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Proteomics of Acute Myeloid Leukemia: Cytogenetic Risk Groups Differ Specifically in their Proteome, Interactome and Posttranslational Protein Modifications

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Dedicated To My Father Mohd Yaseen Balkhi
&
Mother Shahzada Yaseen

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Abbreviations

AML	Acute Myeloid Leukemia
ALL	Acute Lymphoid Leukemia
APL	Acute Promyelocytic Leukemia
BIR	Baculoviral IAP Repeat
BSA	Bovine Serum Albumin
CDK	Cyclin-Dependent Kinases
CARD	Caspase Recruitment Domain
C/EBPα	CCAAT/Enhancer Binding Protein α
CHAPS	3[(3-Cholamidopropyl) dimethylammonio] propanesulfonic acid
CHCA	α-Cyano-4-Hydroxycinnamic Acid
CK	Complex Karyotype
CLL	Chronic Lymphocyte Leukemia
CPC	Chromosomal Passenger Complex
CML	Chronic Myelogenous Leukemia
CLP	Common Lymphoid Progenitor
CMP	Common Myeloid Progenitor
DAPI	4, 6-diamidino-2-phenylindole
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
DTE	1, 4-Dithioerythritol
DTT	Dithiothreitol
DHB	2, 5-Dhydroxy-Benzoicacid
EDTA	Ethylene Diamine Tetra-acetic Acid
EGTA	Ethyleneglycol-bis(2-aminoethylether)-N,N,N',N'-Tetraacetic Acid
EGF	Epidermal Growth Factor
FACS	Fluorescent Activated Cell Sorter

GFP	Green Fluorescent Protein
GMP	Granulocyte Monocyte progenitor
HSCs	Hematopoietic Stem Cells
HDAC	Histon Deacetylase
IAPs	Inhibitor of Apoptosis Proteins
IB	Immunoblot
IPG	Immobilized pH Gradient
IRF-1	Interferon Regulatory Factor-1
ICAT	Isotope-Coded Affinity Tags
LSCs	Leukemic-Stem Cells
LT-HSCs	Long-Term HSCs
MALDI	Matrix-Assisted Laser Desorption Ionization
MLP	Multilineage Progenitors
MEP	Megakaryocyte Erythrocyte Progenitor
µCi	Microcurie
µM	Micromolar
mM	Millmolar
µg	Microgram
µl	Microliter
MAPK	Mitogen-Activated Protein Kinase
MPO	Myeloperoxidase
MS	Mass Spectra
NBM	Normal Bone Marrow
NB	Nuclear Body
N-CoR	Nuclear Receptor Corepressor
NK	Normal Karyotype
NP40	Nonidet P-40
NPM	Nucleophosmin
NuMA	Nuclear Mitotic Apparatus

OR	Oestrogen Receptor
OP18	Oncoprotein 18
PAGE	Polyacrylamide Gel Electrophoresis
PCNA	Proliferating Cell Nuclear Antigen
PML	Promyelocytic Leukemia
PBG	PBS, BSA, fish skin Gelatin
PBS	Phosphate Buffer Saline
PODs	PML Oncogenic Domains
PTM	Posttranslational Modification
PMF	Peptide Mass Fingerprinting
RARE	Retinoic Acid Response Element
Rb	Retinoblastoma
RIPA	Radioimmunoprecipitation Assay
SDS	Sodium Dodecyl Sulphate
shRNA	Short hairpin small interfering RNAs
siRNA	Small Interfering RNAs
SMRT	Silencing Mediator for Retinoid and Thyroid hormone
ST-HSCs	Short-Term HSCs
TFA	Trifluoroacetic Acid
TOF	Time of Flight

1 Introduction

1.1 Acute myeloid leukemia

Acute Myeloid Leukemia is a malignant disorder which affects myeloid development or myelopoiesis. Myelopoiesis begins with the differentiation of a small pool of pluripotent stem cells into the most primitive myeloid progenitors. These progenitors develop into myeloid precursors, which subsequently follow a specific differentiation program and lineage commitment controlled by lineage specific transcription factors and newly discovered micro RNAs (Shivdasani, 2006). This process ultimately results in the development of mature neutrophils, eosinophils, basophils and monocytes, eythrocytes and megakaryocytes. The schematic representation of myeloid development from hematopoietic stem cell is shown as an Akashi-Kondo-Weissman model of adult hematopoiesis (Figure 1).

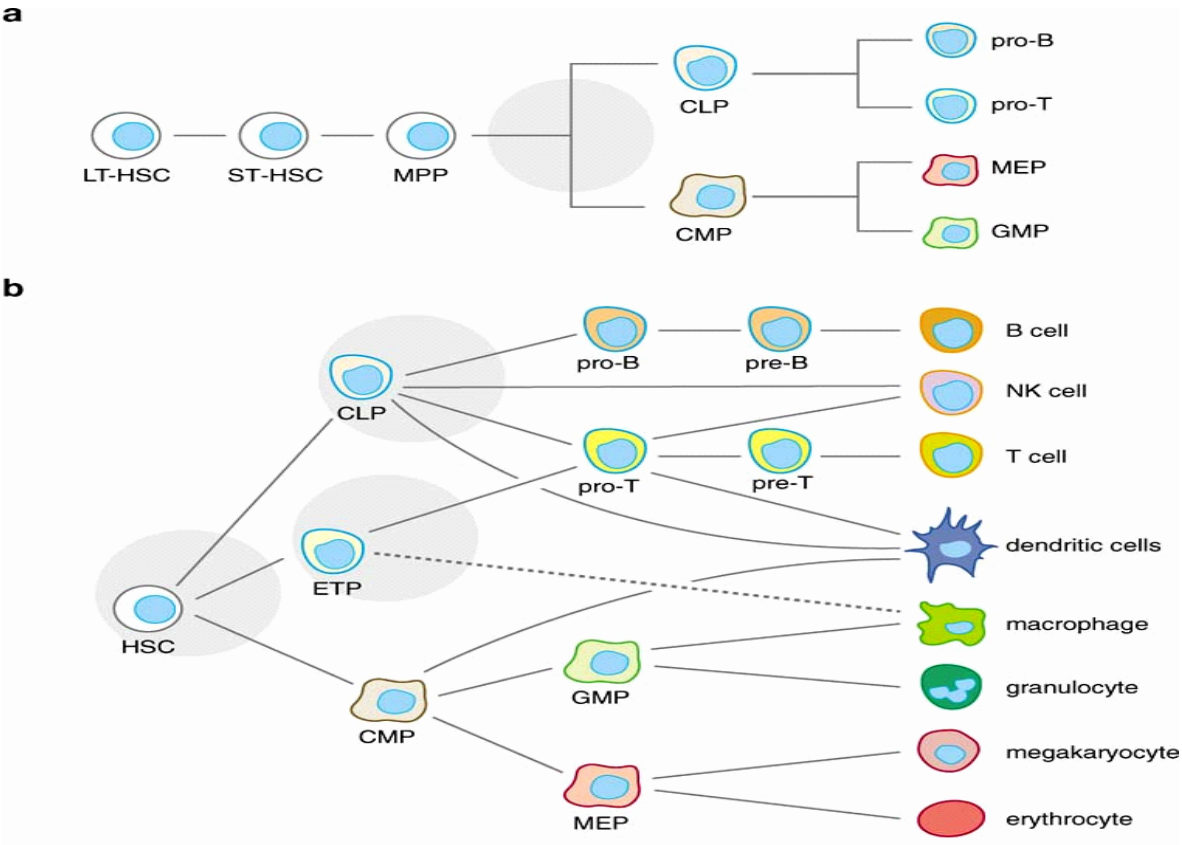


Figure 1. Lineage tree of adult hematopoiesis and lymphoid-myeloid branching points (a) Akashi-Kondo-Weissman model of adult hematopoiesis with the branching point between

lymphoid and myeloid lineages indicated by the gray shaded circle. (b) Revised lineage tree, showing three areas where branching might occur (LT- and ST-HSC, long-term and short-term HSC; MPP, multipotent progenitor; ETP, early T lineage progenitor). Adapted from a proposed model of major hematopoietic maturation pathways from HSCs, proposed by Irving L. Weissman (Akashi et al., 2000).

There have been significant advances in our understanding of hematopoiesis and molecular regulation of myelopoiesis. This has lead to the better understanding of molecular mechanisms regulating the development of acute myeloid leukemia. Several recent reports and studies of over two decades has now confirmed that transcription factors are commonly disrupted in AML either by their fusion as a result of chromosomal translocations or by point mutations. Some of the common transcription factors affected by these chromosomal translocations include the core binding factor complex, AML1, the retinoic acid receptor (RAR), GATA family of transcription factors, the MLL protein, and Hox proteins. Point mutations in myeloid transcription factors like C/EBPα and PU.1 may also lead to loss of normal myeloid differentiation in AML (Tenen, 2003). Besides dominant negative mutations of C/EBPα, which have been reported to occur in 10% of AML patients (Schwieger et al., 2004), also affect myeloid development. The chromosomal translocations, which are often associated with AML, often result in aberrant fusion of these transcription factors with other genes, for example, in t(8;21)-AML, AML1 is fused with ETO gene and in t(15;17)-AML, PML is fused with RARA gene. These chimaeric transcription factors often act as a dominant negative over wild type transcription factors and are widely reported to be co-repressors of gene transcription. The other commonly disrupted transcription factors in AML include GATA1. GATA family of transcription factors is indispensable for hematopoiesis. GATA 1 is expressed at high levels in erythroid cells, megakaryocytes, mast cells, and eosinophils (Evans and Felsenfeld, 1989). GATA-1-deficient erythroid precursors fail to differentiate beyond the proerythroblast stage during the

lineage development of erythrocytes. PU.1 and GATA factors have antagonist functions in the hematopoietic development. For example, GATA-1 blocks PU.1 transactivation by blocking its binding of PU.1 coactivator c-jun. Besides PU.1 inhibits GATA-1 transactivation by inhibiting its binding to DNA (Nerlov et al., 2000). A recently discovered myeloid specific transcription factor MafK has been reported to play a crucial role in specifying transcription of erythroid specific genes. C/EBPα is a well known and extensively investigated lineage specific transcription factor. This transcription factor is required for the development of CMP (common myeloid progenitors) to GMP (granulocyte/ macrophage progenotors). Figure 2; describes the role of various transcription factors in myeloid lineage commitment decisions. C/EBPα inhibits cell cycle progression via interaction with E2F1 and additional mechanisms, and stimulates cell survival by inducing bcl-2 in cooperation with NF-κB (Wang et al., 2003). C/EBPα$^{-/-}$ hematopoietic cells derived form adult bone marrow do not generate granulocyte-monocyte progenitors (GMPs) from the common myeloid progenitor (CMP) (Heath et al., 2004).

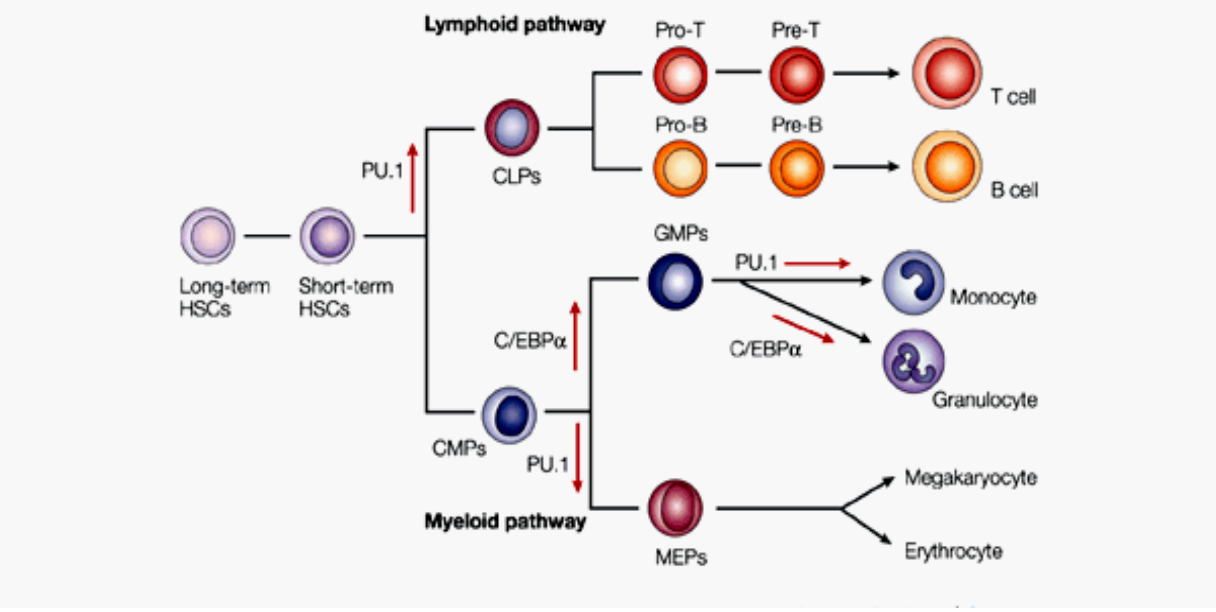


Figure 2. Role of transcription factors in hematopoietic development**.** The common myeloid progenitor (CMP) gives rise to granulocyte/macrophage progenitors (GMPs) and megakaryocyte/erythroid progenitors (MEPs). Upregulation of the transcription factor PU.1 is essential for the transition from HSC to CLP, whereas downregulation of PU.1 is required for the differentiation of CMP to MEP. CCAAT/enhancer binding protein-α (C/EBPα) upregulation initiates the transition from CMP to GMP. One hypothesis is that the 'default' pathway involves differentiation of GMPs to monocytes and macrophages, which depends on PU.1 activity. In this model, C/EBPα subverts this default monocytic pathway and promotes differentiation into granulocytes. Adapted from Nature Reviews Cancer (Tenen, 2003).

1.2 Chromosomal translocations in AML

Acute myeloid leukemia is a disease of molecular alterations and genomic instability that disrupt almost every aspect of cellular biology. These include the regulation of cell proliferation, differentiation, self-renewal, cell cycle checkpoint control, DNA repair mechanism and chromatin stability, and cell migration. One of the important features of acute myeloid leukemia is the occurrence of cytogenetic abnormalities. Cytogenetic analysis for AML patients provides some of the strongest information available for the disease (prognosis), predicting the outcome of the disease remission and effectiveness of the therapy besides overall survival rates (Marcucci et al., 2004).The cytogenetic abnormalities involving translocations of t(8;21), t(15;17) or inv(16) are associated with favourable prognosis that means they respond positively to the therapy and the chances of cure for the disease is higher (Grimwade et al., 1998). The group of AML that is characterized by aberrations of chromosomes 5 and 7 or abnormalities of 11q23 shows a poor response to chemotherapy (Schoch et al., 2005). Finally, there is a heterogeneous group of patients who do not show any apparent cytogenetic aberrations and are grouped as normal karyotype AML. This group is considered an intermediate AML risk group (Bienz et al., 2005). Because of the importance of cytogenetics in AML the classification of AML has been revised by World Health Organization (Cheson et al., 1990). While the elements of the French-American-British (FAB) classification largely based on morphology, immunophenotype, cytogenetics and clinical features have

been retained. The WHO classification incorporates more recent discoveries regarding the genetics, clinical and prognostic features of AML. This is an attempt to classify entities that are homogeneous biologically and that have prognostic and therapeutic relevance. The WHO classification of AML is shown as table1.

Table 1. WHO classification of AML. Adapted from (Bennett et al., 1985) proposed revised criteria for the classification of AML.

AML with characteristic genetic abnormalities
- AML with t(8;21)(q22;q22); AML1/ETO
- AML with inv(16)(p13q22) or t(16;16)(p13;q22); (CBFβ/MYH11)
- Acute promyelocytic leukemia AML with t(15;17)(q22;q12); (PML/RARα) and variants
- AML with 11q23 (MLL) abnormalities
- AML with FLT3 mutation

AML with multilineage dysplasia

AML and MDS, therapy related
- Alkylating agent-related AML and MDS
- Topoisomerase II inhibitor-related AML

AML not otherwise categorized
- Acute myeloblastic leukemia minimally differentiated (FAB Classification M0)
- Acute myeloblastic leukemia without maturation (FAB Classification M1)
- Acute myeloblastic leukemia with maturation (FAB Classification M2)
- Acute myelomonocytic leukemia (AMML) (FAB Classification M4)
- Acute monoblastic leukemia and acute monocytic leukemia (FAB classifications M5a and M5b)
- Acute erythroid leukemias (FAB classifications M6a and M6b)
- Acute megakaryoblastic leukemia (FAB Classification M7)
- AML/transient myeloproliferative disorder in Down syndrome
- Acute basophilic leukemia
- Acute panmyelosis with myelofibrosis
- Myeloid sarcoma

Acute leukemias of ambiguous lineage

The balanced chromosomal translocations as well as aberrant complex translocations (involving multipule translocations and deletions) are the common occurrence in leukemia. These translocations are important prognostic markers and have been used in disease diagnosis and treatment induction. Common targets of these translocations are the transcription factors that are indispensable for hematopoiesis. The abnormal

expression of these chimaeric transcription factors target other oncogenes and leads to disease progression through unknown mechanisms. The targets of these translocations are described below in detail:

1.2.1 The core binding factor complex

The core binding factor complex consists of a heterodimer of the Runx1 (formerly AML1) and CFBß protein and normally activates a number of genes critical for normal myeloid development. In AML this transcription factor is disrupted by at least three different translocations: t(8;21), which generates the AML1-ETO fusion; inv(16), yielding the CBFß-MYH11 fusion; and t(3;21), which generates the RUNX1-EVI1 fusion protein associated with MDS and therapy-related AML. All of these fusions act as dominant negative over the core binding factor complex. AML1 is the DNA-binding subunit of the core-binding transcription factor (CBF) and binds to the enhancer core sequence TGT/cGGT, which has been shown to be important in the transcriptional regulation of a number of viral and cellular genes (Wang et al., 1993); (Meyers et al., 1993). DNA-binding activity of AML1 is mediated through a central 118 amino acid domain that is homologous to the Drosophila pair-rule protein Runt (hence its designation as the Runt homology domain, or RHD) (Crute et al., 1996; Daga et al., 1992) and its affinity for DNA binding is increased through heterodimerization through the RHD with a second non-DNA-binding subunit CBFβ (Figure 3) (Wang et al., 1993).

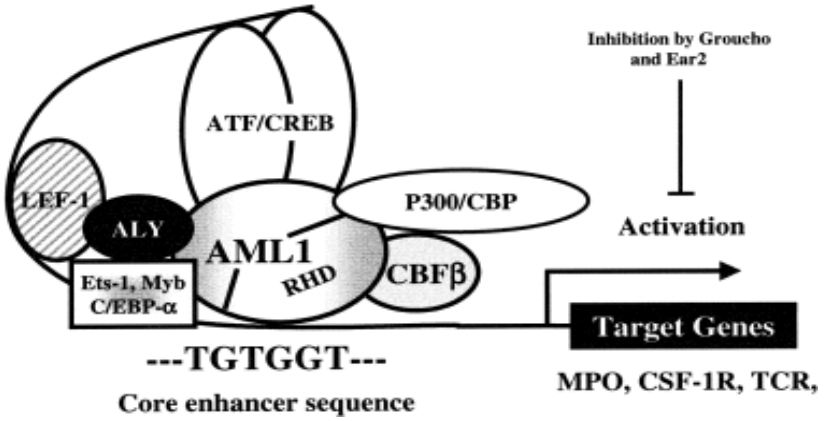


Figure 3. The AML1/CBFβ transcription factor complex binds to the core enhancer sequence and functions as an enhancer organizing factor to induce gene transcription. Other proteins in this complex differ according to the particular promoter or enhancer involved. They include the transcription factors Ets-1 or Ets family members, C/EBP-α, the transcriptional coactivators ATEF/CREB and P300/CBP, and the DNA-bending protein LEF-1, which interacts with AML1 through an adapter protein called ALY. Among the genes whose transcription is regulated by AML1 are: myeloperoxidase (MPO), the receptor for colony-stimulating factor 1 (CSF-1R), and the subunits of the T-cell antigen receptor (TCR). Binding of the AML/CBFβ complex to DNA normally leads to transcriptional activation; however, when it is complexed with either Groucho or the Ear2 protein, its activity is converted to that of a transcriptional repressor. Adapted from British Journal of Haematology review (Downing, 1999).

The t(8;21) translocation which involves AML1 transcription factor is reported in 10–15% of AML patients and is frequently the only cytogenetic abnormality present in the leukemic blasts. (Bitter et al., 1987). Patients with this subtype of AML typically present FAB AML-M2 morphology. Patients with this translocations generally respond positively to induction therapy and have prolonged disease free survival (Bloomfield et al., 1998), however, when associated with extra mutations in Flt3 or deletions of chromosomes, the penetration of disease and prognosis are severe. The translocation t(8;21)(q22;q22) involves the AML1 gene and ETO (eight-twenty-one) gene. The transcriptional activation domains of AML1 are deleted and replaced by ETO sequences known to interact with nuclear co-repressors like N-COR or SMRT and HDACs (Figure 4). This associated of ETO with these corepressors produce secondary effects of histone deacetylation and recruits methyltransferase contributing to abnormally stable corepression complex (Ferrara et al., 2001; Racanicchi et al., 2005). This chimaeric protein, AML1-ETO, target the promoters of AML1 target genes and directly represses AML1-mediated transcriptional activation in transient transcription assays (Meyers et al., 1995). It represses genes usually activated by AML1, including c-FMS, (Follows et al., 2003) p14 ARF (Hiebert et al., 2003), and C/EBPα (Pabst et al., 2001). The AML1 family of transcription factors recognize the binding sequence TGT/yCGGT (Erickson et al., 1992) in a

number of genes including M-CSF receptor, GM-CSF, myeloperoxidase and neutrophil elastase through a 117-amino acid region that is highly homologous to the Drosophila segmentation gene runt (Erickson et al., 1992; Meyers et al., 1993). AML1-ETO complexes retain the ability to bind to the core sequence and activates the transcription of number of genes (Yergeau et al., 1997; Zhang et al., 1996). AML1-ETO inhibits gene activation by inactivating hematopoiesis promoting transcription factors like AML1 and C/EBPα. However, little is known about the exact mechanism of AML1-ETO mediated inhibition and/or activation of target genes.

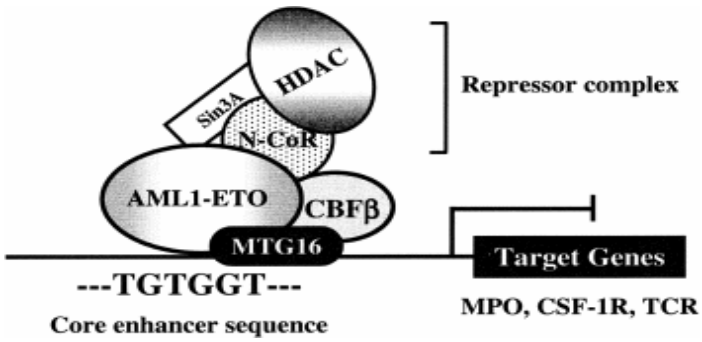


Figure 4. The AML1-ETO fusion protein retains the ability to bind to the core enhancer sequence and to heterdimerize with CBFβ. In contrast to wild-type AML1, this fusion protein binds through its ETO sequences to both ETO/MTG family members such as MTGR1 and MTG16, and to a corepressor complex. This co repressor interaction results in the repression of genes whose transcription is normally activated by AML/CBFβ. Among the target genes affected are: myeloperoxidase (MPO), the receptor for colony-stimulating factor 1 (CSF-1R), and the subunits of the T-cell antigen receptor (TCR). Adapted from British Journal of Haematology review (Downing, 1999).

1.2.2 Acute promyelocytic leukemia

APL occurs in 5%-9% of AML cases and is associated with promyelocytes blasts with a FAB M3 subtype. In APL, the retinoic acid receptor alpha (RARα) gene on 17q12 fuses with a nuclear regulatory factor on 15q22 (promyelocytic leukemia or PML gene) resulting in a PML-RARα gene fusion transcript (Caligiuri et al., 1997). A rare type of masked t(15;17) also occur and is associated with complex variant translocation leading to the

submicroscopic insertion of the RARα gene into PML gene leading to the expression of the PML-RARα fusion transcript called M3v (Le Beau et al., 2002). The translocation t(11;17) results in the PLZF-RARα fusion and is reported in 1% of AML which is a retinoic acid resistant form of disease. APL patients respond positively to the ATRA and arsenic acid treatment and show good prognosis. The other drugs tested are the histone deacetylase inhibitor valproic acid, either alone or in combination with ATRA, has provided evidence of differentiation and decreased blast counts in patients (Kuendgen et al., 2004). PML and all of the fusion partner proteins in APL have been shown to form multimeric complex with RXR. Like AML1-ETO, PML-RARα also acts as dominant negative over RARα. PML-RARα has been shown to recruit DNA methyl transferases to promotors contributing to the transcriptional suppression (Di Croce et al., 2002). Recenty it was reported that a cleavage of the PML-RARα fusion protein by neutrophil elastase (NE) was required for the development of APL like disease (Lane and Ley, 2003).

1.2.3 Acute myeloid leukemia with 11q23 (MLL) abnormalities

AML with 11q23 abnormalities comprises 5% to 6% of cases of AML and is typically associated with acute myelomonocytic, monoblastic, and monocytic leukemia (FAB classifications M4, M5a and M5), respectively. The MLL gene on 11q23, a developmental regulator, has been reported to be involved in translocations with 22 different partner chromosomes. 11q23 is involved with the complex translocations like t(11;22) (q23;q12), t(4;11) (q21;q23), t(9;11) (q22;p13.1) (Giugliano et al., 2002). Patients with t(11;19)(q23;p13.1) have poor outcome and are considered high risk AML (Byrd et al., 2002). Unlike other fusion proteins MLL is an activator protein that binds with the specific DNA sequences notably to the Hox gene promoters. MLL is required for Hoxc8 expression, it binds and methylates histones at the Hox loci (at histone H3 lysine 4), leads to the activation of target genes (Milne et al., 2002). Hox gene expression in turn is associated with increased self-renewal by

hematopoietic cells. MLL-fusion proteins have the ability to dimerize with themselves and wild-type MLL. Some MLL fusion proteins may directly target Hox genes for activation by random recruitment of activators, for example, the MLL-CBP fusion can directly target histone acetyl transferases to target genes (Wang et al., 2005). The distribution of AML cytogenetic among the population is shown (Figure 5).

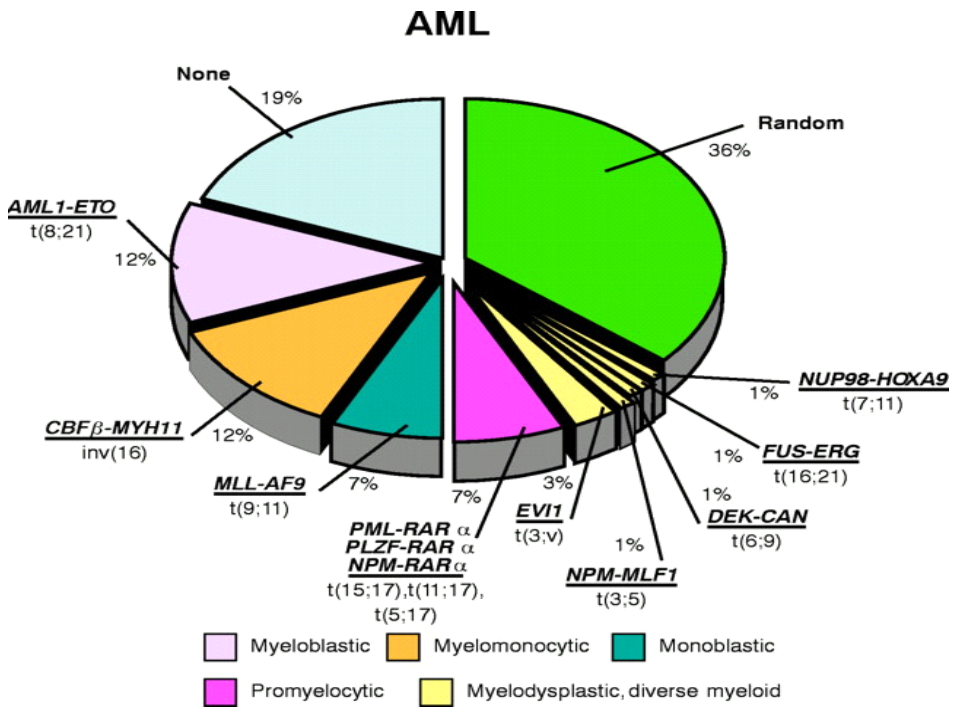


Figure 5. Shows the distribution of AML subtypes with recurrent cytogentic abnormalities and FAB subtype (Rabbitts and Stocks, 2003). Adapted from Science Magazine (Look, 1997).

1.3 Survivin

Survivin belongs to the family of IAPs (inhibitor of apoptosis) proteins (Li et al., 1998b) and is characterised by the presence of 70 amino acid Zinc-finger fold called Baculovirus IAP repeat (BIR). IAP family members besides BIR domain possess a caspase recruitment domain (CARD) and a C-terminal RING FINGER domain. Survivin is the only member of the family which lacks C-terminal RING FINGER domain (Salvesen and Duckett, 2002). A single copy of survivin gene transcribes two other transcript variants in addition to 426 bp survivin. One transcript variant is generated by the

insertion of an alternative exon 2 (survivin 2B) and the third variant is generated by the deletion of exon 3 (survivin ΔEx3) (Mahotka et al., 1999) (Figure 6).

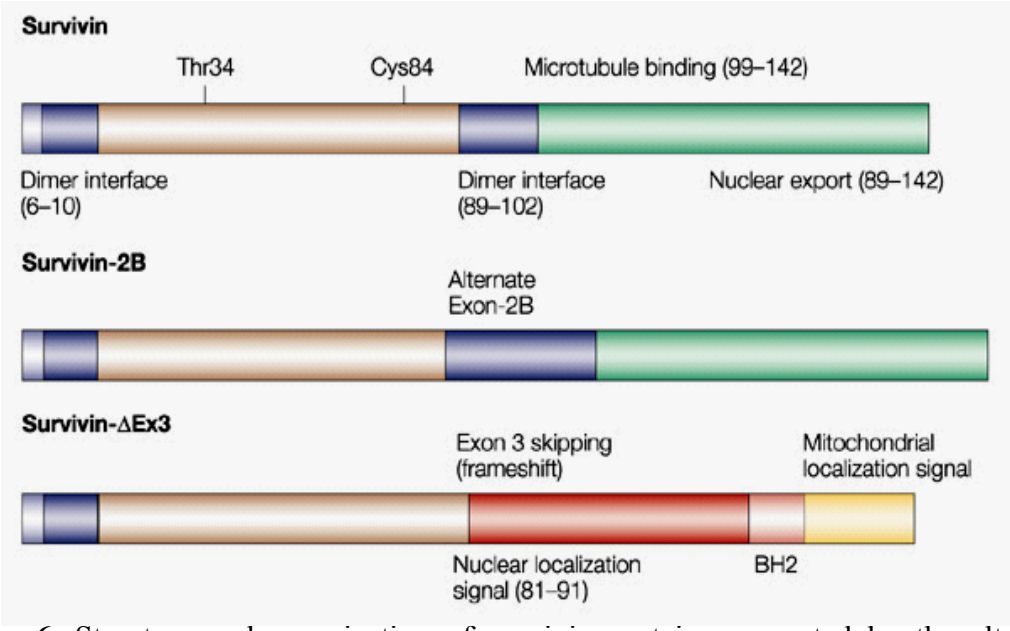


Figure 6. Structure and organization of survivin proteins generated by the alternative splicing of survivin gene. Survivin-2B is generated by the alternative insertion of exon 2 and survivin ΔEx3 is result of deletion of exon 3. Discrete regions are indicated that are implicated in dimerization with other isoforms, microtubule binding, nuclear localisation etc. Figure adapted from Nature Reviews Cancer (Altieri, 2003).

Survivin expression is regulated by many signalling pathways and its expression goes up during the G2/M phase of cell cycle. It is a microtubule regulatory protein and is a part of the chromosomal passenger complex (CPC) consisting of the serine/threonine kinase Aurora B, the inner centromere protein INCENP and Borealin/DasraB which has essential functions at the centromere in ensuring correct chromosome alignment and segregation (Vagnarelli and Earnshaw, 2004). Survivin is an antiapoptotic protein and is involved in many apoptotic pathways in conjunction with bcl2. In general mammalian cells use two main pathways to undergo apoptosis. An extrinsic pathway initiated by the ligation of cell-surface death receptors, including the tumour necrosis factor-α (TNFα) receptor and CD95 (Krammer, 2000). An intrinsic pathway mostly initiated by

intracellular and environmental signals and is centred on dysregulation of mitochondrial functions (Wang, 2001). The result is increase of mitochondrial membrane permeability and release of proteins into cytoplasm that facilitate caspase activation, most notably SMAC/DIABLO (Zhou et al., 2005) (Figure 7).

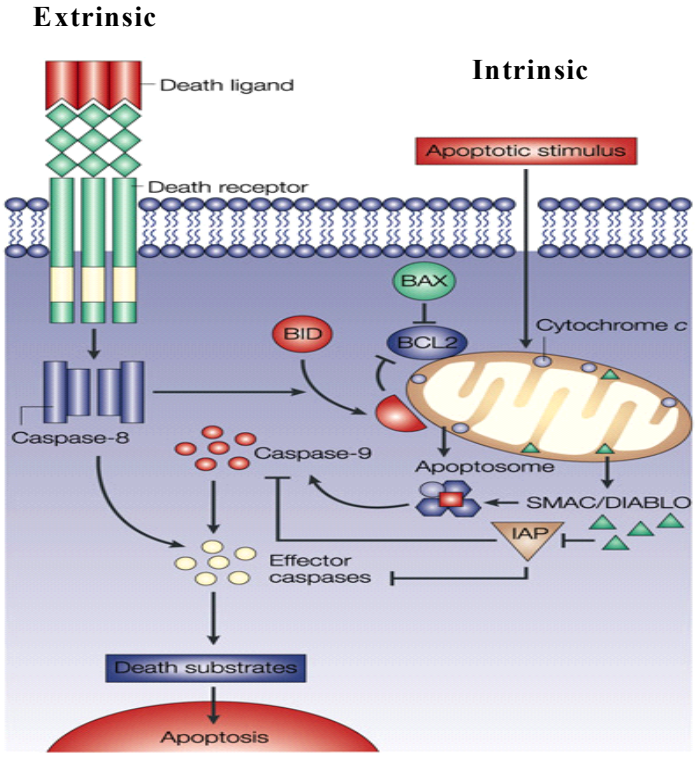


Figure 7. Apoptosis can be initiated by the intrinsic pathway governed by mitochondria and acts through caspase 9 and extrinsic pathway regulated by caspase 8. Both pathways converge to activate the effector caspases, which act on the death substrates. In addition, cell death is regulated by the BCL2 and inhibitor of apoptosis (IAP) proteins. BCL2 act to regulate mitochondria permeability by activating BAX and BID; whereas IAP proteins act downstream to prevent processing of initiator caspase-9 from the apoptosome. Proteins that are released by mitochondria during the permeability transition, including cytochrome *c* and SMAC/DIABLO, facilitate caspase activation by forming the apoptosome or relieving the caspase-inhibitory function of IAP proteins, respectively. Adapted from Nature Reviews Cancer (Altieri, 2003).

Interestingly, survivin expression is undetectable in most normal adult tissues, however, its expression enhances dramatically with tumor progression and also in human leukemia including AML (Wagner et al., 2006). With respect to hematopoietic system a low level of survivin expression has been shown to be important for megakaryopoiesis. During the course of this study we further report that AML1-ETO fusion specifically

activates survivin expression. Further investigation revealed that by knock down of survivin expression, AML1-ETO mediated inhibition of granulocytic differentiation (Pabst et al., 2001) was overcome significantly through the restoration of C/EBPα activity (Balkhi et al; unpublished data).

1.3.1 Suvivin as a potential therapeutic target for AML

Survivin expression is undetectable in most normal adult tissues, however, its expression enhances dramatically in tumor tissues and human leukemia including AML (Wagner et al., 2006). Survivin also serves, in some instances, is an independent prognostic marker. Survivin expression, however, is not involved in major differentiation steps within myeloid cell maturation which is in contrast to XIAP whose expression correlates with monocytic differentiation in therapy related AML (Tamm et al., 2004). With respect to hematopoietic system survivin has been shown to express at low levels in human umbilical-cord blood and bone-marrow derived CD34+ cells and is rapidly enhanced with cytokine incubation (Fukuda and Pelus, 2001). Survivin also has an important role in the survival of terminally differentiated neutrophils and is highly expressed in immature neutrophils (Altznauer et al., 2004). In many instances the clinical outcome of patients with AML is correlated with altered levels of pro-apoptotic and pro-survival molecules in leukemic cells. Expression of the bcl-2 survival molecule, the survivin and the extrinsic death pathway protein FADD are predictive of clinical response rates and survival in AML. Survivin has assumed a great therapeutic significance because of two main reasons; it is differentially expressed in cancer and is required to maintain cancer viability. Survivin based therapy would thus be expected to exert limited toxicity on normal tissues and at promoting the leukemic cell death. These approaches has been independently validated by several groups reporting that T cells mount a vigorous cytolytic response

against survivin peptides in vitro and in vivo (Hirohashi et al., 2002);(Rohayem et al., 2000).

