with (lane 5) and without anti-PU.1 antibody (lane 4). To investigate if this binding and supershift is specific for PU.1, similar experiments were performed with rabbit reticulocyte lysate (lanes 6, 7, 8, 9). In presence of AML1-ETO, PU.1 still binds to its DNA (lane 10) and supershifts with anti-PU.1 antibody (lane 11).

**Figure 5: AML1-ETO downregulates PU.1 s transactivation capacity in myeloid cells and the expression of PU.1 s target genes in AML patients with t(8;21)**

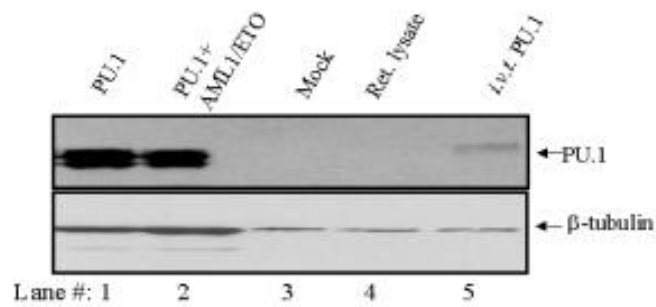
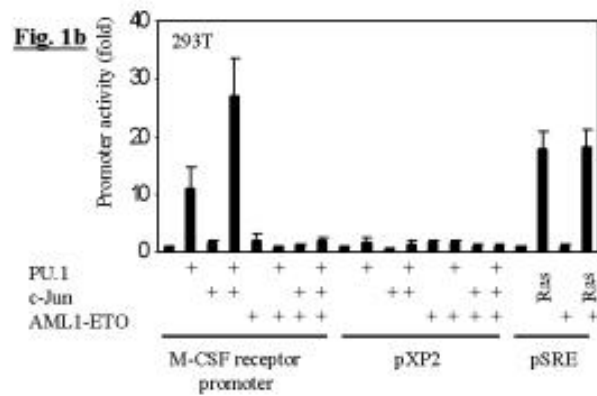
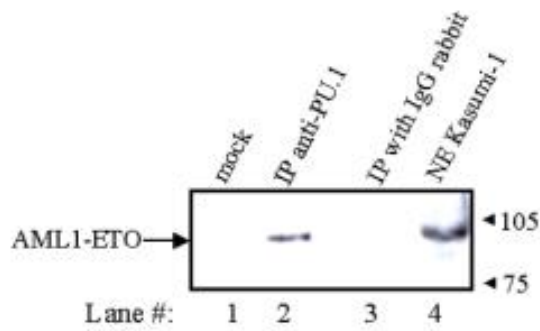
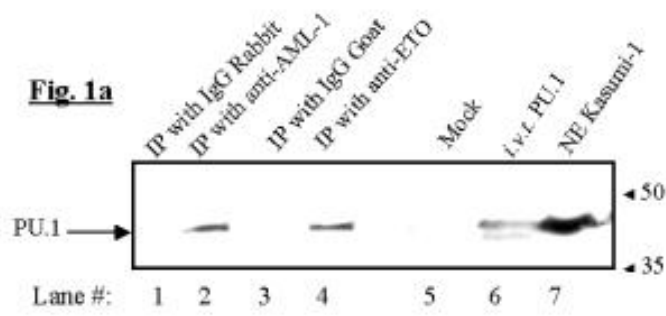
- a. **AML1-ETO downregulates PU.1 s transactivation in myeloid cells:**  
U937 cells were electroporated with wild type M-CSF receptor promoter, M-CSF receptor promoter without (w/o) AML1 binding site, p(PU.1)4TK, p(mutPU.1)4TK or pXP2 with and without AML1-ETO.
- b. **Low expression of PU.1 target genes in patients with t(8;21):** AML patients (n = number of patients) with t(8;21) have less positive cells for cell surface markers regulated by PU.1 as compared to patients without t(8;21). CD14 and CD64 promoters have PU.1 binding sites, but no putative C/EBP $\alpha$ , AML1 or MEF binding sites.