1.4 Self-renewal and AML

The normal progenitor cells from the hematopoietic system are committed to a particular hematopoietic lineage, however, the hematopoietic stem cells like long term HSCs maintain a colony of stem cells that have a potential to self renew. Leukemic cells from patients with AML can undergo extensive self-renewal rather than lineage-specific commitment. Moreover, the leukemic stem cell population in AML is functionally heterogeneous with differing capacities for self-renewal (Falini et al., 2005). Various explanations have been put forward to explain the process of self-renewal. Nucleophosmin (NPM) is reported to be mutated in approximately one-third of newly diagnosed AML, and the expression of this cytoplasmic NPM variant is associated with expression of genes thought to support maintenance of the stem cell phenotype (Alcalay et al., 2005). The FLT3-ITD mutant of AML and enforced expression of it, which activates proliferative and survival pathways, also confers the property of self-renewal in human CD34+ cells (Chung et al., 2005). The Wnt/ß-catenin signaling is a critical element in the control of self-renewal of normal and cancer stem cells. AML1-ETO fusion protein is known to induce the extensive self renewal of human erythroid cells (Tonks et al., 2003) and in human CD34+ cells (Mulloy et al., 2003). Our results further suggest that activation of survivin by AML1-ETO may be involved in the extensive self renewal property of AML1-ETO positive cells (Balkhi et al; unpublished data). AML1-ETO and PML-RARα induce the expression of ß-catenin and γ-catenin proteins (Muller-Tidow et al., 2004). Thus, the expression of tyrosine kinase mutant and fusion genes in AML seems to enhance the self-renewal to give a survival advantage to the leukemic blasts.

1.5 Proteomics based on mass spectrometry

Proteome represent the complex set of all proteins in a cell that could be interacting with other proteins or proteins with specific posttranslational modifications. It also can be an activated set of specific genes of a given cell at any given time. Proteome, thus, represents a functional state of a cell and could provide valuable information regarding the pathological, physiological and differentiation state of a cell. Proteomics based on mass spectrometry (MS) has become an important tool for molecular and cellular biology. It promises the study of protein-protein interactions via affinity-based isolations and to precisely quantify thousands of proteins from complex samples through quantitaive proteomics like ICAT (Isotope-Coded Affinity Tags) and SILAC (stable isotope labelling of amino acids in culture). Protein analysis that includes the primary protein sequence, post-translational modifications or protein−protein interactions has been successfully used through Mass spectrometry. An overview of a typical proteomic experiment based on mass spectrometry is shown (Figure 8)

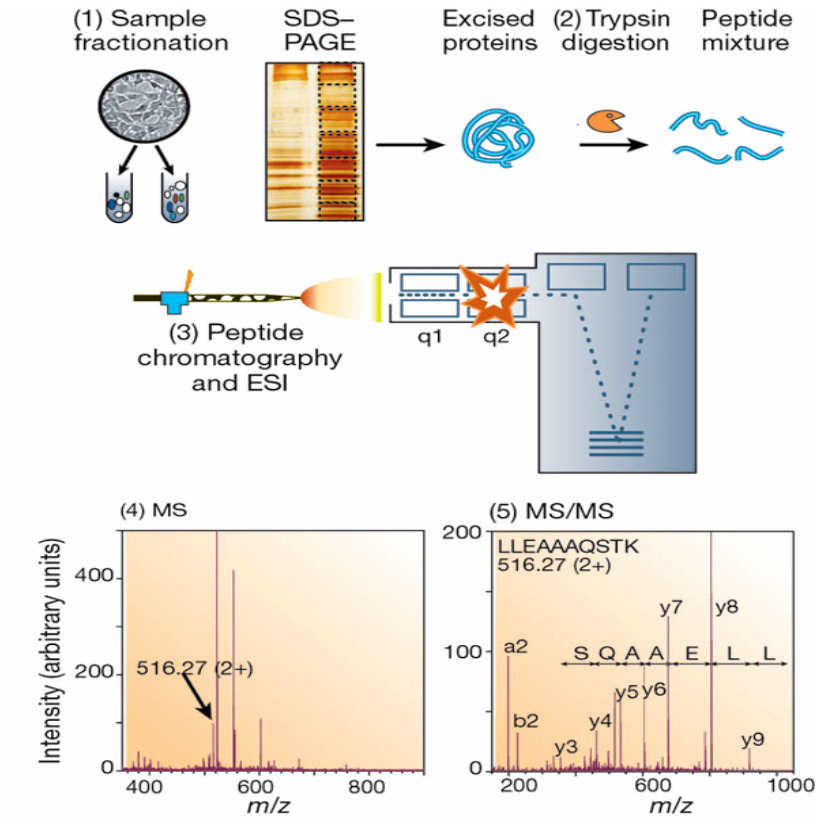


Figure 8. The figure gives an overview a typical proteomics experiment which consists of five stages. Stage 1, the proteins to be analysed are isolated from whole cell lysate or tissues by sample fractionation. This often includes a final step of one-dimensional gel electrophoresis where proteins are separated by Isoelectric focussing using different pH range immobiline dry strips. Strips are loaded and separated by SDS PAGE in a 2nd dimension and gels are stained. Spots are cut and digested enzymatically to peptides in stage 2, usually by trypsin, leading to peptides with C-terminally protonated amino acids, providing an advantage in subsequent peptide sequencing. In stage 3, the peptides are loaded on a MALDI target or processed by high-pressure liquid chromatography. After evaporation, multiply protonated peptides enter the mass spectrometer and, in stage 4, a mass spectrum of the peptides eluting at this time point is taken (MS1 spectrum, or 'normal mass spectrum'). The computer generates a prioritized list of these peptides for fragmentation and a series of tandem mass spectrometric or 'MS/MS' experiments ensues (stage 5). These consist of isolation of a given peptide ion, fragmentation by energetic collision with gas, and recording of the tandem or MS/MS spectrum. The MS and MS/MS spectra are typically acquired for about one second each and stored for matching against protein sequence databases. The outcome of the experiment is the identity of the peptides and therefore the proteins making up the purified protein population. Figure adapted from Nature (Aebersold and Mann, 2003).

The main approaches to mass spectrometric protein identification are the 'peptide-mass mapping'. In the 'peptide-mass mapping' approach proposed by Henzel and co-workers (Henzel et al., 1993), the mass spectrum of the eluted peptide mixture is acquired, which results in a 'peptide-mass fingerprint' of the protein being studied. This mass spectrum is obtained by a relatively simple mass spectrometric method — matrix-assisted laser desorption/ionization (MALDI) or Nanospray ionization— which results in a time-of-flight distribution of the peptides comprising the mixture of protonated ions. Advances have been made in automation of the MALDI identification procedure whereby hundreds of protein spots can be excised, digested enzymatically, their mass spectra obtained and automatically searched against databases (Berndt et al., 1999; Jensen et al., 1997). In order to obtain the sequence of individual ions or informtaion about its post-translation modification, specific ions from mass spectra are selected and fragmented either by an inert gas or by radiomagnetic waves (FTICR or Q-TOF mass spectrometry) and captured by a 2nd time of flight. This gives a MSMS of the selected ions comprised of series of y, a, m, b, ions. From the molecular mass estimation of these ions, a sequence of a given peptide or the

posttranslational modification, if any, could be calculated. In recent years several techniques for protein quantitation by mass spectrometry have emerged. The accurate quantitation of proteins could provide us the valuable information regarding the state of a given protein. There are number of proteins which dramatically enhance in expression during the disease state or transformation. These proteins could serve as a potential drug target or prognostic marker. However, their accurate quantitation remains elusive. Quantitative mass spectrometry based on affinity tag methods like isotope-coded affinity tags (ICAT) and metabolic labelling and stable isotope labelling of amino acids in culture (SILAC) (Gygi et al., 1999; Krijgsveld et al., 2003) are methods of choice. These techniques allow the peptides derived from two samples to be distinguished by mass spectrometry. The proteins are labelled with isotopically distinct tags (ICAT) through the incorporation of isotopically distinct amino acids (SILAC) in a cell culture. Protein quantitation can then be achieved by comparing the mass spectrum intensity of the peptides (MS peaks) derived from the two samples.

Proteomics based on two-dimensional gel electrophoresis or 2DE has a potential clinical application to investigate differential expression data. The comparative two-dimensional gel approach which means the comparison between the two conditions can be used for the investigation of the differential regulation, up- or downregulated, in a disease-specific manner. This information can then be used for diagnostic, prognostic and therapeutic purposes. Besides the conventional staining techniques, new approaches have been in use for the 2DE approach. For example, to quantitate the protein targets, Differential ingel Electrophoresis (DIGE), which makes the use of flourescent probes provides a better alternative to quantiate and differentiate proteins between diseases versus normal state. The important aspect of this approach is to control the experimental conditions and variations among the samples. The identification of the differential proteome between

normal versus diseases could provide us a significant amount of information regarding the protein interaction networks and the signalling pathways that are dysregulated within a cell (Petricoin et al., 2005). An overview of differential proteomics is shown (Figure 9).

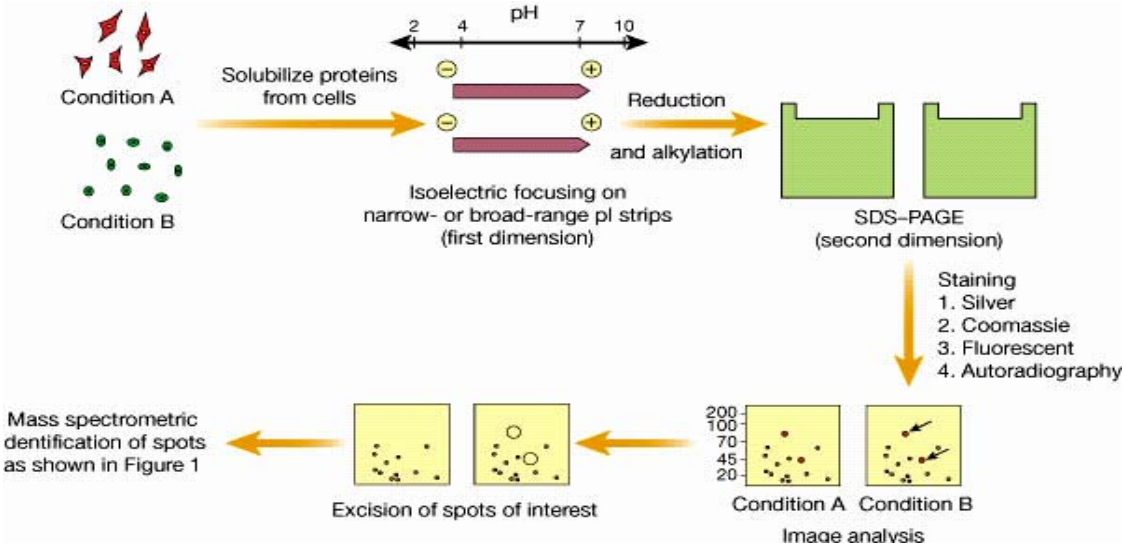


Figure 9. Differential two dimensional gel approaches to investigate disease versus normal. Cells from two different conditions, A and B, are lysed and protein solublised. Samples are then applied to a 'first dimension' gel strip that separates the proteins based on their isoelectric points. After this step, the strip is subjected to reduction and alkylation and applied to a 'second dimension' SDS–PAGE gel where proteins are denatured and separated on the basis of size. The gels are then fixed and the proteins visualized by silver staining or coomassie or labelled with flourescent dyes. After staining, the resulting protein spots are recorded and quantified using sophisticated image software.The spots of interest are then excised and subjected to mass spectrometric analysis. Figure adapted from Nature (Pandey and Mann, 2000)

1.5.1 Post-translational modifications (PTMs)

One of the unique features offered by proteomics based studies is the ability to identify the post-translational modifications of proteins. These modifications occur posttranslationally and are not posttranscriptionally apparent from genomic sequence or mRNA expression data. Phosphorylation, glycosylation, acetylation, methylation and sulphation as well as many other modifications are extremely important for protein function as they affect the protein activity, stability, localization and turnover and could be an important target for therapy. One mechanism explains PTMs induce new conformational state of a

protein to alter its functions, for example, phosphorylation of protein kinase reorganises the active site of this kinase into productive conformation (Yang, 2005). A single polypeptide can get phosphorylated at several sites; likewise the lysine residue of a peptide can be mono- di- or trimethylated, or mono- or polyubiquitylated. Furthermore, the same polypeptide chain can be modified by different classes of PTM, which generates an even larger number of possible variants (Yang, 2005). Mass spectrometry is the proteomic method of choice to determine these protein modifications; tandem mass spectrometry (MS/MS) provides a series of analytical features that are highly useful for the characterization of modified proteins via amino acid sequencing and specific detection of posttranslationally modified amino acid residues. Large-scale, quantitative analysis of proteins by MS/MS is beginning to reveal novel patterns and functions of PTMs in cellular signaling networks and biomolecular structure (Larsen et al., 2006). An overview of PTM detection is shown (Figure 10).

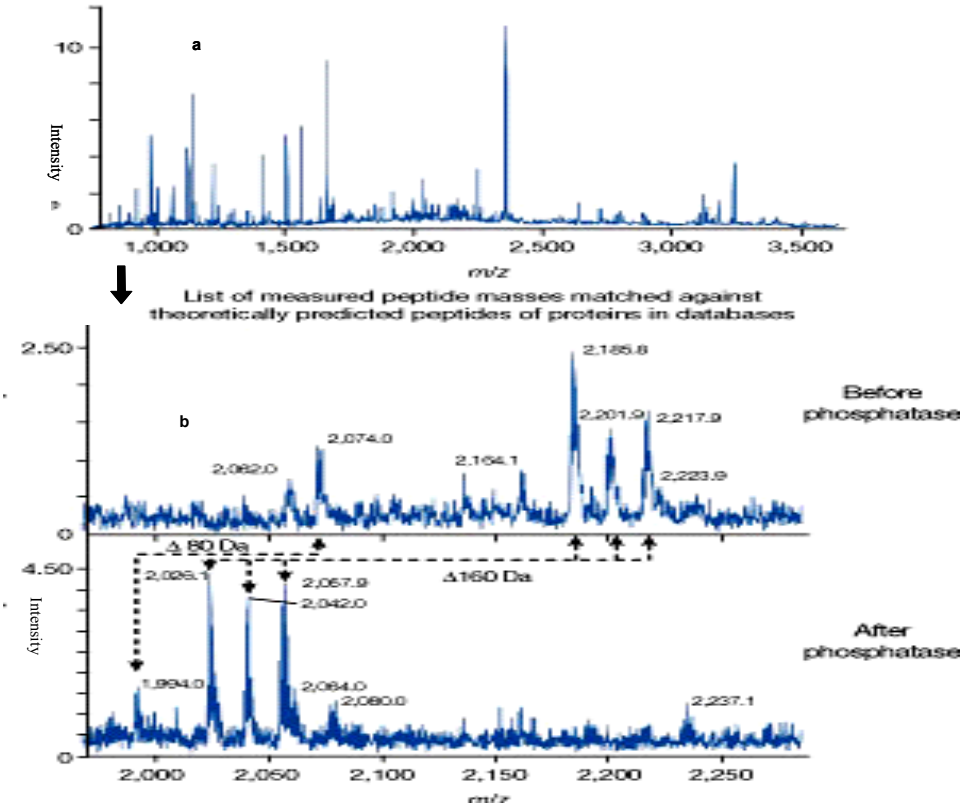


Figure 10. An overview of detection of post-translational modification by MS and MSMS; a) shows the individual peptide peaks in the mass spectrum. The bottom panel shows how

sequence can be derived by fragmentation of the chosen peptide (m_2) by tandem mass spectrometry. Phosphopeptides may be enriched by purifying the peptide mixture over a metal resin microcolumn. The resulting peptides can then be analysed by MALDI as shown before and after treatment with alkaline phosphatase. The panel shows a singly phosphorylated (showing a shift of 80 Da) and a doubly phosphorylated (showing a shift of 160 Da) peptide in the MALDI spectrum. Figure adapted from Nature (Mann and Jensen, 2003; Pandey and Mann, 2000).

1.6 Aim of the study

Advances in the diagnosis of cytogenetic abnormalities of AML and refinements in the therapeutic approaches have improved the cure for patients with AML. However, the survival rate among AML patients is only 30%, which can be attribute to not so well defined sub type specfic therapy which demand new findings that promises to improve further the cure rate. Recent advances in gene expression profiling using oligo c-DNA microarray have been used to differentiate cytogenetic groups and to identify new subgroups within the intermediate risk group of AML with normal karyotype (Bullinger et al., 2004). Even though transcriptomics have provided relevant information regarding the gene activity and cell function, however, they do not necessarily correlate with levels of protein expression. More importantly, such analysis cannot detect important posttranslational modifications of proteins (PTMs), such as acetylation, phosphorylation, or glycosylation. Proteomics has emerged as an indispensable technique to identify the proteome and posttranslational modifications of proteins which indeed represents the functional state of a cell. By undertaking such investigation an understanding of physiological and pathological state of a cell can be elucidated. The identified proteome leads to a new way of understanding the biological interaction in determining the disease relevant targets and pathways for the primary drug targets (Cristea et al., 2004). This result of this study, thus, provides a platform for distinguishing AML cytogentic subgroups according to their protein expression pattern and MS peak pattern corresponding to PTM differences. This might improve our ability to understand the molecular

differences between AML cytogenetic subgroups and would be precisely used for designing subtype-specific therapies. Further, by the identification of direct protein targets of the fusion proteins and their functional and biological characterisation may improve our understanding of the molecular dys-regulation of leukemogenesis.

2 Materials and methods

2.1 Material

2.1.1 Mammalian cell line:

Kasumi 1, human acute myeloid leukemia (AML FAB M2) established from a patient carrying t(8;21). Positive for AML1-ETO fusion gene (from DSMZ ACC 220)

U937 (Human myeloid cell line, monoblastic)

U937T-A/E cells (U937 cells stably transfected with AML1/ETO c-DNA, a tetracycline inducible cell line), kindly provided by Dr. Dong-Er Zhang.

Human kidney 293T cells; a mouse embryonal carcinoma F9 cell line.

HL-60 (human acute promyelocytic leukemia cell line)

AML patient samples were kindly provided by Leukemia Diagnostic Laboratory, Medicine III Klinikum Großhadern, Munich.

2.1.2 Cell culture

RPMI (PAA, Cölbe, Germany)

Foetal bovine serum (Invitrogen/GIBCO, Germany)

Penicillin/Streptomycin (GIBCO, Germany)

DPBS (PAN, Germany)

DMEM (PAN, Germany)

G-418, tetracycline, puromycin (Sigma).

Trypsin EDTA (GIBCO, Germany)

GlcNAc (Sigma, catalog no. A6919)

2.1.3 Immunoblots

Phosphatase inhibitor Cocktail I and II (Sigma, Germany)

Protease inhibitors (Sigma, Germany)

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

Bradford assay buffer (Biorad Laboratories, Germany)

Nitrocellulose membrane (Millipore, Germany)

ECL detection kit (Amersham Biosciences, Germany)

ECL hyperfilm (Amersham Biosciences, Germany)

2.1.4 Antibody

Anti- Survivin (Novus Biologicals, USA, NB 500-201)
Anti-HSC 70 (Santa Cruz Biotechnology, CA, USA, sc-7298)

Anti-hnRNPA2/B1 (Santa Cruz Biotechnology, CA, USA, sc-10036)

Anti-Casein Kinase 1α (Santa Cruz Biotechnology, CA, USA, sc-6477)

Anti-O-GlcNAc (Covance research products, USA, MMS-248R)

Anti- Calreticulin (sigma-aldrich, C-4606)

Anti- Prohibitin (Santa Cruz Biotechnology, CA, USA, sc-18196)

Anti- ETO (Santa Cruz Biotechnology, CA, USA, sc-9737 and sc-9737x)

Anti- hnRNPH/F (Santa Cruz Biotechnology, CA, USA, sc-15387)

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

2.1.5 Plasmid constructs and transient transfections

PINCO AML1/ETO retroviral plasmid (kindly provided by Dr.Pellicci)

pLuc 1430c-survivin promoter luciferase construct (kindly provided by Dr. Altieri)

GFP (Invitrogen, Germany)

Nucleofector kit (AMAXA, Cologne, Germany)

2.1.6 Chemicals

Acetonitrile (Sigma Aldirch, USA)

Acetic acid (Merck, Darmstadt, Germany)

Ammonium bicarbonate (Sigma, USA)

Dimethyl sulfoxide (DMSO) (Sigma, USA)

Ethanol (Merck, Darmstadt, Germany)

Formaldehyde (Merck, Darmstadt, Germany)

Glycine (ICN Bio-medicals)

Isopropanol (Merck, Darmsadt, Germany)

Methanol (Merck, Darmstadt, Germany)

Propidium iodide (Sigma, USA)

Silver nitrate (Merck, Darmsadt, Germany)

Sodium carbonate (Merck, Darmstadt, Germany)

Sodium thiosulfate (Merck, Darmstadt, Germany)

Sodium dodecyl sulphate (SDS) (Sigma, USA)

Trifluoroacetic acid (TFA) (Merck)

Triton X-100 (Sigma, USA)

Zinc sulphate (Sigma, USA)

2.2 Methods

2.2.1 AML patient samples

42 bone marrow samples of the following AML subtypes were used in this investigation: t(8;21) (n=6), t(15;17) (n=7), inv(16) (n=6), 11q23 (MLL/PTD) (n=6), normal karyotype (n=11), complex aberrant karyotype (n=6). The studies were conducted according to the rules of the local ethical committee and the revised Helsinki protocol. Written informed consent from the patients was obtained. At the time each AML patient was diagnosed, mononuclear cells from the bone marrow aspirate with more than 90% blast cells were purified by Ficoll density centrifugation. The healthy bone marrow cells were purchased from stem cell technologies (cell systems Biotechnologie Vertrieb GmbH). CD34+ cells were isolated from the remains of leukapheresis

products and sorted by MACS by a positive selection using CD34+ progenitor cell isolation kit, human, Militeny biotech.

2.2.2 Two dimensional gel electrophoresis and DIGE

Five master gels with gradients pH 3-10 and 4-7 were made from each cytogenetic group : t(8;21) (4 gels pH 3-10 and 1 gel pH 4-7), t(15;17) (2 gels pH 3-10 and 3 gels pH 4-7), Inv(16) (3 gels pH 4-7 and 2 gels pH 3-10), complex aberrant karyotype (4 gels pH 3-10), 11q23 (3 gels pH3-10) and normal karyotype(10 gels pH 3-10 and 3 gels pH 4-7), healthy bone marrow (2 gels pH 3-10) and CD34+ cells (2 gels pH 4-7 and 1 gel pH 3-10). In order to control the gel to gel variability in staining and running conditions between cytogentic groups, four patient samples belonging to different cytogenetic groups were processed together. 300µgs of protein in all the experiments were subjected to IEF and a minimum of four 2D gels were run and stained together. 10^7 cells from each patient sample were carefully thawed and dissolved in 10ml of IMDM, supplemented with 20% FCS, heparin 200IE per ml, and DNAse. After centrifugation the pellet was lysed in a sample buffer containing 7M urea, 2M thiourea, 4%(w/v), 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid (CHAPS), and 1% (w/v) DTT for all silver stained gels. For DIGE experiments, samples were dissolved in a DIGE compatible lysis buffer containing 25 mM Tris, 4% CHAPS (w/v), 8M urea, and 2M thiourea. Protein labelling with cyanine dyes (Cy3 or Cy5) was performed according to the manufacturer's (Amersham biosciences) instructions. 300µgs and 50µgs of protein respectively were used for all silver stained gels and DIGE experiments, respectively. The proteins were subjected to Isoelectric focusing for the first dimension of protein separation which was performed with a Multiphore II electrophoresis system (Amersham Biosciences, Piscataway, NJ). Immobiline Dry IPG strips (for DIGE: 24 cm, pH 4–7 linear IPG strips, Amersham Biosciences) were rehydrated overnight with the sample/rehydration buffer mixture. For silver staining 18cm, pH 3-10

and 4-7 linear IPG strips were directly rehydrated with the sample buffer. The strips were subjected to electrophoresis using a ramping IPG strip (200–8000 V) focusing algorithm. After the isoelectric focusing, the gel strips were electrophoresed in vertical SDS-PAGE gels containing 12.5% acrylamide. The Cy2, Cy3, Cy5 labelled gels were scanned at 473nm, 532nm and 635nm excitation laser filter using laser based FUJI scanner (Fluor imager, FLA 5100 version 1.0).

2.2.3 Statistical analysis

For evaluating the mean expression volumes (mean intensity values) of ingel spots, gels were matched from within a particular cytogentic groups and from other cytogenetic groups. ProteomWeaver software, version 2.0, Definiens AG, Munich, was used. Standard statistical methods were used to analyse the plots includes mean, standard deviation and coefficient of variation calculated as standard deviation/mean. Changes in spot intensity was considered significant where the average normalized volume altered by greater than 1.5-fold between samples. P values were calculated from the student's t-test by comparing the means of two different AML subtypes (expressed as the standard deviation of the difference between the two means).

2.2.4 MALDI-TOF mass spectrometry

Spots were excised manually from silver-stained and for fluorescent gels an automatic spot picker (Bruker daltonic) was used. Gel pieces were rehydrated in 50 ng/µl trypsin (Promega, Southampton, UK) in 25 mM ammonium bicarbonate on ice for 20 min. The gel pieces were incubated at 37 °C overnight. The speedVac dried peptides were dissolved in 5µl of 20% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid, sonicated for 5 min, and spotted along with 0.5 µl of 10 mg/ml -cyano 4-hydroxycinnamic acid (CHCA, Sigma) in 50% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid onto a MALDI target plate for analysis. The mass determinations were performed on

the MALDI TOF/TOF mass spectrometer (Applied Biosystems; AB4700) operating in reflectron mode and with ion source pressure ~0.5 μTorr. After a 400-ns time-delayed ion extraction period, the ions were accelerated to 20 kV for time of flight (TOF) mass spectrometric analysis. A total of 600-1000 laser shots were acquired and signal averaged. The Data were analyzed through GPS explorer (AB 4700 inc., USA) using a Swiss/Prot protein database for Mascot search assuming 1) monoisotopic peptide masses, 2) cysteine carbamidomethylation, 3) variable oxidation of methionine, 4) a maximum of one missed trypsin cleavage, and 5) a mass accuracy of 60 ppm or better, minimum Signal/Noise 45. The four high intense peaks were selected in a range between m/z 1500 to 2500 for MS-MS peptide sequencing in an interpretation method. A molecular weight search (MOWSE) score >53 was assumed to indicate a significant match. For detailed MS and MS-MS spectra analysis, the MS spectra of potential proteins to be dissected for possible PTMs were imported to the peak explorer software, and the MS-MS spectra were imported and sequenced in the data explorer software. The posttranslational modifications were included as a variable modification, and searched by MASCOT database. Further to narrow down MASCOT search we created separate path for calreticulin, hnRNPH1 and hnRNPA2/B1 in MSDB. The modified peptides was reanalysed in a peak explorer from their corresponding MS spectra. For this analysis we used all the MS spectra of Calreticulin, hnRNPA2/B1 and hnRNP H identified from different subgroups. The sample corresponding to hnRNPH1 was fractionated by Nano LC (Dionex) using 500 μm i.d. x 5mm, C18 reverse phase column with a flow rate of 200nl/min.

2.2.5 Transient transfections using LipofectAMINE plus and reporter assays firefly and renilla luciferase

293T (1-2x 10^4) cells were seeded in a 24 well plate in 0.5 ml of complete growth medium 24h before transfection and grown to 50-80% confluence. A

total of 0.4µg of various plasmids were cotransfected that include, PINCO-GFP vector, PINCO-AML1-ETO retroviral construct or pCDNA3-AML1-ETO, AML1b plasmid (Grignani et al., 1998) and pCDNA3-C/EBPα were mixed with 20µl of serum free media and incubated for 15 minutes. 1µl LipofectAMINE plus reagent (Invitrogen, Gmbh, Karlsruhe, Germany) was mixed with the 24µl serum free media and combined with the DNA-media complex and incubated for additional 15 minutes. After 3-4 h serum starvation of cells in a serum free medium at 37 °C, the DNA-liposome complex was replaced with complete medium and cultivated for an additional 24h at 37 °C. Cells were washed in PBS, pH 7.4, solubilized in 50µl of lysis buffer (Promega) and scraped with a rubber policeman, and then 10µl aliquots of the supernatant were mixed with 50µl of luciferase assay reagents (Promega). Firefly luciferase activities from the constructs of pLUC survivin promoter construct pLuc-1430c (Li and Altieri, 1999) and p(C/EBP)2TK and renilla luciferase activity from the internal control plasmid PRL-null were determined using the dual Luciferase Reporter Assay system (Promega). Firefly luciferase activities were normalized to renilla luciferase values of PRL-null (Behre et al., 1999) analysed on a Lumat luminometer (LB9510). Results are given as means +/- SEM of at least 4 independent experiments. Following DNA concentrations of the reporter constructs and expression plasmids were used for lipofectAMINE plus transfections, 0.1µg of pCDNA3-human C/EBPα, AML1-ETO, AML1b, pLuc Survivin, p(C/EBP)2TK each; 0.02µg of the internal control plasmid PRL-NULL and the same concentrations of the empty expression vector were used as a control respectively. 1µg and 0.5µg of survivin shRNA was used for all the experiments performed.

2.2.6 shRNA and flow cytometric analysis

Human $CD34^+$ hematopoietic cells were isolated from human cord blood cells. Briefly, CB was collected on delivery with informed consent. The blood

was diluted with MACS-buffer 1:5 and mononuclear cells were isolated by lymphocyte separation medium (LSM 1077, J 15-004, PAA). The lymphocyte ring was collected, transferred to a new tube and washed with MACS-buffer. Pellets were dissolved in 300µl MACS buffer and 100µl FCR blocking reagent and incubated for 10min. at 4^{o}C. 100µl of CD34 microbeads were added and incubated for 30 minutes at 4^{o}C. The cells were washed and CD34+ cells were sorted by MACS system (Miltenyi Biotec). More than 70% of the cells were required to express the CD34 antigen. An aliquot containing 5×10^{5} $CD34^{+}$ cells was cultured initially for 12 hours in IMDM with 10% heat-inactivated FBS, 50 ng/ml Flt3-ligand (Flt3-L), 50 ng/ml stem cell factor (SCF), 50 ng/ml thrombopoietin (TPO), 10 U/ml penicillin/streptomycin and 2 mM L-glutamine. The cells were transfected with survivin shRNA, 1µg, 0.5µg and 0.1µg (cat. No. RHS1764-97182020, Open Biosystems). The short hairpin shRNA is cloned into retroviral backbone named as pSM2C (pSHAG-MAGIC2) with the following sequence primers U6 5' TGT GGA AAG GAC GAA ACA CC. Control shRNA, 500ng (RHS1707 Open Biosystems) using AMAXA nucleofection technology (AMAXA, Gmbh, Cologne, Germany). A 5 µg portion of plasmid DNA constructs was used for each transfection and the transfection efficiency was analyzed using a plasmid with eGFP marker (2 µg). Nucleofector solution kits VPA-1003 was used for CD34+ cells and VCA-1003 for U937 and Kasumi1 with nucleofection programs U-08 (for CD34+) and V-01(for U937) and T-020 (for Kasumi 1). The voltages are automatically adjusted according to the program and are essentially 110 V AC with a frequency of 50–60 Hz and a power consumption of 16 VA/fuse as described by the manufacturer. Transfection efficiency was analyzed using a plasmid with eGFP marker (2 µg). After 12 hours, the cells were spin down and replaced with new media RPMI plus 10% FCS without cytokines. $1x10^{6}$ Kasumi1 cells were transfected with survivin shRNA and control shRNA as described above. After 72 hours, FACS analysis was performed for CD34+

cells for CD15 expression on a flowcytometer (Becton Dickinson) using FITC labeled CD15 antibodies (55401, BD Pharmingen) and IgG-FITC (555742, BD Pharmingen) as an isotype control.

2.2.7 Quantitative real-time PCR analysis

CD34+ cells were transfected with pCDNA3-C/EBPα, PINCO-GFP vector alone and PINCO AML1-ETO retroviral construct and after 24hours cells were transfected with survivin shRNA and control shRNA and incubated for another 48 hours. RNA was isolation by TRIZOL method (Invitrogen, Germany) followed by cDNA synthesis using standard conditions. Equal amounts of cDNA were used and gene expression was quantified by real-time quantitative RT-PCR in a Rotor-Gene RG-3000 (Corbett Research, Germany) using a SYBR Green kit (Qiagen, Germany). The methods were employed according to the manufacturer's protocol. Following PCR primer sequences was used for Myeloperoxidase, MPO, expression, 5`-TCG GTA CCC ATG TCA GGA AG-3` (Forward) and 5`-CCA GGT TCA ATG CAG GAA GT-3` (Reverse). To determine the relative expression level of each sample, GAPDH expression levels were measured as internal controls. The delta ct value (Δct) was calculated from the given ct value by the formula: $\Delta ct = (ct_{sample} - ct_{control})$. The fold change was calculated as $(= 2^{-\Delta ct})$.

2.2.8 Immunoblot analysis

Patient samples were lysed in a lysis buffer containing 50mM Tris pH 8.0, 0.5% Triton X-100, 0.5% Nonidet P-40, 150mM Nacl, 5mM EDTA, 0.5% sodium deoxycholate, 1% protease inhibitor and phosphatase inhibitor cocktail 1 and 2 (Sigma). Protein concentration was determined by the Bradford assay (Bio Rad Laboratories GmbH, Germany). Protein (60µg) were resolved on 6-10% SDS polyacrylamide gel electrophoresis, transferred onto Immobilon-P (Millipore, USA), blocked in 5% nonfat dry milk in TBS with 0.02% Tween 20 for 1 hour at room temperature and then incubated with

primary antibodies, 1:500, in TBS-T (with 2.5% nonfat dry milk) overnight at 4^{o}C. Anti calreticulin was used in a primary dilution of 1:50,000. A secondary incubation in a dilution range 1:1000 and 1:100,000 (for calreticulin) were followed by using donkey anti rabbit IgG-HRP (Amersham Biosciences, UK, catalog no.NA934) and 1:5000 dilutions for donkey anti goat IgG-HRP (Santa Cruz biotechnology, catalog no. sc-2056). For O-GlcNAc (β-O-linked N-acetyl glucosamine detection, membranes were equilibrated in methanol and air dried. The dry membrane was incubated with a 1:1000 dilution of anti- O-GlcNAc antibody in 1% BSA/ PBS with 0.01% Tween 20 for 2 hours and then washed 3 times in PBS. To demonstrate O-GlcNAc specific immunoreactivity, 10 mM GlcNAc was added during the primary incubation (Comer et al., 2001). The membrane was incubated with HRP conjugated goat anti-mouse (1.5000) in PBST for 1 h. After thorough washings membranes were developed using ECL reagents (Amersham Biosciences). For re-probing the blots, stripping solution, 100mM β mercaptoethanol, 2% SDS and 62.5mM Tris-cl pH 6.8 was used. Briefly, membranes were rinsed in methanol for few seconds and washed 3 times in TBST and incubated in stripping solution for 40 minutes at 58^{o}C under rotation. After the incubation membrane was washed 4 times in TBST and blocked fro 2hours in 1% BSA.

2.2.9 Electrophoretic Mobility-Shift Assay (EMSA)

EMSA was performed by using a non radioactive DIG Shift Kit (Roche, Cat. No. 1635 352). Complementary oligonucleotides were annealed at 95^{o}C and labelling was performed according to manufacturer recommendations. Following sequence of the human survivin promoter was used (-1316) 5'-CTG GTG TGG TGC ATG CCT T-3'(-1297); and the sequence of the Sp1 binding site used for non specific competition from the survivin promoter 5'-ATT CGA TCG GGG CGG GGC CAG-3'. Nuclear extracts (20 µg) were incubated with 15 fmol/µL of double-stranded oligonucleotide in a 20µL of final reaction mixture as recommended by the manufacturer. Cells (2-5 x 10^{7}) were

harvested, pelleted and washed 2X in 5ml of ice cold phosphate-buffered saline (PBS), and the cells were resuspended in I ml of cold PBS and centrifuged at 2000rpm for 5 minutes. The following procedures were then carried out at 4°C. Cells were resuspended in 1 volume cold buffer A (20 mM Tris [pH 8.0], 3 mM $MgCl_2$, 10 mM NaCl, 0.2M EDTA, 1 mM DTT, and 0.1% NP40, 10% Glycerol supplemented with protease and phosphatase inhibitors) and were allowed to swell on ice for 15 minutes with shaking in between. The cells were centrifuged at 2000 rpm for 5 minutes and the supernatant fraction was discarded. The pellet was resuspended in 2 pellet volume of cold buffer C (20 mM Tris [pH 8.0], 20% glycerol, 400 mM NaCl, 0.2 mM EDTA, 1 mM DTT, protease and phosphatase inhibitors) and incubated on ice for 10 minutes. Tubes were repeatedly freeze and thaw in liquid nitrogen and 37^{o}C water bath and finally centrifuged at 13000 rpm for 20minutes and the supernatant fraction (containing DNA binding proteins) was stored at -80°C. Protein concentrations were measured by Bio-Rad protein assay reagent (catalog no. 500-0006; Bio-Rad, Hercules, CA). For supershift experiments, anti- ETO goat polyclonal (catalog no. sc-9737x; Santa Cruz Biotechnology), anti-AML1 goat polyclonal (catalog no. sc-8564x; Santa Cruz Biotechnology) respectively, were added and further incubated for 30 minutes.

3 Results

3.1 Mass spectrometry based identification of proteins from different AML cytogentic groups

This investigation was performed on 42 AML patients belonging to different cytogenetic groups. A prior written consent was obtained from each patient. The patients used in the study with their cytogenetic characteristic are given (Table 2). Patients with the MLL (11q23) translocation has been characterised as a specific subtype of AML (Haferlach et al., 2004). The classification of

patients is in accordance with the latest WHO classification of AML where cytogenetics abnormalities have been used as a major criterion. The proteins were identified by MALDI-TOF Tandem MS (MS/MS) analysis. Four peptides (ions) from each MS spectra of a given identified protein were sequenced by MS/MS to determine the amino acid sequence of peptides. This increases the confidence of the identification of a given protein. 2D gels of patient samples from each subtype including a normal bone marrow and CD34+ cells are shown (Figure 11). From the silver stained 2D gel of each patient all the visible spots were cut for protein identification. These include, 340, 300, 372, 551, 854 and 203 spots from t(8;21), Inv(16), t(15;17), complex aberrant karyotype (here after CK), normal karyotype (here after NK) and 11q23 patients respectively. All the spots were analysed and processed for mass spectrometry based identification. This analysis leads to the identification of 32, 37, 32, 55, 60 and 24 proteins, respectively (Table 4). These proteins were ranked as identified with high confidence with a confirmation from MS/MS sequences of minimum 4 peptides, as discussed previously, for most of these proteins excluding a number of false positives. Interestingly, among the 240 proteins identified, 11, 17, 11, 20, 18 and 8 proteins showed differential expression which means a specific expression from each of the cytogenetic group, t(8;21), inv(16), t(15;17), NK, CK and MLL, respectively. This represents 35% of all identified proteome (Table 3). Interestingly, the statistics points towards the fact that each cytogenetic translocation contributes significantly to the altered proteome.

Table 2. Cytogenetic and morphological characteristics of patients used in the study.

Patients with balanced chromosomal translocations

No.	Sex	FAB	Karyotype	FLT3
1	M	M2	46XY, t(8;21)(q22;q22)	
2	M	M2	46XY, t(8;21)(q22;q22)	
3	F	M2	46XX, t(8;12;21)(q22;p13;q22)	FLT3-LM+
4	M	M2	46XY, t(8;21)(q22;q22)	
5	M	M2	46XY, t(8;21)(q22;q22)	
6	F	M2	46XX, t(8;21)(q22;q22)	
7	F	M3	46XX, t(15;17)(q22;q21)	
8	M	M3v	46XY, t(15;17)(p22;q21)	
9	M	M3v	46XY, t(15;17)(q22;q21),	
10	M	M3	46XY, t(15;17)(q22;q21)	
11	F	M3	46XX, t(15;17)(q22;q21)	
12	F	M3	46XX, t(15;17)(q22;q21)	
13	M	M3	46XY, t(15;17)(q22;q21)	
14	F	M4	46XX, Inv(16)(p13;q22)	
15	M	M4	46XY, Inv(16)(p13;q22)	
16	M	M4Eo	46XY, inv (16)(p13;q22)	
17	M	M4Eo	46XY, inv (16)(p13; q22)	
18	F	M4Eo	46XX, inv(16) (p13;q22)	
19	F	M4Eo	46XX, inv(16) (p13;q22)	

Patients with complex abberant Karyotype

No.	Sex	FAB	Karyotype
20	M	M6	50XY+X, t(8;9;11)(q12;p24, p12)
21	F	M2	46XX, inv (3) (q21; q26) (20)46XX (4)
22	F	M1	47XX, t(5;16)(q11;p12),del(9)(q11)(11)
23	M	M1	47XY+13
24	F	M1	42XX, der(1)t(1;6)(p11;q11),del(1)
25	F	M1	47-51,XX, -3, der(5)t(3;5)(q21;q13)

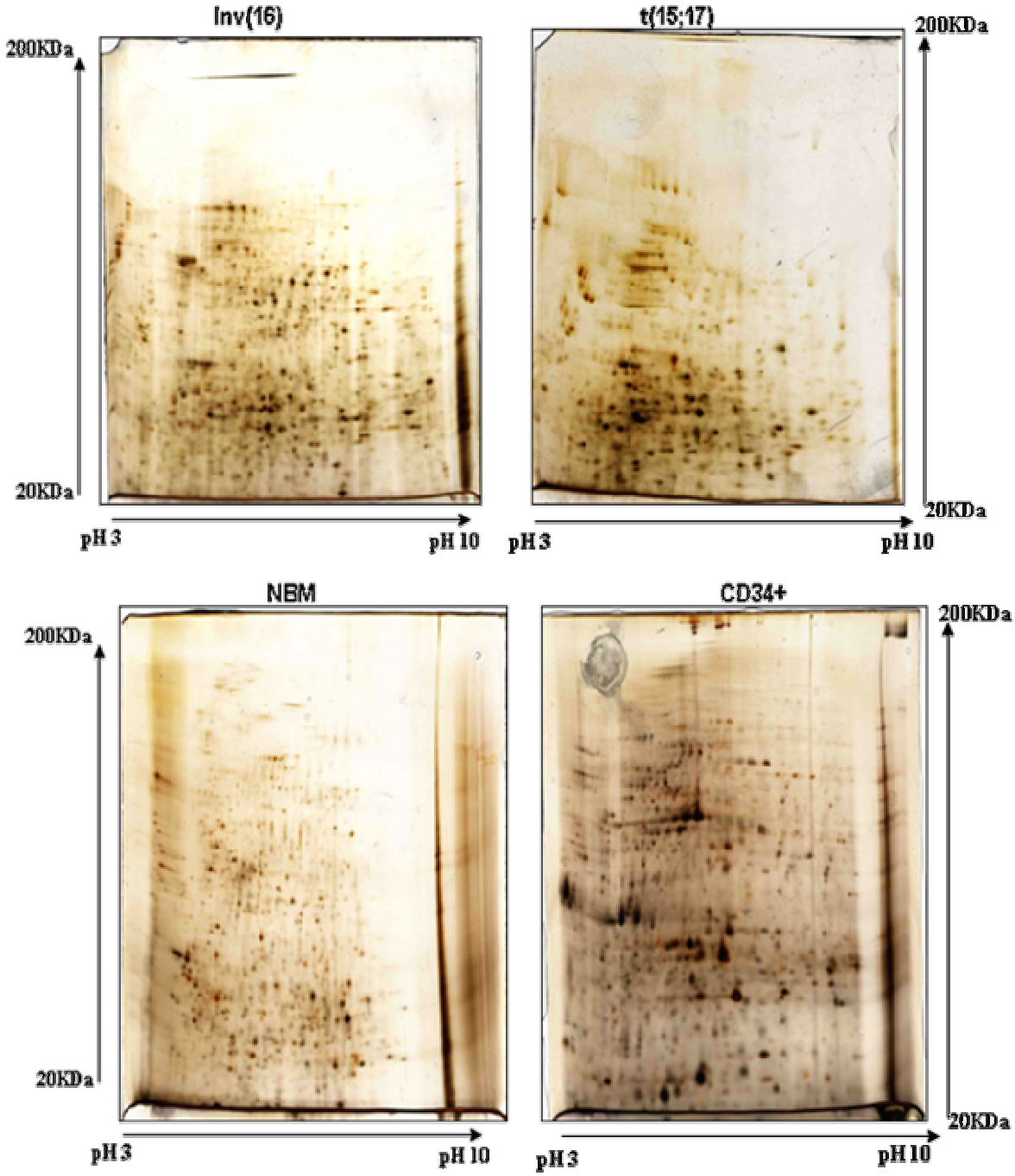
Patients with MLL translocations

No.	Sex	FAB	Karyotype
26	M	t-AML	46XY,t(11;22)(q23;q12)
27	M	M4	46XY,t(11;22)(q23;q12)
28	F	-	46XX,t(4;11)(q21;q23)
29	F	M4	46XX,t(11;19)(q23;p13.1)
30	M	M5a	46XY,t(9;11)(p22;q23)
31	F	M5a	46XX,t(9;11)(p22;q23)

Patients with a normal karyotype

N0.	Sex	FAB	Karyotype	FLT3
32	M	M4	46XX,	FLT 3-LM+
33	M	M5a	46XY	
34	M	M4	46XY	
35	M	M4	46XY	
36	M	M2	46XY CKIT d816+	FLT3 LM+
37	M	M2	46XY	
38	M	M2	46XY	
39	M	M2	46XY	
40	M	M2	46XY	
41	M	M1	46XY	
42	M	M2	46XY	

M, male; F, female, FAB, French-American-British classification; MLL, mixed lymphocyte lineage gene


Inv(16)
200KDa
20KDa
pH 3
pH 10
t(15;17)
200KDa
20KDa
pH 3
pH 10
NBM
200KDa
20KDa
pH 3
pH 10
CD34+
200KDa
20KDa
pH 3
pH 10

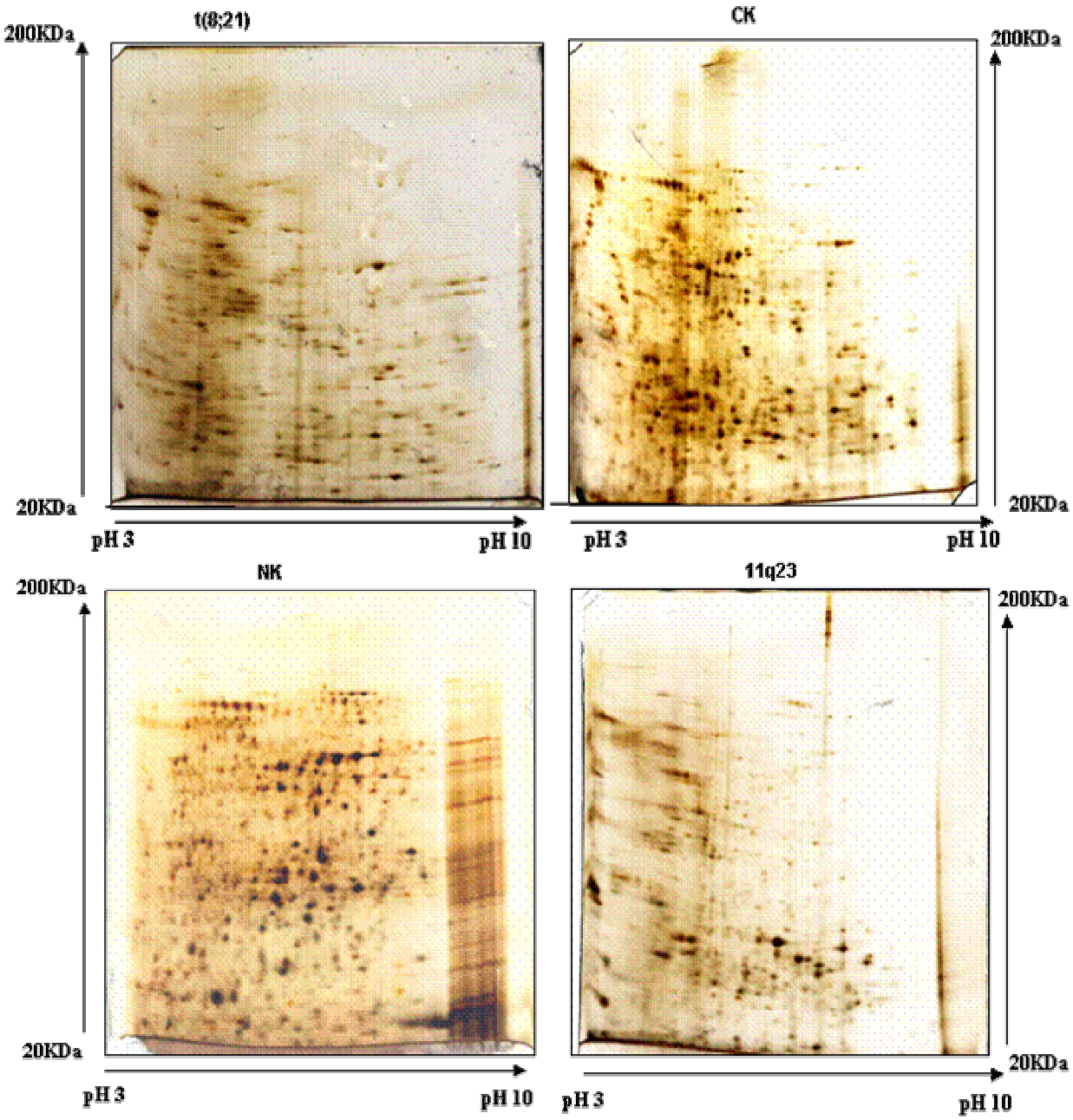


Figure 11. 2D gel images of AML patients with cytogenetic abnormalities. Whole cell lysates from patient samples, NBM and CD34+ cells were urea lysed (described in material and methods). In the first dimension 300µg of soluble proteins were ultracentrifuged and separated on immobiline^{TM} dry strips pH 3-10 (Amersham Biosciences) by isoelectric focusing (IEF) which allows the separation of proteins on the basis of their respective isoelectric point (pI). Proteins were then resolved in the second dimension using 12% SDS PAGE on the basis of their relative size and molecular weight. 2D gels were silver stained to visualize the protein spots. All the visible spots were cut manually (as discussed in results) for the MS analysis.

Table 3. Proteins identified as differential targets of cytogenetic groups

CK

Hepatoma derived growth factor (HDGF)
hnRNP Do (AU rich element R)
Alpha-1-antitrypsin precursor (alpha-1 protease)
Adenosine deaminase
B-cell lymphoma/ leukemia IIA
Diacylglycerol kinase, gamma
ERp29 precursor
Endoplasmic precursor(GRP94
Hypothetical Zinc finger protein KIAA0628
hn RNPK
Alpha -2HS-glycoprotein precursor
Mitogen activated protein kinase-kinase
Sentrin/SUMO specific protease SENP2
Splicing factor, proline and glutamine rich
Sorting nexin II
Splice factor 3B subunit 5
Proline rich protein 4 precursor
Ras related protein Rab-8A
Ruv B-like 2
Splice factor, arginine / serine rich 3

t(8;21)

Transcriptional repressor NF-X1
Transcription elongation factor IIS
FUSE binding protein 2
hnRNPG
Myeloid cell nuclear differentiation antigen
Nucleophosmin
Ras related protein RAB3
Sorcin
Transgelin 2
BMP-3b
Casein kinase II
Apoptosis inhibitor survivin

MLL

HSP70.1
Zyxin 2
L-plastin
Lactotransferrin protein
Phosphatidylethanolamine binding protein
Stress induced phosphoprotein 1
Ubiquitin tropomodulin (U-T mod)
52 kda phosphoprotein lymphocyte

t(15;17)

Splicing factor DNA binding p52/100
Ring finger protein 16
Similar to ATP binding protein (associated with cell differentiation)
Antithrombin
BCL-6 corepressor
Core binding factor beta
Epithelial glycoprotein antigen (GA733-2 precursor)
Guanine nucleotide binding protein G(q)
Myeloperoxidase splice variant H17
Phospholipase D2
Plasminogen related protein A
A 20 binding inhibitor of NF-κB activation 2

Inv(16)

ATP dependant DNA helicase II
Bone marrow zinc finger protein 255
Chaperonin, mitochondrial matrix protein P1
HSPA5
Transcription factor MafK
HSP90 alpha
Leukocyte derived neural aminopeptidase
Myeloblastin precursor (leukocyte proteinase 3)
Melanoma associated antigen B3 (MAGE-B3)
Nucleolar transcription factor 1 (UBF1)
Prolyl 4-hydroxylase, v-erb
Plectin
Serine (or cysteine) proteinase inhibitor
Tranlational control tumor protein(TCTP
KIAA1937
KIAA0141 similar gene product

NK

Cellular glutathione peroxidase
Cargo selectin protein
Casein kinase 1, alpha isoform
Chromatin assembly factor 1 subunit C
CDC 2-related protein kinase
Cathepsin D
UBE3B variant 1
Laminin binding protein
Zeta sarcoglycan
PDZ-LIM protein 1
Similar to nucleolin
T-complex protein 1 beta subunit
Transformation sensitive protein 1EF SSP 3521
Transcription factor MAX, chain B
MRP-14
Nuclear receptor ROR alpha
Apoptosis inhibitor survivin

Table 4. Proteins identified by MALDI-TOF mass spectrometry

Proteins identified from t(8;21) patients

Protein name	Mowse Score	Theoretical p*I*	Theoretical M_r[Da]	Accession[a]	Sequence coverage(%)	MSMS Matche
ATP synthase beta chain mitochondrial	277	5.26	56525	P06576	35	3
Annexin A1	119	6.64	38787	P04083	28	2
Actin cytoplasmic 2, gamma actin	87	5.31	42108	P02571	18	3
BMP-3b	190	9.58	53149	P55107	27	3
Casein kinase II	57	8.65	41358	P19784	34	
Calreticulin precursor	95	4.29	48283	P27797	50	2
Chaperonon GroEL precursor	117	5.70	61187	P10809	40	
DEAD-box protein 4	68	5.38	76571	Q9NQ10	21	
FUSE binding protein 2	73	8.0	72721	Q92945	25	1
Flavoprotein alpha subunit	162	8.62	35400	P13804	46	1
GRP 78	172	5.07	72315	P11021	40	2
GAPHD	230	8.85	36070	P04406	30	2
Gluthione transferase omega	66	6.23	27833	P78417	26	1
HnRNPA2/B1	187	8.97	37464	P22626	42	2
hnRNP F	278	5.38	45985	P52597	41	2
hnRNPG	58	10	42306	P38159	27	
Heat shock cognate 71 KDa	79	5.37	71082	P11142	15	1
60KDa heat shock protein, mitochondrial	225	5.70	61187	P10809	23	3
Lamin B1	388	5.11	66522	P20700	37	3
Leukocyte elastase inhibitor(LEI)	77	5.90	42742	P30740	23	
Myeloid cell nuclear differentiation antigen	70	9.77	46092	P41218	24	
Nucleophosmin	170	4.64	32726	P06748	25	2
Prohibitin	144	5.57	29843	P35232	40	
Ras related protein RAB3	68	6.92	22227	P36409	28	1
Rho-GDP dissociation inhibitor 2	68	5.73	21602	P52566	23	
Sorcin	82	5.3	21947	P30626	31	1
Stathmin (OP18)	105	5.77	17161	P16946	44	1
Superoxide dismutase (Mn)	162	8.35	24878	P04179	34	2
Transgelin 2	181	8.41	22548	P37802	54	2
Tropomyosin alpha 3 chain	224	4.68	32856	P06753	20	2
Transcriptional repressor NF-X1	60	8.47	128656	Q12986	16	1
Transcription elongation factor IIS	64	8.65	33975	P23193	35	

Proteins identified from 11q23 patients

Actin cytoplasmic 1 beta	378	5.29	42052	P60709	24	4
Calnexin precursor	100	4.47	67982	P27824	23	1
Delta 3,5-delta 2,4-dienoyl –CoA isomerase	67	6.61	36314	Q13011	18	1
Destrin (actin depolymerising factor)	66	8.06	18950	P60981	25	
Gluthione S-transferase P	213	5.44	23438	P09211	36	3
GAPHD	122	8.58	36070	P04406	28	1
Hematopoietic lineage cell specific protein	68	4.74	54079	P14307	24	1
hnRNP H	171	5.89	49484	P31943	25	2
hnRNPA2/B1	104	8.97	37464	P22626	26	1
Heat shock cognate 71 KDa protein	199	5.37	71082	P11142	23	1
HSP70.1	78	5.48	70294	O08107	28	
HSP 90 beta	70	4.97	83423	P08238	30	2
52 kda phosphoprotein lymphocyte specific	118	4.69	37397	P33241	23	1
L-plastin	95	5.20	70815	P13796	24	1
Lactotransferrin protein	80	8.56	80170	P02788	24	1
Peroxiredoxin 2	68	5.66	22049	P32119	31	1
Phosphatidylethanolamine binding protein	227	7.42	21027	P30086	43	3
Phosphoglycerate mutase	77	6.75	28769	P18669	26	
Rho-GDP dissociation inhibitor 2	261	5.01	23031	P52566	60	3
Stress induced phosphoprotein 1	83	6.4	63227	P31948	18	
Tropomyosin alpha 3 chain	131	4.68	32856	P06753	19	1
Ubiquitin carboxyl terminal hydrolase 14	94	5.20	56358	P54578	21	
Ubiquitin tropomodulin (U-T mod)	59	5.08	39741	Q9NYL6	16	1
Zyxin 2	108	6.22	62436	Q15942	18	2

Proteins identified from Inv (16)

Alpha enolase	91	6.99	47350	P06733	44	3
Abnormal spindle protein	100	9.0	107719	Q8IZT6	22	1
Annexin 1	108	6.57	38918	P04083	43	
ATP synthase D chain mitochondrial	115	5.22	18405	P30049	38	
ATP dependant DNA helicase II	71	6.23	69717	P12956	24	1
Bone marrow zinc finger protein 255	65	8.99	75063	Q9UID9	26	
BIP protein	76	5.23	71002	Q9UK02	27	
Calreticulin precursor	127	4.29	48283	P27797	29	1
Calnexin precursor	81	4.5	67982	P27824	25	3
C-myc promoter binding protein	243	7.01	47481	P06733	38	3
Chaperonin, mitochondrial matrix protein P1	80	5.7	61187	P10909	20	1
Ferratin light chain	88	5.51	19933	P02792	43	
Glutathione synthetase	120	5.67	52523	P48637	41	1
GRP 78	131	5.1	72402	P11021	27	3
HSPA5	107	5.03	71082	P11021	40	
HSP 90 beta	116	5.0	83423	P08238	27	3
HSP90 alpha	215	4.9	67982	P07900	26	3
KIAA1937	70	6.23	82191	Q96PV1	27	
KIAA0141 similar gene product	70	7.24	56371	Q14154	20	
Leukocyte derived neural aminopeptidase	72	5.78	61412	Q8TD32	23	
Moesin	78	6.08	62004	P26038	38	
Myeloblastin precursor (leukocyte proteinase 3)	63	8.7	28245	P24158	30	2
Monocyte/Neutrophil elastase inhibitor (LEI)	68	9.1	42829	P30740	26	1
Melanoma associated antigen B3 (MAGE-B3)	62	9.8	39326	O15480	32	1
Nucleolar transcription factor 1 (UBF1)	61	5.6	89692	P17480	24	
Protein disulphide isomerase precursor	105	4.76	57146	P30101	21	3
Prolyl 4-hydroxylase, v-erb	76	4.76	57480	P07237	26	1
Plectin	65	5.38	64921	Q15149	26	1
Ribosomal protein L13A	70	6.98	18953	Q8N6Z1	43	1
Serine (or cysteine) proteinase inhibitor	80	5.9	42829	Q4VAX4	27	1
Signal recognition particle 54	77	8.87	55718	P61011	21	1
Tranlational control tumor protein(TCTP)	65	4.98	19696	P13693	20	
Thioredoxin like protein	60	4.8	32630	O43396	27	
Transcription factor MafK	61	6.9	18158	O60675	19	1
Vimentin	170	5.06	53579	P08670	23	3
Valyl 1-t RNA synthetase	80	5.10	105432	Q7TUID	21	1

Proteins identified from complex aberrant karyotype

Alpha -2HS-glycoprotein precursor	74	5.26	39193	P12763	22	1
Annexin II type 1	91	8.60	38618	P27006	26	
Adenylate kinase isoenzyme	105	5.38	22358	Q9Y6K8	40	
Annexin A1	212	6.64	38787	P04083	39	3
Alpha-1-antitrypsin precursor (alpha-1 protease)	71	5.37	46878	P01009	19	1
Alpha enolase	359	6.99	47350	P06733	50	3
Adenosine deaminase	241	5.63	41024	P00813	26	2
ATP synthase alpha chain	83	9.16	59828	P25705	27	1
Actin cytoplasmic 1 beta	110	5.29	42052	P60709	25	1
B-cell lymphoma/ leukemia IIA	71	6.15	92565	Q9H165	19	1
Chloride intracellular channel protein	410	5.09	27248	O00299	56	4
Calnexin precursor	99	4.47	67982	P27824	22	1
Calreticulin	97	4.29	48283	P27797	28	1
Destrin (actin depolymerising factor)	82	8.06	18950	P60981	40	
DEAD box protein 4(VASA homolog)	69	5.67	80113	Q9NQ10	23	
Delta 3,5-delta 2,4-dienoyl –CoA isomerase	93	6.61	36314	Q13011	26	2
Diacylglycerol kinase, gamma	113	6.29	90535	P49619	20	
ERp29 precursor	79	6.77	29032	P30040	22	1
Endoplasmic precursor(GRP94)	76	4.76	92696	P14625	27	1
Glutathione synthetase	132	5.67	52523	P48637	31	3
Glutathione S-transferase P	282	5.44	23438	P09211	55	2
GRP 78	273	5.07	72402	P11021	25	2
Hematopoietic lineage cell specific protein	81	4.74	54079	P14317	20	1
hnRNPF	86	5.38	45985	P52597	22	2
Hypothetical Zinc finger protein KIAA0628	99	8.46	63065	O75123	20	
hnRNP H	291	5.89	49484	P31943	23	2
hn RNPK	117	5.3	51230	P61978	36	1
Hepatoma derived growth factor (HDGF)	65	4.7	26886	P51858	28	
hnRNP Do (AU rich element R)	137	7.6	38581	Q14103	30	2
hnRNP M	114	8.94	77703	P52272	22	
Heat shock cognate 71Kda protein	81	5.37	71082	P11142	20	1

60KDa heat shock protein, mitochondrial	276	5.70	61187	P10809	41
Leukocyte elastase inhibitor(LEI)	81	5.90	42829	P30740	15
Lamin B1	89	5.11	66522	P20700	20
Moesin	85	6.09	67761	P26038	26
Mitogen activated protein kinase-kinase	94	8.28	44716	P45985	23
Peroxiredoxin 6	215	6.02	25002	P30041	30
Pyruvate kinase M1 isoenzyme	88	7.95	58339	P14618	27
Protein disulphide isomerase precursor	313	4.76	57146	P30101	33
Prohibitin	241	5.57	29843	P35232	28
Proline rich protein 4 precursor	60	6.5	15088	Q16378	21
Ras related protein Rab-8A	92	9.15	23824	P61006	25
Ruv B-like 2	63	5.49	51296	Q9Y230	20
Splice factor, arginine / serine rich 3	75	9.3	19546	P23152	27
Stress 70 protein	165	5.87	73920	P38646	27
Splicing factor, proline and glutamine rich	116	9.45	76216	P23246	25
Sorting nexin II	86	6.61	30870	Q9Y5W9	36
Splice factor 3B subunit 5	80	5.89	10243	Q9BWJ5	41
Splice factor, arginine/serine rich 1(SER1)	136	9.12	27711	Q05519	33
Sentrin/SUMP specific protease SENP2	100	8.9	68637	Q9HC62	19
Thioredoxin dependant peroxide reductase	92	7.67	28017	P30048	26
Ubiquitin carboxyl terminal hydolase 14	99	5.20	56358	P54578	27
Vimentin	186	5.06	53579	P08670	28
Zinc finger protein 255	90	8.99	75063	Q9UID9	23

Proteins identified from t (15;17)

Alpha enolase	148	6.99	47350	P06733	23
A 20 binding inhibitor of NF-κB activation 2	65	6.06	48716	Q9BQR6	18
Annexin A4	67	5.85	35751	P09525	20
Annexin A1	215	6.64	38787	P04083	47
Adenylate kinase isoenzyme 5	155	5.38	22358	Q9Y6K8	24
Actin cytoplasmic 1	101	5.31	42108	P02571	29
Antithrombin	63	6.32	52604	P01008	22
BIP protein	84	5.27	70920	Q9UK02	17
BCL-6 corepressor	80	6.06	192829	Q6W2J9	30
Core binding factor beta	55	6.23	21723	Q13951	20
Chloride intracellular channel protein	103	5.09	27248	O00299	41
Calreticulin	330	4.29	48283	P27797	25
Calnexin precursor	92	4.47	67982	P27824	23
Epithelial glycoprotein antigen (GA733-2 precursor)	66	8.14	34923	P16422	26
GRP 78	198	5.07	71402	P11021	39
Guanine nucleotide binding protein G(q)	60	5.58	41726	P50148	26
Growth arrest specific 6	65	5.17	79680	Q14393	52
hnRNP F	100	5.38	45985	P52597	22
hnRNP H	194	5.89	49484	P31943	28
Myeloperoxidase splice variant H17	67	9.19	83891	P05164	26
Protein disulphide isomerase precursor	99	4.76	57480	P07237	21
Profilin IV	61	4.97	14481	Q8NHR9	35
Phospholipase D2	70	6.42	71620	Q86YQ7	25
Plasminogen related protein A	65	8.44	10222	Q9UE70	44
Rho-GDP dissociation inhibitor 3	100	5.45	25367	Q99819	25
Ring finger protein 16	70	6.62	54426	Q9Y577	20
Similar to ATP binding protein- (associated with cell differentiation)	66	5.56	22199	Q8TB70	27
Lamin A/C	103	6.57	74380	P02545	22
Splicing factor DNA binding p52/100	156	7.4	76216	P23246	17
Stress 70 protein	67	5.87	73920	P38646	29
TOB 3	63	7.05	66237	Q9NVI7	25
Vimentin	319	5.06	53579	P08670	36

Proteins identified from Normal karyotype subtype

Actin beta	196	5.29	42052	P60700	23
Annexin A1	142	6.64	38787	P04083	35
ATP synthase beta chain, mitochondrial	145	5.26	56525	P06576	50
Alpha enolase	132	6.99	47350	P06733	23
Annexin 1	103	7.77	35246	P04083	35
Cargo selectin protein	74	5.67	41506	Q9BSO3	33
C-myc promoter binding protein	188	6.79	37862	P06733	19
Cellular glutathione peroxidase	86	6.15	22227	P07203	17
Catalase	61	6.95	59816	P04040	22

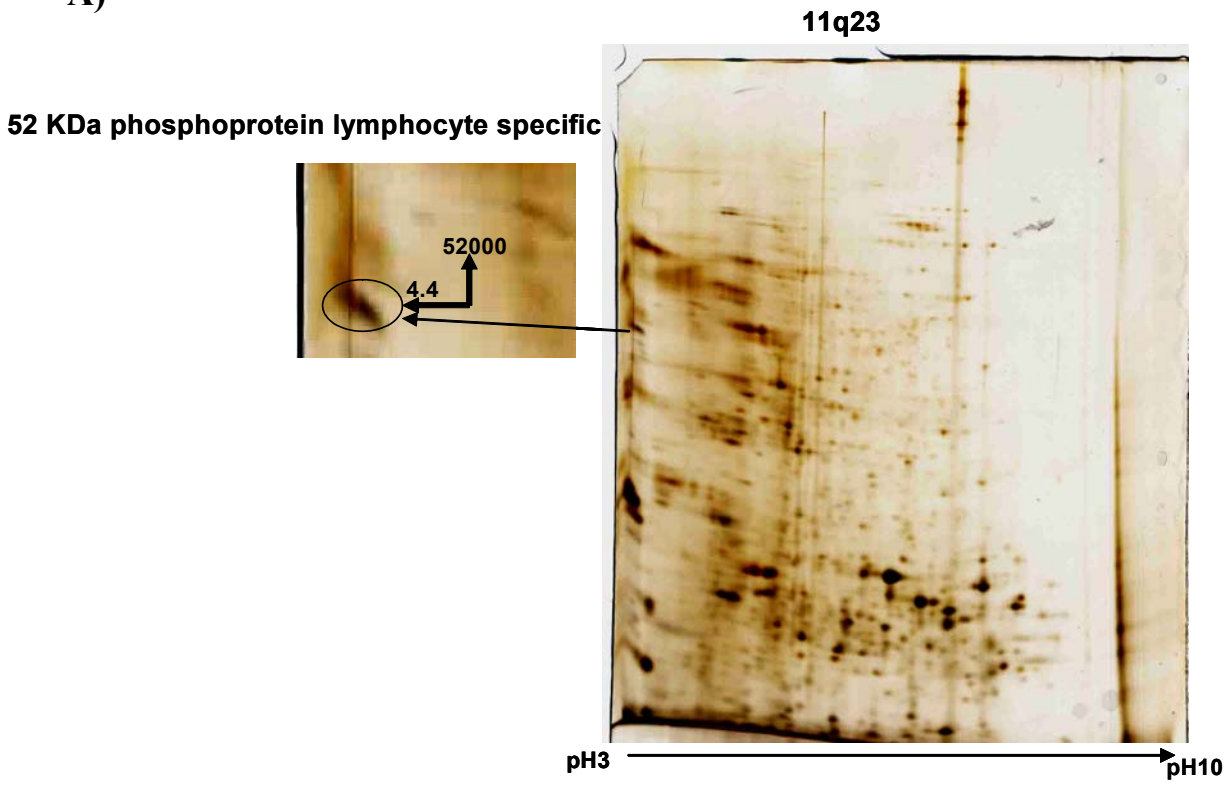
Casein kinase 1, alpha isoform	61	9.59	39118	P48729	29	
Calreticulin	86	4.29	48283	P27797	27	2
Chromatin assembly factor 1 subunit C	102	4.74	47911	Q09028	24	3
CDC 2-related protein kinase	70	9.02	36005	Q9NYV4	20	
Cathepsin D	100	6.1	45037	P07339	30	
Elongation factor 1 beta	65	4.5	24788	P24534	22	
UBE3B variant 1	72	8.45	124457	Q9BXZ4	18	
Elastase inhibitor	79	5.9	42829	P19957	24	
Ferritin light chain	113	5.65	16441	P02792	33	2
Growth arrest specific 6 fragment	68	8.84	79680	Q14393	52	
GRP 78	138	5.07	72402	P11021	15	2
Glutathione transferase omega	101	6.23	27833	P78417	20	1
hnRNP M	81	8.84	77749	P52272	37	
hnRNP H1	108	5.89	49484	P31943	27	1
hn RNPA2/B1	107	8.67	36041	P22626	34	2
hnRNP H´	94	5.89	49517	P55795	29	1
78KDa glucose related protein	88	5.07	72402	P11021	36	2
60KDa heat shock protein	161	5.70	61187	P10809	50	2
Laminin binding protein	64	4.84	31888	P17931	19	
Lamin C	78	6.4	65153	P02545	25	
Lamin B1	138	5.11	66522	P20700	20	1
Myeloperoxidase (1 MYPA)	72	5.28	11998	P05164	30	
MRP-14	68	5.55	12770	P06702	20	
Nuclear receptor ROR alpha	60	5.97	64306	P35398	15	
NADH-Ubiquinone oxidoreductase chain 2	66	8.59	39208	P03891	33	
Peptidyl-prolyl cis-trans isomerase	73	5.36	64242	Q96AY3	39	
Protein disulphide isomerase precursor	183	4.76	57480	P07237	16	3
PDZ-LIM protein 1	79	6.56	36505	O00151	16	
Pyruvate kinase, M1 isoenzyme	117	7.95	58339	P14618	23	2
Peroxiredoxin 3 precursor	79	7.67	28017	P32119	29	
Profilin, chain A	83	8.46	15014	P07737	25	
Prohibitin	122	5.57	29843	P35233	40	
Probable thioredoxin peroxidase	136	8.27	22324	P30044	44	2
Phosphoglycerate kinase1	115	8.3	44967	P00558	40	
Similar to lamin A/C	139	6.03	53222	P02545	40	
Similar to nucleolin	80	4.95	50977	P09405	17	
Stress 70 protein mitochondrial	63	5.87	73920	P38646	17	
Stathmin (OP18)	155	5.77	17161	P16949	29	1
Splice factor, arginine/ serine rich 1	113	9.12	27711	Q07955	22	2
Similar to alpha fetoprotein	80	5.97	48641	Q86YG0	28	
T-complex protein 1 beta subunit	125	5.42	60153	P78371	50	
Transformation sensitive protein 1EF SSP 3521	110	6.40	63227	P31948	26	
Tropomyosin TM30-PI fibroblast	67	4.67	28367	P67936	22	
Tumor metastatic process associated protein	78	5.83	17309	P15531	31	
Transcription factor MAX, chain B	*66*	*6.07*	*17073*	*1HLOB*	*45*	
Ubiquitin carboxyl terminal hydrolase	90	5.2	56358	P54578	21	
Vimentin	603	5.06	53579	P08670	63	3
Zeta sarcoglycan	60	7.59	21501	Q96LD1	23	
Zinc finger protein 255	62	8.99	75063	Q9UID9	21	1