**Figure 6: The anti-proliferative effect of PU.1 is downregulated by AML1-ETO in mouse bone marrow cells**

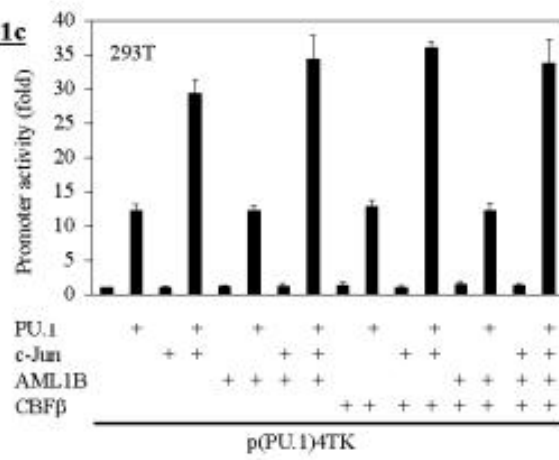
- a. **AML1-ETO causes proliferation in mouse bone marrow cells:** Live transduced mouse bone marrow cells with PU.1, AML1-ETO or PU.1 and AML1-ETO were counted on day 3, 6, and 12 after Trypan blue staining. Since both the empty vectors gave the same cell count, only one (PINCO) vector has been represented as mock.
- b. **Expression of PU.1 in mouse bone marrow cells:** The cells of above transduction were lysed and immunoblot assays were performed for PU.1 and  $\beta$ -tubulin. NGRF (N) (lysate of empty vector of PU.1), N-PU.1 (NGFR-PU.1 transduced cells), PINCO (P) (lysate of empty vector of AML1-ETO transduced cells), P-AML1-ETO (lysate of PINCO-AML1-ETO transduced cells) and P-AML1-ETO+N-PU.1 (lysate of PINCO-AML1-ETO and NGFR-PU.1 transduced cells). The ratio of PU.1/ $\beta$ -tubulin was calculated after densitometric quantification of the bands.

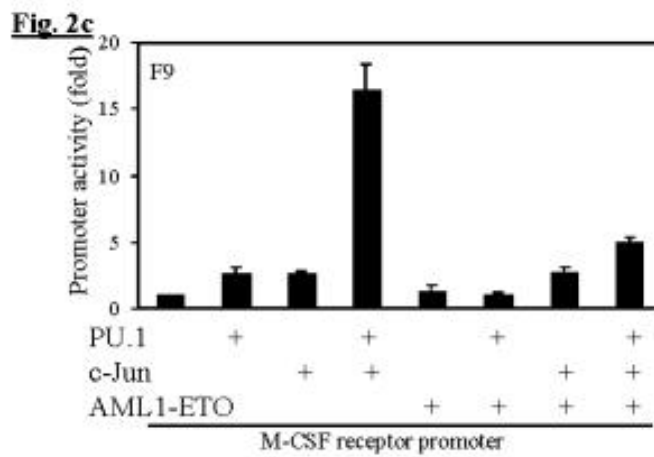
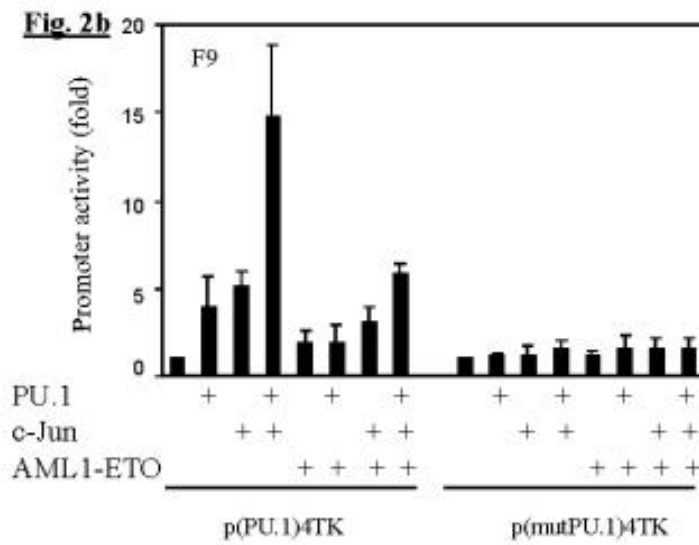
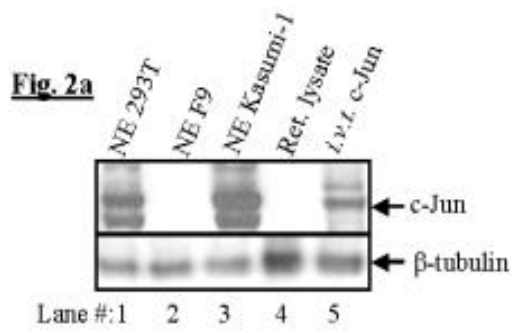
**Figure 7: Transient overexpression of PU.1 induces differentiation towards the monocytic lineage in AML1-ETO positive *asumi-1* cells:**