3.2 Proteins identified as distinguished targets of AML cytogenetic groups

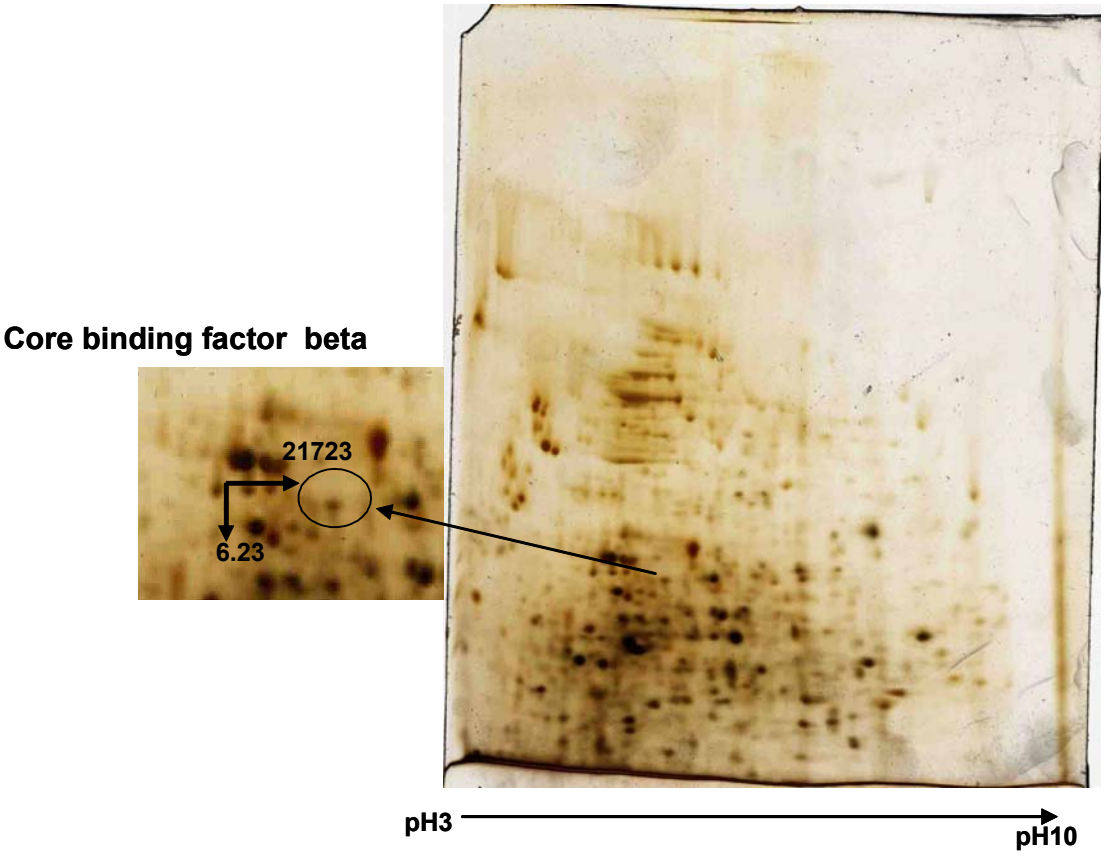
One of our aims of performing the proteomic screen of AML patients was to identify the proteins (diseased proteome) which could serve as distinguished markers for each AML cytogenetic subgroup. Our hypothesis was that since each cytogenetic group possesses different chromosomal fusions which may contribute differentially to the disease progression by altering the proteome differentially. Indeed, we could identify a set of proteins from each cytogenetic group including normal karyotype subtype which were

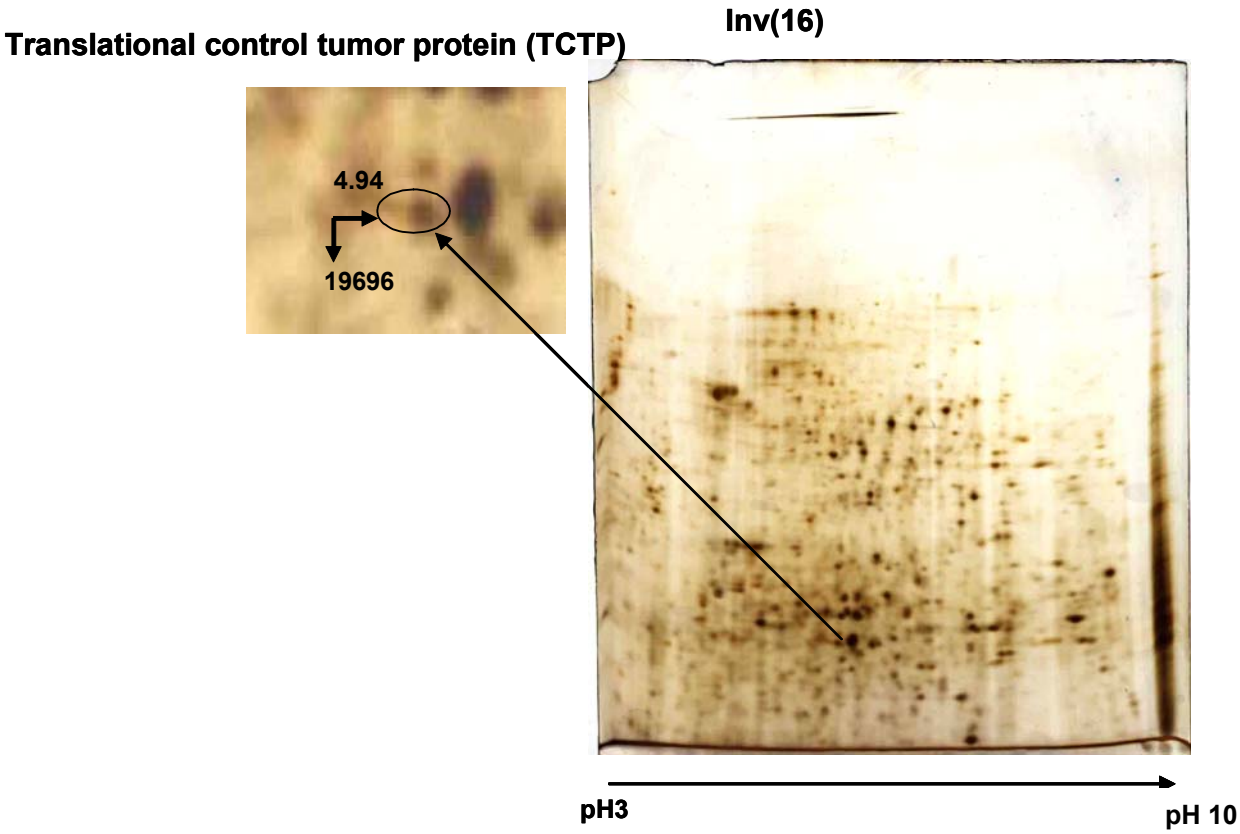
distinguished feature of each AML subtype. These are the proteins which we identify specifically in each subtype. The details of these proteins including the S/T/Y phosphorylation sites for some of the proteins are given (Table 5). Full image 2D gels with the spot position where from these proteins were identified are given separately (Figure 12). Further of our interest was the expression pattern of myeloperoxidase variant H17, sorcin, B-cell CLL/ Lymphoma 11A and transcription factor MafK, identified specifically from t(15;17), t(8;21), CK and Inv(16), respectively. These proteins showed visibly distinct spot position on a 2DE gel. Even though we could reproduce the spot pattern on 2D gels from other subtypes including healthy bone marrow and human CD34+ cells. We could not identify these proteins from rest of other subtypes (Figure 13). As shown in the figure the corresponding spot position in the controlled gels, linear range pH 3-10, was consistently absent, even though the reproducibility in the spot pattern can be seen. These proteins owing to there unique expression in each subtype may serve as an important marker for diagnosis, prognosis and for designing subtype specific therapy.

A)



B) **t(15 ;17)**


Core binding factor beta
21723
6.23
pH3
pH10


Translational control tumor protein (TCTP)
Inv(16)
4.94
19696
pH3
pH 10

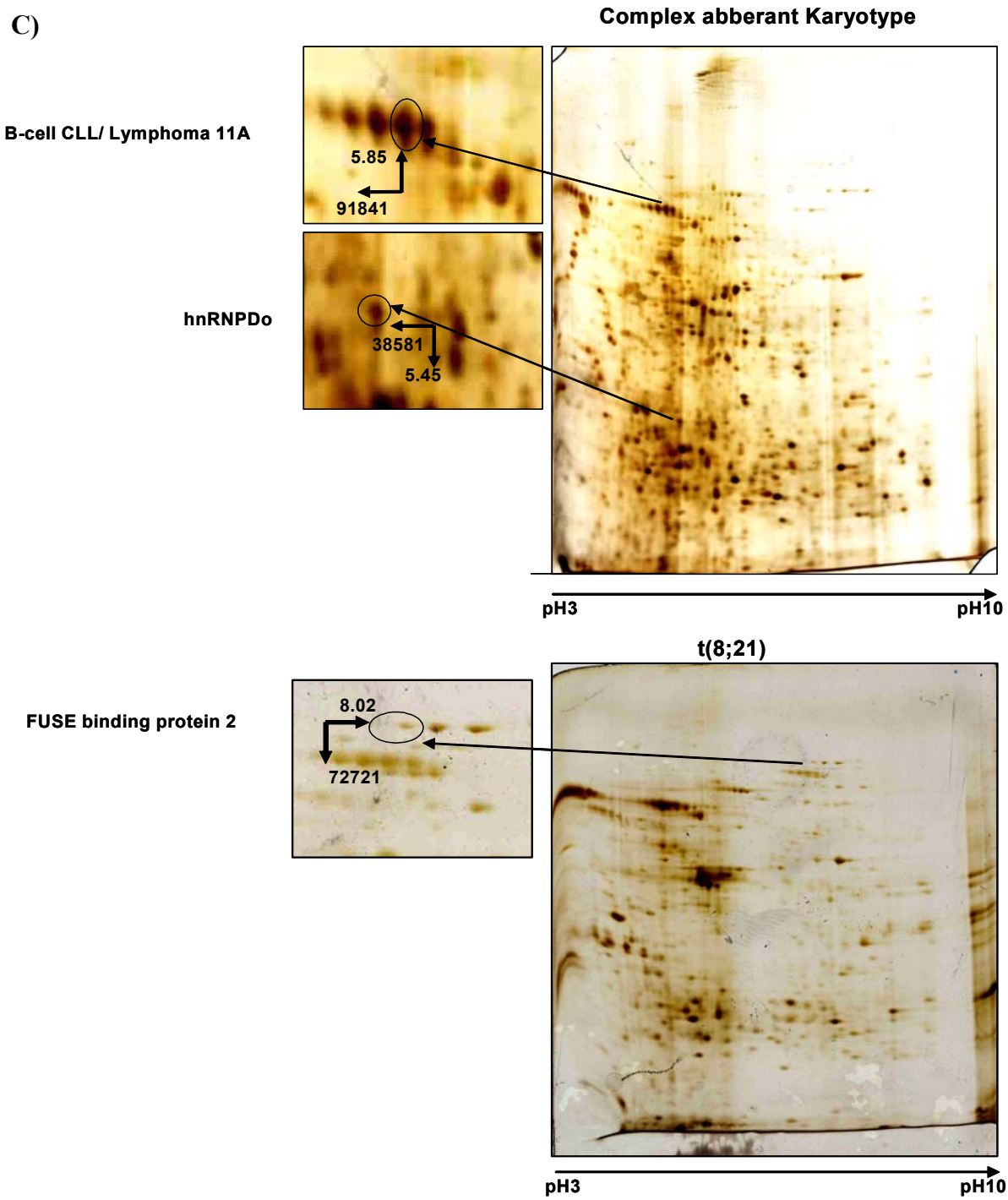


Figure 12. Proteins identified as differential targets of cytogenetic groups**.** A silver stained full image 2D gel from 11q23 patient showing the spot where from 52KDa phophoprotein lymphocyte specific protein was identified (A). Similarly are shown the spots from t(15;17), Inv(16) patients where from core binding factor complex and translational control tumor protein (TCTP) was identified (B). Next, shown are the spot position for B-cell CLL/Lymphoma 11A, hnRNPDo and FUSE binding protein 2 (C). Theoretical pI and Mw are annotated on the image clip.

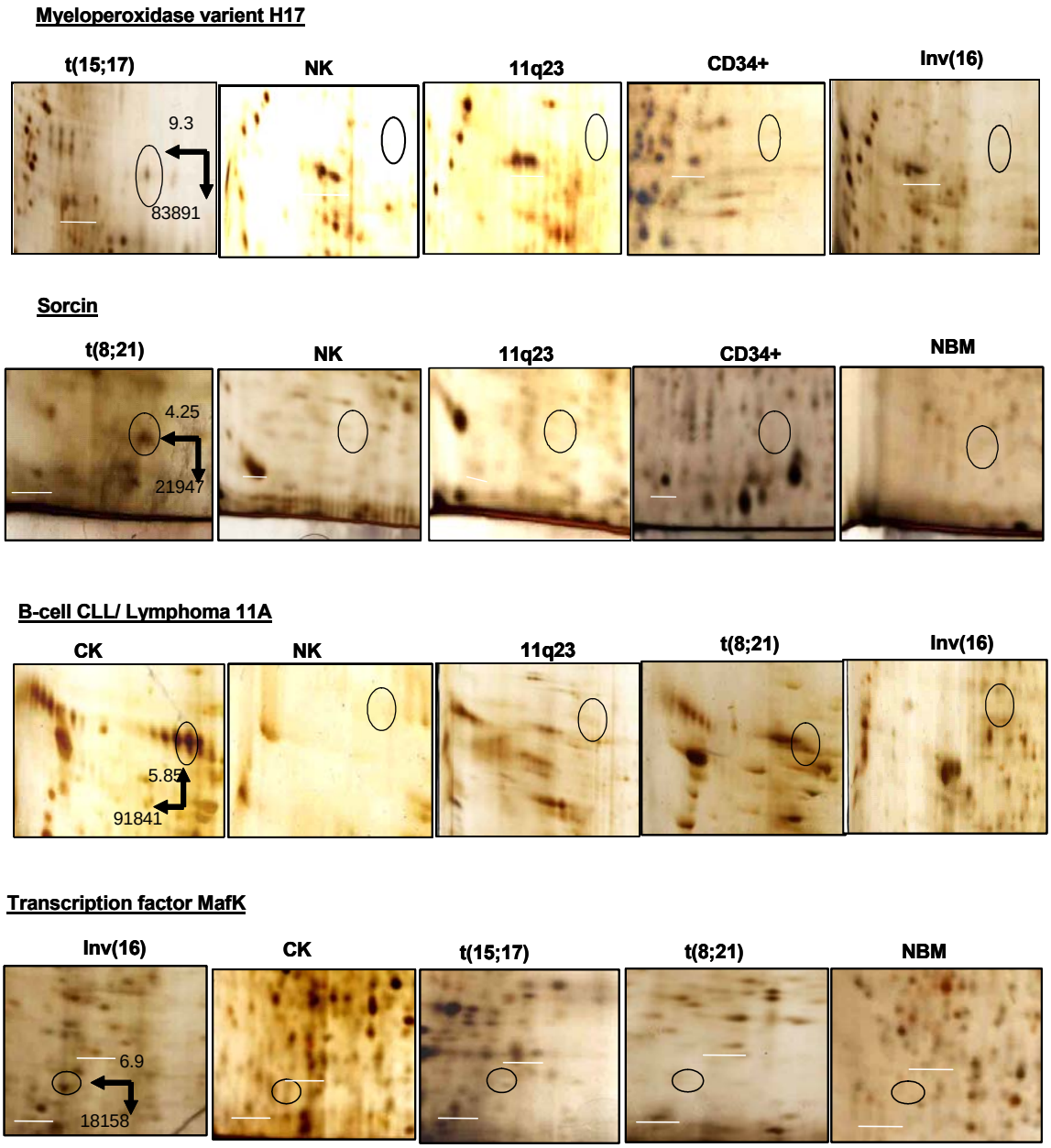


Figure 13. Differential expression of Myeloperoxidase variant H17, Sorcin, B-cell CLL/Lymphoma 11A and transcription factor MafK**.** Close-up sections of spots and a region on a 2D gel identified as Myeloperoxidase variant H17, Sorcin, B-cell CLL/Lymphoma 11A and transcription factor MafK Identified specifically from t(15;17), t(8;21), CK and Inv(16) patients. A close-up comparison on a 2D gel pattern observed between other subtypes, CD34+ and healthy bone marrow are shown with reference spots used as a reference to manipulate the gels. As shown even though the reproducibility in the spot pattern can be observed, the specific protein spots are absent. All the identified proteins which showed specific expression are given in table 5.

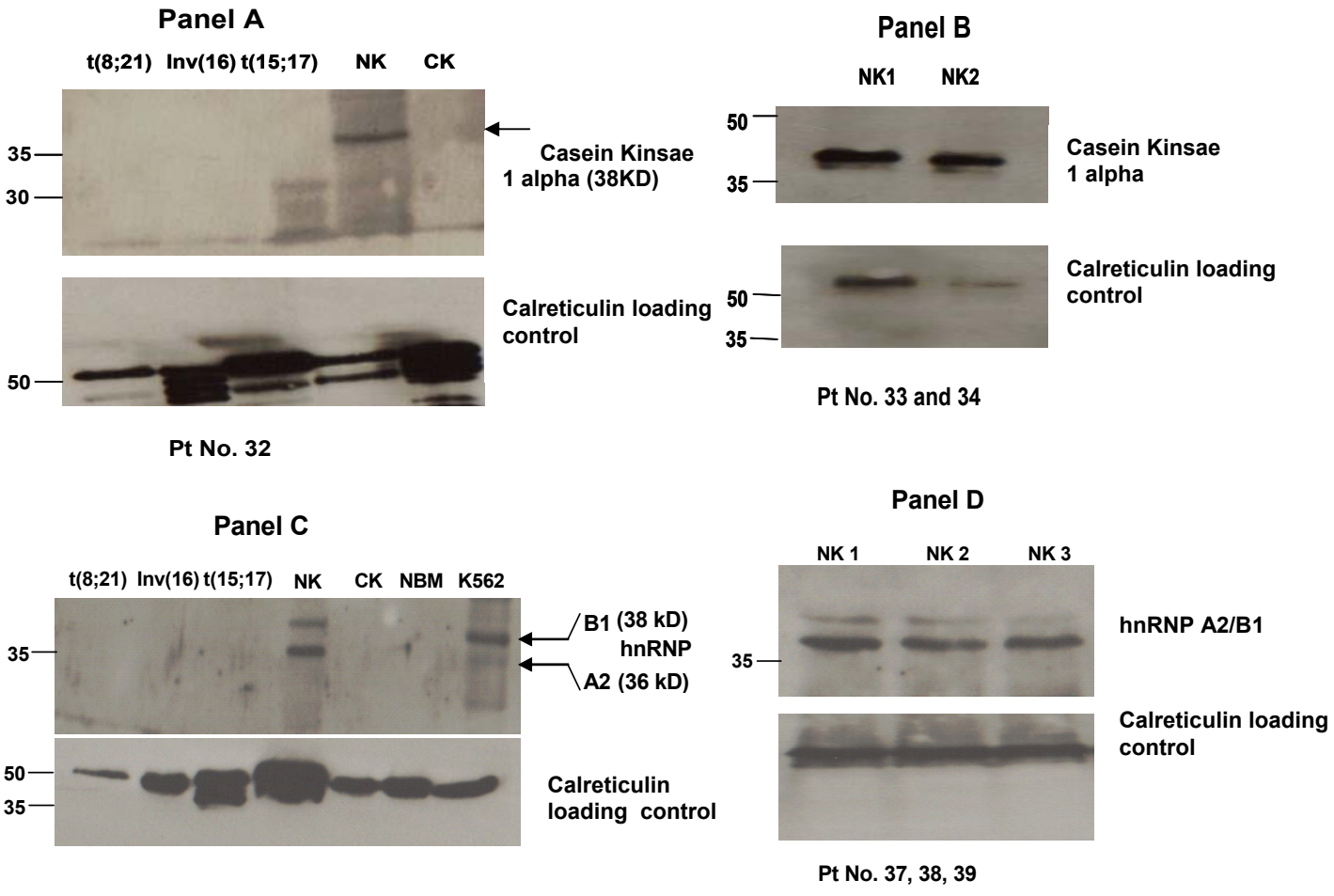
Table 5. Proteins identified as specific targets of Cytogenetic subgroups of AML

Protein name	Accession	p*I*	Sequence Coverage	M*r*[Da]	S/T/Y number	S/T/Y Phosphorylation site
Proteins identified as specific targets of t(8;21)						
Sorcin	P30626	5.3	31	21947		
FUSE binding protein 2	Q92945	8.0	25	72721		
Proteins identified as specific targets of AML patients with a normal karyotype						
Nuclear receptor ROR alpha	P35398	5.97	15	64306		
Growth arrest specific 6	Q14393	8.84	52	56369		
Proteins identified as specific targets of inv(16)						
Nucleolar transcription factor 1(UBF1)	P17480	5.6	14	89692	T117	KHPDFPKKPL**T**PYFRFFM
Transcription factor MafK	O60675	6.9	19	18158		
Translational control tumor protein (TCTP)	P13693	4,9	20	19696		
Proteins identified as specific targets of complex abberant karyotype						
Hepatoma derived growth factor (HDGF)	P51858	4.7	28	26886	S132 T200	DGDKKGNAEG**S**SDEEGKLVID PLPMEVEKNS<u>T</u>PSEPGSGRGP
hnRNP Do (AU rich element R)	Q14103	7.6	30	38581		
B-cell CLL Lymphoma/Leukemia 11A	Q9H165	5.85	19	91841		
Proteins identified as specific targets of 11q23 translocations						
52 Kda phosphoprotein lymphocyte specific	P33241	4.69	23	37397		
Stress induced phosphoprotein 1	P31948	6.40	18	63227		
Proteins identified as specific targets of t(15;17)						
Myeloperoxidase splice variant H17	P05164	9.34	16	83891		
Core binding factor beta	Q13951	6.23	20	21723		

3.3 Validation of differential expression of hnRNPA2/B1, casein kinase 1 alpha, prohibitin and HSC70

Next we performed western blot analysis to confirm the identification of some of the therapeutically relevant proteins previously not reported in the context of AML cytogenetics. Casein kinase 1 alpha which we identified previously by mass spec from normal karyotype patients showed high and specific

expression from 3 different NK patient samples in Immunoblots (Figure 14a, panel A and B). We showed a differential expression of RNA binding protein hnRNPA2/B1 in NK patients (Panel C). This was further confirmed from 3 different NK patients (Panel D). K562 erythroleukemic cell line lysate was used as a positive control for the expression of hnRNPA2/B1. In a similar analysis we further showed a differential expression of cell cycle protein prohibitin in t(8;21) and inv(16) patients which is consistent with the mass spec data (Panel E). Similarly, HSC 70 showed a very high expression in t(8;21), Inv(16), NK and CK patients in comparison to NBM and t(15;17) (Panel F). HL-60 cell line was used as a positive control and 3T3Li preadipocyte cell line as a negative control for HSC 70 expression.



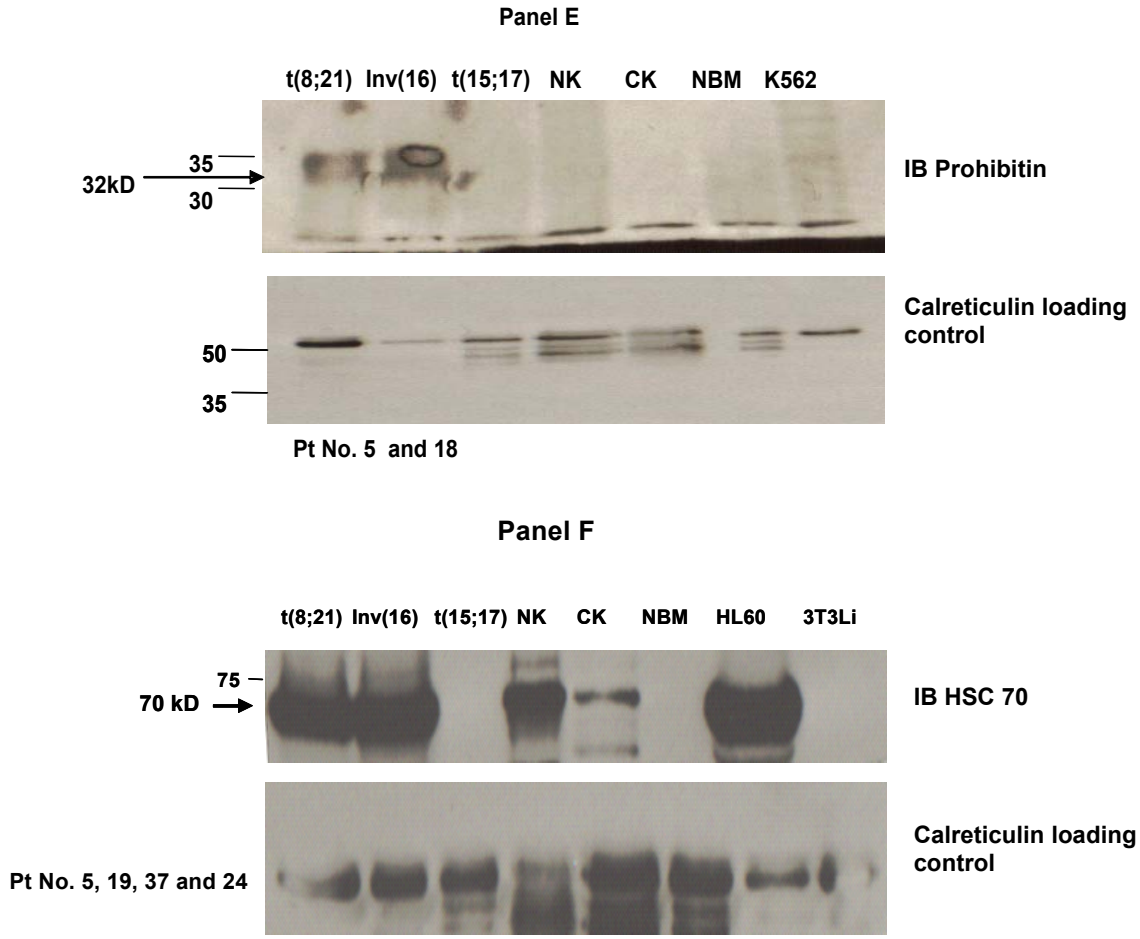


Figure 14a. Validation of mass spec data by western blot. Western blot was performed to confirm the identification of some of the proteins from the whole cell lysates of different patient samples using specific antibodies. Panel A, it shows the expression of casein kinsae 1 alpha which was further confirmed from two different patient samples belonging to normal karyotype (Panel B). The specific expression of hnRNP A2/B1 from NK patients was also confirmed (Panel C & D). Similarly, the differential expression of prohibitin (Panel E) and HSC 70 (Panel F) was confirmed from patient samples in line with the mass spec data. Calreticulin protein was used as a loading control for all cases.

3.4 Survivin identified as a distinguished target of t(8;21) AML

We identified a 16.5KDa apoptosis inhibitor protein (IAP) survivin as a specific target of t(8;21) AML from the proteomic screen. The identification of this protein was further confirmed by the western blot (Figure 14b). Because of the high therapeutic significance attributed to this protein, we characterized survivin both biologically and mechanistically.

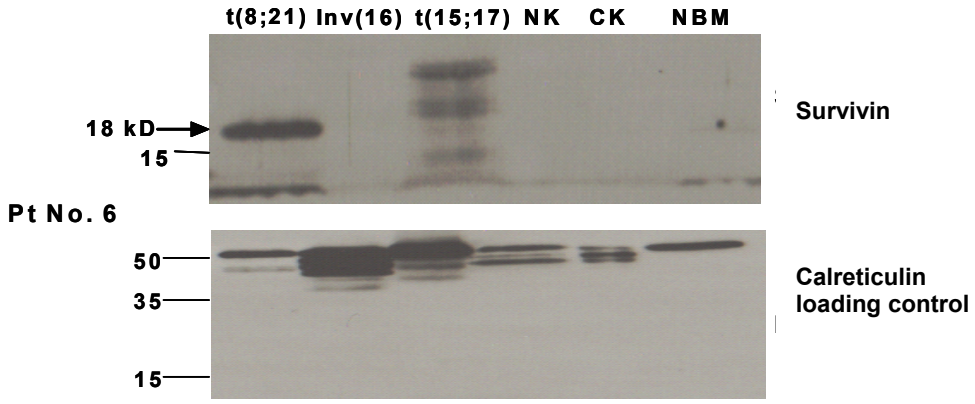
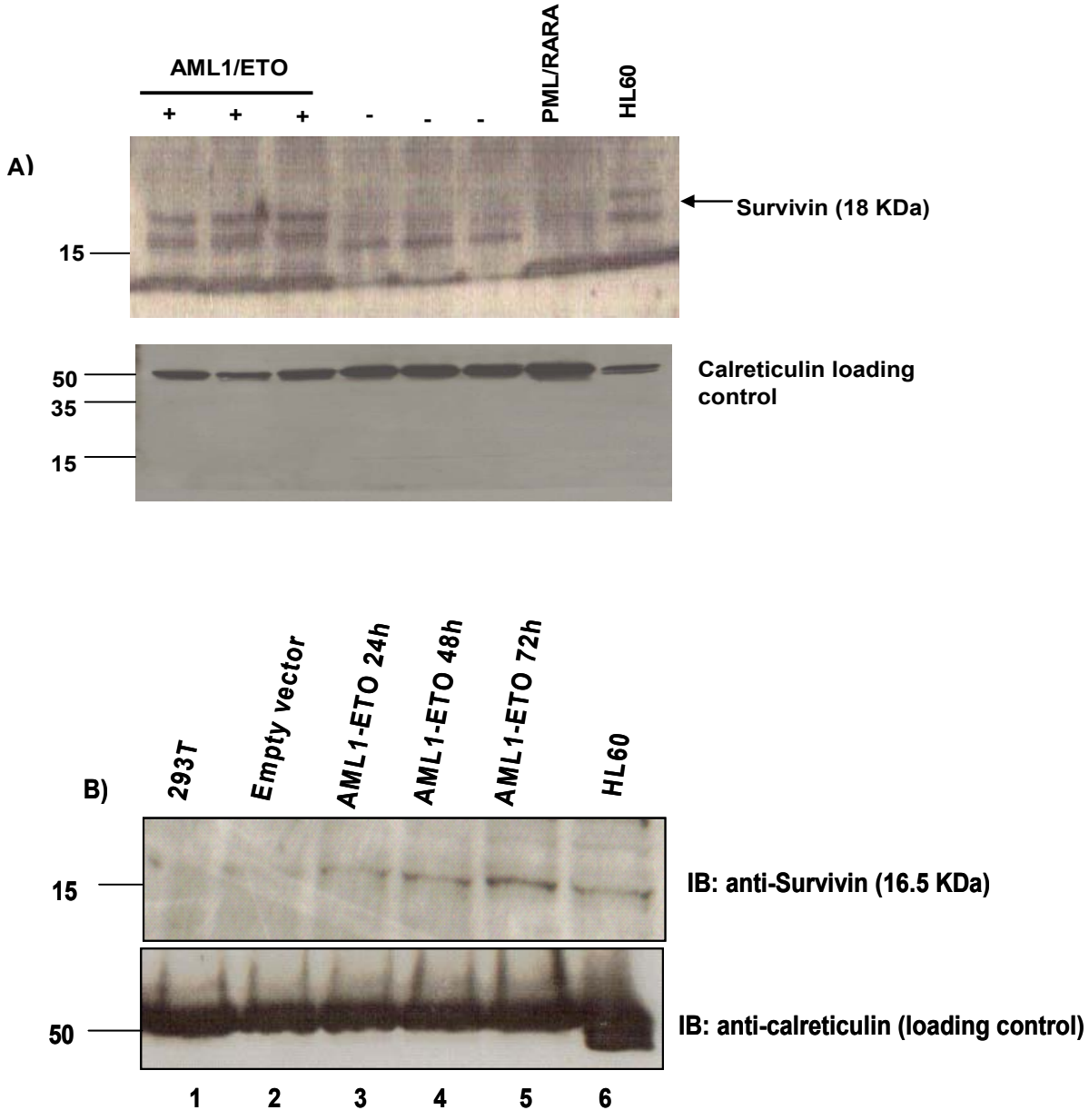


Figure 14b. Western blot performed for the expression of survivin using different AML patient groups. Survivin expression was detected specifically in t(8;21) patient, thus, further confirming the mass spec data.

3.5 AML1/ETO induces survivin expression

Next we asked whether survivin which is a target AML1-ETO fusion protein could induce its expression. To test this hypothesis we used U937 cell line stably transfected with a cDNA of AML1-ETO cloned upstream of a tetracycline (tet)-responsive element. These cells express AML1-ETO after the withdrawal of tetracycline. We performed the cell lyses 48h after the withdrawl of tetracycline and performed a western blot. The results confirmed that U937 T-A/E cells express survivin following the expression of AML1-ETO induced by the withdrawal of tetracycline while as no survivin expression was detected in the presence of tetracycline i.e, without AML1-ETO. HL-60 whole cell lysate was used as a positive control for survivin expression (Fig. 15, panel A). We further confirmed this finding in HEK293T cells which has been extensively used to study AML1-ETO transcriptional mechanism and due to the gross overexpression off of the plasmids (Frank et al., 1999). We could show in 293T cells that AML1-ETO induces survivin expression as early as 24 hours post transfection which enhances subsequently with time (Fig. 15, B). Kasumi-1 cells which harbor endogenous AML1-ETO showed a very high expression of survivin as compared to NB4 cell line (Fig.15, panel E). HL60 cell line was used as a positive control for survivin

expression. This data confirms that AML1-ETO specifically activates survivin expression. The transfection efficiency reached by this method was 85% as confirmed by the expression of GFP plasmid (Fig. 15, C and D). The results strongly suggest that AML1-ETO specifically activates the survivin expression, whose expression is known to enhance with tumor and/or leukemic progression through albeit undefined mechanism. We specifically investigate the underlying mechanism leading to the induction of survivin by AML1-ETO.



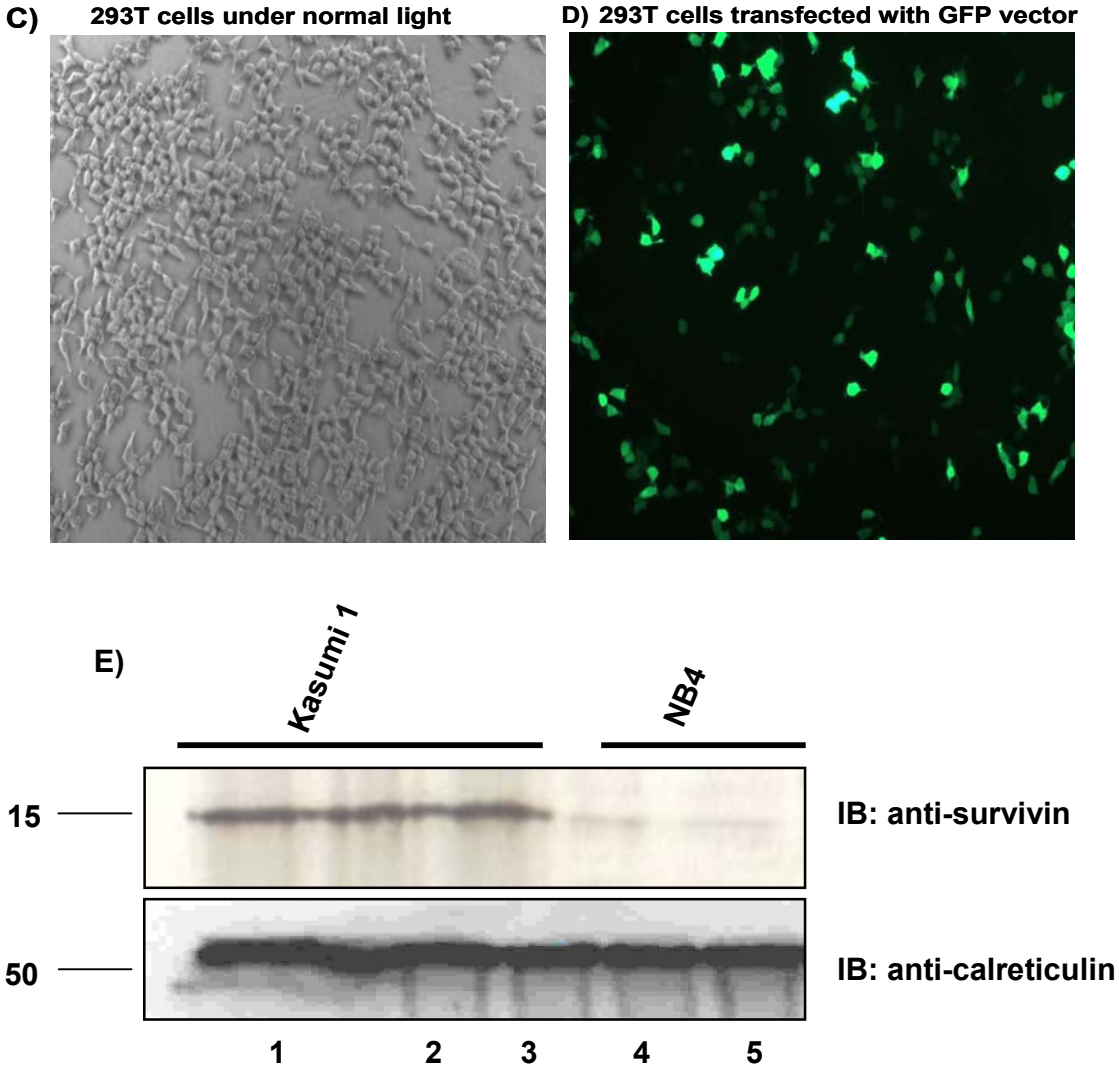


Figure 15. Western blot anaylsis performed for survivin expression in tet off AML1/ETO inducible cell line. Data shows the expression of survivin was induced with the induction of AML1/ETO. HL60 cell line was used as a positive control for survivin expression **(A).**Whole cell lysates from 293T cells were separated on a 12% SDS PAGE and blotted with survivin antibody. The membrane was stripped off bound antibody and reprobed with anti calreticulin antibody to demonstrate equal loading of proteins **(B).** Transfection efficiency observed for 293T cells after the transfection of GFP plasmid (2µg) using lipofectAMINE plus was calculated by counting the cells first under normal light and then under fluorescent light using the same magnification and same field (**panel C & D**). The overall percentage of transfection efficiency reached by this method was 80%. Lysate from Kasumi-1, NB4 and HL60 was resolved on 12% gel to compare the levels of survivin expression which revealed a very high expression of survivin in Kasumi-1 cells; the lower panel shows the equal loading by stripping off the same blot.

3.5.1 AML1-ETO activates basal transcription of the survivin promotor

To define the molecular mechanism of the survivin activation by AML1-ETO, we analysed the sequence of the 5´ flanking region of the survivin gene using the TFsearch. The analysis revealed the presence of a single AML1 binding site (TGTGGT), 1400bp upstream of the initiating ATG (Fig. 16, panel A). We performed a dual luciferase assay to elucidate whether AML1-ETO could activate basal transcription of survivin promotor. Like AML1, AML1-ETO has been reported to act as a transcriptional activator (Rhoades et al., 1996). We transiently transfected 293T cells with a survivin promoter luciferase construct pLuc-1430 which has a single AML1 binding site and co-transfected with an AML1 transcript variant, AML1b, alone or together with AML1-ETO and AML1-ETO alone. AML1b (Tanaka et al., 1995) is a putative transcriptional activator. Both AML1b and C/EBPα are thus supposed to have a negative effect on survivin activation as both supports myeloid differentiation. The results indeed demonstrate that AML1b has no significant effect on the activation of the basal transcription of survivin gene (Fig. 16 panel B, lane 2). However, when AML1b was coexpressed with AML1-ETO survivin activation was enhanced by five fold (lane 3). The activation was maintained when AML1-ETO was expressed alone (lane 4). The activation was repressed by the transfection of granulocytic differentiation transcription factor C/EBPα (lane 6-8). The expression of C/EBPα (lane 5) failed to induce any transcription of survivin promotor. This reduced effect could further be explained as forced expression of AML1 or C/EBPα overcomes the block in differentiation induced by AML1-ETO. This phenomenon explains that AML1-ETO acts as a putative transcriptional activator of survivin gene. In order confirm that effects shown here are specific, the expression of AMLl-ETO and C/EBPα for these conditions are shown (Fig.16, panel C & D)

A)

AAATTGACATCGGGCCGGGCGCAG -1401

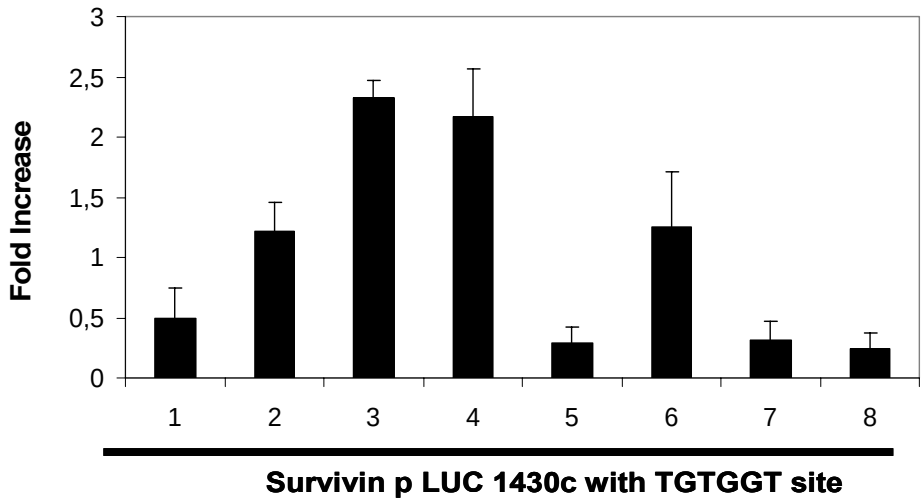
TGGCTCACATCTGTAATCCCAGCACTTTGGGAGGCCGAGGCAG
GCAGATC -1351

ACTTGAGGTCAGGAGTTTGAGACCAGCCTGGCAAACATGGTGA
AACCCCA -1301

TCTCTACTAAAAATACAAAAATTAGCCTGC**TGTGGT**GGTGCAT
GCCT -1251

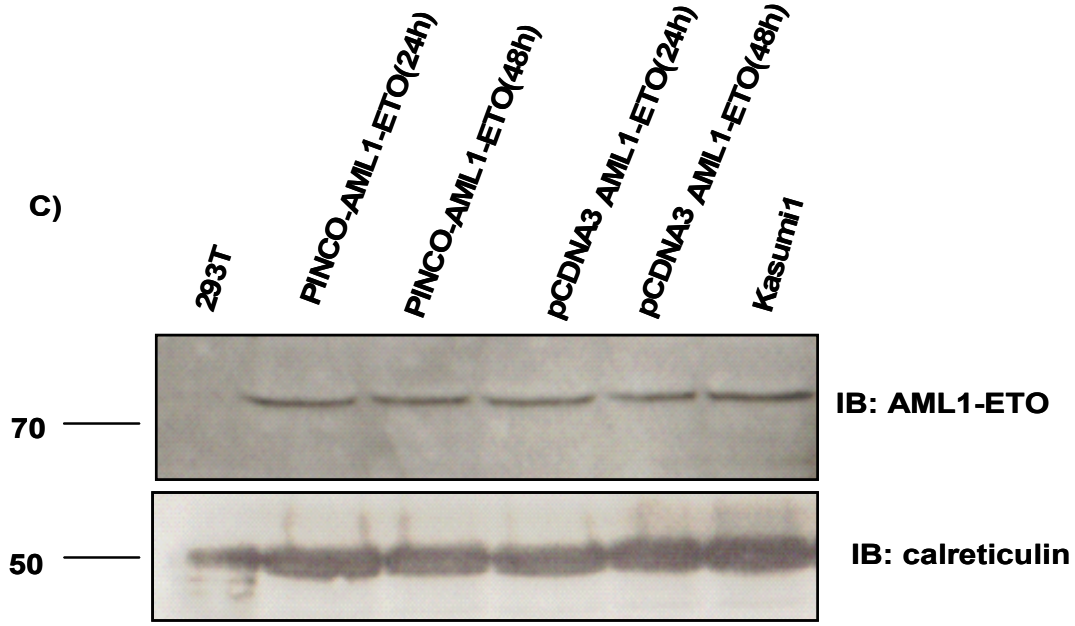
ATCTCAGCTACTCGGGAGGCTGAGGCAGGAGAATCGCTTGAAC
CCGTGGC -1201

B)



PCMV5	+	-	-	-	-	-	-	-
AML1b	-	+	+	-	-	+	-	+
AML1-ETO	-	-	+	+	-	-	+	+
C/EBPα	-	-	-	-	+	+	+	+

C)



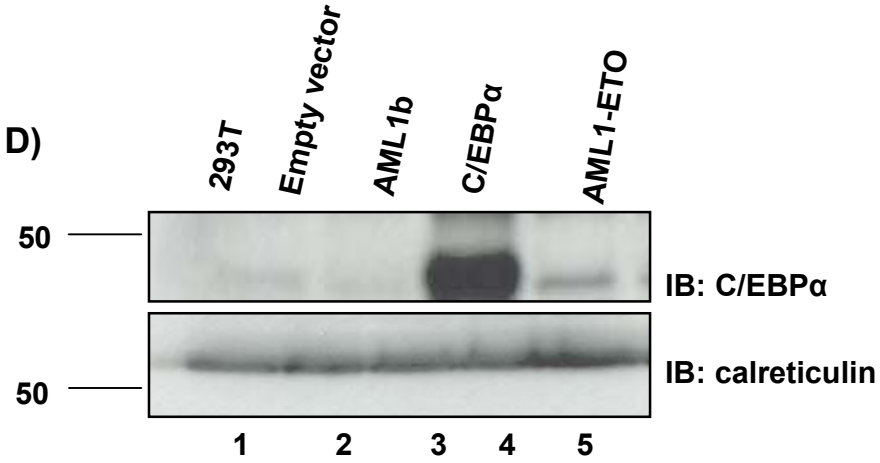


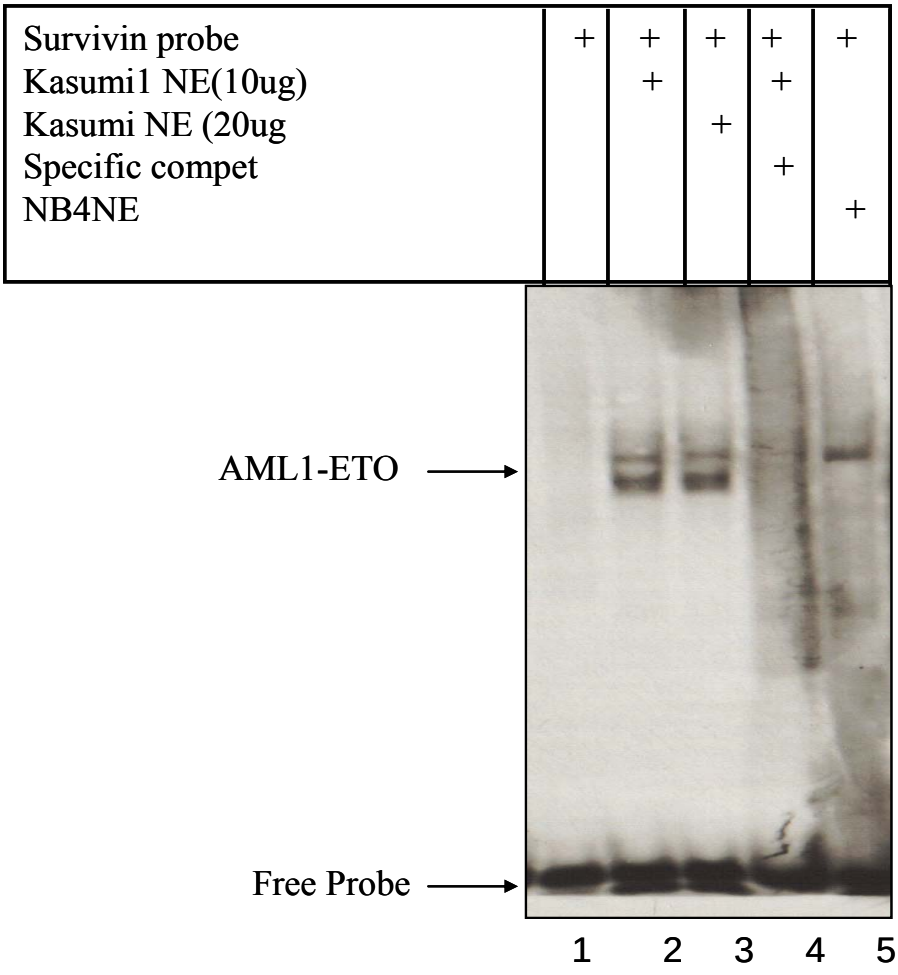
Figure 16. AML1-ETO activates basal transcription of the survivin promotor. DNA sequence analysis of the 5' flanking region of the survivin gene using the TFsearch algorithms (TRASER) revealed the presence of single putative AML1 binding site, TGTGGT. Numbering is from initiating ATG codon. The TGTGGT sequence is highlighted **(A).** Luciferase assay was performed to show that AML1-ETO activates survivin promotor. 293T cells were cotransfected with pLuc-survivin reporter gene construct with pCMV-AML1B or pCDNA3-AML1-ETO expression vectors, pCDNA3-C/EBPα (together with pCMV5 to normalize transfection efficiency). Promoter activity was measured as the ratio of the pLUC/rLUC (rLUC is the renilla luciferase internal plasmid activity). The promoter activity in the presence of the empty vector pCMV5 plasmid was defined as 1 and the promoter activity in the presence of expression vectors for AML1/ETO, AML1B and C/EBPα was defined relative to that value. The results shown are the SEM of four different experiments **(B).** The western blot figure shows the expression of AML1-ETO in 293T cells which were co-transfected with various AML1-ETO expression plasmids (details of these plasmids are given in the section material and methods). Kasumi-1 whole cell lysate was used as a positive control for AML1-ETO expression **(C).** Expression of C/EBPα in 293T cells for above experimental condition (D).

3.5.2 AML1-ETO binds to the survivin promoter

The above data confirmed that AML1-ETO induces survivin expression. We asked next whether AML1-ETO could bind to the AML1 core enhancer binding sequence, TGTGGT, on the survivin promotor. To address this possibility we performed an electromobility shift assay. We used the nuclear extracts from the Kasumi- 1 cells which has an endogenous AML1-ETO. The probes were labeled with non radioactive DIG flour (non- radioactive dye) and were incubated with the Kasumi-1 nuclear extract to identify the sequence specific binding. We also incubate the probe with NB4 promyelocyte cell line nuclear extract used as a negative control for AML1-ETO binding. The data strongly revealed that AML1-ETO binds to the core enhancer sequence of TGTGGT (Fig. 17 panel A, lane 2 & 3). The binding was competed away by a

125 fold excess of specific competitor (lane 4). The absence of a dimmer from NB4 nuclear extract (lane 5) further suggests the binding is specific for AML1-ETO. We further confirmed the data by using specific antibody, while as AML1-ETO binds to the core enhancer sequence of TGTGGT (Fig. 17 panel B, lane 1), the bound complex was shifted by anti-AML1 antibody (lane 2). The binding was competed away by a 125 fold excess of unlabelled specific competitor probe (lane 3). The data suggest while as AML1 binds to the core enhancer sequence, AML1-ETO retain the ability to bind to this sequence. This is the first report of its kind where we show that AML1-ETO binds to the core enhancer sequence of survivin promotor and activates it.

A)



B)

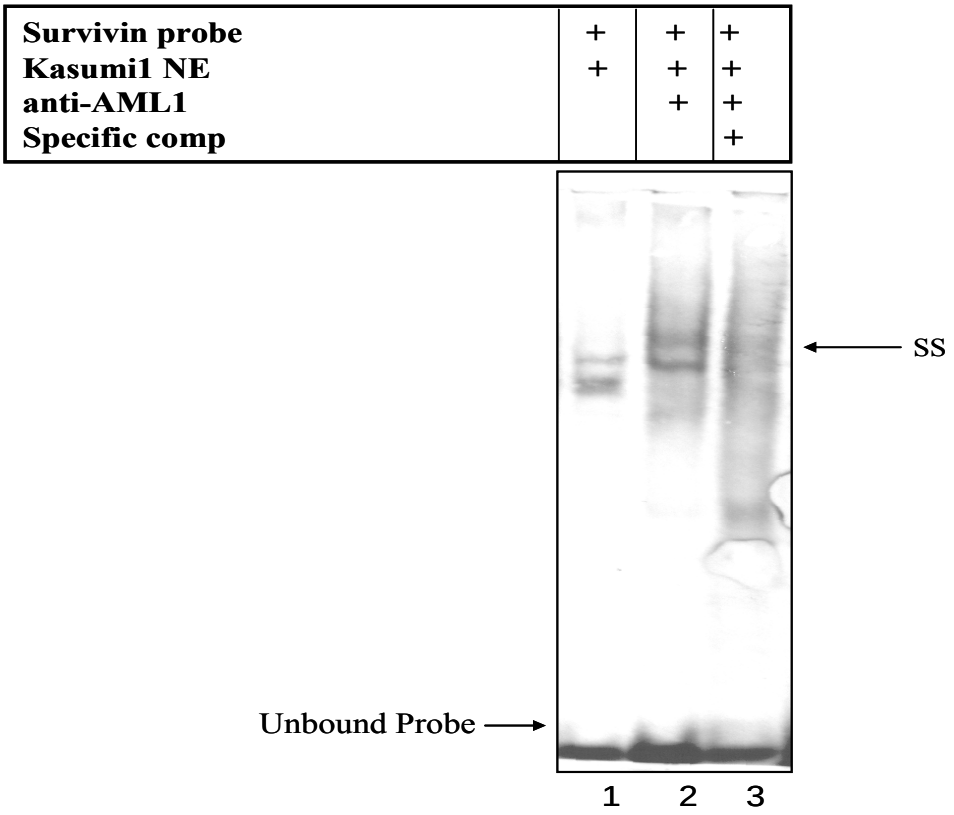


Figure 17. AML1-ETO binds to the survivin promotor. (A) Kasumi 1 nuclear extracts (lane 2, 3 & 4) and NB4 nuclear extract (lane 5) were subjected to EMSA using AML1 core enhancer binding sequence as an oligonucleotide probe (lane 1-5). Oligos (15.5 fmol) were DIG labelled (non radioactive). Probe was run alone (lane 1), or incubated with 10µg and 20µg of Kasumi-1 nuclear extract (lane 2 & 3) respectively. The reaction mixture was preincubated with a 150-fold molar excess of specific unlabeled oligonucleotide (lane 4), and nuclear extract from NB4 (lane 5). Position of free probe is indicated by arrow. (B) The physical binding of AML1-ETO fusion protein to survivin promoter was again analyzed by EMSA using specific antibody. The labelled oligonucleotide probe (lane 1-4) was incubated with 20 µg of kasumi1 nuclear extract (lane1); bound complex was incubated with anti-AML1 antibody respectively (lane 2). The specific binding was competed away by a 150-fold molar excess of unlabeled survivin oligonucleotide probe (lane 3). The position of the free probe and supershift are indicated by arrows.

3.6 Knockdown of survivin expression overcomes AML1-ETO mediated inhibition of C/EBPα autoregulation

The activation of survivin by AML1-ETO may act as a critical mediator of downregulation of granulocytic differentiation factor C/EBPα (Pabst et al., 2001) and C/EBPα-dependent gene activation and autoregulation (Westendorf et al., 1998). The known functions of C/EBPα serve readout for our experiments. We analysed the effect of survivin knockdown on the transactivation potential of C/EBPα on its own promotor by performing a

dual luciferase assay. To test this, we used 293T cell line which lack endogenous C/EBPα. (Erickson et al., 2001) and transiently transfected the cells with a minimal pTKα promoter construct containing two C/EBP sites cloned upstream of the luciferase reporter gene. Along with Luc- pTKα, we cotransfected C/EBPα expression plasmids either alone or with AML1-ETO vector plus shRNA against survivin or a control shRNA. Expression of the luciferase reporter gene was determined 24h post transfection. The results demonstrate that where as C/EBPα alone activates its own promoter (Fig. 18A, lane 2); as expected the transactivation was inhibited by the expression of AML1-ETO (lane 3). However, when co-expressed with the shRNA against survivin the block in transactivation was significantly overcome (lane 4). The control shRNA (lane 5) as well as vector alone and vector plus surviving shRNA had no significant effects (lane 6-7). To further confirm that these effects were specifically mediated by survivin knockdown, degradation of survivin by shRNA in 293T cells transfected with AML1-ETO is shown (fig. 18B, lane 1, 2 & 3). Unrelated control shRNA do not produce any degradation of survivin (lane 5). While as AML1-ETO significantly induces survivin expression (lane 4) in consistent with our finding. HL60 cell line lysate was used as a positive control for the experimentThe results demonstrate that AML1-ETO induced survivin expression that leads to the transcriptional inhibition of C/EBPα and its target genes which in turn is overcome by silencing survivin expression. The data points towards the critical role of survivin in mediating the AML1-ETO induced block of C/EBPα autoregulation.

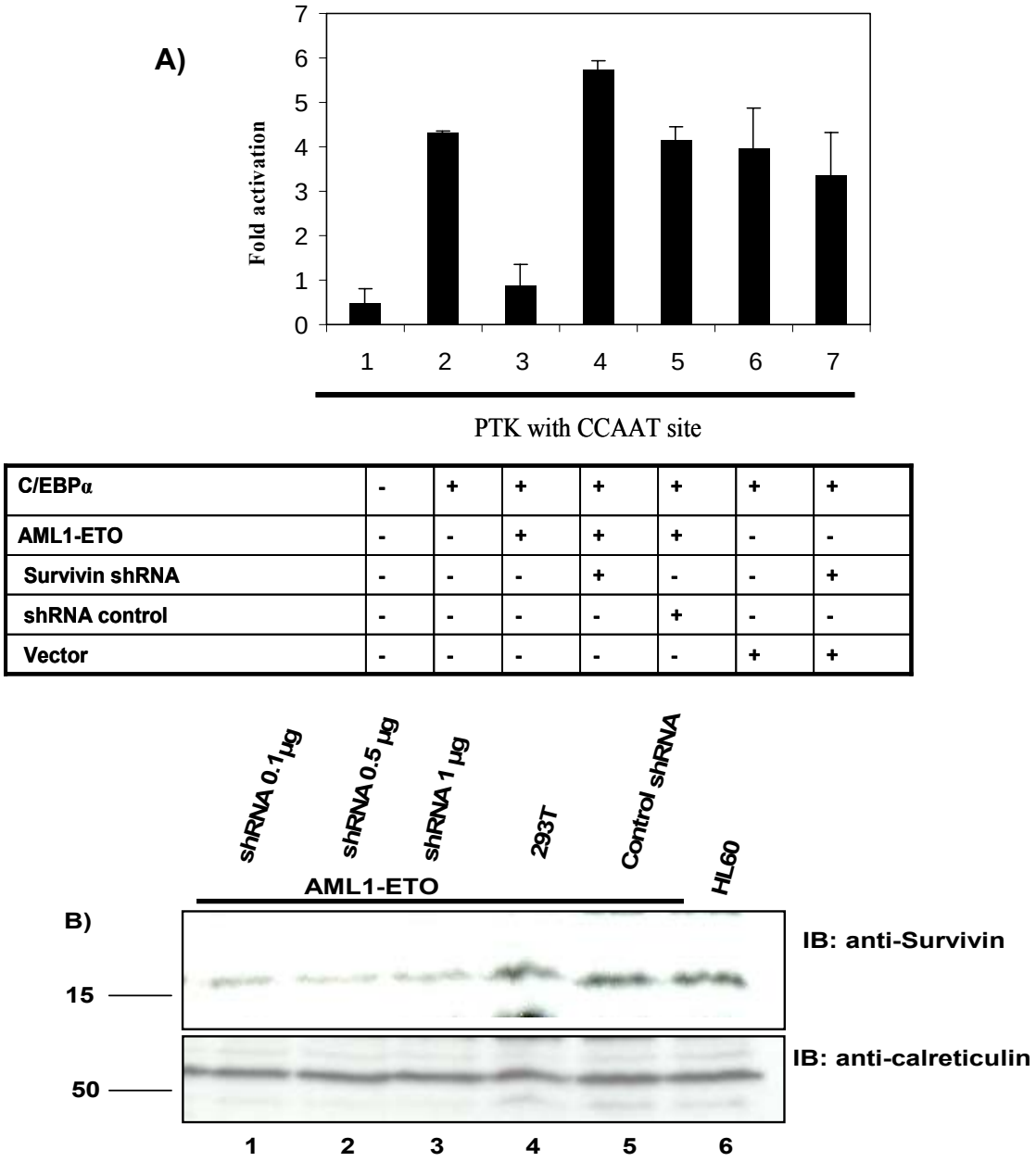


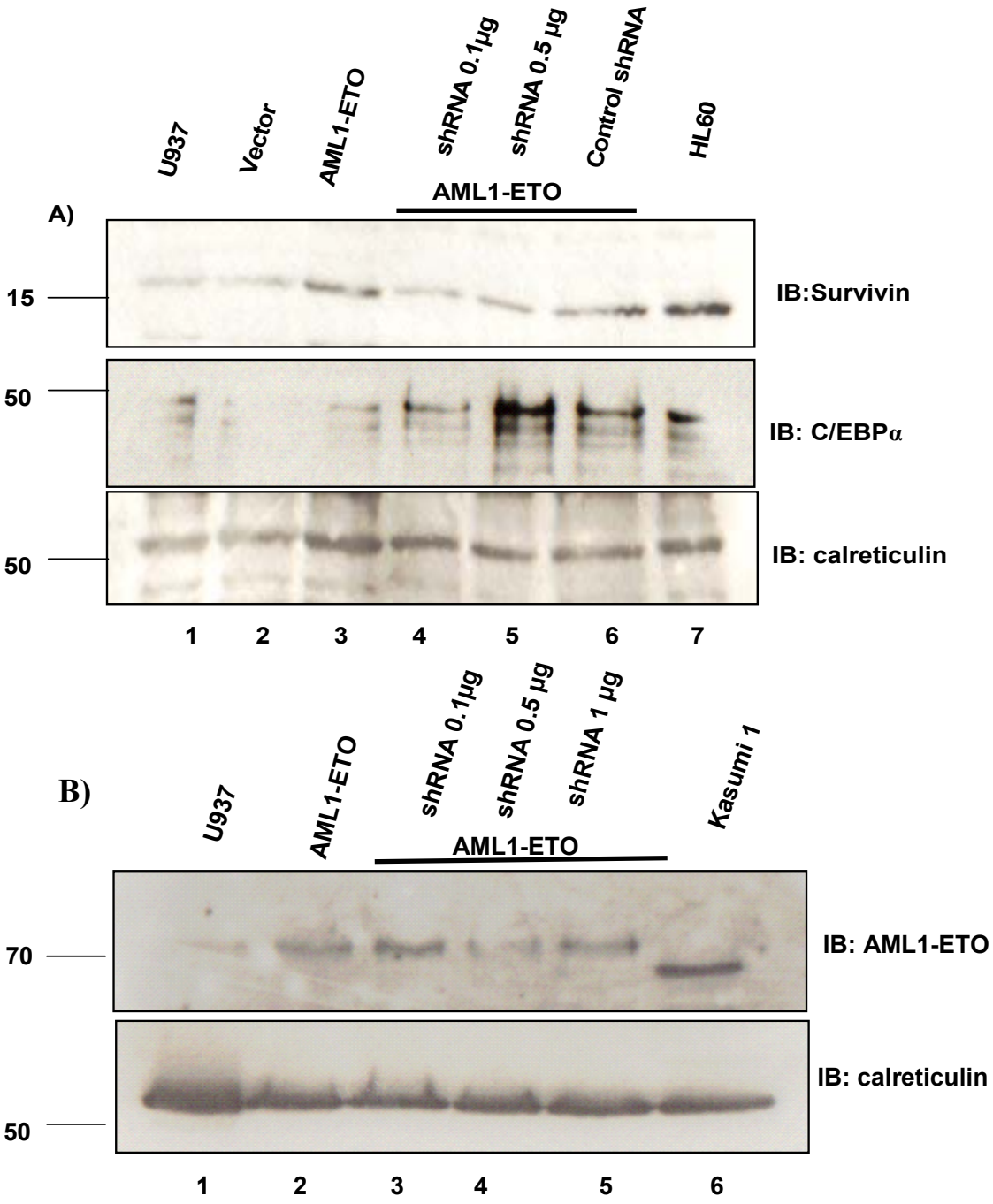
Figure 18. Knockdown of survivin expression overcomes AML1−ETO mediated inhibition of C/EBPα autoregulation. A) Transient cotransfection of a p(C/EBP)2TK-luciferase reporter construct with the cotransfection of pCDNA3-C/EBPα (lane 2-7). pCDNA3-C/EBPα alone (lane 2) plus pCDNA3-AML1-ETO (lane 3) and shRNA against survivin (0.5µg) (lane 4). A control shRNA (lane 5) and a pCDNA3 empty vector (lane 6) with survivin shRNA (lane 7). The pRL-0 renilla luciferase construct was cotransfected to normalize for transfection efficiency. Error bars indicate standard errors of the means. (B) Shows the depletion of survivin achieved in 293T cells after transfection of various amounts of shRNA against surviving and unrelated control shRNA.

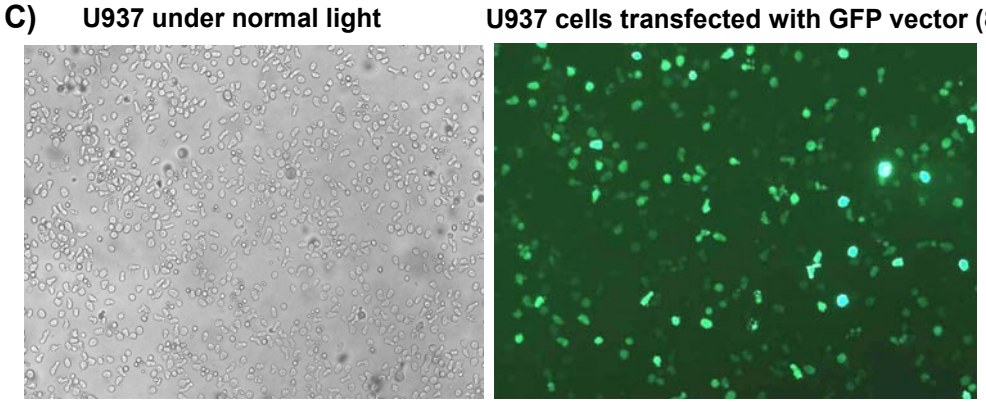
3.7 Repression of survivin expression by shRNA restores C/EBPα expression inhibited by AML1/ETO

Next we investigated the effects of functional and stable knock-down of survivin by shRNA on the CEBPα expression. We cotransfected the AML1-

ETO into U937 cells together with shRNA designed to target survivin. We observed the expression of survivin 48 hours after the transfection of survivin shRNA construct which revealed that survivin was effectively repressed (Fig. 19 panel A, lane 4 & 5) as compared to the U937 transfected with control shRNA (6), HL60 cell line lysate was used as a positive control. The same blot was stripped off bound antibody and blotted with C/EBPα to check the expression. The data suggests as AML1-ETO effectively reduces C/EBPα expression (lane 3). The C/EBPα expression was dramatically enhanced by the survivin knockdown (lane 4 & 5). The control produces no substantial effect (lane 6). The expression of AML1-ETO and the transfection efficiency in U937 in this case are shown (Fig. 19 panels B & C). As we assume the restoration of C/EBPα drives U937 cells to terminal granulocytic differentiation. To confirm this aspect of the investigation we performed FACS staining for the surface expression of granulocytic marker, CD15, on U937 cells after 48 and 72 hours, the results are shown for 48h. Data revealed that CD15 expression enhances to a 10 fold when U937 cells were cotransfected with AML1-ETO and 0.5μg of survivin shRNA (fig 19D panel V), calculated after gating on CD15+ fraction from U937 cells (panel II). U937 transfected with AML1-ETO (panel IV) and AML1-ETO plus control shRNA (panel VI), empty vector (panel III) does not produce any significant increase. This enhancement of terminal differentiation leads to growth arrest as indicated by the cell count monitored for day 3 and day 5. The transfection of (0.5μg) of survivin shRNA leads to significant decrease in cell growth at day 5 as compared to controls (fig 19E). Further, cytospin preparation for similar conditions showed signs of apoptosis at day 8 (fig 20A panel b), untransfected U937 cells (panel a) or AML1-ETO transfected cells (panel b), AML1-ETO plus control shRNA (panel d) does not show any nuclear fragmentation indicative of secondary necrosis. These results, thus, confirmed that AML1-ETO induced survivin expression interferes with granulocytic program.

Removal of survivin restores biological function of C/EBPα protein inactivated by AML1-ETO, which is sufficient to induce terminal differentiation and growth arrest of myeloid leukaemia cells. Further, we asked how survivin depletion restores biological activity of C/EBPα. We designed synthetic oligos derived from Ubc9 promotor which possess a single CCAAT site (-500bp upstream of the initiating ATG). Ubc 9 is a well known SUMO conjugating enzyme which covalently links SUMO moiety to C/EBPα and activates it. We used U937 cells which express low levels of endogenous C/EBPα and transfected into these C/EBPα expression plasmid or cotransfected with AML1-ETO and survivin shRNA (0.5μg and 1μg), besides a control shRNA (fig. 20B). Whole cell extract was prepared 48h posttransfection and lysate was incubated with oligo probe and subjected to EMSA. No sequence specific binding was observed for condition where probe alone or U937 cell lysate transfected with AML1-ETO was incubated with probe (lane 1 & 2). However, when coexpressed with shRNA against survivin, sequence specific binding of C/EBPα increases on the probe dramatically (lane 3), binding enhances quantitatively with increased amount of transfected shRNA (lane 4). Sequence specific binding was effectively competed away by incubation of 125 fold excess of unlabelled probe (lane 5). Control shRNA do not show any significant increases comparable to that of survivin specific shRNA (lane 6). C/EBPα sequence specific binding was confirmed by the transfection of C/EBPαp42 expression plasmid which results in a sequence specific binding which was shifted by C/EBPα specific antibody (lane 7). This in vitro data indicate that survivin knockdown enhances C/EBPαp42 binding on the potential CCAAT binding sites including promoter of SUMO conjugating enzyme, Ubc9.