- a. **PU.1 induces differentiation in t(8;21) positive *asumi-1* cells:** Kasumi-1 cells were transiently transfected with PU.1 (pGCsam-PU.1-ires-NGFR) or the empty vector (pGCsam-ires-NGFR) and morphological changes were observed on day 5. Arrows indicate the differentiating cells.
- b. **PU.1 overexpression in *asumi-1* cells:** Western blot showing PU.1 expression and  $\beta$ -tubulin in transfected Kasumi-1 cells after day 5.
- c. **PU.1 induces CD11b expression in *asumi-1* cells:** FACS analysis for the cell surface expression of CD11b, in Kasumi-1 cells transfected with empty vector or PU.1.
- d. **PU.1 induces CD14 expression in *asumi-1* cells:** In the same experiment FACS analysis for the cell surface expression of CD14, in Kasumi-1 cells transfected with empty vector or PU.1.
- e. ***asumi-1* cell number decreases in PU.1 transfected cells:** The above transfected cells were counted by trypan blue staining on day 1, 2, 3, 4, and 5 after transfection.
- f. **Model of AML1-ETO blocking PU.1 function:** Model of AML1-ETO interacting with PU.1 and displacing its co-activator c-Jun. This downregulation of PU.1's transcriptional activity by AML1-ETO results in downregulation of PU.1 target genes important for myeloid differentiation.