A)
U937
Vector
AML1-ETO
shRNA 0.1µg
shRNA 0.5 µg
Control shRNA
HL60
AML1-ETO
15
IB:Survivin
50
IB: C/EBPα
50
IB: calreticulin
1 2 3 4 5 6 7
B)
U937
AML1-ETO
shRNA 0.1µg
shRNA 0.5 µg
shRNA 1 µg
Kasumi 1
AML1-ETO
70
IB: AML1-ETO
IB: calreticulin
50
1 2 3 4 5 6


C)
U937 under normal light
U937 cells transfected with GFP vector (85%)

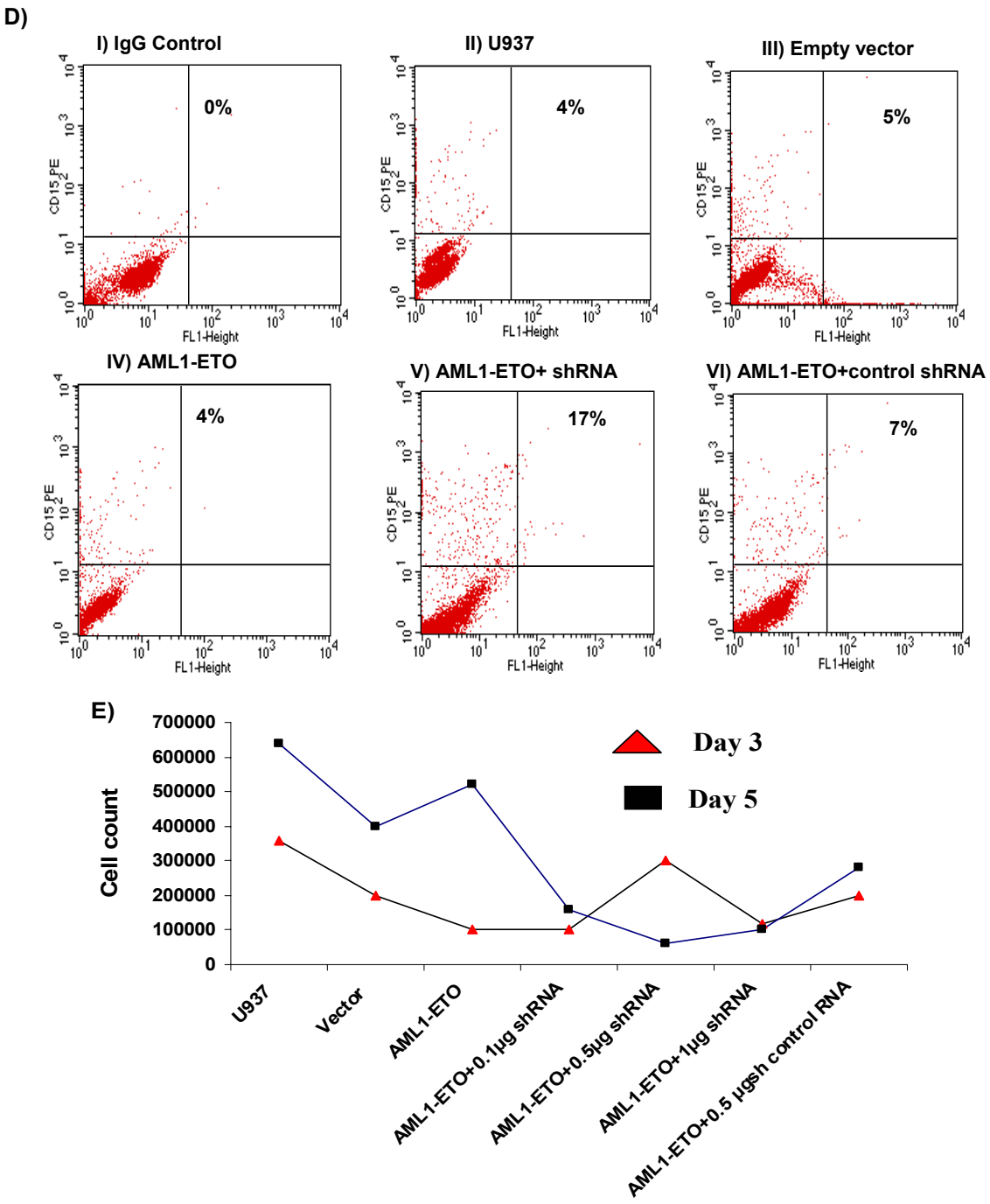


Figure 19. Inhibition of survivin expression by shRNA restores CEBPα protein expression. Western blot analysis performed 48 h after transfection on the whole cell lysates of U937 cells cotransfected with AML1-ETO and various amounts of shRNA designed to knock-down survivin and an unrelated control shRNA. **(A)** As shown the expression of survivin is inhibited by the shRNA while as unrelated control shRNA had no effect. The same blot was stripped and reprobed with anti C/EBPα (lower panel). To check the equal loading, same blot was again stripped off bound antibody and reprobed with anti-calreticulin (lowest panel). **(B)** The expression of AML1-ETO in U937 from the lysate of fig. 5A, Kasumi 1 lysate was used as a positive control for AML1-ETO expression. **(C)** The transfection efficiency in U937 cells reached by AMAXA was 85% which was calculated by counting the cells first under normal light and then under fluorescent light using the same magnification and same field. **(D)** FACS analysis performed on U937 cells for CD15 expression after 48 hours**. (E)** Trypan blue staining showing the number of viable cells under different conditions

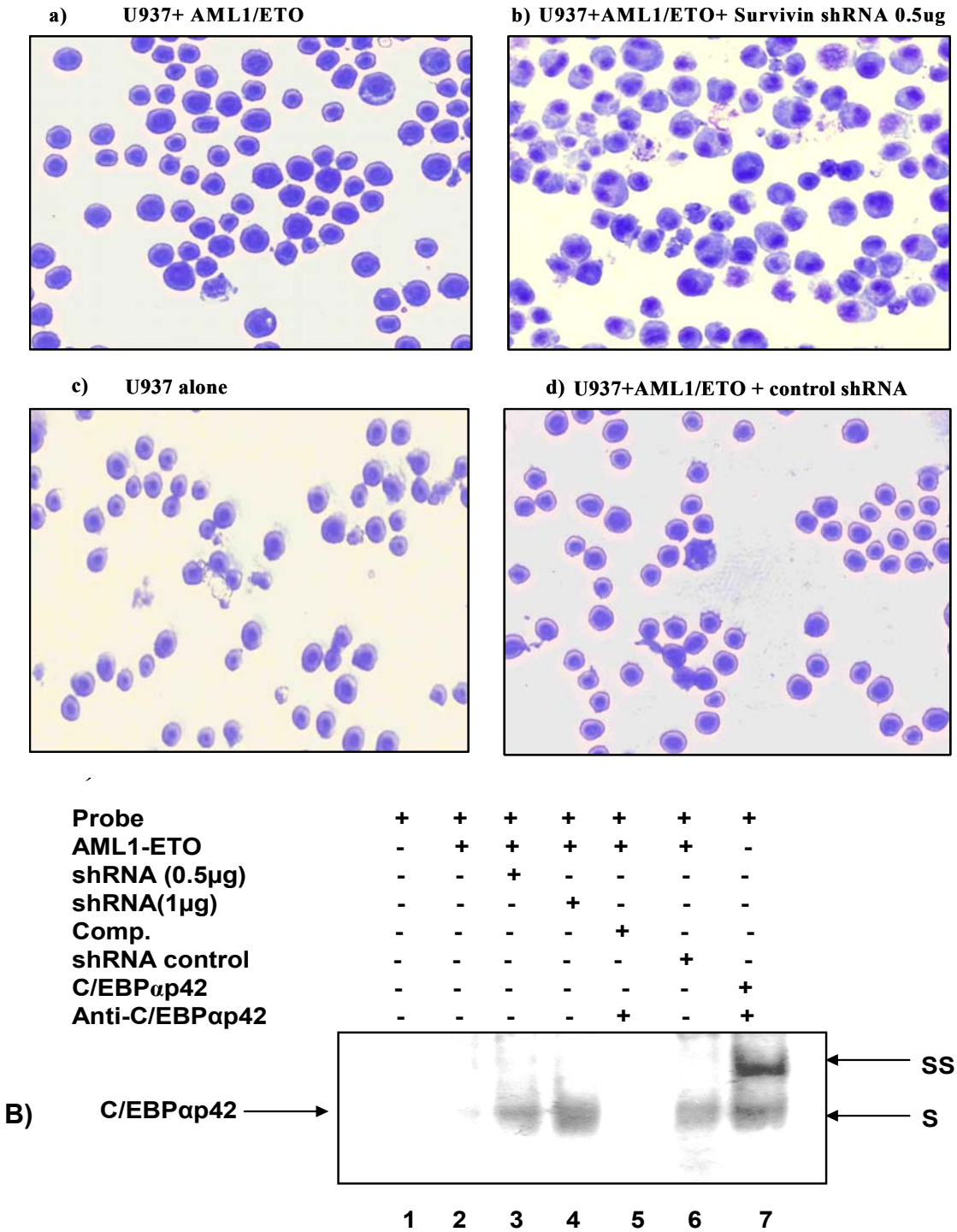


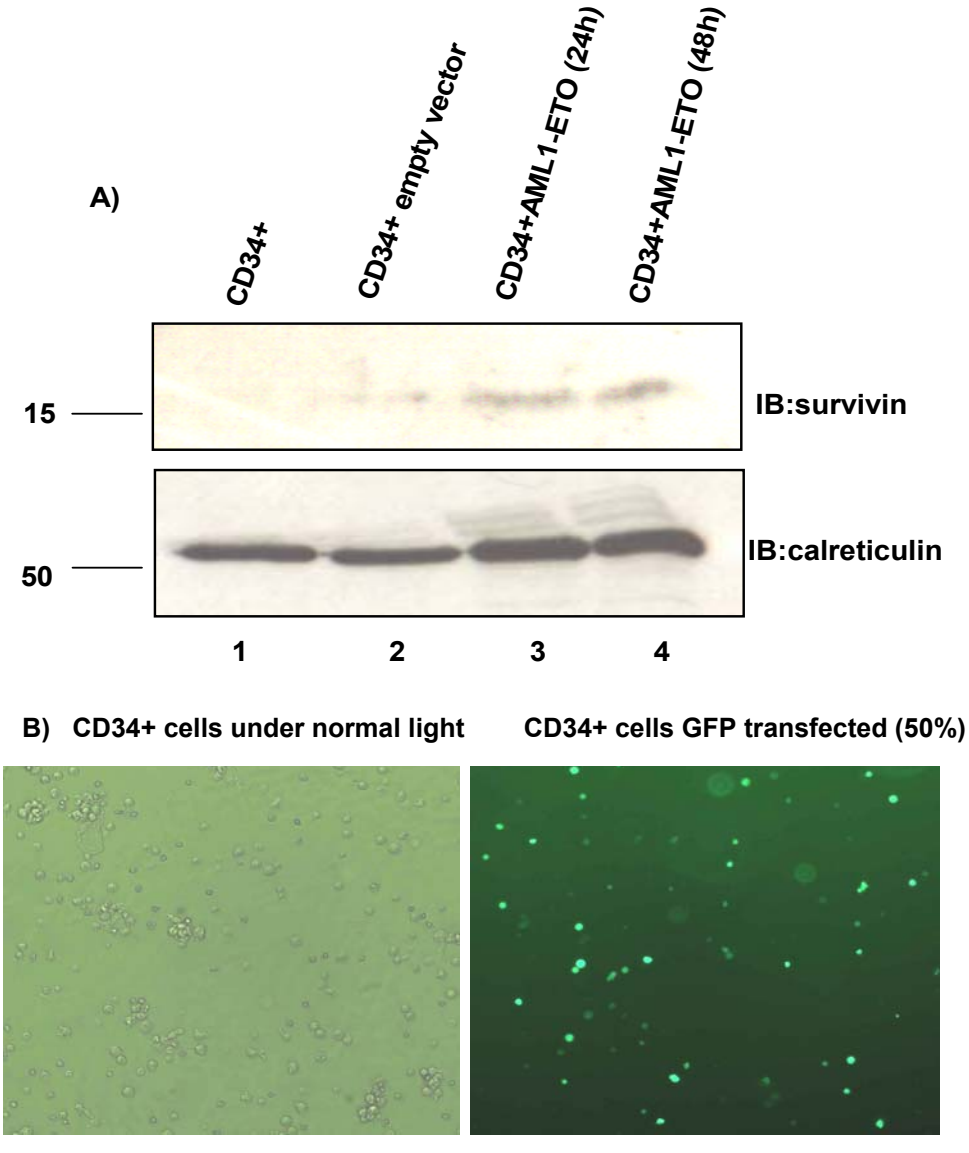
Figure 20. Morphology of U937 cells transfected with various plasmids. Day 8 cytocentrifuge preparations of U937 cells stained with Wright–Giemsa. U937 transfected with AML1-ETO (panel a) plus shRNA against survivin (panel b) showing immature as well as mature neutrophils or U937 alone at day 4 (panel c) plus control shRNA (panel d), empty vector (panel e) and empty vector plus survivin shRNA (panel f). Original magnification × 400. **(B)** EMSA for increased binding of C/EBPα on Ubc9 promotor. The labelled oligonucleotide probe 5'-AACTGGCCAATTGCAAGGGGTT-3' (lane 1-7) were incubated with 20 µg of U937 whole cell lysate prepared at 48h time point after transfection with various combination of plasmids (lane 2-6). Lysate were prepared by using 50mM Tris-Cl, pH 8.0, 1% NP40, 150mM NaCl, 100µg/ml leupeptin, 1mM PMSF, and 5mM orthovandate. Knockdown of survivin by shRNA, 0.5µg and 1 µg concentration is shown to result in enhanced binding to the probe. The bound complex of probe plus C/EBPα (introduced by transfection) was shifted by the incubation of 2 µg anti-C/EBPα

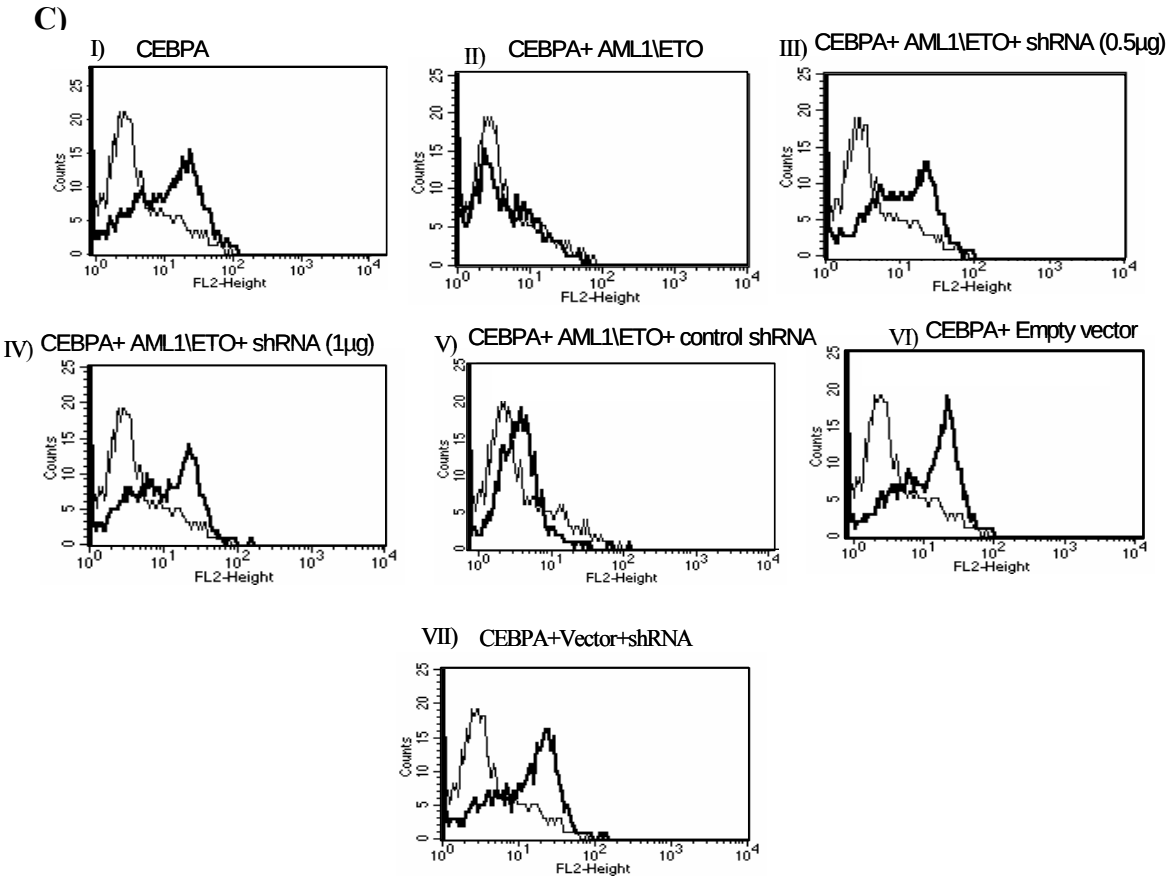
antibody (lane 7). The specific binding was competed away by a 150-fold molar excess of unlabeled Ubc9 oligonucleotide probe (lane 5). The position shift and supershift are indicated by arrows.

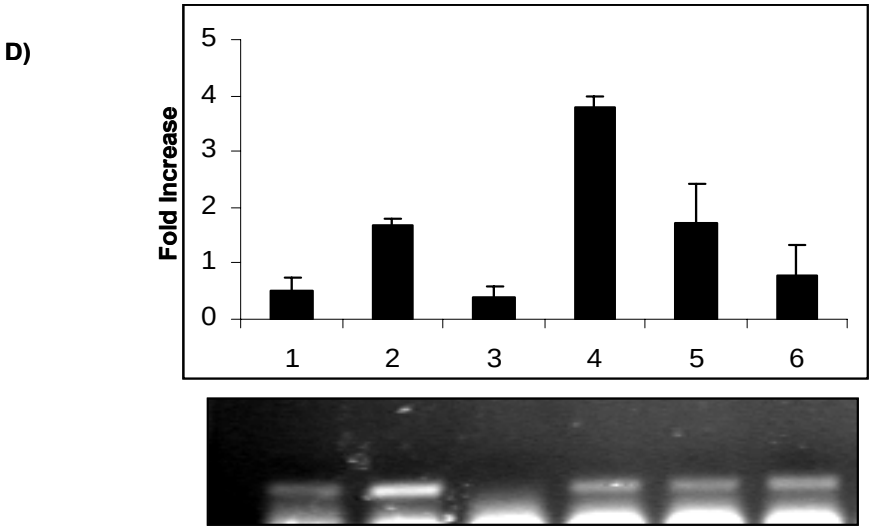
3.8 Repression of survivin expression overcomes granulocytic differentiation block induced by AML1/ETO in human CD34+ cells

Next we asked whether downregulation of survivin may have an effect on the AML1-ETO mediated inhibition of granulocytic differentiation in primary hematopoietic CD34+ cells. We used human CD34+ cells isolated from cord blood which shows 70%-80% positivity for CD34+ marker after MACS enrichment (Data not shown). These cells were grown in IMDM alone to block the background induction of survivin by cytokines and transfected with C/EBPα to induce granulocytic differentiation (Radomska et al., 1998) and cotransfected with PINCO-AML1-ETO expression plasmid alone or together with shRNA against survivin; besides the empty vector and control shRNA. The expression of survivin was detected as early as 24 post transfection of AML1-ETO (Fig. 21, A).The transfection efficiency reached by the AMAXA nucleofection in CD34+ was 50% (Fig. 21, B). Further we performed the FACS analysis on these cells 72 hours after the transfections. The data shows while as C/EBPα alone could induce significant increase of CD15 expression (Fig. 21 C, panel I), however, when co-transfected with AML1-ETO, CD15 expression was significantly inhibited (panel II). This block in differentiation was overcome by the shRNA against survivin, 0.5µg and 1µg (panel III and IV). The control shRNA had no effect and AML1-ETO could effectively block the CD15 expression (panel V). The vector alone and the vector plus shRNA had no effect (panels VI and VII). We further validate this interesting finding by performing quantitative real time PCR for the expression of another granulocytic marker, myeloperoxidase (MPO). AML1 is known to activates transcription from enhancer core motifs (TGT/cGGY) which are present in a number of genes relevant to myeloid development including

myeloperoxidase (Frank et al., 1995). AML1-ETO, however, inhibits the activation of AML1 genes in a dominant negative manner. To investigate whether knockdown of survivin could overcome the dominant negative effect of AML1-ETO on the MPO expression. We again performed the transient transfections in CD34+ cells as described previously and isolated the RNA after 72 hours of transfection. Q-PCR data for MPO expression revealed that shRNA against survivin was able to overcome AML1-ETO mediated inhibition of the expression of MPO (Fig. 21 D, lane 4) while as it was able to inhibit the C/EBPα induced expression of MPO (lane 3). C/EBPα alone induces a significant increase in the expression of MPO (lane 2). The empty vectors and the control shRNA had no effect (lanes 5 and 6).







1.	**C/EBPα**	**-**	**+**	**+**	**+**	**+**	**+**
2	**AML1-ETO**	**-**	**-**	**+**	**+**	**-**	**+**
3	**Survivin shRNA**	**-**	**-**	**-**	**+**	**-**	**-**
4	**Empty vector**	**-**	**-**	**-**	**-**	**+**	**-**
5	**Control shRNA**	**-**	**-**	**-**	**-**	**-**	**+**

Figure 21. Knockdown of survivin expression overcomes granulocytic differentiation block induced by AML1-ETO in human CD34+ cells. A) Western blot for the expression of survivin from the whole cell lysates of human CD34+ grown in IMDM

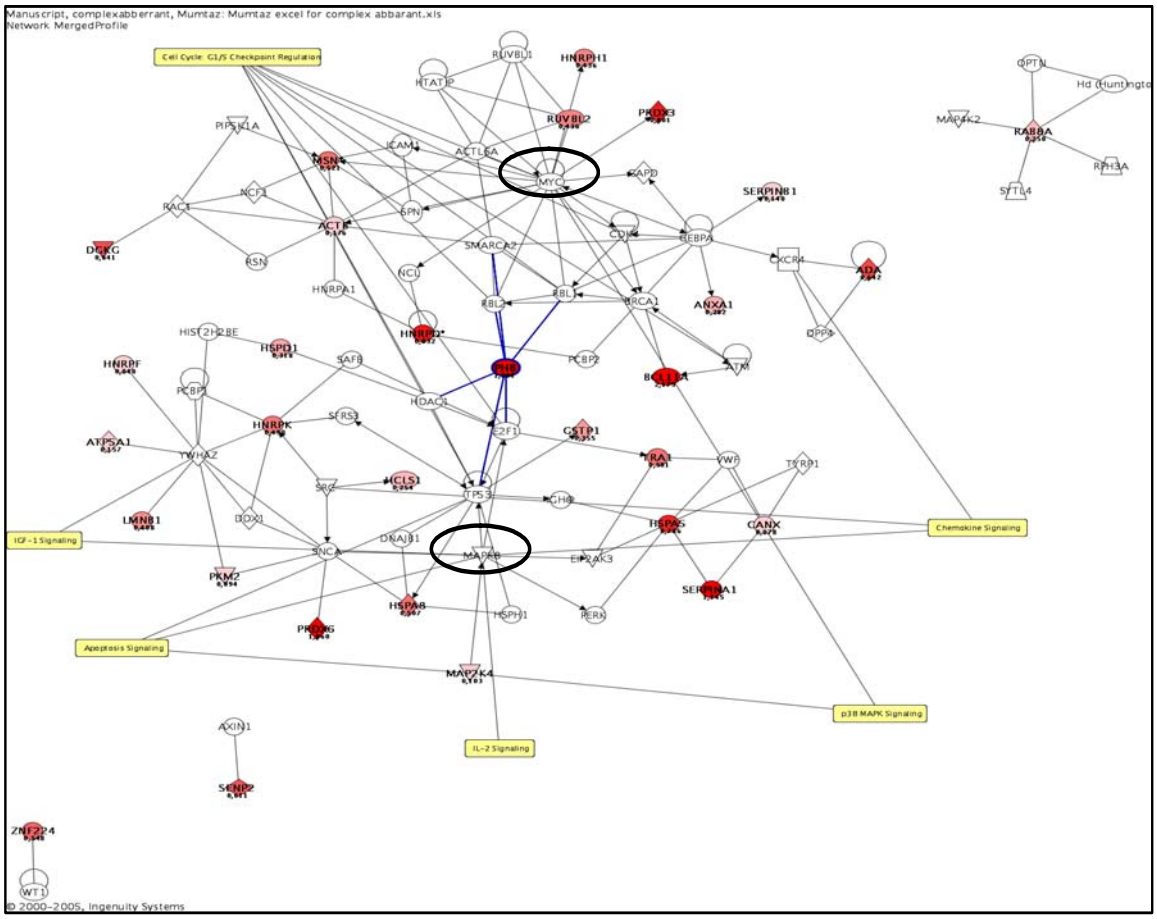
without cytokines and transfected with AML1/ETO (lane 3 & 4). The same blot was striped and reprobed with anti calreticulin to show equal loading (lower panel). **B)** Shows the transfection efficiency in CD34+ was calculated to be 50 %. **C)** Surface levels of CD15 on human CD34+ cells (thick line) 72h following cotransfections with C/EBPα (I-VII) and AML1-ETO (II); AML1-ETO plus shRNA against survivin (III- IV) or a control shRNA (V) or with an empty vector (VI) and empty vector plus survivin shRNA (VII). The thin lines show staining with isotype-matched control antibody on untransfected CD34+ cells**. D)** Q-PCR for the expression of MPO in CD34+ cells 72 h post transfection, the values on Y-axis are the fold increase. MPO expression is shown after the PCR samples were run on a 1% agarose gel, lower panel.

3.9 Cytogenetic groups differ in their protein interaction networks

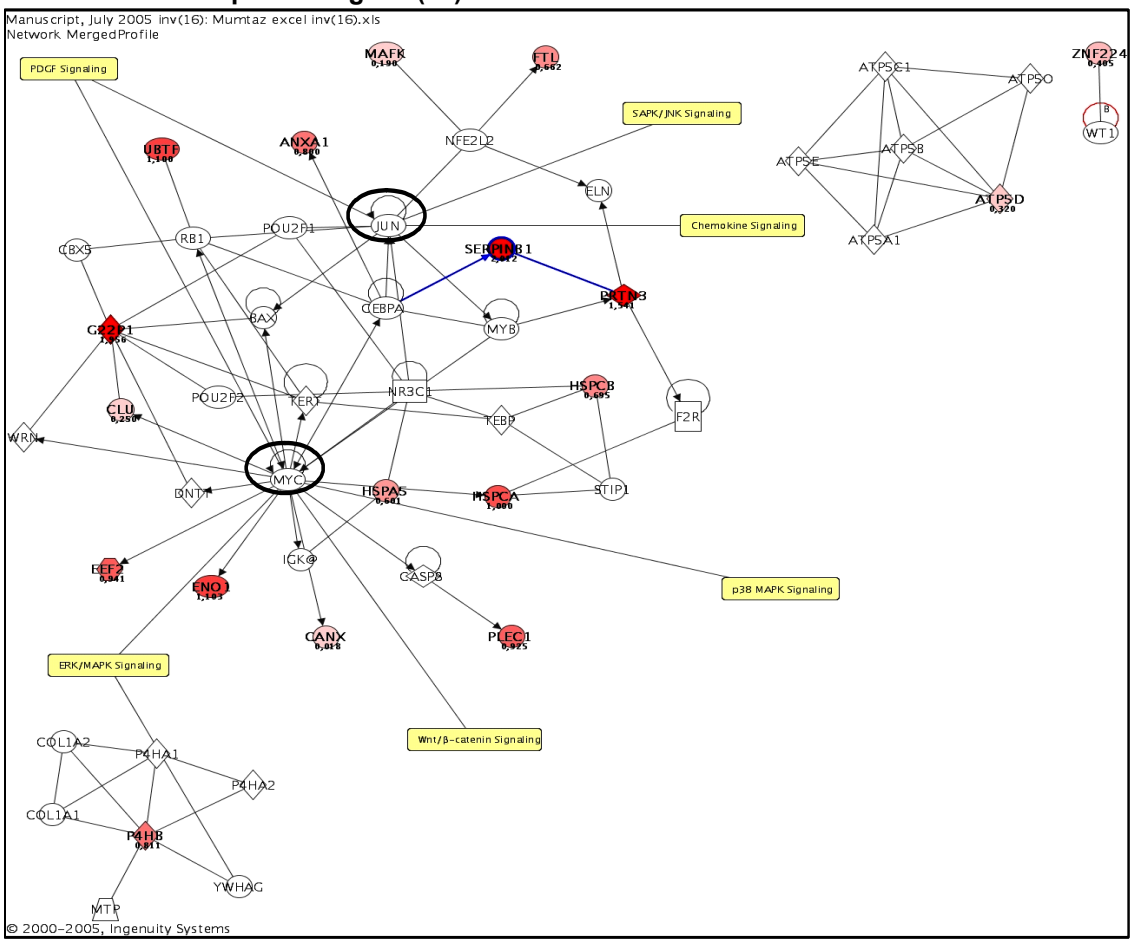
All the proteins identified by mass spec screening from different AML cytogenetic groups were used to generate a wider protein interaction map by employing an ingenuity pathway finder software program, (www.ingenuity.com). Ingenuity database provides the protein-protein interaction maps on the basis of known literature which has been sited. These interactions of proteins are either confirmed by yeast hybrid screens or other methods. We used this software to investigate the protein-protein interactions among the proteins we identified from AML cytogenetic groups. This would give us an idea about the signaling pathways that may be involved in the process of leukemogenesis. Among the proteins matched to this database to generate Protein protein interaction maps (PPI) include, 19, 14, 18, 18, 40 proteins identified from t(8;21), t(15;17), Inv(16), complex aberrant, 11q23 and normal karyotype, respectively. The details of these proteins with their Ingenuity code names are ginven (Table 6). These proteins are represented by coloured shapes (nodes) in a network (Figure 22). Separate subnetworks for each cytogenetic group were generated; subnetworks from each AML subgroup were merged to obtain a wider network of interactions. The major regulating network and the signalling pathways contributed by these networks appeared to be MAPK8 and MYC for complex aberrant karyotype, JUN and MYC for Inv(16), TP53 for t(8;21), TP53, MYC, PRKAC (cAMP dependant kinase) for 11q23, (Figure 22, panel A, B, C and D). We identified different

hyperactive modules in the networks by singling out those proteins which, by a Proteomweaver analysis, expressed highest mean expression value in a network indicated by red colour intensity of a node (highlighted modules in Figure 22). In a complex aberrant karyotype, a module comprised of HDAC, E2F, TP53 and RBL2 is activated in the signature network of CK (Panel A). Similarly, in a signature network of Inv (16), serine (or cysteine) proteinase inhibitor (SERPINB1) is highly upregulated (mean spot intensity of 2.412 is highest in the network (refer to Figure 22, panel B). The module comprised of transcription factors C/EBP alpha (CEBPA), C-MYB and proteinase 3 (PRTN3) may be a dysregulated module which is more likely the case as C/EBP alpha is mutated in 10% of all AML cases. Similarly for t(8;21) a module comprised of transcription elongation factor A (TCEA1) and FOS which belongs to the family of AP1 transcription factors appeared to be hyperactive. Interestingly the activated modules appear to be different in each cytogenetic group.

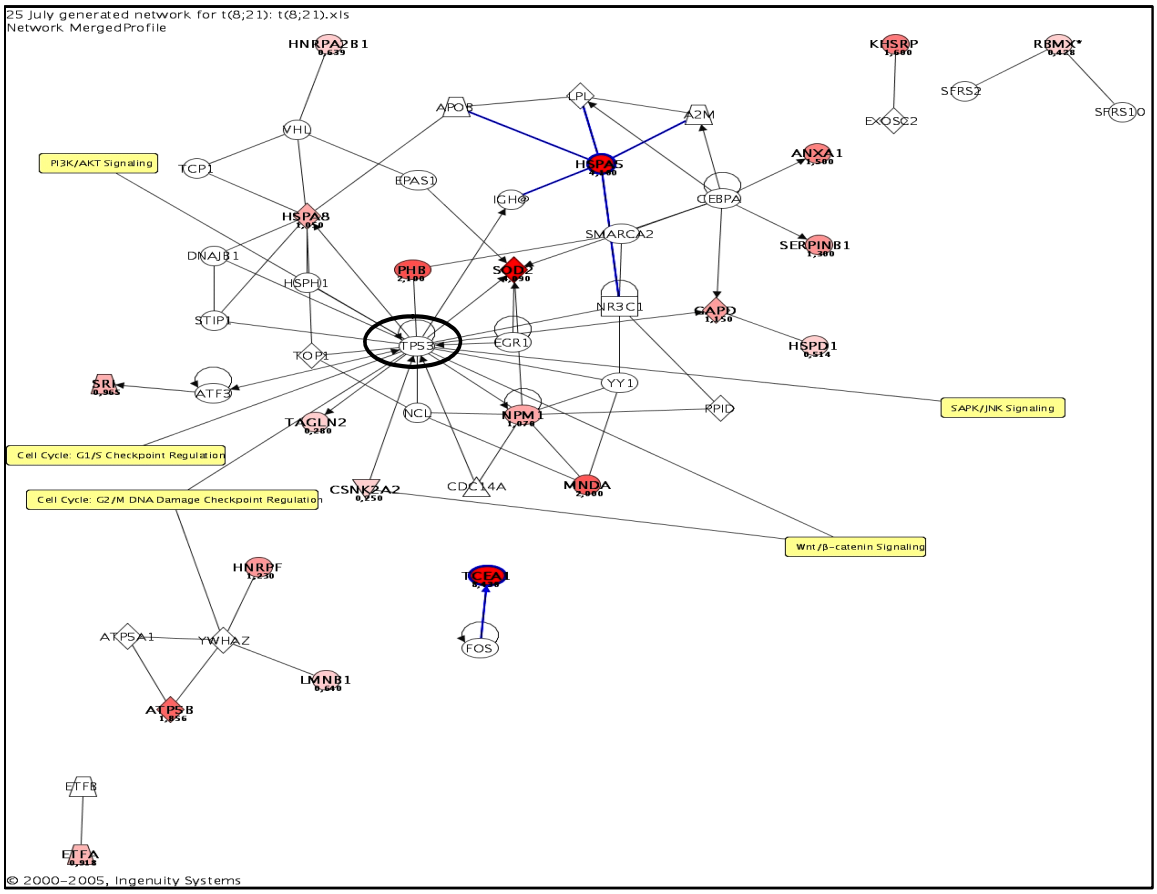
A. Network representing Complex aberrant karyotype



B. Network representing Inv (16)



C. Network representing t(8;21)



D. Network representing 11q23

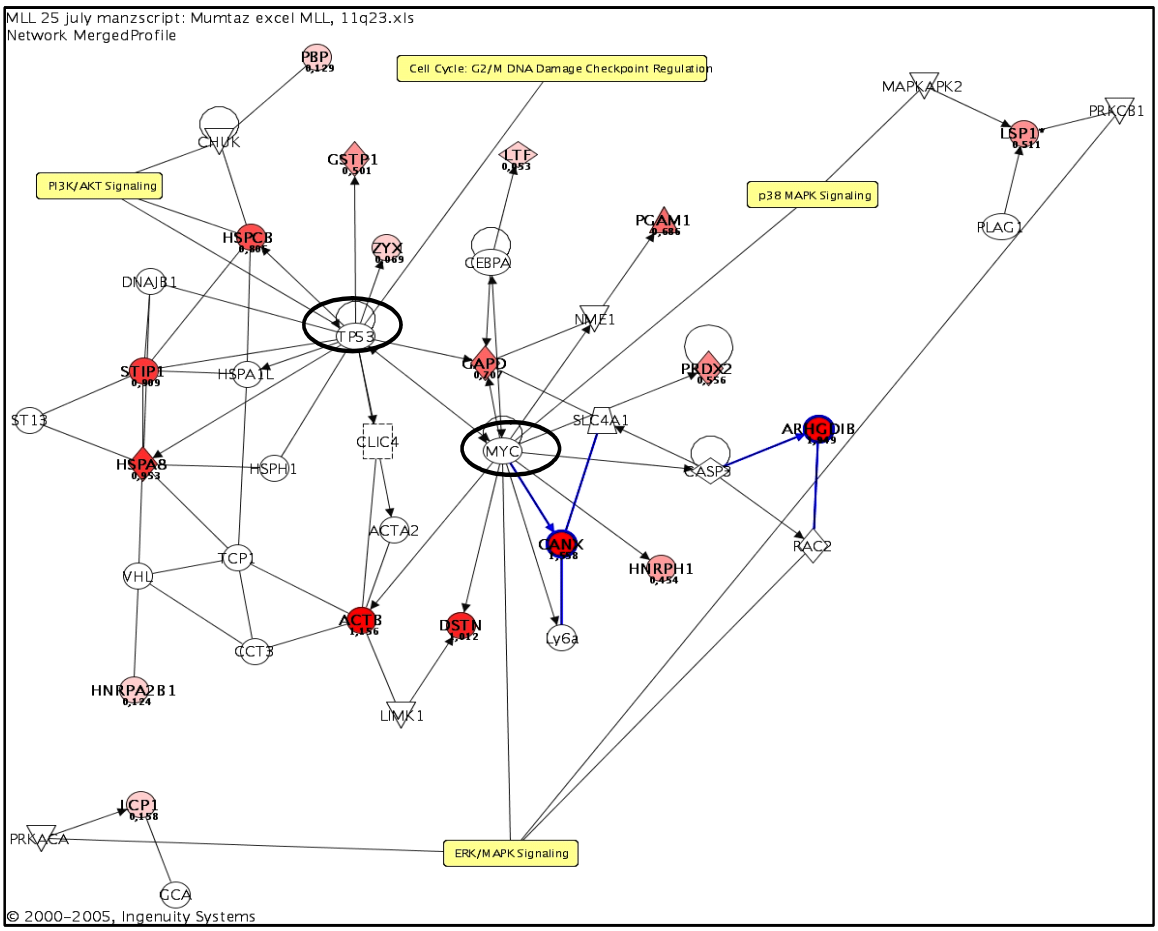
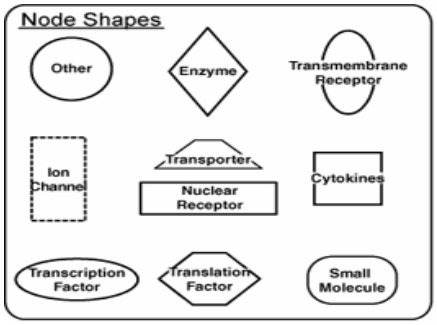


Figure 22. Biological networks generated for cytogenetic groups through Ingenuity database**.** Panels A, B, C, D, shows the molecular interaction networks generated for Complex aberrant karyotype, Inv(16), t(8;21), and 11q23 subtypes. The coloured nodes (shapes) are the proteins which we identify and that matched (Table 6) to the database of known proteins which are involved with the protein- protein interactions. The intensity of the node colour means the expression levels of a protein which we calculated from the gel spot analyser programme (ProteomWeaver as described in the material and methods) and used for the generation of these networks. Rectangular boxes represent the signaling pathways influenced by these interactions. The highlighted lines represent the module (a small interaction network) highly active in the network. Circular arrows or a line originating from one node and pointing back at that same node in a network arise from the ability of a gene product to act upon itself.



This legend describes the Canonical pathway node shapes of the interaction networks. An arrow pointing from A to B in a network signifies that A causes B to activate.

Table 6. The proteins identified from various cytogenetic groups which showed significant expression levels were used for generating protein interaction networks.

t(8;21)

1. Annexin A1 (ANXA1)
2. Casein kinase 2
3. GAPHD
4. HNRPA2B1
5. HSPA5
6. HSPA8
7. Myeloid cell nuclear differentiation antigen (MNDA)
8. Nucleophosmin (NPM1)
9. Prohibitin (PHB)
10.Heat shock 60KDa protein 1 (HSPD1)
11. Seine (or cysteine) proteinase inhibitor (SERPINB1)
12.Superoxide dismutase 2 (SOD2)
13. Sorcin (SRI)
14. Transgelin 2 (TAGLN2)
15. ATP synthase H+ transporting, mitochondrail (ATP 5D)
16. HNRPF
17. Lamin B1
18. FUSE binding protein 2(KHSRP)
19. Electron transfer flavoprotein (ETFA)
20. Transcription elongation factor A (TCEA1)
21. HNRPG (RBMX)

Inv(16)

1. Annexin A1 (ANXA1)
2. Calnexin (CANX)
3. Eukaryotic translational elongation factor 2 (EEF2)
4. Enolase (ENO1)
5. Apolipoprotein J ((CLU)
6. Ferritin light chain (FTL)
7. DNA helicase II (G22P1)
8. HSPA5
9. HSPCA
10. HSPCB
11. Mafk (MAFK)
12. Serine (or cysteine) proteinase inhibitor (SERPINB1)
13. Upstream binding transcription factor, UBF1 (UBTF)
14. Zinc finger protein 255 (ZNF 224)
15. ATP synthase H+ transporting, mitochondrail (ATP 5D)
16. Protein disulphide isomerase (P4HB)

Complex aberrant

1. ATP synthase H+ transporting, mitochondrail (ATP 5D
2. Calnexin (CANX)
3. Glutathione-S transferase Pi (GSTPI)
4. Hematopoietic cell- specific lyn substrate 1(HCLS1)
5. HNRPK
6. HSPA5
7. HSPA8
8. HSPD1
9. Lamin B1 (LMNB1)
10. Mitogen-activated protein kinase kinase 4 (MAP2K4)
11. Prohibitin (PHB)
12. Pyruvate kinase (PKM2)
13. Peroxidoredoxin 6 (PRDX6)
14. Seine (or cysteine) proteinase inhibitor (SERPINB1)
15. Endoplasmin (TRA1)
16. Actin Beta (ACTB)
17. Adenosine deaminase (ADA)
18. Annexin 1 (ANXA1)
19. B-cell CLL/Lymphoma 11A (BCL11A)
20. Diacylglycerol kinase, gamma(DGKG)
21. HNRPD
22. HNRPH1
23. Moesin (MSN)
24. Prohibitin (PHB)
25. Peroxidoredoxin 3 (PRDX3)
26. Ruv B-like 2 protein (RUVBL2)
27. Zinc finger protein 224 (ZNF224)
28. SUMO1/sentrin/ SMT3 specific protease 2(SENP2)
29. RAB8A

11q23

1. Actin beta (ACTB)
2. Rho-GDP dissociation inhibitor(GDI) beta (ARHGDIB)
3. Calnexin (CANX)
4. Actin depolymerising factor, destrin (DSTN)
5.Glutathione S-transferase pi (GSTP1)
6. HNRAPA2B1
7. HNRPH1
8. HSPA8
9. HSPCB
10. Lactotransferrin (LTF)
11.Phosphatidylethanolamine binding protein (PBP)
12.Phosphoglycerate mutase 1 (PGAM1)
13. Peroxidoredoxin 2 (PRDX2)
14. Stress induced phosphoprotein1 (STIP1)
15. Zyxin (ZYX)
16. Lymphocyte cytosolic protein 1 (LCP1)
17. Lymphocyte specific protein 1(LSP1)

3.10 Cytogenetic groups showed significant differences in their protein expression patterns

From the proteomic screen of various AML cytogenetic groups we identified a set of proteins as common targets which means they were identified among all groups of AML. These protein targets contribute to 65% of all identified proteome; however, they showed a significant quantitative variation in their expression pattern. For comparing the quantitative variations we used gel spot analyser software, ProteomeWeaver. This software measures protein expression levels of a spot by calculating its volume after reducing the background effect of staining. The spots representing common proteins were matched with the different gels from other AML cytogenetic groups and the standard deviation of matched spots were estimated. From this analysis we could identify significant differences between normal karyotype, CD34+ and healthy bone marrow. For example, growth arrest specific 6 protein identified from Normal karyotype shows a quantitative variation of 4 fold up in comparison to CK. Similarly c-myc promoter binding protein identified from NK and hematopoietic lineage specific protein identified from 11q23 patients shows a 2 fold enhanced expression in comparison to Inv (16) and CK respectively. hnRNPF identified from t(8;21) exhibits 1.5 fold increased expression in comparison to CK and t(15;17), (data not shown). The expression of highly abundant proteins like alpha enolase, vimentin, GRP 78 and protein disulphide isomerase shows highly significant quantitative variation between cytogenetic groups. P values calculated from the student's t-test showed the mean deviation of alpha enolase and protein disulphide isomerase from other cytogenetic groups is significantly different. However, the data for GRP 78 and vimentin was determined as insignificant (Figure 23, panels A, B, C and D). This holds promise as these proteins can significantly distinguish t(8;21) and inv(16) from other subtypes.

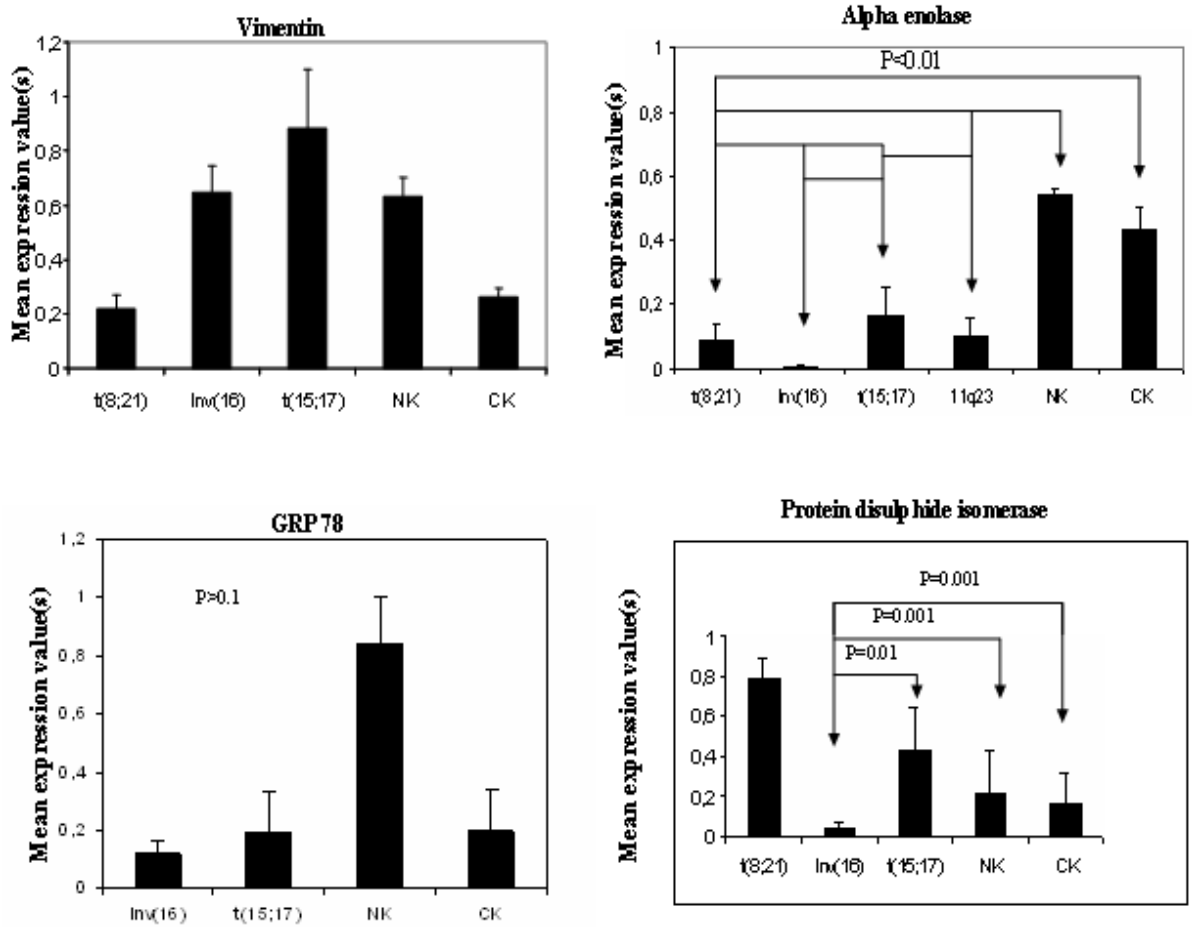
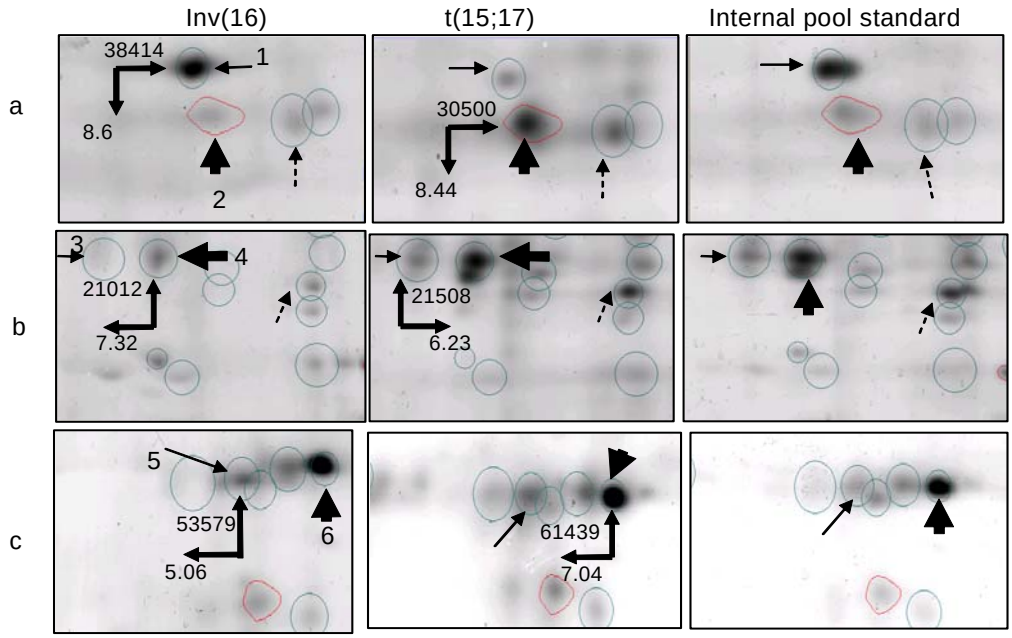


Figure 23. Quantitative variation in the protein expression pattern for common targets between cytogenetic risk groups of AML**.** The calculated expression level(s) are the mean normalised spot volume(s) and standard deviation was calculated from a minimum of 3 replicates of each protein. Each histogram represents coefficient of variation calculated as standard deviation/mean for alpha enolase (A), vimentin (B). GRP 78 (C), protein disulphide isomerase (D) calreticulin (E). Calculated P value are obtained from the student's t-test by comparing the means of two different AML subtypes, $P<0.05$ was considered significant.

3.11 DIGE method identifies significant quantitative variation of expression pattern between t(15;17)- and inv(16)-AML

We used a fluorescent based DIGE approach to detect the expression differences between t(15;17) and inv(16) patients. This method uses the fluorescent probes to label the protein. One of the advantages of this method is the accurate estimation of protein quantitation. Equal amount of protein extracts from t(15;17), inv(16) and a control comprising of protein mixture from both t(15;17) and Inv(16) (an internal pool standard) were labelled with Cy dyes and co-separated and visualised on one single 2D gel. Three

reciprocal gels were run in tandem. This analysis showed that some of the proteins exhibit visible quantitative variation in their expression (Figure 24). These spots were cut and later subjected to the mass spectrometry based identification. The details of these proteins with the mean spot volume(s) are given in the table below. Annexin II type I shows enhanced and significant quantitative variation from t(15;17), panel a. Here again, vimentin shows the expression level higher in t(15;17) in comparison to inv(16) panel c, which further validate our method of quantisation of silver stained gels using proteomweaver, which showed almost similar results.



Details and the mean expression volume(s) of the identified proteins from 2D-DIGE

No	Accession	Protein Name	Theoretical *pI*	Observed *pI*	Theoretical M*r[Da]*	Observed M*r[Da]*	Mean Spot Volume Inv(16)	Mean Spot Volume t(15;17)
1.	P27006	Annexin II type I	8.6	8.6	38414	38414	0.60	0.20
2.	Q8N912	Hypothetical protein	11.21	8.44	18051	30500	0.30	2.10
3.	Q13951	Core Binding factor 2	6.23	6.23	21508	21508	0.14	0.81
4.	Q525Q1	Stress 27KDa protein	7.32	7.32	20934	21012	0.24	0.88
5	P08670	Vimentin	5.06	5.06	53579	53579	1.20	0.75
6	O75123	KIAA0628	8.46	7.04	61439	61439	1.05	0.60

Figure 24. 2D-DIGE based expression pattern of proteins identified as common target of t(15;17)- and inv(16)-AML. Protein samples from t(15;17) and inv(16) were differentially labelled with Cy3 and Cy5. An internal pool standard was included which comprised equal amount of proteins from either sample and was labelled with Cy2. Panel a, b and c shows the differential expression of spots showing a visible expression differences. The Images are grey scale TIFF acquired from ProteomWeaver operated in a MFA mode. The table gives the details of the proteins with the mean spot volume identified from these spots.

3.12 Calreticulin, hnRPH1 and hnRNPA2/B1 showed a difference in their posttranslational modifications between cytogenetic risk groups

Next we hypothesized whether the proteins identified as common targets of cytogenetic abnormalities show any differences between their MS data. The mass spectrometer acquires ions (peptides) and generates a mass spectrum on the basis of mass to charge ratio of ions. We were interested to identify the differences in the raw mass spectrum of some of the common proteins identified. These differences can be mainly posttranslational modifications. In our proteomic screen we have identified calreticulin from t(8;21), t(15;17), inv(16), complex karyotype and normal karyotype. We analysed 5 different calreticulin MS spectra from 5 different patients with t(8;21), 4 MS spectra from Inv(16), 4 from t(15;17), 7 from normal karyotype and 4 from Complex aberrant karyotype. Figure 25a; shows the signature MS spectra of calreticulin labelled with the tryptic ions (the ions digested by trypsin). In order to identify the possible posttranslational modification differences of calreticulin between AML subtypes we searched calreticulin by MASCOT database and matched to our created database path in MSDB (a theoretical trypsin digestion database of calreticulin protein which includes an acetylation modification); a variable modification of acetylation was included. Among few potential peptides searched was a peptide *(m/z)* 2433AcetK, of the sequence ^{186}IDNSQVESGSLEDDWDFLPPK^{206}. This comprises the peptide region between I186K206. The peptide has only one C-terminal lysine residue, K206 that could serve as site for the acetylation. The Peptide Cutter theoretical digestion database (http//prospector.ucsf.edu) (Lominadze et al., 2005) revealed the presence of another ion *(m/z)* 2391 with the same sequence as that of 2433 ion. We suspect it to be the acetylated peptide which is shifted by 42Da from a peptide *(m/z)* 2391. The reanalysis in a peak explorer for calreticulin MS spectra identified from t(8;21) discovered the acetylated peak

(*m/z)* 2433 to be present (Figure 25a, panel A and B). However, the similar procedure followed for MS spectra of calreticulin in other subtypes like t(15;17), complex karyotype, inv(16), and normal karyotype could not detect the acetylated peptide even though the peak *(m/z)* 2391 was present. This suggests that this modification is unique to t(8;21)-AML. This is a first report where we have shown that calreticulin posttranslational modification of acetylation could serve as a distinguished feature of t(8;21) AML.

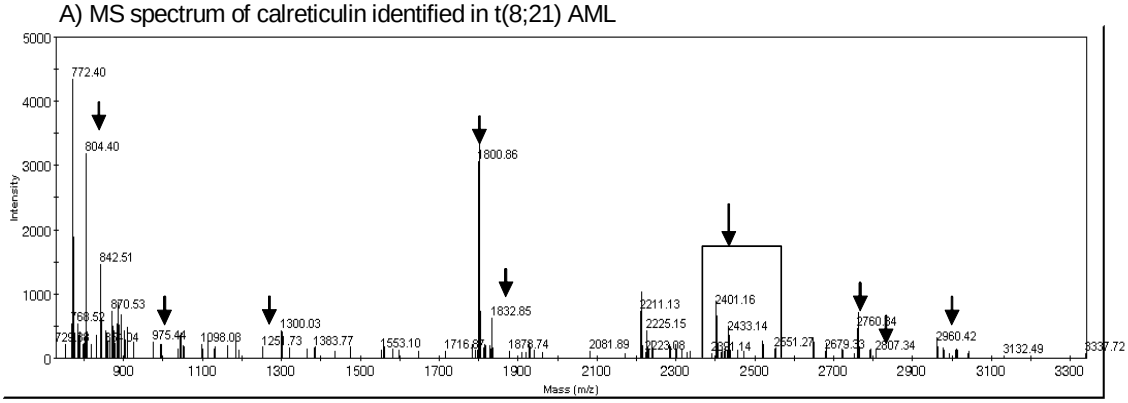
Next we selected hnRNPH1 which is already known to be modified by alternative processing of pre-mRNA splicing and by posttranslational modifications (Wang et al., 2004). We searched this protein within t(15;17), complex karyotype, 11q23, and t(8;21) AML cytogenetic groups for possible posttranslational modification. We analysed for hnRNP H1, 2 different MS spectra from t(15;17) patients, 3 spectra from 11q23, 2 spectra from complex aberrant, 3 spectra from normal karyotype. Unique to 11q23 patients a high intense peak, 2044, was consistently found which showed a difference of 203Da from a genuine tryptic ion, 1841, (Figure 25b, panel A). This corresponds to the mass of *O*-linked acetyl hexosamine (*O*-GlcNAc) adduct, a modification covalently binding to hydroxyls of threonine or serine residues (Sagi et al., 2005). To further confirm whether the sequence of MH+1841 and 2044 are matching, we sequenced their MS/MS spectra in data explorer (Figure 25b, B and C). The marked y and b ions confirm to the sequence ^{150}STGEAFVQFASQEIAEK^{166} between the region 150S151T166K for the 1841 and 2044 ions. Further MS analysis of hnRNPH1 from other cytogenetic groups failed to show the presence of glycosylated *(m/z)* 2044 ion from the cytogenetic subgroups of t(15;17), t(5;6)del(9) and t(8;21) (Figure 25b, panel D, E and F). The peptide has typical RSTG motif binding sites for *O*-GlcNAc modification. To map the site for *O*-GlcNAc we sequenced the *(m/z)* 2044 by MS-MS. We suspect it to be either serine150 or threonine151 known sites for *O*-GlcNAc modifications. The N-terminal b1 ion which is serine 150, a signal

corresponding to *(m/z)* 392 (monoisotopic) will correspond to serine residual mass plus 203Da mass of *O*-GlcNAc, if present in MS/MS. However, we could not detect the corresponding signal instead the signal corresponding to b2 ion which must include the masses of ST+203Da mass *(m/z)* 448 (monoisotopic) is present (Figure 25b, D). This confirms the site to be threonine 152. To further confirm the neutral losses of immonium and other ions, which should match for both the ions, we dissected the low *(m/z)* region and acquire the similar pattern (data not shown). Further, we confirm the *O*-GlcNAc posttranslational modifications by Immunoblot analysis of whole cell lysates from 3 different 11q23 positive patients using *O*-GlcNAc specific antibody CTD110.6, which has been successfully used previously in immunoblots and ELISA. We detected a signal for *O*-GlcNAcylation at 48kD, (Figure 25b, panel G). In each of these experiments the specificity of CTD110.6 immunoreactivity was established by competitively blocking antibody binding with free GlcNAc. The same gel was reprobed for hnRNPH; we could confirm the detection of hnRNPH (48kD) in the same region where GlcNAcylation was detected (Figure 25b, panel H).

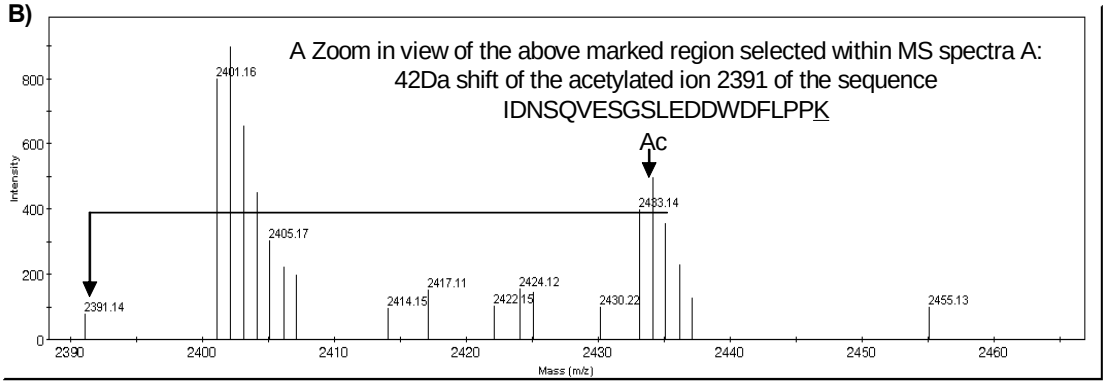
In a further analysis we investigated the methylation pattern of a protein hnRNPA2/B1 identified from various cytogenetic groups. We could identify hnRNPA2/B1 from NK, normal bone marrow, t(8;21) and Inv(16) patients. For hnRNPA2/B1 we analysed 2 MS spectra from t(8;21), 1 from 11q23 patient, 1 from normal bone marrow, 4 from normal karyotype. The representative MS spectra of this protein with tryptic ions are shown (Figure 25c). We investigated the spectra of hnRNPA2/B1 for methylation modification. We included methylation as a variable modification in a MASCOT search (data not shown); peptide cutter theoretical digestion database revealed ion *(m/z)* 1879 to be di-methylated. The detailed peak dissection of the real MS spectra of hnRNPA2/B1 discovered this peptide with a di-methylated signature with a sequence RGFGFVTFDDHDPVDK, a

peptide region between R153K168 (Figure25c, panel B, C and D). Methylation occurs predominantly on arginine and glycine rich RGG residues (Li et al., 1998a; Lin et al., 2000). This ion has N-terminal R153G154 motif which are the sites for di-methylation, $R_{Me}G_{Me}$FGFVTFDDHDPVDK. The ion must have shifted by a 28Da mass from *(m/z)* 1852. The detailed dissection across MS spectra of this protein revealed the presence of this ion, panel D. The theoretical digestion database revealed this ion has the same sequence as that of 1879 ion (data not shown). A similar analysis for hnRNPA2/B1 failed to detect the di-methylated ion from NK patients (data not shown).

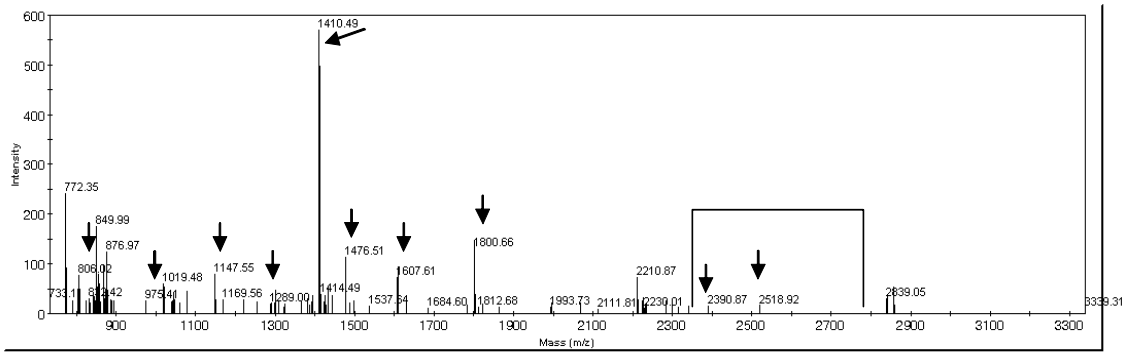
A) MS spectrum of calreticulin identified in t(8;21) AML



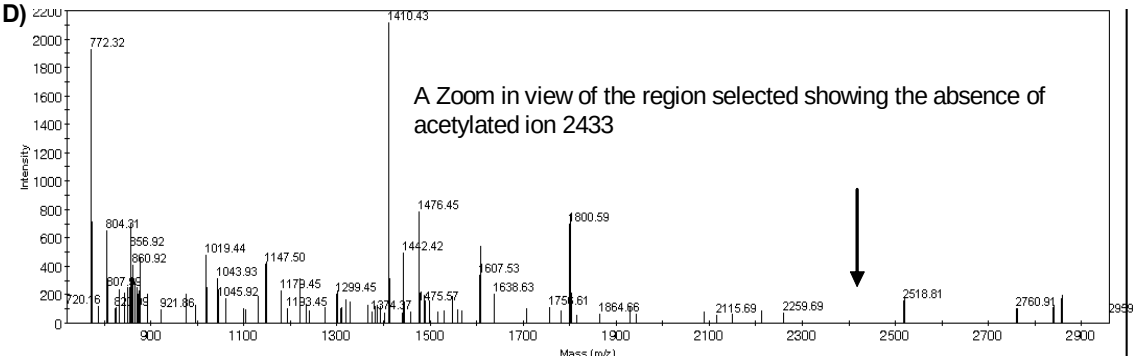
B)



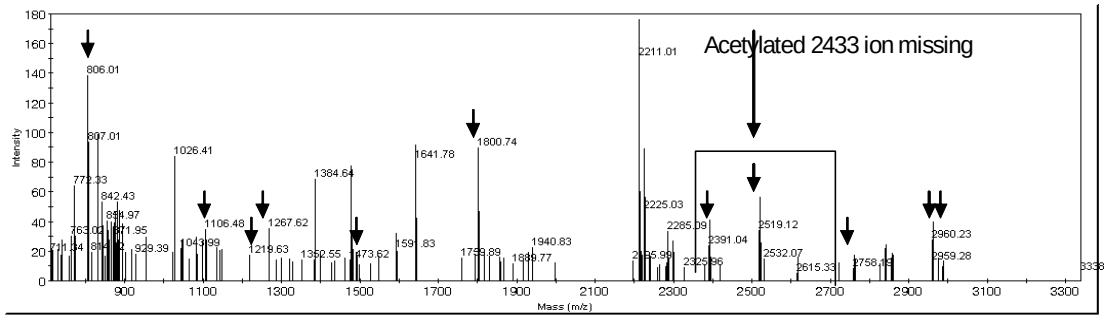
C) MS spectrum of calreticulin identified from t(15;17) AML showing absence of 2433 ion



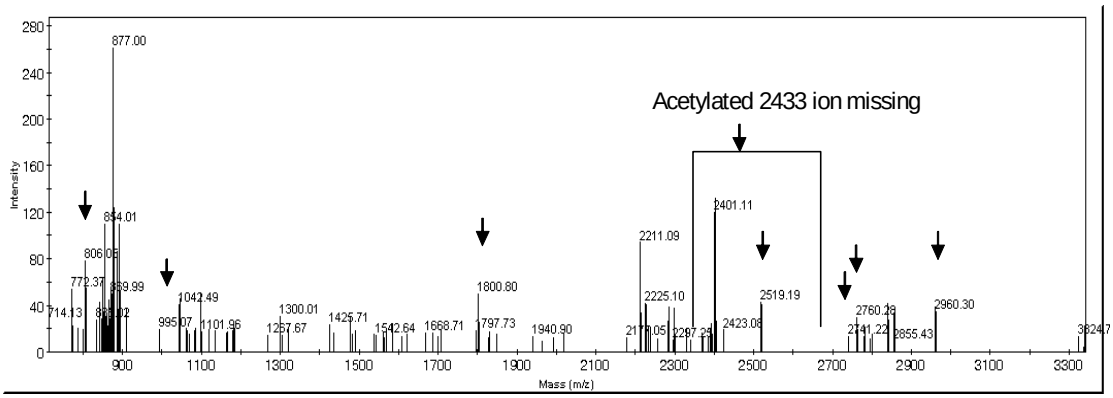
D)



E) MS spectrum of calreticulin identified from complex karyotype AML



F) MS spectrum of calreticulin identified from inv(16) AML



G) MS spectrum of calreticulin identified from normal karyotype AML

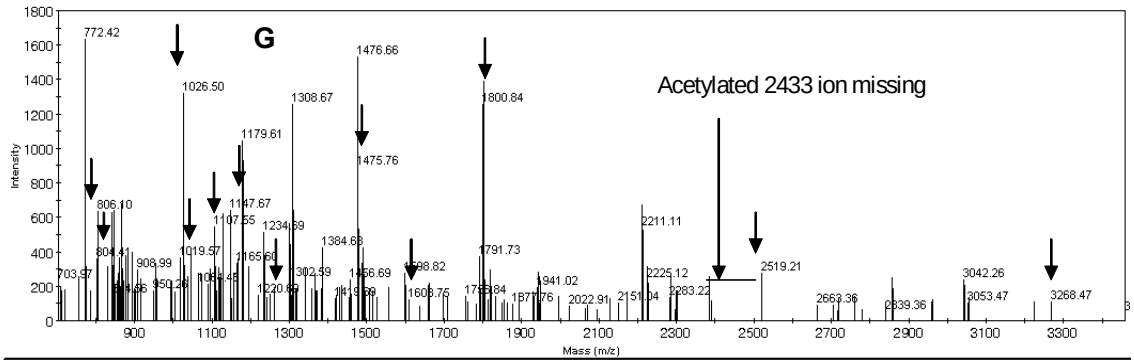
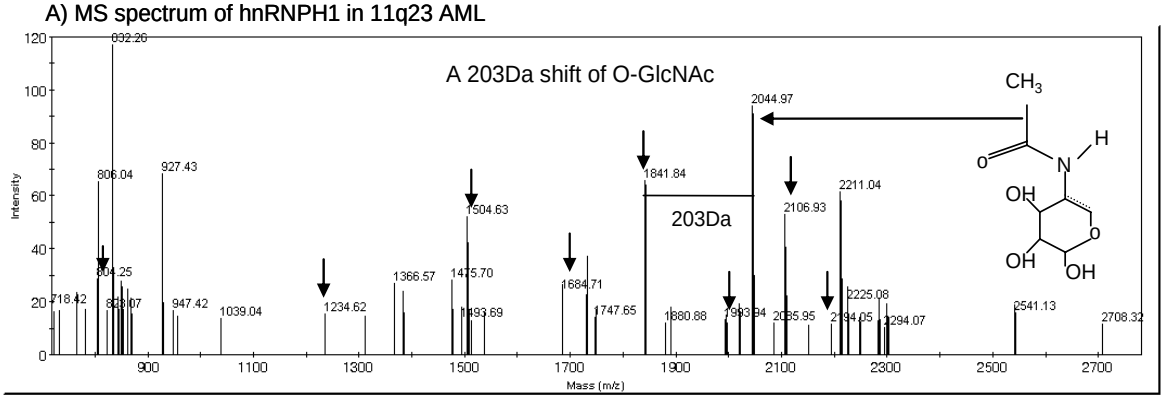
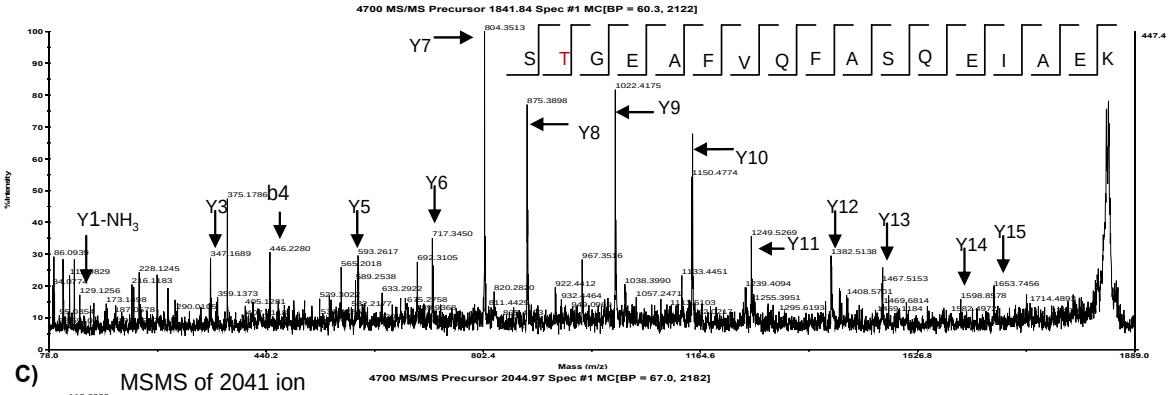


Figure 25a. Calreticulin acetylation in t(8;21) AML patients. Panel A shows the signature MS spectrum of calreticulin identified from t(8;21) AML. Each arrow represents the tryptic ion (peptide) matched to the theoretical database of trypsin digestion for calreticulin. Panel B shows the zoom in view of the MS spectrum of calreticulin identified from t(8;21) AML. *(m/z)* 2433 represents the acetylated ion. The m/z 2391 represents the ion of the same sequence shifted by a 42Da mass. Panel C, D, E, F and G for t(15;17), complex karyotype, inv(16), and normal karyotype AML show the calreticulin spectra where the acetylated 2433 ion could not be detected even though the peak *(m/z)* 2391 is shown to be present.

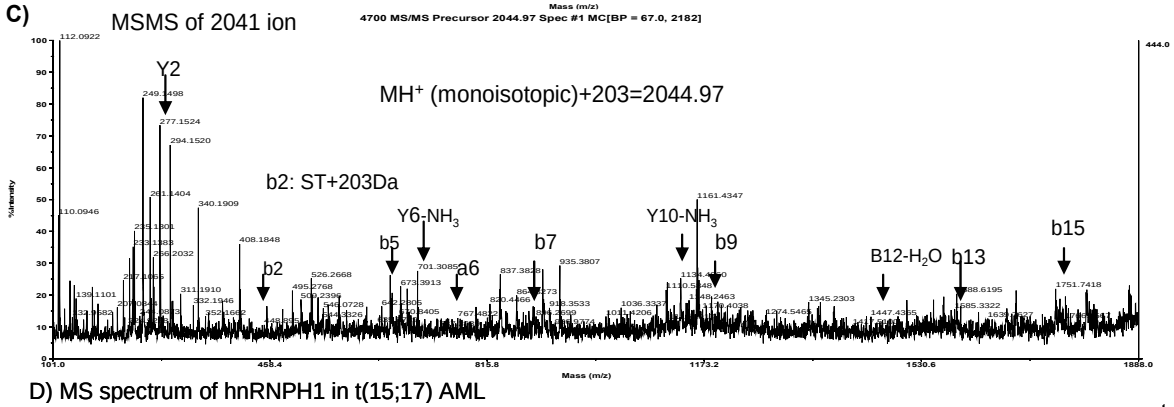
A) MS spectrum of hnRNPH1 in 11q23 AML



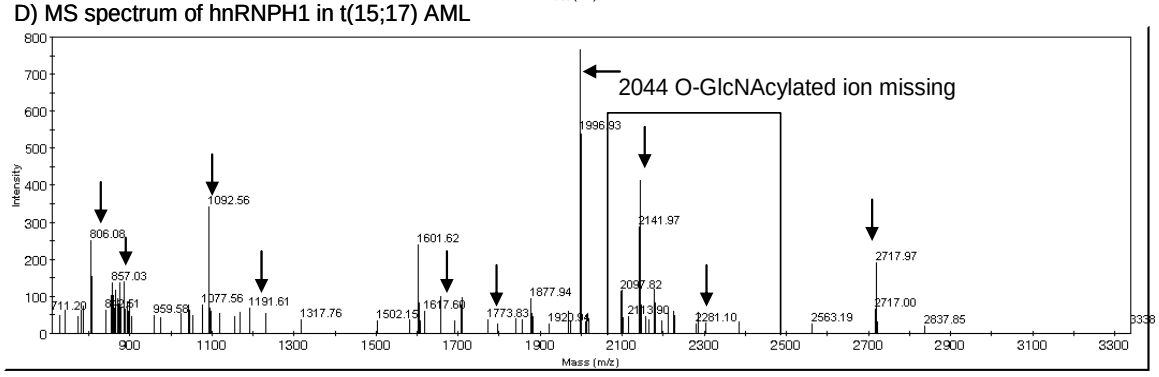
B) MSMS of 1841 ion



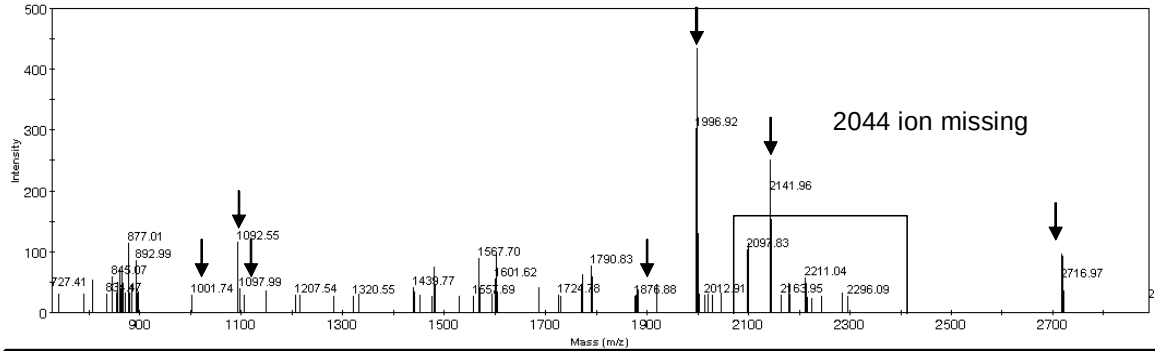
C) MSMS of 2041 ion



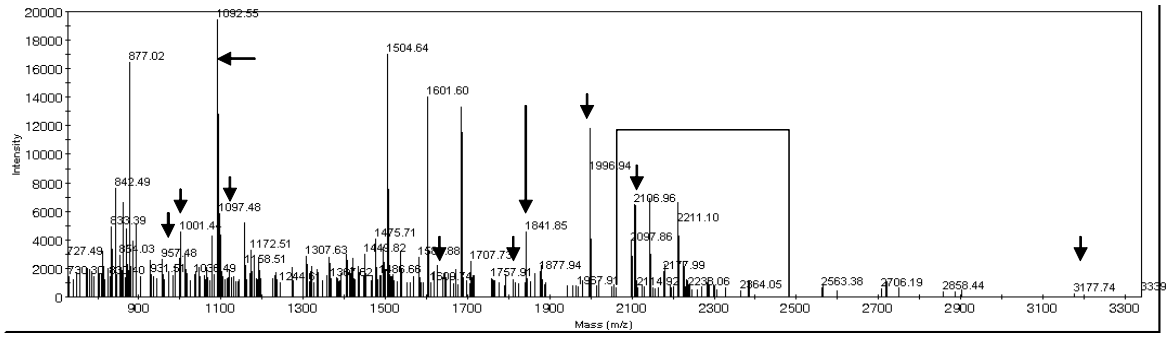
D) MS spectrum of hnRNPH1 in t(15;17) AML

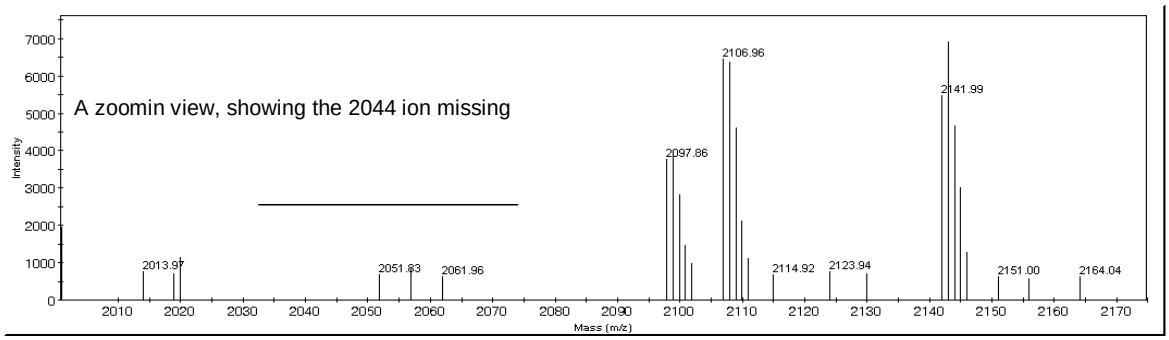


E) MS spectrum of hnRNP H1 identified from an AML patient with a t(5;16),del (9)

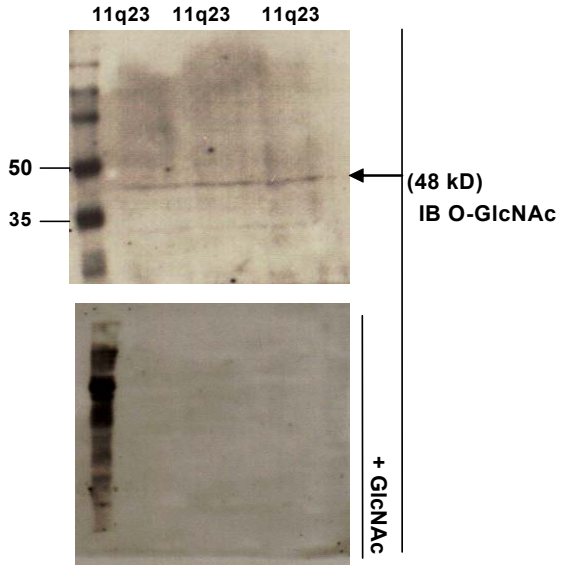


F) MS spectrum of hnRNPH1 identified from an AMLpatient with t(8;12;21)

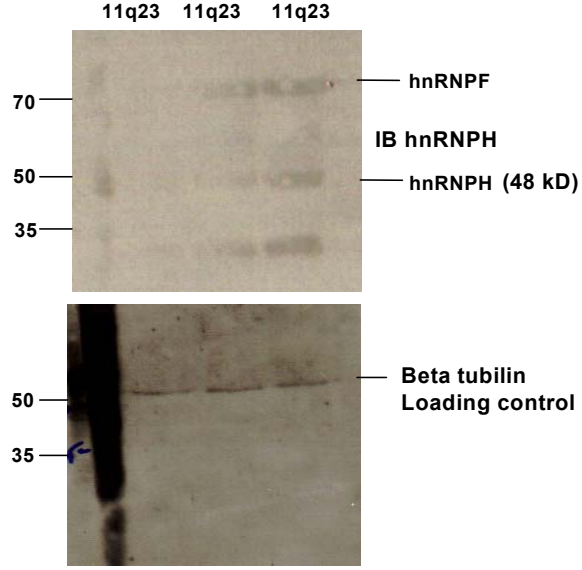




G)



H)



Pt No. 29, 30 and 31

Figure 25b. Modification of O-linked acetyl glucosamine of hnRNPH1 in 11q23 AML Patients**.** Panels A represent the MS spectra of hnRNPH1 identified from 11q23 showing the O-GlcNAcylation. The 203Da mass shift between *(m/z)* 1841 and 2044 corresponds to addition of O-GlcNAc. Panels B and C represents the MS-MS spectra of ions (*m/z)* 1841 and (*m/z)* 2044 sequenced in data explorer. Panels D, E and F show the absence of 2044 ion corresponding to O-GlcNAc modification from t(15;17), t(5;16)del(9), t(8;12;21) . Panel G & H, validation of O-GlcNAcylation of hnRNPH1 detected by O-GlcNAc specific immunoblot from 11q23 patients**.** O-GlcNAc specific immunoblots were preformed using fresh protein RIPA lysates from 3 different 11q23 patients using anti-O-GlcNAc antibody CTD110.6. The specificity of the antibody for O-GlcNAc was tested by adding 10mM GlcNAc to the primary antibody dilution, **Panel G**. hnRNPH was detected from the same gel after reprobing with anti hnRNPH, **Panel H**. β- tubilin was used as a loading control.