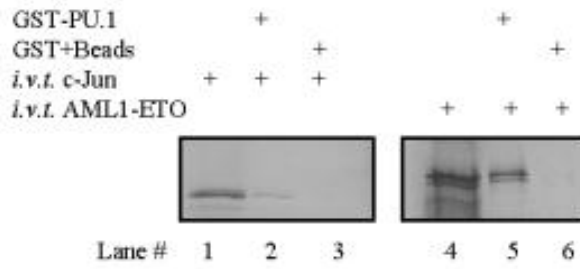


**Fig. 1c**

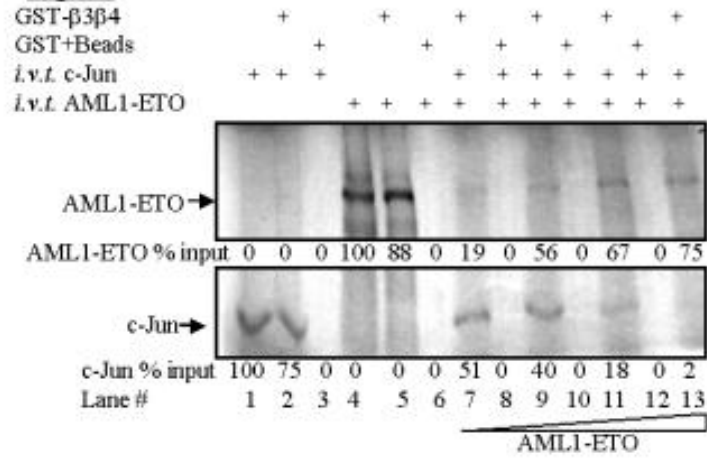




**Fig. 3a**



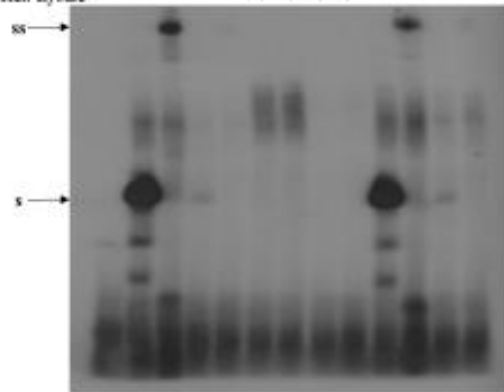
**Fig. 3b**





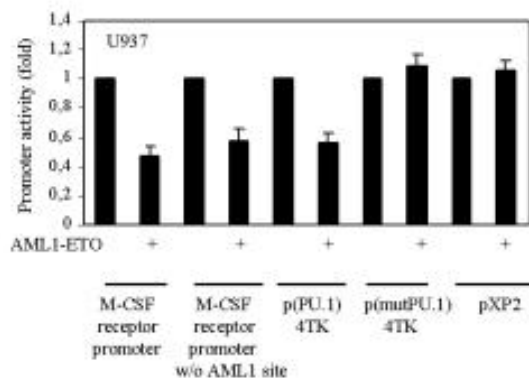
**Fig 4**

Lane	1	2	3	4	5	6	7	8	9	10	11	12	13
PU.1 probe	+	+	+	+	+	+	+	+	+	+	+	+	+
PU.1		+	+	+	+					+	+	+	+
anti-PU.1			+	+		+		+			+		+
Coempt				+	+			+	+			+	+
AML1/ETO										+	+	+	+
Ref. Lysate						+	+	+	+				