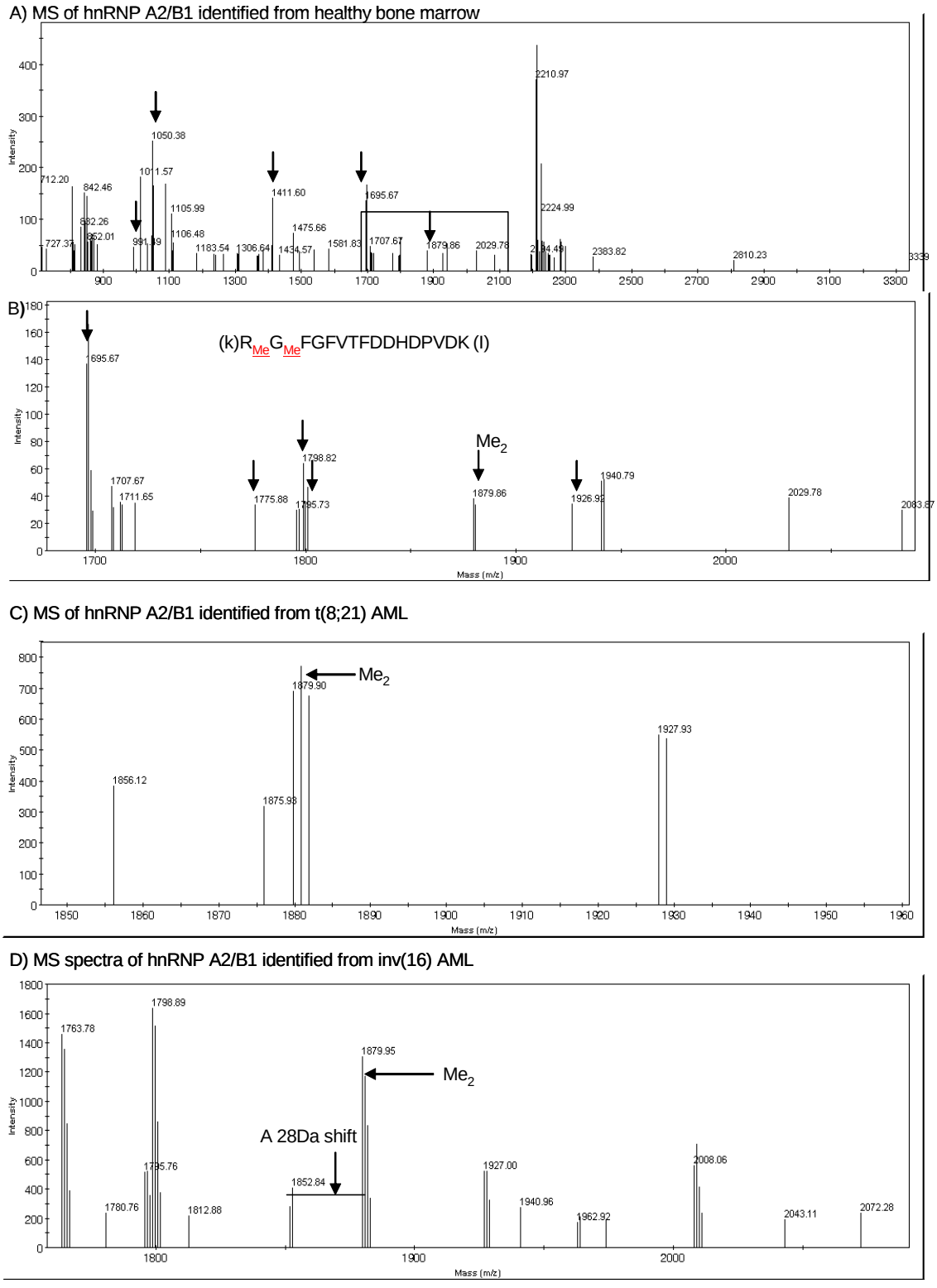


Figure 25c. Di-methylattion of hnRNPA2/B1 in t(8;21) and inv(16) AML patients. Panels A, B, C and D represent the MS and Zoom in view of the MS spectra of hnRNPA2/B1 identified from healthy bone marrow, t(8;21) and inv(16). The ion 1879 represents the di-methylated ion which is shifted by 28Da from *(m/z)* 1852.

4 Discussion

The systematic investigation of proteomic differences between various cytogenetic risk groups of AML has not been fully investigated. The mechanism how fusion proteins specifically alter the proteome of a normal cell is a subject of intense investigation. Characterisation of proteomic differences between set of AML patients with balanced chromosomal translocations is crucial given that any observed variation in its expression pattern may have prognostic and disease relevance (Nedelkov et al., 2005). Besides the identification of proteins/ and protein interaction pathways dysregulated by fusion proteins could provide disease relevant information regarding the signalling defects. Moreover, the biological characterisation of a potential therapeutic target could provide critical insight into the mechanism of the disease progression. The present study involves the mass spectrometry based characterisation of proteomic, bioinformatics and MS based analysis of posttranslational modifications of cytogenetic groups of AML. In this investigation we applied state of the art proteomic technology that includes the characterisation and identification of MS based peak pattern analysis to identify posttranslational protein modification differences between cytogenetic subgroups, differential gel electrophoresis (DIGE) to quantify the differential expression data and biological characterisation of AML1-ETO induced inhibitor of apoptosis protein (IAP) survivin given its immense therapeutic potential for AML.

We could identify significant differences in the proteome pattern which may discriminate cytogenetic risk groups with in themselves and from healthy bone marrow and CD34+ cells. The expression pattern of some of the proteins may correlate for diagnosis, prognosis and pathogenesis. The expression of hnRNPA2/B1, the RNA binding protein (Wang et al., 2006) and casein kinase 1 alpha identified as a distinguished target of NK patients shows higher expression in NK patients in comparison to other subtypes where it is either

less expressed or absent may mean a positive discriminatory diagnostic marker for NK patients. Casein kinase 1 alpha have been reported to be involved in diverse cellular processes including cell cycle progression, apoptosis and cellular differentiation (Knippschild et al., 2005). However, for many of the other proteins which we report here as showing discriminatory pattern of expression among AML cytogenetic groups, their clinical significance remain to be investigated. For example, transcription factor MafK which we identified as a distinguished target of Inv(16) is known to promote the erythroid differentiation program of erythroleukemic cells and acts as a crucial regulator of many erythroid specific genes (Igarashi et al., 1995). B-cell CLL Lymphoma/Leukemia 11A, identified as a differential target of CK, functions as a myeloid and B-cell proto-oncogene. Its high expression in AML points towards a probable role in leukemogenesis and hematopoiesis (Satterwhite et al., 2001). During the proteomic screen of t(8;21) patients we identified the differential expression of apoptosis inhibitor protein (IAP) survivin from t(8;21). Because survivin has received great attention due to its potential therapeutic role in cancer we characterised this protein both mechanistically and biologically. We could show that by the knock down of survivin expression AML1-ETO mediated inhibition of granulocytic differentiation (Pabst et al., 2001) was overcome marked by the restoration of C/EBPα activity. C/EBPα in the absence of AML1-ETO autoregulates its own promotor (Legraverend et al., 1993; Smith et al., 1996) which is completely abolished by the presence of AML1-ETO (Timchenko et al., 1995). By the repression of survivin using short hairpin RNA we showed that AML1-ETO mediated inhibition of C/EBPα autoregulation of its own promotor was restored in a reporter assay. This restoration of C/EBPα activity and protein levels were sufficient to drive AML1-ETO positive leukemic blasts to neutrophilic differentiation and growth arrest. Thus explaining the lack of C/EBPα observed in AML1-ETO cells as critically mediated by the survivin

induction. AML1-ETO inhibits AML1-dependent activation of a granulocytic promoter like M-CSF (Westendorf et al., 1998). Interestingly, our results showed that AML1-ETO induces the expression of survivin in hematopoietic CD34+ cells and inhibition of survivin in these cells restores AML1 dependent transcription as confirmed by the Q-PCR data for the expression for MPO gene (Fig. 21, panel D). The induction of survivin by AML1-ETO may explain the phenomenon of extensive self renewal capacity observed in primary hematopoietic cells transformed with AML1-ETO. Besides the differential requirement of survivin for hematopoietic cell development in switching off the arm of definitive granulopoiesis (Gurbuxani et al., 2005; Tonks et al., 2003). Further, we showed that AML1-ETO directly activates the basal transcription of the survivin gene in a transcriptional assay and that could explain the mechanism behind the overexpression of survivin in t(8;21) leukemia and in addition to higher expression in immature neutrophils (Altznauer et al., 2004). Not surprisingly, the higher expression of survivin in AML1-ETO transformed cells may explain that AML1-ETO like AML1 can act as a transcriptional activator (Frank et al., 1999; Klampfer et al., 1996; Yergeau et al., 1997) of survivin. AML1-ETO, otherwise, is perceived to be a well known transcriptional repressor of granulocytic genes (Meyers et al., 1995). With the alteration of one allele of AML1 gene in t(8;21) leukemia resulting in AML1-ETO can efficiently inhibit AML1-dependent transcriptional activation as well as AML1 transcript variant AML1b (Frank et al., 1995; Meyers et al., 1995). However, AML1b effect on the survivin activation was negative providing a further poof of survivin is being involved principally in transformation program. This was further substantiated by a transcription assay that showed AML1-ETO in the absence of AML1b activate the transcription of survivin gene by 5 fold and the activation was repressed to 2 fold in the presence of AML1b (Figure 16 panel B). The therapeutic potential of survivin as a targeted cancer therapy has been variedly exploited

in number of other cancers (Altieri, 2006). Molecular antagonists of survivin, including dominant-negative mutants and antisense have been reported to be associated with induction of apoptosis and inhibition of tumor growth in vivo, without going against normal cells (Altieri, 2003). Antisurvivin therapy is potentially a novel and attractive cancer treatment strategy for AML. Thus for, in various preclinical animal models trials, targeted anti survivin therapies have shown significant efficacy. However, the consequences of prolonged and stable survivin disruption (Altieri, 2006; Fukuda and Pelus, 2006) in t(8;21) leukemia which comprises of 40% AML have not been fully investigated.

Molecular interaction maps are particularly useful for networks that include protein-protein binding and posttranslational modifications (e.g., phosphorylation). Both are important for nearly all of the proteins involved in DNA double-strand break signaling. Visualizing the regulatory circuits underlying cellular signaling may help identify key regulatory reactions and defects that can serve as targets for anticancer drugs (Pommier et al., 2005; Pommier et al., 2006). Mapping protein networks for various cytogenetic risk groups in vivo is critical for realizing the promise of subtype specific patient-tailored molecular therapy. The proteins whose expression is significantly altered between cytogenetic groups could explore therapeutically relevant networks by providing an idea of dysregulation or hyperactivity in the network of intracellular signalling cascades (Petricoin et al., 2005). We applied this approach by employing one of the publicly available databases for generating the protein interactions. This approach is a robust assay for protein-protein interaction (Parrish et al., 2006). Our results present cytogenetic specific signature networks and hyperactive modules. This provides valuable information regarding the dysregulation of signalling pathways based on differential proteome data. We used, from our proteomic data all the expressed proteins with the expression values, identified from cytogenetic groups to generate the protein interaction networks (Fig. 22). This data

provides the relevant information regarding the signalling networks that are influenced by the proteins showing abnormally higher expression. This information could also be exploited to investigate the signalling defects associated with the specific fusion proteins that could be important for the outcome of leukemia. Many proteins with oncogenic potential undergo a complex series of posttranslational modifications. Post-translational modifications are an important biological mechanism to produce various protein species with entirely different biological roles from a single gene (Schoneich, 2006). PTMs affect significantly a given protein in terms of its stability, function, half life, complex formation with other proteins the proteins, etc. Some of the common but important posttranslational modifications include phosphorylation, glycosylation, acetylation and methylation. Identification of these modifications presents a formidable challenge, but their determination could provide an indispensable insights into disease progression and possible key therapeutic and prognostic targets (Mann and Jensen, 2003). Proteomics combined with tandem LC/ MSMS and other online separation method makes it possible to dissect a given MS spectra of a protein and characterize the individual parts of post-translational modifications. Systemic analysis of post-translational modifications in various signaling pathways has been applied to illustrate the kinetics of modifications (Seo and Lee, 2004). Information regarding the PTM differences which may correspond for prognosis, pathogenesis and therapeutic relevant targets has not been fully investigated in the context of cytogenetic risk groups of AML. We analysed the MS spectra of some of these commonly regulated proteins which we could identify among all the cytogenetic groups to look into possible differences in their MS spectra which may correspond for PTM differences (Fig. 25). Generally this information has been overlooked in majority of other high throughput proteomic screens reproted. For this analysis we selected calreticulin, hnRNPH1, and hnRNPA2/B1. Here we

show that calreticulin is modified by acetylation specifically in t(8;21) AML. Increased calreticulin expression is correlated with the suppression of the granulocytic differentiation factor CEBPA in AML with inv(16) and t(8;21); calreticulin is also reported to interact with C/EBP alpha and C/EBP beta m-RNA and represses them at protein level (Helbling et al., 2004; Helbling et al., 2005). AML patients positive for t(8;21) have undetectable C/EBPalpha protein as compared to other subgroups of AML patients (Timchenko et al., 2002; Timchenko et al., 1996). These effects may be in part due to the acetylation of calreticulin in t(8;21) which we reported here and not because of the nascent calreticulin protein alone. Posttranslational modification of glycosylation is an important regulatory modification of proteins. Altered glycosylation patterns are a hallmark of many types of cancers. Glycosylation modifications frequently affect expression, metabolism, functions, stability and/or cellular localization of glycoproteins in cancer cells contributing to their extensive proliferation. Therefore identification of glycosylation pathways has a potential for innovative anti cancer therapies (Kukuruzinska and Lennon, 1998). hnRNPs are differentially regulated by alternative processing of pre-mRNA and by posttranslational modifications. However, among AML cytogenetic subgroups the significance at the posttranslational level is unknown except for hnRNP p43 which is known to get modified by *O*-GlcNAc (Vosseller et al., 2001; Wells et al., 2003). Here we could show that hnRNPH1 is reversibly modified by *O*-linked acetyl hexosamine (GlcNAc) in 11q23 positive patients. The covalent modification of intracellular proteins by *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) is a crucial regulatory posttranslational modification which play a role in apoptosis and modulate transcriptional and signal transduction events (Khidekel et al., 2004), and may be associated with high therapeutic significance for AML in general and 11q23 translocations in particular. Methylation is a reversible and most stable posttranslational modification that occurs predominantly on

arginine and glycine rich RGG residues (Aoki et al., 2002; Li et al., 1998a; Lin et al., 2000). Methylation affects a variety of protein-protein interactions with distinct functional outcomes that make this modification potentially valuable in signaling networks not only for histone but also for non-histone proteins, such as p53 and TAF10 and hnRNPs (Chuikov et al., 2004; Kouskouti et al., 2004). Methylation pattern constitutes an important regulatory mechanism, large number of genes have been reported to be methylated in AML, and differences in the pattern of methylation has been reported between various subtypes of leukemia (Herman and Baylin, 2003; Wolffe and Matzke, 1999). The expression of hnRNPA2/B1 is a prognostic marker for early lung cancer detection (Zhou et al., 2001). Interestingly we report for the first time differences in the methylation pattern of hnRNPA2/B1 in AML patients. Interestingly, the intensity of this peptide in t(8;21) and inv(16) is 10 and 20 fold higher than NBM. Even though the technique in itself is not quantitative unless the peptides are tagged, however, growing the blasts in culture and tagging invivo to accurately quantitate is the limitation to achieve this end. Nonetheless the intensity value of methylated ion of hnRNPA2/B1 in t(8;21) and Inv(16) is in itself highly significant than NBM.

Our findings support the rational hypothesis that AML cytogenetic subgroups could be distinguished by proteome analysis. AML which is long believed to be heterogeneous in nature and we showed it differed at the basic biological setup and fusion proteins fundamentally alters the proteome. We further showed in our validation and characterisation experiments that survivin is a novel target of t(8;21) leukemia and AML1-ETO directly regulates its expression to induce the differentiation block that could be overcome by silencing its expression. Furthermore, the detection of modified peptides by MS peak import analysis could provide new insights into the functional modulation of these disease relevant proteins.

5 Summary

Acute Myeloid Leukemia (AML) is characterized by specific cytogenetic aberrations that are strong determinants of prognostic outcome and therapeutic response. Because the pathological outcome of AML patients with cytogentic abnormalities differs considerably we hypothesized that their proteome may also differ specifically in their expression pattern, protein interaction pathways and posttranslational modifications. We performed this study using 42 AML patients diagnosed for various cytogenetic abnormalities based on two-dimensional gel electrophoresis and MALDI TOF Tandem MS (MS/MS) analysis. We could identify significant differences in the proteome and posttranslational modifications of peptides, later confirmed by other methods, between cytogenetic groups. The interactome analysis based on computational bioinformatics reveals a major regulating networks, MAPK8 and MYC for complex aberrant karyotype, TP53 for t(8;21), TP53- MYC- PRKAC for 11q23, JUN and MYC for Inv(16). We could show in our validation and characterisation experiments that survivin is a novel target of t(8;21) leukemia and AML1-ETO directly regulates its expression to induce the differentiation block that could be overcome by silencing its expression. Further, we analysed 42 MS spectra representative of hnRNPH1, Calreticulin and hnRNPA2/B1 in a peak explorer which reveals a cytogenetic specific posttranslational modification of β-O-linked N-acetyl glucosamine (O-GlcNAc) of hnRNPH1 in AML patients with 11q23 translocation, an acetylation of calreticulin in t(8;21) translocation and methylation of hnRNPA2/B1 in patients with translocations of t(8;21) and inv(16). This report may lead to a new thinking about the AML pathogenesis as differences at PTM level could be used to distinguish different subtypes of AML besides for testing the therapeutic significance. Further, we characterised the biological role of survivin identified specifically from t(8;21) patients. We could show that AML1-ETO induces the expression of survivin both in a cell

line model and in primary human hematopoietic precursors. AML1-ETO activates the basal transcription of the survivin promoter and binds to the only AML1 core enhancer binding sequence, TGTGGT, in survivin promotor. Repression of AML1-ETO mediated induction of survivin expression by a specific short hairpin RNA restores C/EBPα protein and its basal transcriptional activity on its own promotor. This restoration differentiates AML1-ETO positive leukemic cells to terminal granulocytic differentiation and growth arrest. These observations indicate that the antiapoptotic survivin protein, which holds a great therapeutic promise, is a critical mediator of AML1-ETO induced defective granulopoiesis. Thus, proving that AML1-ETO induces inhibition of granulocytic differentiation by activating survivin expression

6 Zusammenfassung

Akut Myeloische Leukämien (AML) sind charakterisiert durch spezifische zytogenetische Veränderungen, die entscheidende Faktoren hinsichtlich der Prognose und dem Ansprechen auf Therapien darstellen. Es gibt beträchtliche Unterschiede im klinischen Bild von AML Patienten, die verschiedene zytogenetischen Abnormalitäten tragen. Aufgrund dieser bekannten Differenzen zwischen den einzelnen Subtypen, stellen wir die Hypothese auf, dass das Proteom dieser Patienten ebenfalls spezifische Unterschiede in seinem Expressionsmuster aufweist und des Weiteren, dass auch nachweisbare Unterschiede in den Interaktionen der einzelnen Proteine und deren posttranslationalen Modifikationen vorliegen. Unsere Studie umfasst 42 AML Patienten, bei denen verschiedene zytogenetische Veränderungen, basierend auf 2D-Gelelektrophorese und MALDI TOF Tandem Massenspektrometrie (MS/MS) Analyse, nachgewiesen wurden. Mit Hilfe verschiedener Methoden konnten wir zwischen den einzelnen zytogenetischen Gruppen in diesem Patientenkollektiv signifikante Unterschiede in deren Proteom und in den posttranslationalen Modifikationen verschiedener Proteine identifizieren. Bei der Analyse der Interaktionen der einzelnen Proteine, basierend auf bioinformatischen Berechnungen, konnten verschiedene bedeutende Netzwerke aufgedeckt werden, die jeweils spezifisch für die einzelnen zytogenetischen Gruppen sind. So involviert dieses Netzwerk beim komplex-aberranten Karyotyp die Proteine MAPK8 und MYC, in der Translokation t(8;21) das Protein TP53, in der Translokation 11q23 die Proteine TP53, MYC and PRKAC und in der inv(16) die Proteine JUN und MYC. In unseren Charakterisierungs- und Bestätigungsexperimenten konnten wir zeigen, dass Survivin ein neues Zielprotein in Leukämien mit t(8;21) Translokationen ist. Das Fusionsprotein AML1/ETO reguliert dabei direkt die Expression von Survivin und induziert dadurch einen Differenzierungsblock, welcher bei Ausschaltung der

AML1/ETO Expression überwunden werden kann. Des Weiteren ergab die Analyse von 42 Massenspektren repräsentativ für die Proteine hnRNPH, Calreticulin und hnRNPA2/B1 in einem Peak Explorer, dass bei AML Patienten mit 11q23 Translokation ß-O-gebundene N-acetyl-Glucosamine (O-GlcNAc) des Proteins hnRNPH1 spezifische posttranslationale Modifikationen aufweisen und dass bei Patienten mit t(8;21) Translokationen Acetylierungen im Calreticulin nachweisbar sind. Außerdem weisen sowohl Patienten mit t(8;21) Translokationen als auch Patienten mit Inv(16) Methylierungen im Protein hnRNPHA2/B1 auf. Diese Erkenntnisse können zu neuen Ansätzen hinsichtlich der Diagnostik und Therapie von AML Patienten führen, indem Unterschiede auf der Ebene translationaler Modifikationen von Proteinen zur Unterscheidung verschiedener AML Subtypen verwendet werden kann und daneben auch die therapeutische Signifikanz getestet werden kann. Darüber hinaus charakterisieren wir die biologische Rolle von Survivin und diese speziell bei Patienten mit t(8;21) Translokationen. Das Fusionsprotein AML1/ETO aktiviert die basale Transkription des Survivin Promotors und bindet dabei an die einzige spezifische AML1 Bindungs-Sequenz, TGTGGT, im Survivin Promotor. Die Unterdrückung der AML1/ETO mediierten Induktion der Survivin Expression mit Hilfe einer spezifischen „short hairpin RNA“ führt zur Normalisierung des Transkriptionsfaktors C/EBPα auf Proteinebene und es zeigt sich auch die Rückkehr von C/EBPα zu seiner basalen Transkriptionsaktivität am eigenen Promotor. Diese Wiederherstellung der C/EBPα Funktionen führen zur Differenzierung leukämischer Zellen, die das Fusionsprotein AML1/ETO tragen, in neutrophile Granulozyten. Diese Beobachtungen deuten darauf hin, dass das antiapoptotische Protein Survivin, das große therapeutische Möglichkeiten verspricht, einen kritischen Faktor in der Vermittlung AML1/ETO induzierter Störung der Granulopoese darstellt. Dabei zeigt sich

das die AML1/ETO induziert Hemmung der granulozytären Differenzierung mit einer Aktivierung der Survivin Expression einhergeht.

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9 Appendix

1. Cell culture

RPMI (PAA)

10% Foetal bovine serum (Invitrogen)

100µM Zinc sulphate ($ZnSO_4$) (Sigma)

1% Penicillin/Streptomycin (Gibco)

2. Immunoblot

RIPA laysis buffer (1% NP40, 0.5% Sodium deoxycholate, 0.1% SDS, 0.15M NaCl, 5mM EDTA, 50mM Tris pH8.0 and dH_2O)

Phosphates inhibitor cocktail I and II (Sigma, USA)

Proteinase inhibitor (Sigma)

Bradford assay (BioRad Laboratories, Germany)

2x loading dye

1x running buffer and 1x Transfer buffer

Nitrocellulose membrane (Millipore)

1xTBST and 5% milk (Blocking buffer)

2.5% milk in TBST (Washing buffer)

ECL detection Kit (Amersham Biosciences, UK)

Hyper film ECL (Amersham Biosciences, UK)

3. 2D-gel electrophoresis

Urea (Amersham bioscience, EU)

Urea Lysis buffer (9.8M urea, 1% DTE, 4% CHAPS, 2.5mM EDTA and 2.5mM EGTA)

Resolyte (Amersham Biosciences)

Bromophenol blue (Sigma, USA)

IPG strips (pH 3-10 and pH 4-7) (Amersham Bioscience, Sweden)

SDS PAGE (Gel running buffer 192mM Glycin, 25mM Tris and 0.5% SDS)

DTE buffer and carbamylation buffer (1M Tris pH6.8, urea, 80% Glycerol)

20% SDS and ddH_2O and add one half 0.2g DTE and to the other half 0.25 iodoacetamide)

4. Colloidal Coomassie blue stain (Sigma, USA)

Fixing: 50% methanol and 10% acetic acid

Staining: 0.1% Colloidal Coomassie, 2% H_3PO_4 , 10% Ammonium sulfate and 20% Methanol

Destining: 25% Methanol

5. Silver nitrate (Merck, Darmsadt, Germany)

Silver stain

50% Methanol, 12% Acetic acid and 37% Formaldehyde

50% Ethanol

200mg/L Sodium thiosulfate ($Na_2S_2O_3$ $5H_2O$)

2g/L Silver nitrate ($AgNO_3$) and 750µl/L 37% formaldehyde

60g Sodium carbonate (Na_2CO_3), 5mg $Na_2S_2O_3$ $5H_2O$ and 500µl formaldehyde

50% Methanol and Acetic acid

20% Ethanol and 12% Glycerine

6. Peptides Extraction

Acetonitrile (Aldirch)

50% and 70% Acetonitrile

50mM Ammonium bicarbonate (NH_4HCO_3)

Trypsin enzyme reconstituted with ammonium bicarbonate (NH_4HCO_3)

DHB Matrix

20% Acetonitrile and 0.1% Trifluoroacetic acid (TFA) (Merck)

20 mg/ml 2.5-dhydroxy-benzoicacid (DHB) (Merck)

20 mg/ml 2-hydroxy-5-methoxy-benzoicacid (Merck)

mix 9:1 DHB:HMB

take 1µl of matrix with 1µl of peptide and spot 0,8µl on an anchor chip plate

CHCA Matrix

50% Acetonitrile and 0.05% TFA (Solvent solution)

8mg of CHCA (Sigma) in 200µl solvent solution

2/3 of solvent solution mix 1/3 of CHCA solution

Apply 0.5µl of matrix to the plate

Add 0.5µl of the sample and dry

7. Mass spectrometry

PMF Reflex III MALD-TOF (Bruker Daltonics)

MS/MS AB4700 and GPS explorer software (Applied Biosystems)

Mascot database search (Matrix Science)

8. Immunofluoresent

Cytocentrifuge

Glass slides

P/R9 cells

PBS (PAN)

99% methanol and Acetone (Fixing)

Permeabilized with 0.3% Triton X

Block with 5% FBS in PBG (PBG=% BSA, 0.045% Fish-gelatine in 1X PBS)

Primary antibody (Diluted in PBG and 2.5% FBS) anti-rabbit sc20796 Op18 (Santa Cruz Biotechnology Inc.)

Secondary antibody (Diluted in PBG and 2.5% FBS) (anti mouse α tubulin) Alexa Fluor (Molecular probes)

488 Chicken anti-rabbit and Alexa Fluor 894 chicken anti-mouse IgG (Molecular probes)

4′, 6′-Diamidino-2-phenylindole dihydrochloride(DAPI) (Molecular probes)

9. FACS PI-cell cycle analysis

Propidium Iodide (Sigma, USA)

U937 and P/R9 cells

Cell lysis buffer

0.1% Sodium citrate

0.1% Triton X-100

20µg/ml Propidium Iodide

10. *In-vitro* kinase

Protein A agarose beads (Roche)

Kinase buffer (150mM NaCl, 1mM EDTA, 50mM tris-HCl, pH 7.5,

10mMMgCl_2 and 10mMDTT)

Histone H1 (Upstate, Germany)

Rb-fusion protein (Santa Cruz Biotechnology Inc.)

ATP and [γ-32p] ATP

11. siRNA

Designed 2 Oligos (Gene accession number NM-203401)

RNAiFect (Qiagen)

Non silencing siRNA

siRNA conjugated to rhodamine

10 Lebenslauf

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1.47th Annual meeting of the American Society of Hematology (ASH), Atlanta Georgia, USA **Dec. 2005**. **Title:** "Proteomic of Acute myeloid leukemia: Cytogenetic Risk Groups Differ specifically in their proteome, Interactome and posttranslational protein Modification. " Blood, Nov. 2005 (Abstract).

2.Gemeinsame Jahrestagung der Deutschen, Österreichischen und Schweizerischen Gesselschaften für Hämatologie und Onkologie, Innsbruk, Ösraisch **Oct. 2004. Title**: "Proteomic of Acute myeloid leukemia: Cytogenetic Risk Groups Differ specifically in their proteome, Interactome and posttranslational protein Modification." (Abstract).

3**.**Gemeinsame Jahrestagung der Deutschen, Österreichischen und Schweizerischen Gesselschaften für Hämatologie und Onkologie, Leipzig, **Oct. 2005. Title**: "Proteomic of Acute myeloid leukemia: Cytogenetic Risk Groups Differ specifically in their proteome, Interactome and posttranslational protein Modification." (Abstract).

4.Participated in an international workshop on immunology held at International Centre for Genetic Engineering and Biotechnology (ICGEB) New Delhi, **08/2004**.

Kurs Teilgenomen

1. Completed a one week Advanced Course on, "Mass Spectrometry based proteomics and identification of posttranslational modification." at the University of Lund, Sweden.

2. Completed a course on 4700 Proteomic Analyzer with TOF TOF Optics from 17-20 August at Klinikum Grosshadern und GSF Hämatologikum, KKG Leukemia, München.

Publikationsliste:

1. Mumtaz Yaseen Balkhi, Maximilian christopeit, Mulu Geletu and Gerhard Behre. AML1-ETO induces inhibition of granulocytic differentiation by activating survivin expression (manuscript under preparation)

2.**Mumtaz Yaseen Balkhi**[1], Arun Trivedi[1], Mulu Geletu[1], Maximilian Christopeit[1] Stefan K Bohlander[2], Herman Behre[3], Gerhard Behre[1]. Proteomic of Acute myeloid leukemia: Cytogenetic Risk Groups Differ specifically in their proteome, Interactome and posttranslational protein Modification. **Oncogene. 2006 May 29**.

3.Mumtaz Yaseen Balkhi, Sinha A, Natarajan K**.** Dominance of CD86, transforming growth factor- beta 1, and interleukin-10 in Mycobacterium tuberculosis secretory antigen-activated dendritic cells regulates T helper 1 responses to mycobacterial antigens. **J Infect Dis. 2004 May 1; 189(9):1598-609**.

4.Mumtaz Yaseen Balkhi, Latchumanan VK, Singh B, Sharma P, Natarajan K. Cross-regulation of CD86 by CD80 differentially regulates T helper responses from Mycobacterium tuberculosis secretory antigen-activated dendritic cell subsets. **J Leukoc Biol. 2004 May; 75(5):874-83.**

5.Latchumanan VK, **Mumtaz Yaseen Balkhi**, Sinha A, Singh B,Sharma P, Natarajan K. Regulation of immune responses to Mycobacterium tuberculosis secretory antigens by dendritic cells.**Tuberculosis. 2005 Sep-Nov;85(5-6):377-83**

6. Mulu Geletu, **Mumtaz Yaseen Balkhi**[1], Abdul A Peer Zada[1], Arun Trivedi[1] Maximilian Christopeit[1], Herman Behre[2], Gerhard Behre[1]. Identification of C/EBPα-p30 target proteins in acute myeloid leukaemia: C/EBPα-p30 enhances Sumoylation of C/EBPap42 via upregulation of Ubc9 (Manuscript submitted)

7. Abdul A Peer Zada[1], John A Pulikkan[1], Deepak Bararia[1], Mulu Geletu[1], Arun Trivedi[1], **Mumtaz Yaseen Balkhi**[1], Daniel G Tenen[2] Herman Behre[3], Gerhard Behre[1]. Proteomic discovery of Max as a novel interacting partner of C/EBPα: A Myc/Max/Mad link (In Press, Leukemia).