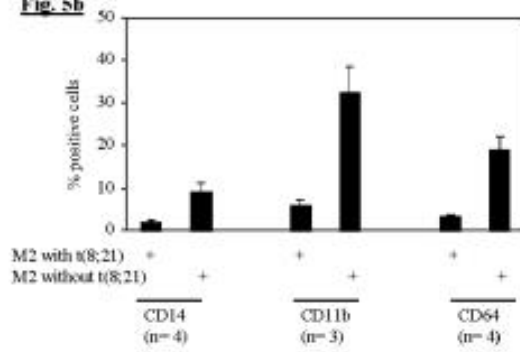


s Shift  
ss Super shift

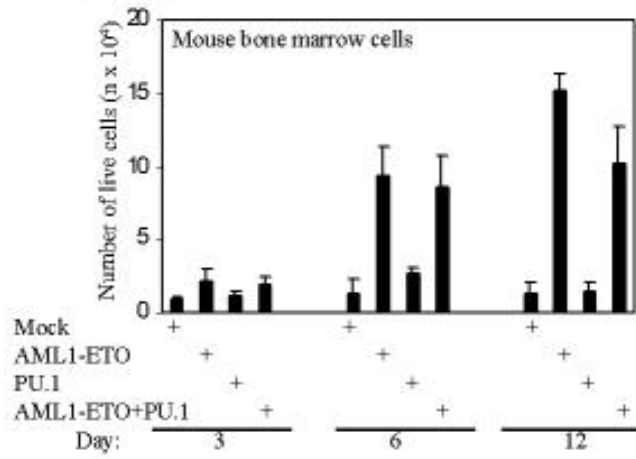
**Fig. 5a**



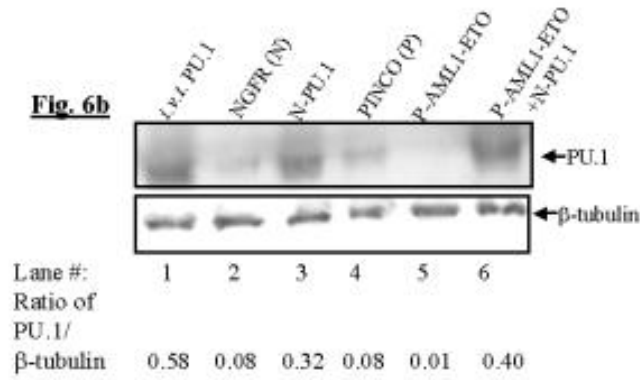
**Fig. 5b**

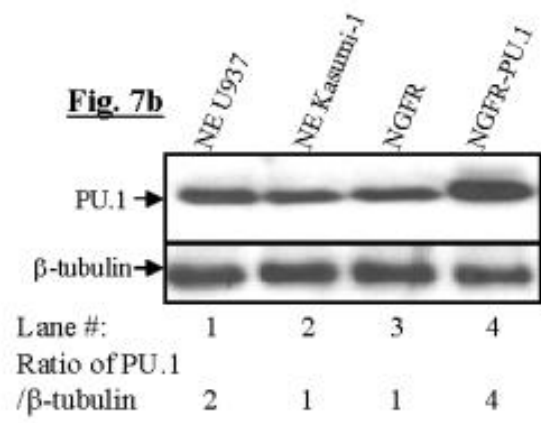
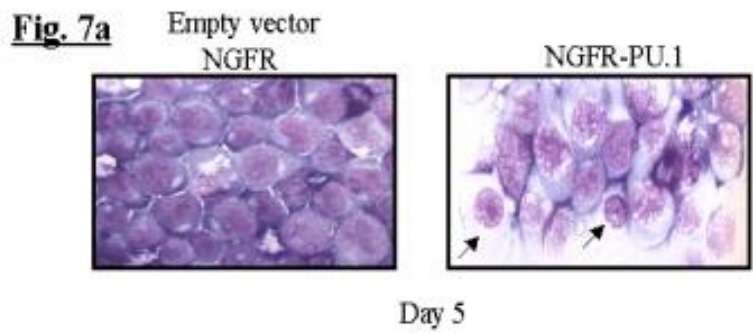


**Fig. 6a**

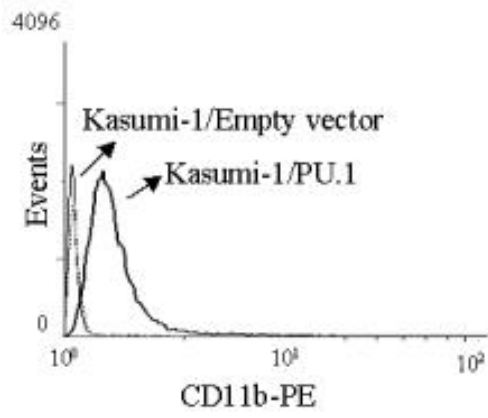


**Fig. 6b**

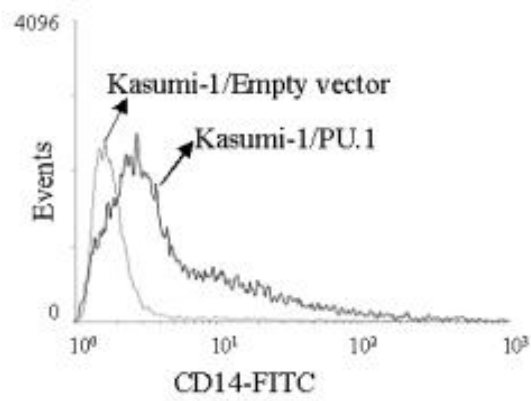




**Fig. 7c**



**Fig. 7d**



**Fig. 7f**

